UNIVERSITY OF GHANA

COLLEGE OF BASIC AND APPLIED SCIENCES

GENOTYPIC DIVERSITY OF *MYCOBACTERIUM TUBERCULOSIS* COMPLEX FROM THE SOUTHERN HALF OF THE VOLTA REGION, GHANA

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DECLARATION

This is to certify that this thesis is the result of research undertaken under supervision by Selassie Louis Ameke towards the award of Master of Philosophy in Molecular Cell Biology of Infectious Diseases in the Department of Biochemistry, Cell and Molecular Biology, University of Ghana.

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ABSTRACT

Tuberculosis (TB) still remains a major global health problem particularly in resource-limited settings despite the availability of treatment regimen for decades. Although two pathogens, *Mycobacterium tuberculosis* sensu stricto (MTBss) and *Mycobacterium africanum* (Maf) are responsible for TB incidence in humans, more research attention has been accorded MTBss to the neglect of Maf, which is reported to be geographically restricted to West Africa and its immigrants. This thesis sought to determine the phylogenetic population structure of *Mycobacterium tuberculosis* complex (MTBC) isolates from the southern half of Volta region, which has never been done.

Sputum samples were cultured and the obtained mycobacterial isolates confirmed as MTBC by PCR amplification of insertion sequence, IS*6110.* Non-MTBCs were characterized by amplifying mycobacterial heat shock protein 65 (*hsp65*). The MTBCs were then genotyped by spoligotyping and their drug susceptibility profiles determined based on line probe assay using GenoTypeMTBDRplus. The MTBC lineage associations were assessed.

Of the 270 sputum samples collected, acid fast bacilli (AFB) positivity recorded was 183 (67.8%) and mycobacterial isolation positivity recorded was 125 (68.3%) out of the 183 AFB positive confirmed samples. In all, 117 isolates were characterized as members of the MTBC with 8 as non-tuberculous mycobacteria (NTMs). The odds of finding a multi-drug resistant (MDR) MTBss infection was relatively higher than finding an MDR Maf infection (OR=1.54, CI=0.07-33.13). This study thus, confirms the importance of *M. africanum* lineages in Ghana and should be considered in the development of new diagnostics, drugs, and vaccines.

DEDICATION

I dedicate this work to the Lord God Almighty for granting me strength and to my wife Mrs. Portia Adomaa Ameke and my beautiful God-given kids, Selinam, Sedem and Setor for making me appreciate life outside medical research.

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LIST OF ABBREVIATIONS

AMK	Amikacin
BCG	Bacillus Calmette Guérin
DOTS	Directly Observed Short Course Therapy
DST	Drug Susceptibility Test
EMB	Ethambutol
INH	Isoniazid
KAN	Kanamycin
MDR	Multi- Drug Resistance
MTBC	*Mycobacterium tuberculosis* complex
MTBss	*Mycobacterium tuberculosis* sensu stricto
Maf	*Mycobacterium africanum*
NCBI	National Center for Biotechnology Information
NTMs	Non-tuberculous Mycobacteria
NTP	National Tuberculosis Control Programme
PCR	Polymerase Chain Reaction
PZA	Pyrazinamide
RIF	Rifampicin
SNPs	Single Nucleotide Polymorphisms
STR	Streptomycin
TB	Tuberculosis
WHO	World Health organization
XDR	Extensively Drug Resistance

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Tuberculosis (TB) still is a major health problem globally, but particularly in resource-limited settings (Dirlikov *et al*., 2015; Lewandowski *et al*., 2015) in spite of treatment availability for decades. In the year 2015 an estimated 10.4 million new TB cases and 1.8 million (including 0.4 million among people living with HIV) deaths due to TB occurred worldwide (World Health Organization, 2016). New (incident) TB cases recorded were 5.9 million (56%) among males, 3.5 million (34%) among females and 1.0 million (10%) among children (World Health Organization, 2016). TB control is limited by socio-economic factors such as overcrowding and stigma. This leads to late reporting of cases which facilitates community transmission as well as non-compliance to treatment resulting in the emergence of drug resistant mutant bacilli which become difficult to treat (Velayati *et al*., 2009). If the emergence of TB resistance is not controlled, a once treatable disease will be rendered untreatable, as we have been unsuccessful over the past decades with the development of new effective TB drugs. An additional important factor hampering TB control is the strong TB- HIV infection synergy, which is a great drawback to the control of TB in sub-Saharan Africa. HIV infections secondary to TB infection leads to a compromised immune system, which facilitates early progression to an active TB disease phase. With 30% burden of tuberculosis cases and 80% of all HIV co-infection, Africa is the most burdened of the two diseases (World Health Organization, 2016).

In Ghana, the disease has been of great concern to stakeholders in the health sector. With a prevalence rate of 286/100,000, and mortality rate of 7.5/1,000 among the infected, Ghana is now considered a high TB burden country in sub Saharan Africa (Ntoumi *et al*., 2016). TB burden in Ghana is on the upsurge as 14,632 cases were reported in the year, 2016 as against 9,714 in 2015 (NTP, 2016). Out of the 14,632 cases recorded in 2016, six hundred and ninety-nine (699) were children, 5,011 were women with 8,922 being men (NTP, 2016).

Despite the challenges facing TB control in Ghana**,** there was an 85% treatment success in the year 2014. This was among 14,662 new and relapse cases registered in 2014 (Uplekar *et al*., 2015; World Health Organization, 2015) from which lessons could be drawn.

Tuberculosis is caused by a group of closely related aerobic, acid-fast bacteria, referred to collectively as *Mycobacterium tuberculosis* complex (MTBC) (Gagneux and Small, 2007). The MTBC is made up of *M. tuberculosis* sensu stricto (MTBss), *M. africanum* (Maf), *M. microti*, *M. bovis*, *M. caprae*, *M. mungi*, *M. suricattae*, *M. orygis* and *M. pinnipedii* (Halse *et al*., 2011; Safianowska *et al*., 2009; Richter *et al*., 2004, 2003). Human TB is caused mainly by MTBss and Maf with the remaining seven species as animal adapted (Brosch *et al*., 2002; Meyer *et al*., 2008) although there are occasional cross species infection. The human adapted MTBC consists of seven main phylogenetic lineages, which have been shown to exhibit a strong phylogeographical structure, and suggests specific lineages are closely associated with distinct geographic regions, and preferentially infect persons originating from these regions (Gagneux *et al*., 2006). Five

of the seven lineages, L1-L4 and L7 are MTBss with the remaining two L5 and L6 being Maf. With the exception of L7, all MTBss lineages have been isolated from different human populations, but Maf is restricted to individuals originating from West Africa where it causes about 50% of all TB cases in some countries (de Jong *et al*., 2008; de Jong *et al*., 2009; Gagneux *et al*., 2006).

Case detection, the main TB control strategy in low resourced settings, is supported by microbiological methods such as smear microscopy, mycobacteria culture, DNA based assays followed by treatment of identified cases by the Directly Observed Treatment Short course strategy (DOTS) (Lienhardt and Ogden, 2004; Zalesky *et al*., 1999). Furthermore, the public health laboratories in resource-constrained countries usually do not have the infrastructure and expertise to carry out drug susceptibility testing (DST) and molecular epidemiological analysis. Thus, this strategy does not identify circulating strains, groups most at risk and an indication of the transmission dynamics, though this is a useful public health intervention measure to reduce source of infection (Asante-Poku *et al*., 2015; Gehre *et al*., 2013; Yeboah-Manu *et al.*, 2011). Molecular techniques such as large sequence polymorphism (LSP) analysis (Du *et al.*, 2011; Yeboah-Manu *et* al., 2011), and spoligotyping (Driscoll, 2009) have been applied successfully in developed countries and recently in developing countries to address epidemiologic questions. Furthermore, these tools have been used to understand the transmission dynamics of the disease (Du *et al*., 2011; Hershberg *et al.*, 2008; Yeboah-Manu *et al*., 2011).

1.3 Rationale

Tuberculosis still remains one of the leading causes of infectious diseases related morbidity and mortality worldwide. In 2015, the incidence of TB was estimated to be

10.4 million with associated 1.8 million deaths worldwide (World Health Organization, 2016). One of the main drawbacks to the fight against TB is the emergence of MTBC drug resistant strains. Case detection is one of the key strategies for the disease control; however, the detection of these drug resistant strains has been a challenge in health facilities of low resource settings since the main diagnostic tool, smear microscopy, has limited sensitivity and cannot detect drug resistance. Moreover, microscopy has low sensitivity detecting only 50% of TB cases and cannot distinguish between the MTBC and non-tuberculous mycobacteria, which has implications on treatment regimen.

Also, the only vaccine, Bacillus Calmette–Guérin (BCG) administered for preventive purposes has been reported to be effective against TB meningitis in children beyond which its potency decreases making many more adults prone to the infection (Roth *et al*., 2006). This may have contributed to the fact that one-third of the world's population is latently infected (Sharma *et al*., 2005).

To effectively control the TB disease, several resources are being invested into studies that aim to understand the biology of the causative agents to allow development of new effective drugs, vaccine and diagnostic tools. However, all work is mainly concentrated on MTBss. Albeit two pathogens MTBss and Maf are responsible for the incidence of TB disease in humans, MTBss has been accorded more attention in terms of research to the neglect of Maf, which is reported to be geographically found in West African sub-region and its immigrants (de Jong *et al*., 2010). The reason Maf has been neglected could be attributed to the notion that the MTBCs are monomorphic and there is no diversity between the pathogen with functional implications. Also Maf is considered less virulent so would be outcompeted by MTBss, however, current epidemiological studies still

indicate significant presence of Maf within the transmission cycle of the West African sub-region (de Jong *et al*., 2008; Gehre *et al*., 2013). The geographical restriction is interesting hence understanding of Maf pathogen biology could contribute to more information on evolution, host-genetics and susceptibility to TB drugs as well as contribute to development of control tools especially vaccines.

Asante-Poku *et al*., (2015, 2016) in two molecular epidemiological studies indicated a strong association between Maf and the Ewe ethnicity. This observation needs to be confirmed; Volta Region is the home of the Ewe Ethnic group and to the best of my knowledge, there has not been any study to characterize the population structure of circulating MTBC and their susceptibility to the two most potent first-line drugs; rifampicin and isoniazid.

1.5 Aim

The main purpose of my masters' thesis is to study the population structure of clinical MTBC isolates obtained from smear positive pulmonary patients attending public health facilities in the Southern half of Volta Region.

Specific Objectives

The specific objectives are:

- To isolate mycobacteria from smear-positive patients' sputum samples collected from the Southern half of Volta Region.
- To characterize isolated mycobacteria by polymerase chain reaction, target sequencing and genotype MTBC isolates using Spoligotyping.
- To determine drug susceptibility profile of MTBC isolates using a line probe assay, Genotype MTBDRplus.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Historical facts of TB.

Tuberculosis (TB) is an ancient disease and has been described in almost all cultures of the world, even before Christ. In the old testament of the Bible various quotations such as "The LORD will strike you with wasting disease, with fever and inflammation, with scorching heat and drought, with blight and mildew, which will plague you until you perish" (Deut. 28:22) are found. Analysis of specimens obtained from individuals from different tomb complexes (as far back as 2050 BC) in Thebes West, Egypt, for the presence of *Mycobacterium tuberculosis* (the causative agent) specific DNA sequences were found to be positive for *M. tuberculosis* DNA(Matheson *et al.*, 2009; Zink *et al.*, 2003). Millions of illness and associated deaths that occurred mostly among the youth in Europe and North America between the 18th century to the 19th century earned TB the name, the '*the robber of youth'* (Segen, 1992). Other names include *phthisis* (Greek), *consumptione* (Latin), *yaksma* (India), and *chaky oncay* (Incan), each making reference to the "drying" or "consuming" effect of the illness (Daniel, 2006). The first mention of TB in non-Western world was when it was found in tubercular decay in the spines of Egyptian mummies dating from 3000–2400 BC (Zink *et al.*, 2003, 2007). The disease was at first thought as of non-infectious aetiology and believed to be hereditary according to Hippocrates until Aristotle proposed its contagious nature in the 4th century B.C and years later was supported by Jean Antoine-Villemin in 1865 (Cambau and Drancourt, 2014). This understanding led to the use of methods including breathing of fresh air at

high altitudes and surgical operations for case management. A major contribution in understanding the disease came with the invention of the stethoscope in 1816 by Rene Laennec to listen to sounds from the lungs as an individual breathes (Daniel, 2000; Daniel, 2005).

The date March 24, 1882 was when clearer understanding of TB and its control was found, with the remarkable presentation of Robert H.H. Koch, "Die Aetiologie der Tuberkulose", in Germany during which he reported *M. tuberculosis* as the causative agent (Koch, 1932)**;** thus March 24th is still celebrated as the world TB day. Von Pirquet and Mantoux in 1907-1908 developed the tuberculin skin test, and Seibert in 1931 prepared the purified protein derivative (PPD) of tuberculin and used it 3 years later to demonstrate latent tuberculous infection in asymptomatic children (Bates and Stead, 1993).

Public Health measures to combat the spread of the disease intensified following the discovery of its bacterial origin. Until the 1940s, there was no effective chemotherapy against TB. The first anti-mycobacterial agent, streptomycin was identified in 1944 by Albert Schatz and his colleagues (Kingston, 2004; Schatz *et al*., 1944), and subsequently by the simultaneous discovery of isoniazid in 1951, by chemists working at Bayer Laboratory in Wuppertal, Germany, at Hoffman-La Roche in Basel, Switzerland, and at Squibb Institute for Medical Research in New Brunswick, New Jersey (Barnard, 2001; Squibb *et al*., 2008). Subsequently in 1965, rifampicin was discovered by the research group of Piero Sensi and Maria Teresa Timbal in Millan, Italy (Riccardi and Pasca, 2014). Albert Calmette and his associate Camille Guerin working at the Pasteur Institute

in Lille then attenuated *M. bovis* by several subcultures to produce the Bacillus Calmette–Guérin (BCG) vaccine (Luca and Mihaescu, 2013). The BCG vaccination has been widely employed following the World War I for prevention of TB and it is the first vaccination for most parts of the world. Despite all these efforts, humankind has never been free from TB (Colditz *et al*., 1994; Luca and Mihaescu, 2013).

2.2 Global burden of TB

In resource-limited settings, TB ranks alongside the human immunodeficiency virus (HIV) as a leading cause of death worldwide as it remains one of the major public health issues globally. In 2015, an estimated 10.4 million new (incident) TB cases (5.9 million (56%) males, 3.5 million (34%) females and 1.0 million (10%) children) and 1.8 million (including 0.4 million deaths among people living with HIV) deaths due to TB occurred worldwide. Moreover, a third of the world population estimated as over 2 billion individuals are latently infected (World Health Organization, 2016). As a disease of poverty, the TB cases are commonly found in low-income and developing economies with Southeast Asia and sub-Saharan Africa (more specifically India, Indonesia, China, Nigeria, Pakistan and South Africa) accounting for more than 61% of all cases (Maitra and Bhakta, 2014). Africa accounts for more than one quarter of all incidence, and its associated mortality (World Health Organization, 2016) as shown in **Figure 2.2.**

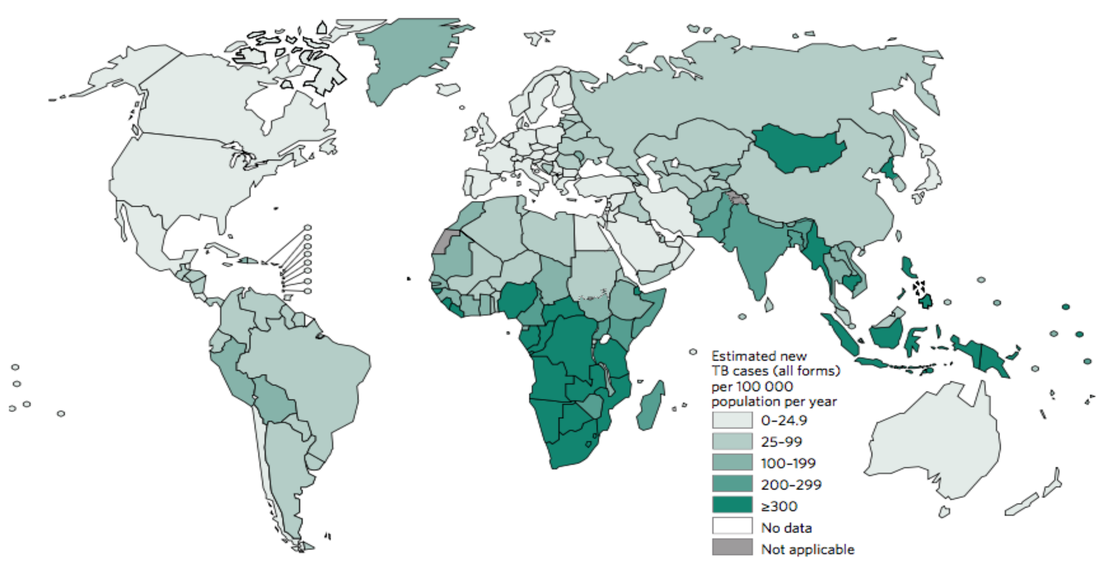

Figure.2.2: New Global estimated TB cases in 2015 (World Health Organization, 2016).

The rapid upsurge of TB cases has been attributed to the upsurge in HIV infections, non-compliance to treatment regimen which together with irregular supplies of drugs contributes mainly to the increasing emergence of anti-TB drug resistant strains (Matteelli *et al*., 2014; Wells *et al*., 2007)**.** The HIV infection has a direct correlation with progression from latent TB to full blown disease state (Sullivan *et al.*, 2015). In 2015, 80% of the TB/ HIV co-infected people globally, were concentrated in Africa (**Figure 2.2.1**) (World Health Organization, 2016). Screening for HIV in TB patients in health facilities has now become a key component of the routine algorithm used in most African countries (Corbett *et al*., 2006).

The rise in drug resistant (DR) TB especially MDR-TB cases is very alarming. Individuals with DR-TB take drugs for longer times and have reduced positive outcomes. Globally, in 2015, 480,000 new cases of MDR-TB (3.5% of new cases, 20.5% of

previously treated cases) with an estimated 210,000 deaths occurred (Global TB report, 2014). Notwithstanding, the reported MDR numbers may have fallen short of input from most TB burden countries, especially in Africa with scarce resources which are unable to perform routine drug susceptibility testing (DST) in order to detect most MDR-TB cases.

The rate of decline in TB incidence remained at 1.5% in 2015, far from the 4–5% annual decline needed to reach the first milestones of the End TB Strategy, which aims to achieve a decline of 20% by 2020.

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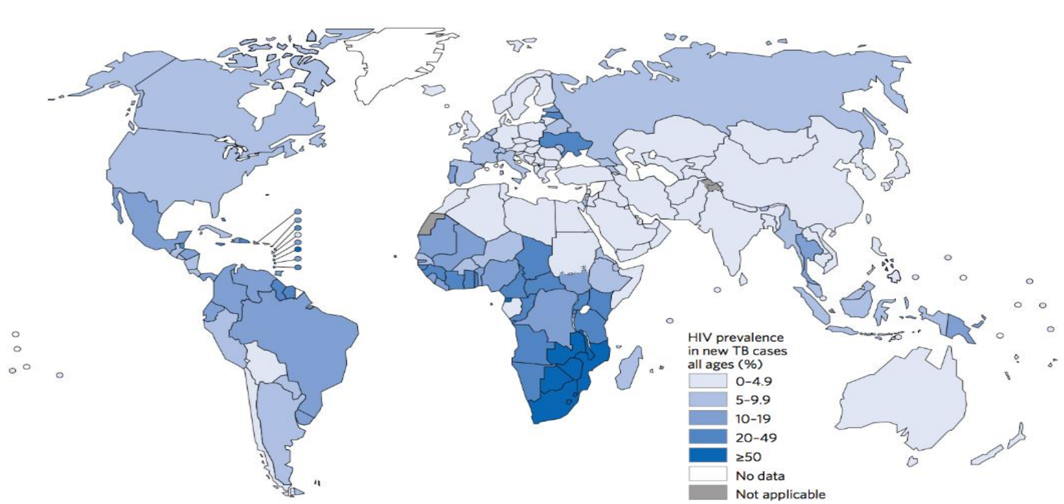


Figure 2.2.1: Global Estimated HIV prevalence in new and relapse TB cases, 2015 (World Health Organization, 2016).

2.3 Tuberculosis in Ghana

Treatment of TB in Ghana began as early as 1950s before independence, with the springing up of care-giving units. On the whole, efforts at curbing the disease were uncoordinated thereby disenfranchising most TB patients (Dodor, 2012; Dodor and Kelly, 2009). Following the adoption of the Directly Observed Treatment Short Course

(DOTS) strategy in the early 1990s (National tuberculosis annual report, 2008), the service became free and accessible to all patients. The introduction of the DOTS strategy contributed greatly to reduce patient defaulter rate in Ghana from 11% in 2005 to 2.6% in 2010 below WHO target of 5% (World Health Organization, 2016). Currently, there are over 700 public treatment centers, about 1000 sub-treatment centers and many private health facilities that offer TB treatment.

Despite much effort to control the disease, TB has become an unending public health issue. The annual incidence as of the year 2015 stood at 160/100,000 population, and with a TB/HIV coinfection rate of 24%, Ghana is ranked amongst high TB/HIV burden countries in Africa by WHO (World Health Organization, 2016). Nevertheless, some successes have been achieved in the control of the disease in terms of seeking early treatment which resulted in boosting cure rates for new smear positive TB increasing from 22% in 1996 reaching the recommended WHO level of 85% in 2010 (World Health Organization, 2016). Treatment success for MDR-TB also increased from the year 2010 - 2012 with a decline in 2013 (**Figure 2.3**) (World Health Organization, 2016)

Despite these achievements, Ghana's TB detection rates of 31% falls short of the recommended seventy percent WHO standard (Jones-López *et al*., 2011; Woldeyohannes *et al*., 2011). This means that more than 60% of the cases are missed which must be of great concern since they are of great threat to the control of the disease (World Health Organization, 2016).

One major factor for the low detection rate could be the association of the disease with death resulting in stigmatization of the disease. It is worth noting that, the disease TB is

referred to as '*Yomekpe'* among the people of the Volta Region of Ghana, (grave yard cough), literally meaning once infected, the individual would die of the cough (Dodor and Kelly, 2009; Dodor, 2008). Similarly, across the country, different names are given to the disease which leads to stigmatizing people living with the disease and prevents them from seeking treatment.

The TB/ HIV associated mortality increased from 3.2% in 1987-88 to 5.1% in 1997-98 and as of 2014 it was 7%, second to malaria (Bjerrum *et al.*, 2015). The above notwithstanding, increasing proportion of individuals with TB/HIV co-infection have been treated of TB successfully since 2009 (**Figure.2.3**) (World Health Organization, 2016).

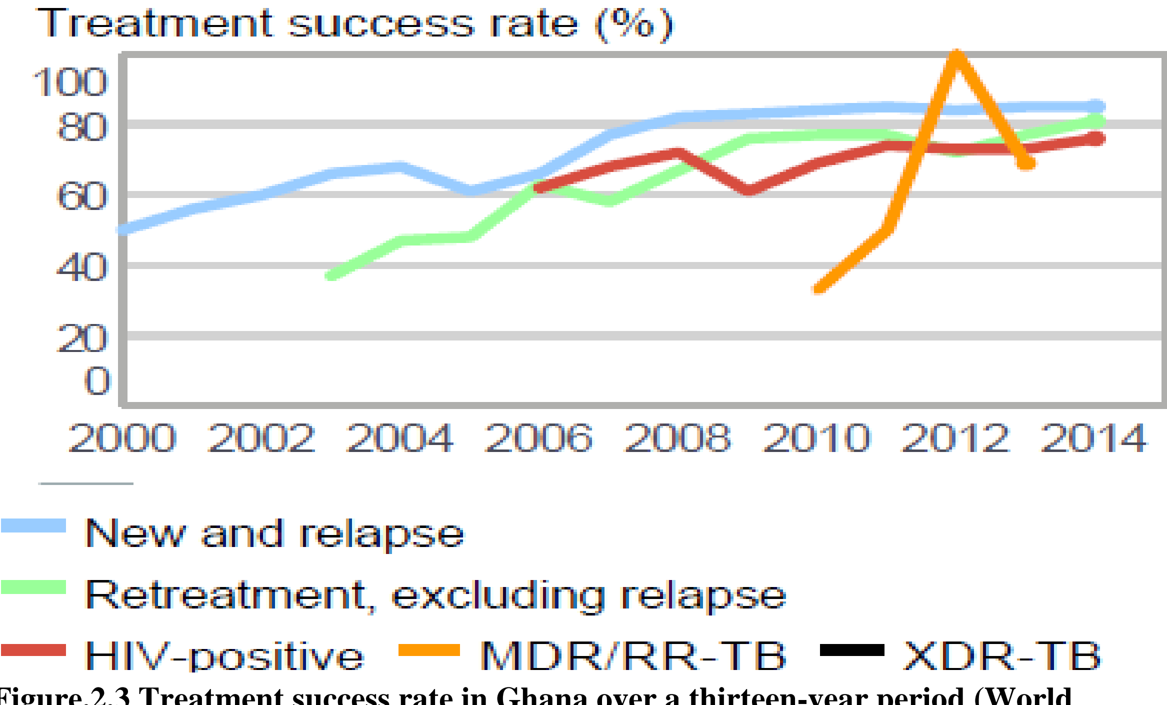


Figure.2.3 Treatment success rate in Ghana over a thirteen-year period (World Health Organization, 2016)

2.4 The TB causative agent

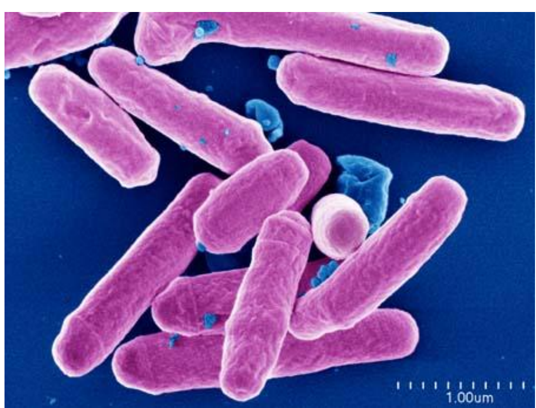


Figure 2.4: Rod-like mycobacterium species

The disease TB is caused mainly by closely related bacteria collectively known as the *Mycobacterium tuberculosis* complex (MTBC). The MTBCs belong to phylum actinobacteria, order actinomycetales, suborder corynebacteriaceae and the genus mycobacterium (**Figure 2.4)** (Polkade *et al.*, 2016; Scherr and Nguyen, 2009; Ventura *et al.*, 2007). Some mycobacteria are fast growers that produce macroscopic growth within seven days, and the others, slow growers that form colonies after seven days which comprise of well-known pathogenic organisms such as *M. tuberculosis*, *M. bovis* and *M. leprae* (Stahl and Urbance, 1990; van der Wel *et al*., 2007a, 2007b).

The members of MTBC share genome identity of (more than 99.99%) and this makes them appear monomorphic genetically (Kempsell *et al*., 1992; Wirth *et al*., 2008), but differ in terms of host tropism. *Mycobacterium tuberculosis* sensu stricto (MTBss) and *M. africanum* (Maf) are the main causative agents in humans (Brosch *et al*., 2002; Zink *et al.*, 2003). *Mycobacterium microti* affects voles, (Cavanagh *et al.*, 2004), *M. caprae* affects goats and sheep (Aranaz *et al*., 2003). *M. mungi:* Mangoose pathogen, *M. orygis* pathogen of antelope (van Ingen *et al*., 2012) and *M. pinnipedii* a pathogen of seals and

sea lions (Riedman, 1990). Amongst them, *M. bovis* shows the widest spectrum of host as it infects animals and occasionally Humans (Vasconcellos *et al.*, 2010). Three mutations in the PhoP regulon gene of *M. bovis* leads to reduced expression of its virulent product hence its reduced potential to transmit among humans (Smith *et al*., 2006; Berg and Smith, 2014). The MTBss is found in all regions of the world unlike Maf, which is restricted to West Africa where it causes about 50% of all TB cases in some countries (de Jong *et al*., 2010).

A unique feature of *Mycobacterium* species is the cell wall; a complex structure that is thicker than the cell walls of other bacteria. More than half of the mycobacterial cell wall is made up of covalent mesh of lipids, arabinogalactan, and peptidoglycan which are surrounded by a non-covalently linked outer network of proteins and polysaccharides (**Figure 2.4.1**) (Kieser and Rubin, 2014). Notably, the core peptidoglycan essential for cell viability has a covalent attachment with the heteropolysaccharide arabinogalactan which is in turn esterified at its non-reducing ends to long-chain (C70-C90) mycolic acids (Brennan, 2003; Daffé, 2015). The high mycolic acid content of the cell wall of mycobacteria offers several properties to the bacteria; responsible for their acid-fastness, (Apers *et al*., 2003; Tansuphasiri *et al*., 2004); resistances to potent antibiotics and attack by lysosomes. (Boshoff and Barry, 2006; Cho *et al*., 2014).

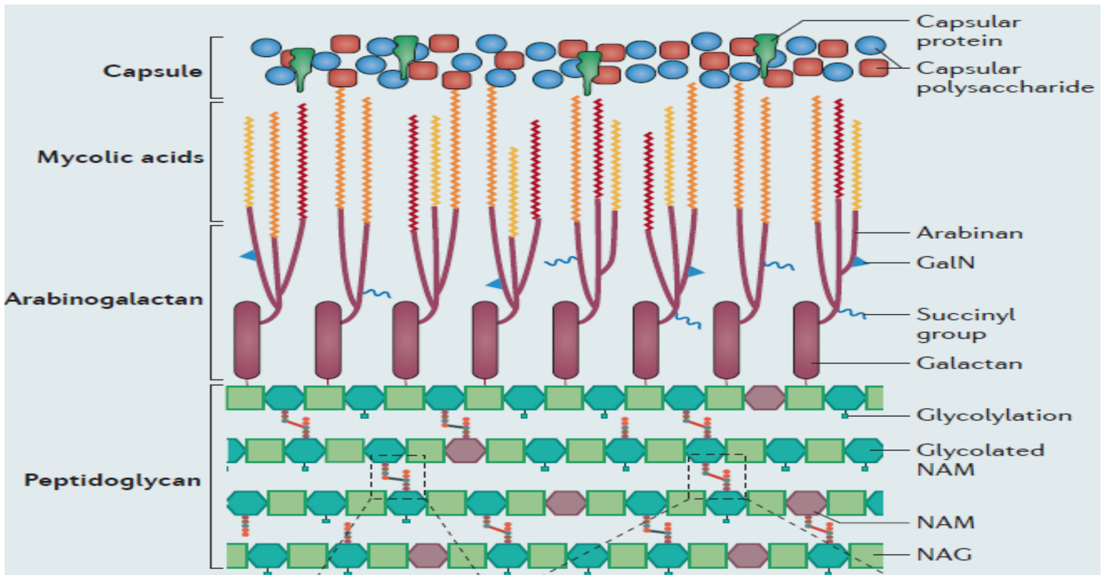


Figure 2.4.1: Structural composition of mycobacterial cell wall (Kieser and Rubin, 2014)

2.5 Facts about *Mycobacterium africanum*

Mycobacterium africanum (Maf) was discovered in 1968 by Castets *et al*., (1968), and was reported to exhibit characteristics between *M. tuberculosis* and *M. bovis*. Notably, *M. africanum* tends to be nitrate negative and a weak producer of niacin like *M. bovis* (Yeboah-Manu *et al.*, 2011), and grows slowly on Lowenstein Jensen (LJ) media supplemented with pyruvate since it lacks pyruvate kinase (Gehre *et al*., 2013; Casal *et al*., 1997; Chew *et al*., 1998). On the other hand, Maf shares pyrazinamide sensitivity with MTBss (Frothingham *et al*., 1999; Huang *et al*., 2013; de Jong *et al*., 2008).

Initial biochemical characteristics identified two major *M. africanum* subgroups: the West Africa- subtype I (MAF1) and Eastern Africa -subtype II (MAF2) (Frothingham *et al*., 1999). The MAF1was more closely related to *M. bovis*, MAF2 was more related to MTBss (Viana-Niero *et al.*, 2001). However, based on recent DNA-based analyses including large sequence polymorphism and single nucleotide polymorphism (Mostowy

et al., 2004; Brosch *et al*., 2002) the East-African genotype, MAF2 was found to display the TbD1 deletion, a distinguishing feature of modern MTBCs. Subsequently was reclassified as Uganda" genotype and member of MTBss (Brosch *et al*., 2002; Niemann, 2004). The true Maf was then identified as the West-African variant. The true Maf is also subdivided into 2 phylogenetic lineages called West African 1 (Maf-WA1) and West-Africa genotype II (Maf-WA2) which are found more in eastern part of West-Africa and western part of West-Africa respectively. Few countries like Ghana and Cote d'Ivoire have been reported to harbour both genotypes (Mostowy *et al*., 2004; Vasconcellos *et al*., 2010).

As first described by Mostowy *et al*., (2004) in a genome comparative study with H37Rv, Maf-WA1 strains were found to uniquely possess the large sequence polymorphism (LSP) RD713, RD711 while Maf-WA2 (also known as clade 2) (Smith *et al*., 2006) carried the defining LSPs RD701 and RD702. Additionally, Maf strains have been reported to show a characteristic gyrase subunit B (*gyrB)* DNA sequence that permitted their clear distinction from *M. tuberculosis* (Bentley *et al*., 2012; Brosch *et al*., 2002). A further genomic region that allows the differentiation of Maf from *M. bovis* was RD12, which was present in the Maf but not *M.bovis* (Bentley *et a*l., 2012; Halse *et al.*, 2011; Huard *et al*., 2003).

In addition to the above genomic differences, Maf-WA1 showed more than five IS*6110* copies whereas Maf-WA2 generally had five or fewer IS*6110* copies (Agasino *et al*., 1998; Isabel *et al.*, 2008; Kent *et al.*, 1995). Similarly, the two variants differ in the presence or absence of spacers within the MTBC specific direct repeat region and this allows separation using the oligonucleotide typing, Spoligotyping. Spacers 8 through 12

and 37 through 39 are absent in Maf-WA 1 whereas spacers 7 through 9 and 39 are absent in Maf-WA 2 (de Jong *et al*., 2008; Gagneux and Small, 2007; Gori *et al*., 2005; Kamerbeek *et al.*, 1997) . Outside of West Africa, there have been reported cases in England (Grange *et al.*, 1989), and other parts of Europe. All these cases notably, were from recent immigrants from West Africa.

Even though Maf is primarily a human-adapted TB pathogen, there are many schools of thought about its possibility as an animal ecotype (Dharmadhikari and Nardell, 2008; Gagneux, 2012). This is due to the proximity of Maf-WA 2 to this MTBC ecotype on the evolutionary tree (Broset *et al.*, 2015; Coscolla *et al*., 2013).

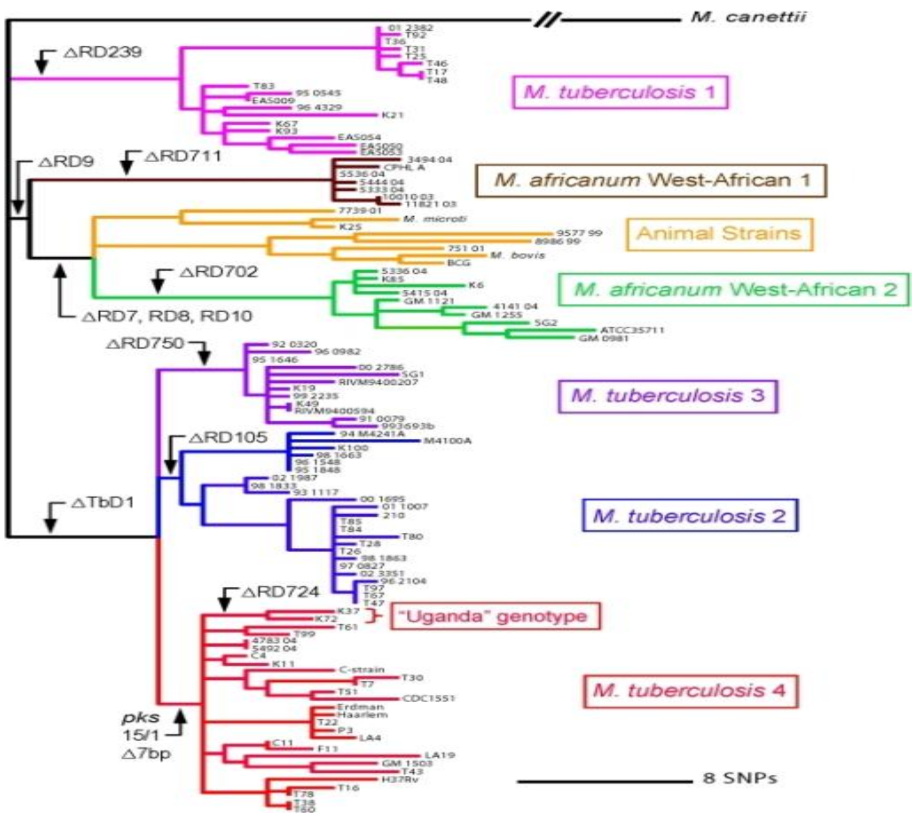


Figure 2.5: The global phylogeny of MTBC indicating position of Maf (Gagneux, 2012).

2.6 Tuberculosis Pathogenesis and Clinical Presentation.

2.6.1 Pathogenesis of Tuberculosis

Tuberculosis is aerosol transmitted and an infection occurs when a person inhales aerosolized droplet containing live tubercle bacilli released through cough by an infectious person (Gagneux, 2012). However, in order to establish infection, factors such as mycobacterial load, ventilation and the extent of proximity to an infectious individual come to play. The bacilli after inhalation, is transported into the lungs where they are internalized by phagocytic-macrophages of alveoli through receptor-mediated phagocytosis, with several different receptors like toll-like receptor 2 (TLR2) (Kang *et al.*, 2011) (Ernst, 1998; Lin and Flynn, 2010) as shown in (**Figure. 2.6**). Once internalized, *M. tuberculosis* actively interferes with phagocytosis and phagosome-lysosome fusion, by inducing the production of anti-inflammatory cytokines such as interleukin 10 by dendritic cells. It also interferes with interferon-γ signaling, and subsequently results in the preferential expression of interleukin 4 (IL-4), thereby directing the immune response from a type 1 T-cell response towards a non-protective type 2 T-cell response (Giacomini *et al.*, 2006; Huizinga *et al.*, 2011; Spellberg and Edwards, 2001). *M tuberculosis* also has the capacity to inhibit MHC-II expression in antigen presenting cells (Malik *et al.*, 2001) and spread into the lymph nodes via the lymphatic system. Through the activity of the ESX1 secretion system, *M. tuberculosis* then disrupt the phagosomal membrane, causing the release of bacterial products, including mycobacteria DNA, into the macrophage (Ates *et al.*, 2015; MacGurn and Cox, 2007; Guth *et al.*, 2009; Keane *et al.*, 1997). To prevent the invading pathogen from spreading, lymphocytes and other cells join the fight in an attempt to eliminate the

pathogen (Serbina *et al*., 2003) where they engulf the bacilli leading to granuloma (giant wall) formation (Ernst, 2012; Russell *et al.*, 2010). The granuloma is made up of phagocytic-macrophages in the center and lymphocytes at the periphery (Ulrichs *et al*., 2006**).** In spite of these robust immune defenses, some bacilli with effective evading strategies escape killing and go into dormancy, to avoid elimination by the immune cells (Finlay and McFadden, 2006; Hampshire *et al*., 2004). This stage is the hallmark of latent TB where the bacteria is contained. The contained bacilli remain in the granuloma for decades creating a balance between the host and the bacilli (Ahmad, 2010; Vernon, 2013). However, when the strength of the immune system goes down as in the case of HIV co-infection, the center of the granuloma undergoes necrosis and eventually leads to the release of live bacilli into the airways as result of cough (Russell *et al*., 2009; Vicenzi *et al*., 2007). It has been shown that as a result of the immune response, only 5-10% of these latent infections lead to disease state for unknown reasons (Kang *et al.*, 2011**).**

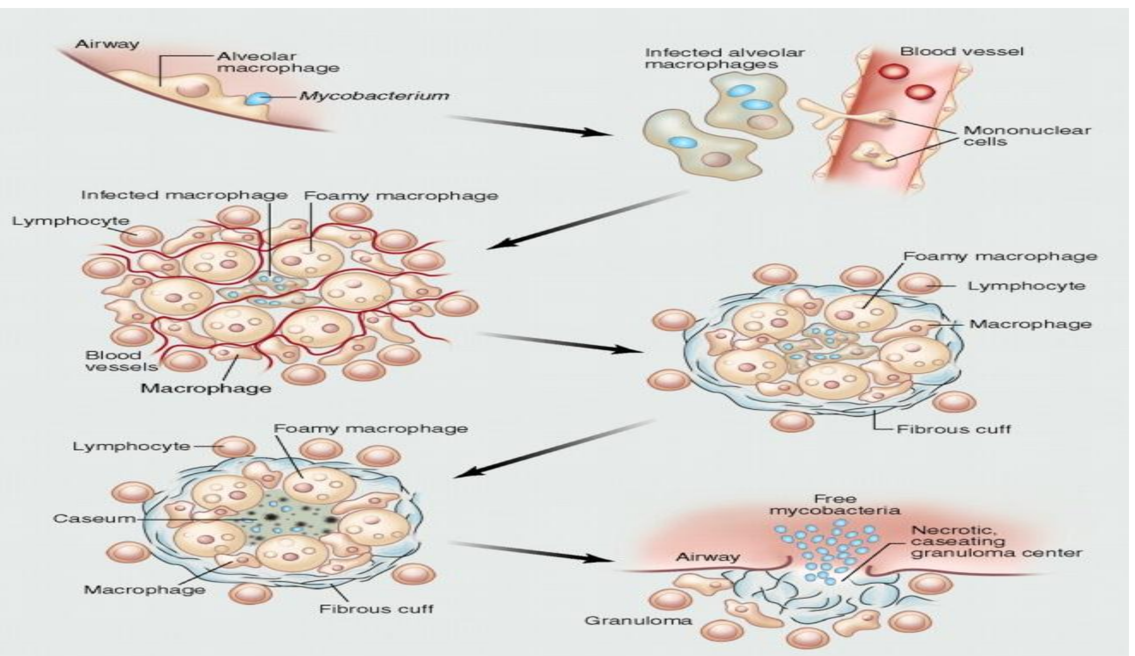


Figure 2.6: ***M. tuberculosis*** **Pathogenesis and granuloma mass formation (Ramakrishnan, 2012)**

2.6.2 Clinical Presentation

During the stone ages, tuberculosis (TB) was referred to as “consumption” indicating the severe wasting and the coughing of blood associated with later stages of the disease. Notably, tuberculosis can affect any part of the body but predominantly affects the lungs. Tuberculosis in the lungs is termed pulmonary TB whereas in parts of the body other than the lungs is termed extrapulmonary TB (EPTB). Symptoms of pulmonary TB include chronic cough, chest pains, fatigue, lack of appetite and weight loss. Other symptoms such as fever, haemoptysis (sputum with streaks of blood), night sweats, recurring colds and shortness of breath are also associated with the disease (Holmes and Faulks, 1981; Marais *et al*., 2005). Though EPTB has symptoms similar to that of pulmonary TB, there can be specific symptoms that relate to the particular site affected (Sharma and Mohan, 2004).

2.7 Tuberculosis Diagnosis and Treatment

2.7.1 TB Diagnosis

Tuberculosis is diagnosed based on clinical presentations and confirmed by microbiological identification of mycobacteria in a clinical sputum sample obtained from a presumptive TB patient. Chest radiographs also help identify abnormalities in the lungs. At present, bacteriological methods that have proven useful in clinical practice identifying the disease TB include, microscopy, commercial kits with probes specific to bacilli DNA sequences, and culture.

2.7.1.1 Direct Smear Microscopy

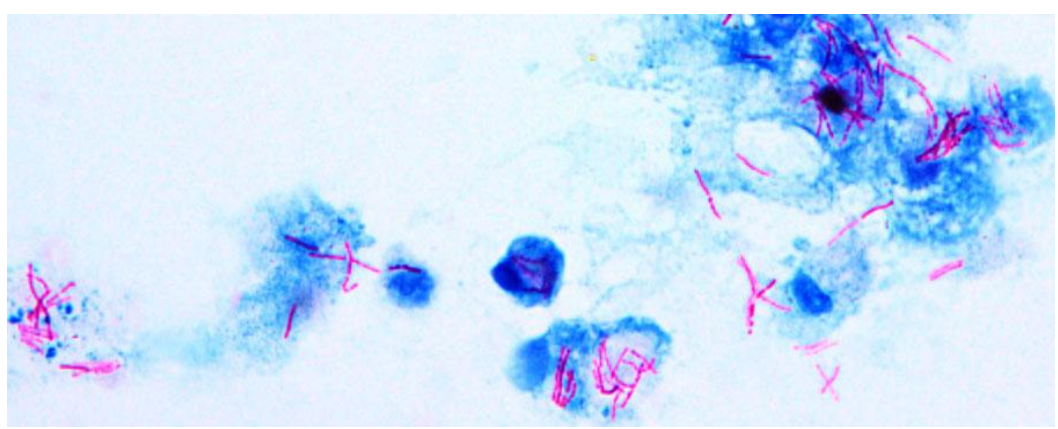


Figure 2.7.1.1: A picture of acid-fast bacilli under the light microscopy (X1000) **(Bhonsle & Karpe, 2007)**

Microscopy is the routinely and most widely used diagnostic tool for the detection of active pulmonary TB in most resource-limited-settings like Ghana. This technique is based on the impermeable nature of the cell wall of *Mycobacterium spp*. which allows it to resist acid-alcohol decolourisation after staining (Forrellad *et al*., 2013)**.** As a result of this property, all members of *Mycobacterium spp*., have been classified as acid-fast bacilli (AFB).

Generally, smear microscopy is inexpensive, simple, and efficient in detecting active cases of pulmonary tuberculosis that are most infectious. Unfortunately, it has a challenge of low sensitivity as it requires on the average about 10^4 bacilli per milliliter to be positive for smear microscopy and also, the need for quality sputum production limits its use especially in people living with HIV and children who often have difficulty in producing sputum resulting in low bacillary load (Getahun *et al*., 2007; Shingadia and Novelli, 2003)**.** This notwithstanding, fluorescence microscopy technology is gradually

being proposed as an alternative to basic fuchsin Ziehl Neelsen (ZN) staining procedures. This procedure has been reported to have increased sensitivity compared to ZN staining (Prasanthi and Kumari, 2005; Mfinanga *et al*., 2007; Myneedu *et al*., 2011; Olaru *et al.*, 2014)**.** The main advantage of sputum smear microscopy is its rapidity, requiring less infrastructure and expertise making it suitable for resource limited endemic regions such as Africa.

2.7.1.2 Chest radiology

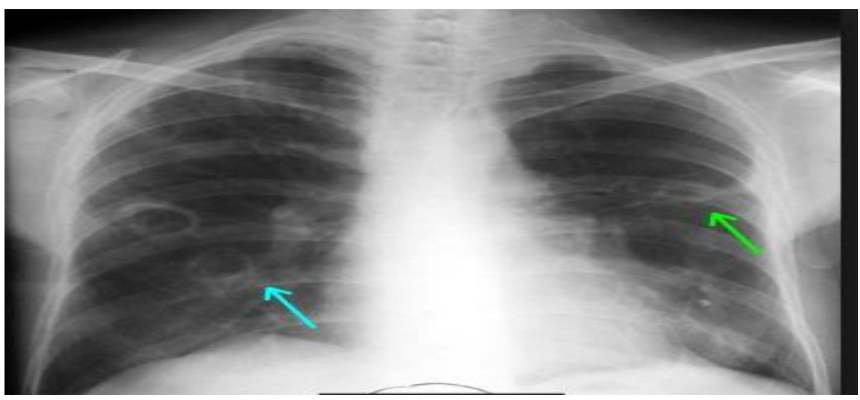


Figure 2.7.1.2: A radiograph showing abnormalities in the lung of TB patient

Chest X-ray to detect abnormalities in the upper lungs (infiltration or cavities) is occasionally depended upon to complement findings of smear microscopy for identifying pulmonary TB cases. Although this procedure is not necessarily conclusive for TB infection, it is often useful to individuals with high reactive Mantoux results and asymptomatic of TB (Wanchu *et al.*, 2008)**.**

2.7.1.3 Cultivation of Mycobacteria from Sputum

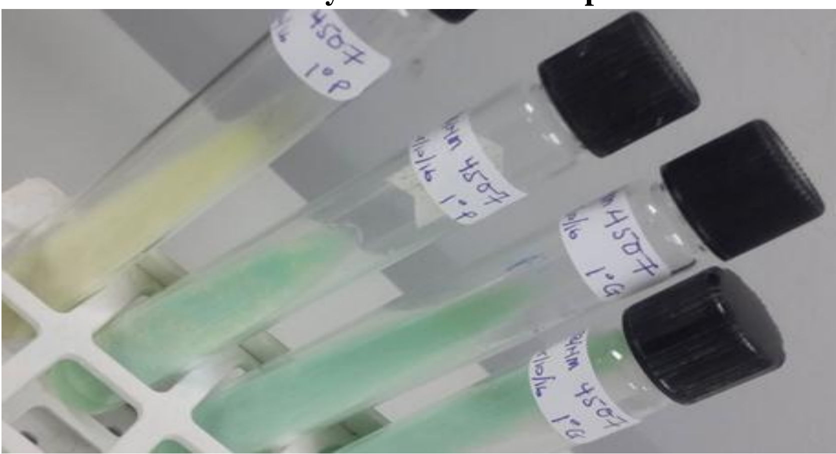


Figure.2.7.1.3: Macroscopic mycobacterial growth on pyruvate supplemented Lowenstein Jensen media-TB/Nm4507 P (Bacteriology Department, Noguchi Memorial Institute for Medical Research, Legon).

The microbiological recovery of live mycobacterium bacilli gives definitive case indication hence its usefulness in research laboratories. The bacterial isolates obtained also offers opportunity for in depth analysis. This procedure is highly sensitive as it detects very low numbers of bacilli (approximately 10 bacilli/ml of sputum compared with sputum microscopy which requires at least 5000 bacilli/ml for detection (Kleger and Kantor, 1996). The slow growth rate of MTBC and the need for sophisticated equipment prevents its routine use as a first line rapid diagnostic tool for active TB (Palomino *et al*., 1998).

2.7.1.4 DNA-Based Methods

Currently, several rapid kits with probes specific to bacilli DNA sequences have been introduced for diagnosis of TB with an added advantage of identifying drug resistance. Notably of such WHO approved assays are GeneXpert MTB/RIF (Cepheid, Sunnyvale, USA) and the GenoType MTBDRplus line probe assays from HAIN Lifescience (Nehren, Germany). These two are currently being employed even in high burdened low-

resourced settings. The GeneXpert MTB/RIF, which is based on a semi real-time amplification of *rpoB*, identifies the MTBC and its resistance to rifampicin directly from sputum, in less than 2 hours. The GenoType MTBDRplus on the other hand uses a reverse hybridization principle that diagnoses and detects MTBC resistance to the two main anti-TB drugs: isoniazid and rifampicin.

This method produces results within 5 hours in a day, however, common with any DNA based assay, this technology screens only the nucleic acid sequences and not the amino acid sequence. It is therefore possible silent mutations would be captured in the probe region as absence of wildtype band and might result in false resistance output for an MTBC isolate.

2.7.1.5 Immunological Assays

Several antigen-antibody centered assays have been introduced mainly for picking up latent TB infection. The Mantoux test is the most common (Mendel, 1908). In this test, delayed hypersensitivity is measured after intradermal administration of PPD of tuberculin. The main disadvantage of this test is the false positives among individuals vaccinated with Bacille Calmette-Guerin (BCG) and the high degree of interference with mycobacteria other than MTBC, makes it not suitable for countries where Bacille Calmette-Guerin (BCG)–vaccination is a requirement for all children.

To overcome this problem, alternative methods centered on measuring interferon-gamma (IFN-γ) released from T lymphocytes have been introduced (Becker *et al*., 2001). These assays are based on the released interferon-gamma by the human host in response to the MTBC antigens, early secretory antigen target 6 (ESAT-6) and culture filtrate protein 10

(CFP-10). Because ESAT-6 and the CFP-10 are absent in mycobacteria other than MTBC and *M. bovis* vaccine strain (Pittius *et al*., 2001; Waters *et al*., 2004), it makes these assays ideal for detecting MTBC infection (Brodin *et al*., 2006). As a limitation, they are unable to distinguish between latent TB and active MTB infections (Rangaka *et al*., 2011) and the need for sophisticated instruments and training limiting their potential usage as point of care diagnostic in resource limited settings. Its usefulness for diagnosis of TB in children and immunocompromised individuals is being assessed.

Table 2.7.1.4: WHO approved TB diagnostic tools (Muller *et al.*, 2013)

Approach	Output	Strengths	Weakness
Sputum smear Microscopy	Detects acid fast bacilli	Very few resource needed to operate	Lacks specificity
In vivo solid LJ culture	Live TB bacilli isolation	Detects as low as 10 bacilli/mL	Takes long to show macroscopic growth
liquid Culture	Determines isolate susceptibility profile	Shorter time to produce results	Easily contaminated
Chest radiology	Detects abnormalities in the lungs	Shows granuloma mass	Expensive
Tuberculin skin test (Mantoux)	Detection of latent TB	Very few resource needed to operate	False positives in persons with BCG vaccination
Interferon -γ release assay	Detection of Latent *M. tuberculosis* infection	Highly specific for *M. tuberculosis*	Formal training and expensive equipment
Line probe assays	TB case detection and drug susceptibility testing	Generates results in 5 hours	Requires training and Space.

2.8 Treatment of Tuberculosis

Figure 2.8: Some drugs used in treatment of TB

TB treatment is based on combination therapy for several reasons: 1) reduction in the chances of acquiring drug resistance 2) the combined modes of action of the drugs aid in effectively clearing the bacteria and helps to reduce the duration of treatment. Rifampicin (RIF) inhibits RNA synthesis and has a sterilizing effect (McClure and Cech, 1978). Pyrazinamide (PZA) although weakly bactericidal, is very effective against bacteria located in acidic environments found inside macrophages, or in areas of acute inflammation (Zhang *et al*., 2003). Ethambutol (EMB) inhibits the polymerization step of arabinogalactan synthesis (Mikusova *et al*., 1995). Isoniazid (INH) is a pro-drug and bactericidal against replicating bacteria by inhibiting mycolic acid synthesis (Zhang *et al.,* 1992), para-aminosalicylic acid inhibits folic acid synthesis (Rengarajan *et al*.,2004*)*, fluoroquinolones act on DNA replication (Drlica *et al.,* 2008) whiles Ethionamide (ETD) also a prodrug, inhibits fatty acid synthesis required for mycolic acid synthesis (Banerjee *et al*., 1994).

The standard treatment for new TB patients with drug susceptible bacteria (patients with no prior anti-TB treatment or with previous anti-TB treatment for less than 1 month) consists of two months intensive phase with daily INH/RIF/PZA/EMB, followed by a 4 months continuous phase of daily INH/RIF (Kleger and Kantor, 1996). The drugs INH/RIF are the most important drugs for TB treatment: The effort of INH in killing the organisms during the first days of treatment, is complemented by RIF and PZA during the remaining intensive phase, whilst for the continuation phase, RIF is the main active drug against persisters from the intensive phase (World Health Organization, 1994). In most people with active TB, treatment with appropriate regimen for at least 2 weeks renders them no longer contagious (Kleger and Kantor, 1996).

However, for patients infected with multi-drug resistant (MDR) bacteria, a 9–12-month regimen (WHO recommendation) might be used in selected patients, in appropriate settings, taking into account previous treatment and local resistance profiles of infecting bacteria (Nikaido, 2009; World Health Organization, 2010). If patients are not eligible for the shorter regimen, a longer treatment regimen is used. The composition of the regimen includes pyrazinamide in addition to at least four second-line drugs to which the organism is likely or proven to be susceptible for a duration of ≥20 months (Di Perri and Bonora, 2004). The second-line drugs should include a later-generation fluoroquinolone (such as moxifloxacin, levofloxacin or gatifloxacin), an injectable agent such as amikacin (AMK), kanamycin (KAN)) and two or more core second-line agents (such as ethionamide, prothionamide, cycloserine, terizidone, clofazimine or linezolid) (World Health Organization, 2016). First-line drugs (such as isoniazid or ethambutol) could be added to strengthen the regimen. However, when toxicity or resistance occurs, additional agents could be introduced, and this may include bedaquiline and delamanid, such that four drugs that are likely to be effective are being used (CDC, 2014; Kleger and Kantor, 1996). Previously treated patients are globally 5 times more likely to present with TB caused by multidrug-resistant (MDR) strains, and therefore, should be treated based on drug susceptibility test (DST) results (Kimerling *et al*., 1999; Nagaraja *et al*., 2011).

All together, these drugs work against different targets in order to effectively clear the bacteria. Total duration of therapy for treatment of drug-resistant TB is at least 18 months.

Table 2.8: Anti-TB drugs and their mechanism of action (Muller *et al.*, 2013)

Drug	Year of discovery	Effect on bacterial cell	Mechanism of action	Targets
		First line drugs		
Streptomycin	1944	Bactericidal	Inhibition of protein synthesis	Ribosomal S12 protein and 16SrRNA
Isoniazid	1952	bacteriocidal against replicating tubercle bacilli	Inhibition of cell wall mycolic acid synthesis and other multiple effects on DNA, Lipids, carbohydrates and NAD metabolism	Multiple targets including acyl carrier protein reductase (InhA)
Pyrazinamide	1952	Bacteriostatic/ bacteriocidal against slow replicating bacilli in acidic lesions	Disruption of membrane transport and energy depletion	Membrane energy metabolism
Ethambutol	1961	Bacteriostatic	Inhibition of polymerization of cell wall arabinogalactan	Arabinosyl transferase
Rifampicin	1966	A semi derivative of Rifamycin. Bacteriocidal activity against tubercle bacilli	Inhibition of RNA synthesis	RNA polymerase β subunit
		Second line drugs		
ρ-aminosalicylic acid (PAS)	1946	Bacteriostatic	Inhibition of folic acid and iron metabolism synthesis	
Cycloserine	1952	Bacteriostatic	Blocks enzyme of cell wall biosynthesis	D-alanine racemase
Ethionamide	1956	Bacteriostatic	Inhibition of mycolic acid synthesis	Acyl carrier protein synthesis (InhA)
Kanamycin	1957	Bacteriocidal	Inhibition of protein synthesis	16S rRNA
Capreomycin	1960	Bacteriocidal	Inhibition of protein synthesis	30s ribosomal subunit
Quinolones	1963	Bacteriocidal	Inhibition of DNA synthesis	DNA gyrase

2.9. Drug resistance TB

Drug resistance in TB is increased capacity of the bacteria to tolerate high doses of specific antibiotics at any given time compared with drug-susceptible bacilli (Ferdinand *et al*., 2005; Somoskovi *et al*., 2003). Drug resistance in MTBC is classified into two groups depending on how the resistance emerged: primary resistance and acquired resistance. Primary resistance is defined as a new patient infected with already drug-resistant strains. In contrast, acquired resistance occurs in a patient previously infected with a sensitive strain that mutated during the course of treatment (Canetti *et al*., 1969). Of the two forms of resistance, primary resistance poses the biggest challenge largely due to the lack of appropriate laboratory infrastructure in low-income endemic areas (Franzblau *et al*., 1998; Malhotra *et al*., 2010; Somoskovi *et al*., 2008). As such less than 20% of the estimated drug resistant cases in the world are properly diagnosed (Migliori *et al*., 2008). Drug resistance in TB unlike other bacteria is conferred by specific chromosomal mutations and promoted either through environmental/extrinsic effect or bacterial factors. These factors can either be as a results of delay in diagnosis, inadequate or interrupted drug supply, patient non-adherence to treatment (Perlman *et al.,* 2005*;* Muller *et al.,* 2013) or through 'persisters' (bacterial cells that phenotypically tolerate high levels of drug concentration, prolongs the average lifetime of bacteria exposed to drugs) (Day, 2016; Payne *et al*., 2007).

The two most important forms of drug resistance are MDR-TB and extensively drug-resistant TB (XDR-TB). MDR-TB is defined as resistance to at least RIF and INH, the two most potent anti-TB drugs (Valway *et al*., 1994). The XDR-TB is MDR-TB case that is additionally resistant to at least one injectable drug (AMK, KAN or CAP) and one

fluoroquinolone (Migliori *et al.*, 2008; Velayati *et al*., 2009). The XDR-TB is treated with a designed regimen based on drug susceptibility testing (DST) results that includes bedaquiline (Chan *et al*., 2013; Diacon *et al.*, 2014).

2.9.1 Diagnosis of drug resistance TB (DR-TB)

The current gold standard for diagnosing drug resistance-TB is Phenotypic drug susceptibility testing (DST) by the "proportion method" which is based on determining the proportion of mycobacterial growth on media containing the relevant drug at a critical concentration (Gurung *et al*., 2010). However this method is becoming obsolete due to the long incubation time, even weeks.

In addition to the proportion method, micro-plate alamar blue assay, which monitors the reducing environment of the living cells of the testing pathogen, is increasingly being used due to its rapidity and low cost. The method uses the active water-soluble, stable in culture medium, non-toxic ingredient resazurin (IUPAC name: 7-hydroxy-10-oxidophenoxazin-10-ium-3-one) (Page *et al*., 1993) as an indicator for growth. Resazurin, blue non-fluorescent dye is reduced to the pink-colored, highly fluorescent resorufin in the presence of reducing agents. The dye accepts electrons from reducing agents NADPH, FADH, FMNH, NADH, cytochromes and many more in the electron transport chain and changes from the oxidized, non-fluorescent, blue state to the reduced, fluorescent, pink state (Page *et al*., 1993). This change from oxidized to reduced state allows for detection by quantitative colorimetric and/or fluorometric measurements or detected qualitatively as a visible change in color indicating presence or absence of viable cells (Page *et al*., 1993).

A fully automated Mycobacteria Growth Indicator Tube (MGIT) is now an alternative to proportion method. It has the added advantage of detecting mycobacterial growth within 7-14 days and drug resistance reducing the delay for reporting results (Abe *et al*., 2001; Ardito *et al.*, 2001).

An alternative to phenotypic DST is the detection of chromosomal mutations using PCR-based methods (Ramaswamy, 1998; Sandgren *et al*., 2009). These techniques have the added advantage of being fast and sensitive enough and work directly on sputum positive samples, circumventing the need for growing of the bacilli and are designed based on earlier DNA sequencing analyses and target specific genes where mutations of interest occur. Of these, the best known are Xpert MTB/RIF and GenoType MTBDRplus for first line drugs and GenoType MTBDRs*l* for second line drugs (Hain Lifescience, Germany). Xpert MTB/RIF detects mutation in the *rpoB* gene regions. On the other hand, GenoType MTBDR*plus* in addition to detecting resistance to RIF by targeting the *rpoB* mutant genes and absence of *rpoB* wildtype genes. It also detects resistance to INH by targeting mutant genes of *inhA* and or *katG* gene together with the absence of their wildtypes. GenoType MTBDRs*l* identifies mutations in the gyrA gene (coding for DNA gyrase) for fluoroquinolones resistance, mutations in 16S rRNA gene (rrs) for detection of resistance to aminoglycosides/cyclic peptides and mutations in the embB gene for resistance to ethambutol, (which, together with the genes embA and embC, codes for arabinosyl transferase).

2.10 Genetic diversity within *Mycobacterium tuberculosis* complex

The MTBC are genetically monomorphic organisms; they exhibit low DNA sequence variability compared to other bacteria such as *Vibrio cholerae*, and *Salmonella typhi* and there is no evidence of horizontal gene transfer (Brosch *et al*., 2000; Supply *et al.,* 2003; Ozcaglar *et al.*, 2011). Diversity within the MTBC is driven mainly by large sequence deletions also referred to as 'region of difference' (RD), single nucleotide polymorphisms (SNPs) and repetitive elements and insertion sequences (Huard *et al.,* 2006).

2.10.0 Large Sequence Polymorphism (RD)

Large sequence deletions usually lead to loss of genes, some of which encodes for virulence factors or immunogenic molecules. For example RD1 contains genes that belong to the ESAT6 gene cluster encoding a type 7-secretion system, a potent stimulator of the immune system (Abdullah *et al*., 2007; Tekaia *et al*., 1999; Elhay *et al.,* 1998; Rosenkrands *et al.,* 1998; Horwitz *et al*., 1995). This 10-kb region is absent from all BCG strains tested so far, but present in virulent *M. bovis,* MTB and Maf and the loss of RD1 is thought to account for attenuation of BCG.

2.10.1 Single Nucleotide Polymorphisms (SNPs)

Research into genetic polymorphisms at the nucleotide level has revealed certain genetic markers that help in differentiating clinical isolates. The SNPs can be categorized into two groups namely: nonsynonymous SNP (nsSNP) and synonymous SNP (sSNP) (Homolka *et al*., 2012). Nonsynonymous SNP genes conferring drug resistance can aid in understanding the spread and the nature of the drug resistant isolates within the populations. In contrast, sSNPs are considered functionally neutral as these do not alter

the amino acid profile and hence the protein structure (Mathema *et al*., 2006). These neutral changes, when in structural or housekeeping genes, can provide the clue to study genetic drift and evolutionary relationships among mycobacterial strains (Mathema *et al*., 2006). The sSNP also help determine MTBC epidemiology in a given population (Mathema *et al*., 2006).

2.11.1 Genotyping Techniques for Identification of MTBC

Several tools have been developed for characterization of the MTBC strains and to study genetic diversity among members of MTBC. Depending on the discriminatory and stability, different markers have been used for species identification, phylogenetic studies, and in support of epidemiological studies to analyze transmission dynamics, risk factors for infection and disease and even delineate relapse from new infections. Genomic markers for genotyping include: single nucleotide polymorphisms (SNPs), direct repeats (DR) regions, insertion sequences (IS*6110*) and methods employed include restriction fragment length polymorphism (IS*6110*-RLFP), spoligotyping, and mycobacterial Interspersed Repetitive Units-Variable Number of Tandem Repeats (MIRU-VNTR). As the cost of high throughput DNA sequencing is decreasing, whole-genome sequencing is gradually replacing these conventional genotyping tools.

2.11.2. Insertion and repetitive sequences

2.11.2.1 IS*6110*-RFLP

The IS*6110*, first described by Thierry *et al*., (1990) is a mycobacterial insertion element that has been consistently used as a genetic marker for typing of MTBC species. The 1.4kb size IS element belongs to the IS3 insertion sequence family that contain three

independent sequences and differ only in a few base pairs. The IS3 family was originally discovered in *Escherichia coli* and *Shigella* species (Hermans *et al*., 1990; Thierry *et al*., 1990). The basis for genotyping is due to differences in copy number and locations within the genome of distinct MTBC strains. The technique involves the digestion of genomic DNA with *Nru* I, *EcoN* I, and *Pst* I restriction enzymes that cleaves the IS*6110* sequence at different sites, creating several DNA fragments that are separated through gel electrophoresis. The main advantages of the IS*6110*-RFLP method are its high discriminatory power. The main limitation of this method is its requirement of 2–3 μg of high quality DNA from prior culture of isolates, and has low discriminatory power in isolates presenting five or fewer IS*6110* copies. **Figure.2.11.2.1** shows band patterns for *Eco*N I. *Nru* I and *Pst* I endonuclease digestion of H37Rv (virulent *M. tuberculosis* isolate H37 with rough morphology) and H37Ra (attenuated *M. tuberculosis* isolate H37 with rough morphology) genomic DNA.

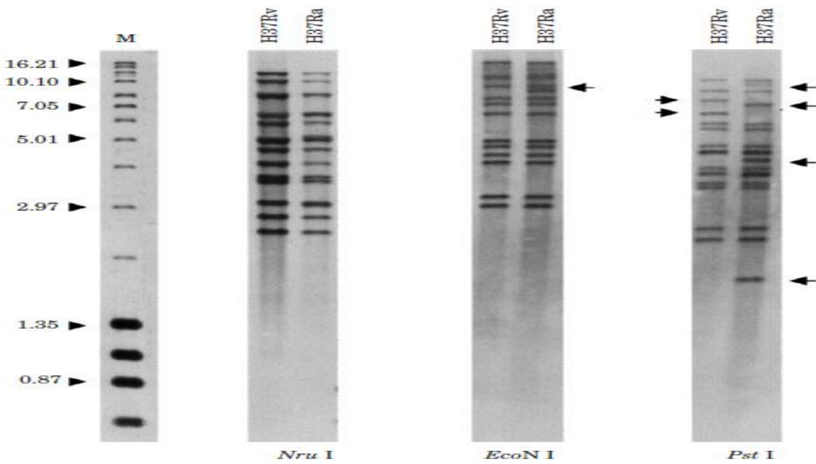


Figure 2.11.2.1: Restriction endonuclease activity of EcoN I, Nru I and Pst I on genomic DNA in H37Rv and H37Ra and IS6110 fingerprints generated (Lari *et al*., 1999).

2.11.3 Mycobacterial interspersed repetitive units-variable number tandem repeat

Mycobacterial interspersed repetitive units-variable number tandem repeat (MIRU-VNTR) typing is gradually becoming the most reliable and efficient genotyping system for TB transmission studies and is the new gold standard replacing *IS6110*-RFLP (Mazars *et al.*, 2001; Supply *et al.*, 2000). This is a PCR-based technique that detects polymorphisms based on the number of tandem repeats of distinct repetitive elements dispersed in intergenic regions of the MTBC genomes. Currently, over 40 different MIRU/VNTR loci have been identified with at least 24 of them considered as polymorphic. Protocols used for this assay is based on PCR amplification of different proposed formats: 8, 12, 15 or 24 loci using primers positioned in flanking DNA sequences.

The MIRU-VNTR is labour intensive due to a high number of individual PCRs required (Comas, 2009). To overcome this, several minimal sets of loci have been designed to provide maximum discriminatory power and to minimize genotyping costs for geographically restricted MTBC lineages (Murase *et al.*, 2008; Shamputa *et al.*, 2010, Dong *et al.*, 2012). **Figure.2.11.4** shows some MIRU-VNTR loci A, B, C and D utilized to genotype MTBC strain.

2.11.4 Spoligotyping

Spoligotyping which stands for “spacer oligonucleotide typing” is gradually gaining grounds after the widely used IS*6110*-based approaches for studying the molecular epidemiology and phylogeography of MTBC (Brosch *et al*., 2002; Huard *et al*., 2003). The spoligotyping assay detects the presence or absence of specific DNA spacer sequences within the direct repeat (DR) locus also known as the Clustered Regularly

Interspaced Short Palindromic Repeats (CRISPRs) genomic region. This is composed of identical 36 base pair repeats interspersed by 94 unique “spacers” of 35-41 base pairs in length (Beggs *et al*., 1996; Warren *et al*., 2002). The standard assay employs the presence or absence of selected 43 spacers (Brudey *et al*., 2004; Van Embden *et al.*, 2000). The assay involves immobilization of amplified illuminescent PCR products on nitrocellulose membrane, followed by detection using sensitive x-ray films which captures blue light produced as a result of chemiluminescence reaction to indicate oligonucleotide spacers amplified.

Identified patterns are then converted into a numerical code for easier analysis, comparison; and data exchange using web-based international databases such as SpolDB4.0, SITVITWEB and MIRU-VNTRplus (Allix-Béguec *et al*., 2008). **Figure.2.11.4** shows the direct repeat locus and how it can be explored to determine MTBC strains. One advantage of the this platforms is that theoretically spoligotyping can be performed directly on clinical sample (Augustynowicz-Kopec *et al*., 2007).

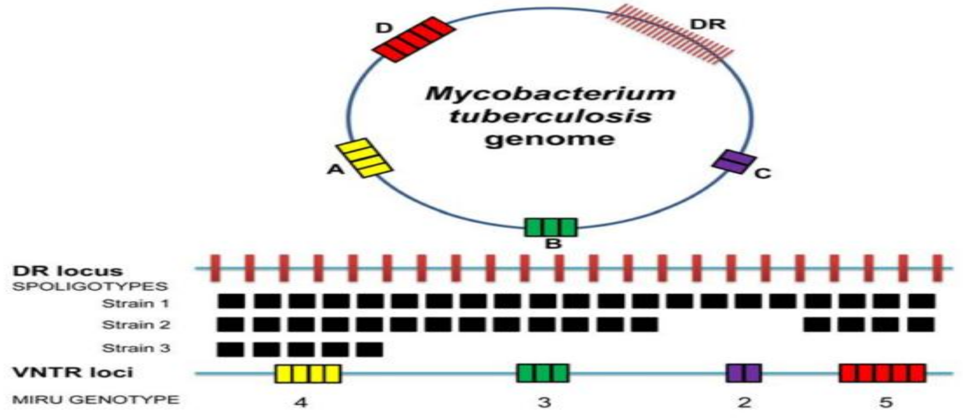


Figure 2.11.4: Principle of CRISPR and VNTR-based genotyping in MTBC (doi:10.1371/journal.pone.0007815.g001).

2.12 Consequences of genetic diversity within MTBC

Host, environmental and pathogen factors have been identified as major determinants driving infection course. Due to the monomorphic genome of the MTBC, previous thought was that the diversity in outcome of TB infection is due to environmental and host factors. However, several recent studies have demonstrated that the genetic diversity of the pathogens of the MTBC is higher than previously anticipated that may have functional implication (Gagneux and Small, 2007; Nicol *et al.*, 2008; Kato-Maeda *et al.*, 2001; Coscolla and Gagneux 2010**)**.

Unlike other pathogenic bacteria like *Streptococcus pneumoniae* that exhibit virulence by possessing capsule and *V. cholera* that produces toxins, MTBCs do not show a clear cut virulence factor (Faruque *et al.*, 1998; Mitchell and Mitchell, 2010**)**. Several experimental studies have provided evidence that clinical strains of MTBC differ in virulence**.** Disease outcome however have been shown to depend strongly on immune response (Gagneux and Coscolla, 2014).

For instance, several animal models gave a clear indication on the difference in virulence between MTBC strains where some animal studies comparing Maf from Senegal to MTBss showed that *M. africanum* (Maf) was less virulent (Castets and Sarrat, 1969). Though transmit equally, have a longer latency period compared to MTBss (de Jong *et al.*, 2008). In another study, Aguilar *et al*. (2010) demonstrated that highly transmissible Lineage 2 of Beijing sub-lineages from South Africa were more virulent in mice than less transmissible strains.

The strain HN878 (also known as strain 210), a clinical strain was associated with disease outbreaks in Los Angeles and Houston in the USA (Tsenova *et al.*, 2005). Studies in

different infection models found the strain HN878 which belongs to Lineage 2 together with the Beijing strain to have constantly been associated with delayed inflammatory immune response and hyper virulence (Tsenova *et al*., 2005).

The "modern" Lineages 2–4 which are more globally wide-spread than other lineages, have been reported to show a lower early inflammatory response compared to Lineages 1 and 6 (Portevin *et al*., 2011). Hence, the observation that "modern" strains are associated with a delayed inflammatory response (i.e. higher virulence) might be linked to the global success of these strains (Comas and Gagneux, 2011).

In 1995, strain CDC1551 caused an outbreak in a rural community near the Kentucky-Tennessee border of the USA, however, studies in human monocytes have shown that lineage 4 strain CDC1551 (or its lipid extracts) induced strong expression of proinflammatory cytokines, including TNFα, interleukin 1β, interleukin 12, and interferon-γ, all of which are characteristic of a protective immune response (Manca *et al*., 1999, 2004). By contrast, in another ex-vivo study using the same cell lines, the strain HN878 of lineage 2 was found to be associated with reduced expression of proinflammatory cytokines and increased production of macrophage deactivating cytokines such as interleukin 11 and interleukin 13 (Manca *et al*., 1999, 2004).

On the other hand, Maf have been reported to be less virulent than MTBss, and tend to affect more HIV co-infected patients, and was more likely to respond to ESAT 6 (de Jong *et al.*, 2005; de Jong *et al.*, 2006).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Disposables

Disposable laboratory coats (Medline, France), powder free nitrile examination gloves (Polyco-Bodyguard, UK), powdered latex examination gloves (Digamed, South Africa), Sterile disposable inoculating loops (Thermo Scientific, USA), medical grade cotton wool (Robinson Healthcare, USA), particulate respirator (3M, UK), 1.5 mL sterile microfuge tubes (Sarstedt, Germany), autoclave bags (Greiner Bio-One, Germany), 15 mL sterile falcon tubes (Greiner Bio-One, Germany), flat-bottom flask (Pyrex, USA), Bench guard (Sterilin Ltd., England), sterile single wrapped disposable pasteur pipette (Alpha Laboratories, UK), Plate cushions for miniblotter (Isogen Life Science, Maarssen, The Netherlands), Hyperfilm (X-ray films) Enhanced Chemiluminiscence (18 x 24 cm), (Amersham Biosciences, UK), Exposure cassette (24 cm X 30 cm) (SIGMA, USA).

3.1.2 Reagents

Glycerol (Timstar Laboritories, UK), Middlebrook OADC (Becton, Dickinson and Company, USA), Tween 80 (Sigma, USA), Primers for PCR (primer sequences) (Macrogen), Hot-start Taq polymerase (Qiagen), 20 x SSPE, (Gibco BRL Life Technologies Inc.), SDS (BDH Laboratory Supplies), Middlebrook 7H9 broth (Becton, Dickinson and Company, USA), Streptavidin-POD-conjugate (Boehringer), ECL detection liquid (Amersham International), Amplification mixes A and B (Hain Life Sciences GMBH, Germany).

3.1.3 Equipment

Olympus CH30 Light Microscope (Olympus Optical Co.LTD, Japan), Vortex mixer (Scientific Industries, USA),Digital coagulator (Hirasawa works, Japan),Twincubator (Hain Lifesciences GMBH, Germany), Heating block (Heat Labor Consult, Germany), Pipettes, Miniblotter MN45 (Isogen Life Science, Maarssen, The Netherlands), Heratherm Incubator (Thermo Scientific, USA), Chemical balance (A&D Instruments, Japan), Thermal cycler (Applied Biosystems 2720, USA), Autoclave (Tomy Seiko Co. Ltd., Japan), Class 2 Safety cabinet (Air Tech, UK) , Hybrigen oven (Bibby Scientific Ltd, UK).

3.2 Methods

3.2.1 Study Participants and Design

This was a cross-sectional study design in which participants were recruited consecutively. Sputum specimen were collected over a one year period from all consented newly diagnosed smear-positive pulmonary TB patients either before initiation of treatment or have been less than 4 weeks on treatment. Ethical clearance for this study was sought from the Institutional Review Board of Noguchi Memorial Institute for Medical Research (NMIMR) (Appendix 1-8). The procedure for sputum sample collection for routine diagnosis of TB in Ghana was followed. In accordance, patients were made to produce two sputum samples, one early morning and on the spot samples. The objectives of the study, inconveniences and procedures were explained carefully to all study participants before inclusion into the study.

The consent form has been provided in the Appendix 1-7. Demographic, epidemiological, and clinical data were obtained from all study participants' (Appendix 1-3). All collected samples were stored at 4°C and transported within 4 days to NMIMR for laboratory analysis.

3.2.2 Study Area

The participants were recruited from twelve (12) public health facilities in the Southern half of the Volta Region (VR) of Ghana as indicated in **Figure 3.2.2.** These health facilities together attend to more than 90% of all TB cases in the study area and also had a good cooperation with the health workers for case recruitment. The twelve health facilities cover 9 of the 25 administrative districts of the Volta Region of Ghana. According to the 2010 national census, the projected population for the region was 1,901,179 with the largest populated district being Ho municipality and Ho West with a population of 214,612 followed by Hohoe municipal with a population of 181,297. The main ethnic group in the southern Volta is the *Ewe* (68.5%). The other relatively large ethnic groups are the Guan (9.2%), the *Akan* (8.5%) and the *Gurma* (6.5%). The Mole-Dagbon, Grusi, *Mande*, Ga-Dangme and the other smaller ethnic groups constitute 7.3 per cent (Ghana Statistical Service, 2014).

Vegetation in the region is divided into; Savannah grassland along the coast, Semi-deciduous forest in the Middle zone and Semi-savannah woodland in the Northern zone.

Though majority of the indigence engage in crop and fish farming along the Volta Lake, a few compete for positions in government agencies which includes 242 health facilities within the various districts.

Scenic attractions in the region include highest mountain in Ghana, Mount Afadzato located in the Afadzato-South district, the Wli waterfalls close to Mountain Gemi (Amedzofe) and the Tafi monkey sanctuary in the Afadzato-South district.

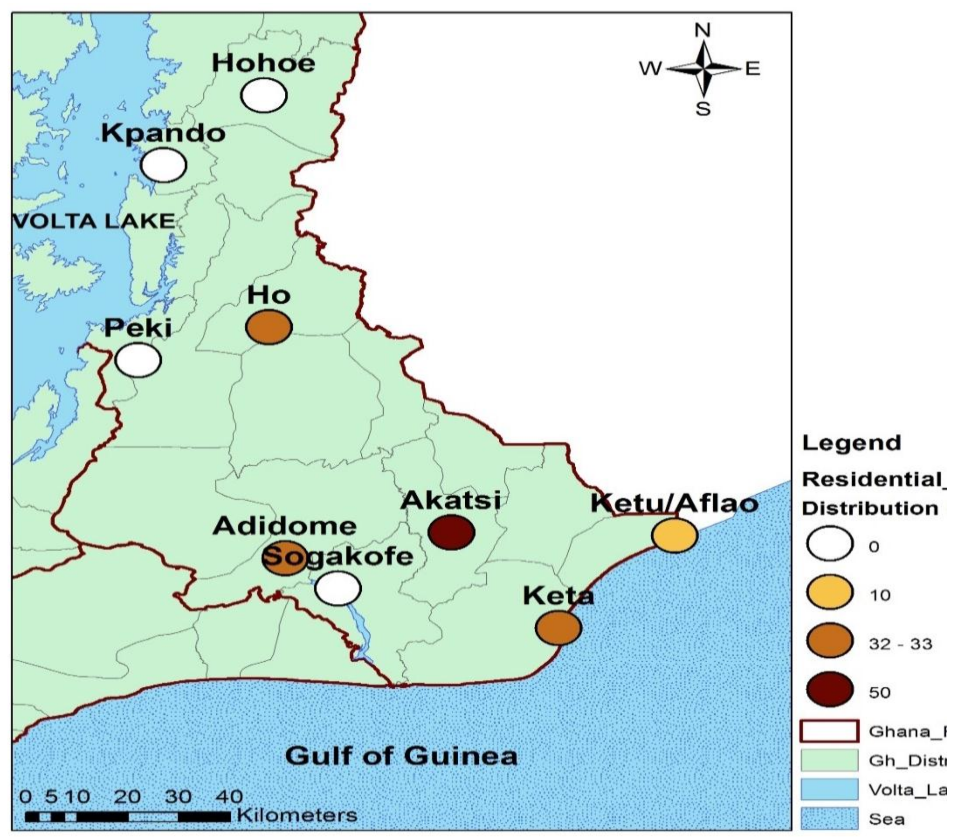


Figure.3.2.2: Districts/Municipalities where the 12 Health facilities are located

3.3. Laboratory Analysis

3.3.1 Media preparation

3.3.1.2 Lowenstein-Jensen media

Lowenstein-Jensen (LJ) media was prepared for primary isolation of mycobacterial species as well as for sub-culturing to obtain pure isolates for downstream analysis. All glassware used for media preparation were washed and sterilized at 160^0C in a hot air oven for 1 hour. Mineral salts (potassium phosphate, magnesium sulphate, magnesium

citrate, L-asparagine, glycerol/sodium pyruvate (Appendix 1-2) for the preparation of L-J media were weighed on a chemical balance and added to 100 mL of distilled water in a flat-bottomed flask before autoclaving at 121^{0}C for 15 minutes. Two types of mineral salts were prepared: one which contained glycerol without pyruvate to facilitate the growth of *Mycobacterium tuberculosis* sensu stricto (MTBss) and the other supplemented with pyruvate without glycerol to promote the growth of *M. bovis* and *M africanum* (Maf). Each salt suspension was cooled to room temperature and egg homogenate volume of 167 mL added to it. Two percent (2%) filtered malachite green solution was then added to the resulting media to prevent the growth of fast growing bacteria. About 5 mL of the liquid medium was aliquoted into sterilized glass tubes and slanted in a coagulator for solidification at 85°C for 55 mins. The freshly prepared LJ slants were incubated overnight for sterility check, the sterile tubes were then stored at 4°C in sealed bags until use.

3.3.1.3 Middlebrook 7H9 Storage Media

Approximately 4.7g of Middlebrook 7H9 powder was weighed and dissolved in 900 mL distilled water containing 2 mL of glycerol. The resulting media solution was autoclaved at 121°C for 10 minutes, cooled to 45°C and 100 mL of Middlebrook albumin dextrose casitone (ADC) enrichment added to the media and mixed thoroughly. The prepared media was used for storing viable isolates for future use at -80°C.

3.4 Primary Isolation.

All processes involving decontamination and inoculation were done in a class II bio-safety cabinet at a Biosafety level 3 laboratory. To remove unwanted fast growing

bacteria, the sputum sample was digested and decontaminated by adding equal volume of autoclaved 5% oxalic acid to about 2 mL sputum sample in 50 mL falcon tubes, mixed by vortexing and incubated at room temperature for 30 minutes. The reaction was stopped by adding phosphate buffered saline (PBS) to the 45 mL mark and the homogenate concentrated by centrifuging for 30 minutes at 3800 rpm. The tubes were left standing for 5 minutes to allow the created aerosol to settle before opening. The supernatant was carefully decanted and the sediment reconstituted in 1 mL of sterilized distilled water, vortexed and left standing for a few minutes to prevent aerosols circulation. A volume of 100 µL of the suspension was inoculated onto 4 Lowenstein- Jensen slants: 2 containing pyruvate and 2 containing glycerol. The suspension was spread evenly to cover the entire surface of the media, slanted on a wooden tray and incubated aerobically in a 37^0C incubator. Using the Pasteur pipette, 3 drops of suspension was placed on a labelled slide for Ziehl-Neelsen (ZN) staining for confirmation of acid-fast bacilli by microscopy. The cultures were observed daily for the first week and then weekly for macroscopic growth. The colony morphology (colour, appearance) and time of viable growth was recorded for each tube. A tube was indicated as positive if the growth on it was stained as acid fast bacilli with ZN technique, contaminated if more than half of the tube is overgrown with non-acid fast bacilli and no growth if after 12 weeks of culture, there were no visible colonies.

3.4.1 Direct Smear Examination by Ziehl-Neelsen Staining

The procedure as detailed by International Union against Tuberculosis and Lung Disease (IUATLD), 1978 was followed. The smeared slides were allowed to dry in the class II biosafety cabinet to prevent risk of infection. The air-dried slides were heat fixed by

passing the slides over a flame, 3 times. The individual slides were arranged on a staining rack, leaving enough space between slides to prevent cross contamination. The slides were then flooded with filtered carbol-fuchsin (Appendix 1-0). To make holes to allow the stain to pass through the waxy impermeable mycobacterial cell wall, a flame was gently passed underneath the slides until steam came from the stain. After 5 minutes, the slides were gently rinsed with tap water to remove residual dye. The slides were decolorized with a 20% H_2SO_4 (see Appendix1-0) solution for 5 minutes and gently rinsed with tap water and counterstained with 0.1% methylene blue (see Appendix1-0) solution. The methylene blue was left for 1 minute, washed with water and the slides left to dry and examined under oil immersion using 100X objective plus 10X eye-piece lens. Acid-fast organisms appeared bright reddish-pink in colour for easy identification against the other cells, which stained blue. The WHO and IUATLD guidelines (see Appendix 1-1) was followed for grading the slides as either negative, scanty, 1+, 2+, or 3+.

3.4.2 Sub-culturing

In order to obtain pure isolates of cultures, the primary cultures that had gotten to log phase of growth at optimum temperature condition of 37°C in the incubator were sub-cultured. This was achieved by taking a colony of primary culture and inoculating on fresh Lowenstein Jensen (LJ) media in a biosafety level 3 (BSL3) safety cabinet II under aseptic conditions. Two LJ tube slant media supplemented with glycerol with the other two supplemented with pyruvate. All tubes were inoculated and incubated at 37°C. Monitoring for growth of distinct mycobacterial colonies was documented daily and weekly.

3.5 Mycobacterial species identification

3.5.1 Acid Fast Staining

A loopful of pure colonies was placed on a labelled microscope slide for smear preparation in the biosafety level 3 (BSL3) safety cabinet II and allowed to air dry on the slide. Ziehl-Neelsen Staining for microscopy was performed as described in section **3.4.1.** Only Isolates identified as acid fast bacilli (AFB) were used for downstream analysis.

3.6 Extraction of Mycobacterial DNA

A loop full of confirmed AFB pure cultures growing at the log phase was suspended in 1 mL of sterile distilled water. The mycobacterial cells were then inactivated by heating at 95^0C for 1 hour which facilitated the disruption of mycobacterial cell wall to release DNA into suspension. The resulting suspension was stored at -20^oC and used for all downstream DNA-based assays.

3.6.1 Genomic analysis

Four rooms were used for all Polymerase Chain Reaction (PCR) assays to prevent contamination. The master mix and the addition of template were all done in a sterilized hood. The function of each room is as follows.

- First room/Clean Room: Preparation of PCR Master mix
- Second room: Addition of DNA template to each reaction mix
- Third room: Amplification of DNA by PCR
- Fourth room: Electrophoresis and Visualization of amplified Products.

3.6.2 IS*6110* insertion sequence Polymerase Chain Reaction

The presence of the insertion sequence *6110*, which is specific for the *M. tuberculosis* complex (MTBC), was determined to confirm an isolate as an MTBC. The IS*6110* region was amplified using the primers (TB284: 5′-GGACAACGCCGAATTGCG-3′) and (TB850 5′-TAGGCGTCGGTGACAAAGGCCAC-3’) to bind the flanking regions.

An edited protocol described by Yeboah-Manu *et al*., (2001) was followed for this assay. The PCR mix contained the following (16.2 µL of H_2O, 5 µL PCR buffer (10X), 10 µL Q Solution, 2.5 µL $MgCl_2$ (25 mM), 1 µL dNTP (10 mM), 2.5 µL primer F (10 pmol/µL), 2.5 µL primer R (10 pmol/µL), 0.3 µL Hot-Start Taq DNA polymerase (5 U/µL), and 5 µL Coral dye (10x) and 5 µL of extracted mycobacterial DNA. Negative controls H_2O and Positive control; *M. tuberculosis* reference strain (H37Rv) were included in each batch to identify possible amplicon contamination and to determine the reproducibility of the assay respectively. Amplification reaction was carried out in a conventional PCR thermal cycler using the conditions shown in **Table 3.6.2.** The amplicons were then resolved on 2% agarose gel at 125 V for 20 minutes followed by gel examination under a UV trans illuminator in the fourth room and samples with amplicons of the expected band size of 550 bp were considered positive.

Table 3.6.2: Cycling conditions for IS*6110* PCR

<table>
<tr><th>Temperature (^{o}C)</th><th>Time (minutes)</th><th>Activity/Step</th><th>Number of cycles</th></tr>
<tr><td>96</td><td>5</td><td>Initial Denaturation</td><td>1</td></tr>
<tr><td>95</td><td>1</td><td>Subsequent Denaturation</td><td rowspan="3">30</td></tr>
<tr><td>65</td><td>1</td><td>Primer Annealing</td></tr>
<tr><td>72</td><td>1</td><td>Extension</td></tr>
<tr><td>72</td><td>10</td><td>Final Extension</td><td>1</td></tr>
</table>

3.6.3 Mycobacterial Specie Identification by *hsp65* Sequence

The AFB positive isolates that were not confirmed as MTBC by the IS*6110* PCR were identified by amplifying the *hsp65* region followed by sequencing using mycobacterial specific primers TB11: 5'-ACC AAC GAT GGT GTG TCC AT-3' and TB12: 5'-CTT GTC GAA CCG CAT ACC CT- 3'. The PCR mix contained 14.8 µL of nuclease free (H_2O), 3 µL of PCR buffer (10X), 3 µL of Q Solution 1.8 µL of $MgCl_2$ (25 mM), 0.6 µL of dNTP (10 mM), 1.8 µL of primer1 (TB11, 10 pmol/µL), 1.8 µL of primer 2 (TB12, 10 pmol/ µL), 0.20 µL of Hot-start Taq DNA polymerase (5 U/µL) and 3 µL of Coral dye (10x) was prepared, and 5 µL of the isolates' DNA was added to the PCR mix. Amplification was carried out using the parameters below (**Table 3.6.3**). The amplicons were resolved on a 2% gel and the expected band size was 439 bp. The positive amplicons were then outsourced for sequencing. The nucleotide sequences obtained for each amplicon was cleaned using the MEGA (open source version 7) software and Staden package to obtain chromatogram of nucleotides with sharp peaks that were utilized in blast search for identification of organism with highest identity score in **National Center for Biotechnology Information** (NCBI) website at https://blast.ncbi.nlm.nih.gov/Blast.cgi.

Table 3.6.3: Cycling conditions for *hsp65* PCR

Temperature (^oC)	Time (minutes)	Activity/Step	Number of cycles
95	10	Initial Denaturation	1
96	1	Subsequent Denaturation	35
60	1	Primer Annealing	
68	1	Extension	
72	10	Final Extension	1

3.6.4 Strain Typing by Spoligotyping

Only MTBC confirmed isolates were spoligotyped and the experiment was carried out according to the method described by Kamerbeek *et al*., (1997). The whole direct repeat region of mycobacterium genome was amplified with two primers DRa (5'-GGT TTT GGG TCT GAC GAC-3' (marked with biotin at the 5'-end), and DRb (5'-CCG AGA GGG GAC GGA AAC-3'. The PCR mix for each isolate was prepared by adding 34.5 µL of dH_2O, 5µL of 10x buffer, 4 µL each of Dra and DRb followed by addition of 0.4 µL dNTPs, 0.1 µL Hot-Start TaqPol, and then 5 µL DNA, however, the PCR mix alone was used as negative control while two positive controls, Bacillus Calmette–Guérin (BCG) and H37Rv DNA was included in each reaction batch. The PCR cycling conditions included initial denaturation for 3min at 96°C and subsequent denaturation at 96°C for the 35 cycles for 1minute, 1min annealing at 55°C, 1min extension at 72°C, whereas final extension was at 72°C for 5min.

The biotinylated amplicons were then hybridized to the immobilized spacer-oligonucleotides that represent the already characterized 43 spacers of known sequence was performed as indicated below. The membrane was washed for 5 minutes at 60°C in 2XSSPE supplemented with SDS (0.1%) to get rid of the EDTA and was placed in the miniblotter in such a way that the slots were perpendicular to the line pattern of the applied oligonucleotides screwed tightly and residual fluid removed by aspiration.

Twenty microliters (20µL) of the PCR products were diluted with 150 mL of 2X SSPE (supplemented with 0.1% SDS) and heat denatured for 10 minutes at 99^{0}C, then placed on ice immediately to cool. The slots of the miniblotter were then filled with the

denatured amplicons, after which hybridization for 60 minutes at 60^0C was performed. Non- hybridized amplicons and excess solutions were removed from the slots by aspiration. The membrane was then washed with 2X SSPE (supplemented with 0.5% SDS) for 10 min at 60^0C after which, the membrane was incubated in streptavidin-peroxidase conjugate (2.5 mL of streptavidin-peroxidase conjugate in 10 mL of 2X SSPE supplemented with 0.5% SDS) for 60 min at 42^0C.

Washing and rinsing of the membrane with 2X SSPE (supplemented with 0.5% SDS) and 2X SSPE respectively was done for 10 minutes at 42^0C and room temperature respectively. The hybridized DNA was detected by using enhanced chemiluminescence detection liquid for 1 minute and the membrane exposed for 5 minutes to X-ray film (Appendix 1-5).

3.6.5 Anti-TB drug susceptibility testing by GenoTypeMTBDRplus

Drug susceptibility patterns of the isolates to rifampicin and isoniazid were determined by Genotype MTBDRplus version 2.0, a line probe assay. The assay was carried out using the provided reagents according to the manufacturer's instructions (Hain Lifescience GmbH, Germany). The assay is made up of two steps: an amplification step which is a multiplex PCR, followed by a reverse hybridization.

The amplification process consists of two commercially prepared reagents, amplification mixture A (AM-A) and B (AM-B). The AM-A consists of buffer, nucleotides and Taq polymerase while AM-B contains salts, dye and a cocktail of primers. The amplification was carried out for each isolate by adding 10 µL of amplification mix A, 35 µL of amplification mix B and 5 µL DNA to give a final volume of 50 µL. H37Rv, a *M.*

tuberculosis reference strain, was used as the positive control. Sterile distilled water was used as the negative control in place of the templates. The PCR was carried out in a thermal cycler under the cycling conditions (Barnard *et al*., 2012) as shown in **Table 3.6.5.**

Table 3.6.5: The cycling condition for LPA amplification

Temperature	Time	Cycles
95^0C	15 mins	1 cycle
95^0C	30 sec	20 cycles
65^0C	2 min	
95^0C	25 sec	30 cycles
50^0C	40 sec	
70^0C	40 sec	
70^0C	8 min	1 cycle

The stringent solution (STR) (containing >25% of a quaternary ammonium compound, <1% anionic tenside and dye) and the hybridization solution (HYB) (containing 10% anionic tenside and dye) were pre-warmed to 45°C. The rest of the reagents, rinse solution (RIN) (containing buffer, <1% NaCl, <1% anionic tenside), conjugate buffer (CON-D) (containing buffer, 1% blocking reagent, <1% NaCl) and substrate buffer (SUB-D) (containing buffer, <1% $MgCl_2$, <1% NaCl) were also pre-warmed to room temperature with the exception of the conjugate concentrate (CON-C) (which contains streptavidin-conjugated alkaline phosphatase, dye) and Substrate Concentrate (SUB-C) (which contents <70% dimethyl sulfoxide, <10% 4-nitro blue tetrazolium chloride, <10% 5-bromo-4-chloro-3-indolyl phosphate). Working concentrations of conjugate and

substrate solutions were prepared by diluting CON-C and SUB-C with CON-D buffer and SUB-D buffer respectively in the ratio of 1:100 in a 15 mL falcon tube. The solutions were mixed well and kept protected from light by wrapping the 15mL falcon tube with aluminium foil.

The hybridization was carried out in a tray on a Twincubator. An aliquot of 20 µL of the PCR product was placed in each of the wells and mixed with 20 µL of denaturation solution (DEN) (containing <2% NaOH and dye) for 5 minutes at room temperature to separate the double-stranded DNA into single strands. Hybridization was achieved by the addition of 1mL of the pre-warmed HYB to each test well. The contents of the wells were then mixed well after which labelled DNA strips containing immobilized complementary single-stranded DNA wild type and mutant probes were placed in their respective wells using the tweezers. The tray in the Twincubator was then incubated for 30 minutes at 45^0C.

After the hybridization step, the HYB solution was completely poured off followed by the addition of 1mL pre-warmed STR solution to each well and incubated for 15 minutes at 45^0C to remove all non-specifically bound DNA. The STR solution was also completely poured off and each strip was rinsed with 1 mL of RIN solution for 1 minute, which was poured off afterwards.

To facilitate detection of amplified biotinylated sequences, 1 mL diluted conjugate solution was then added and incubated for 30 minutes to ensure streptavidin-alkaline phosphatase conjugate binds to the bound biotin labelled strands. The conjugate solution was then poured off after which 1 mL RIN solution was added to wash the strips twice

followed by 1 minute wash with 1 mL distilled water. 1 mL of the substrate solution was then added to each well for 5 minutes at room temperature for coloured bands to develop through enzyme-substrate reaction. The reaction was then stopped by rinsing twice with 1mL distilled water followed by the removal of the strips with the aid of the tweezers and drying on tissue paper. The band pattern was compared to the reference provided by the manufacturer as indicated in (Appendix 1-6). Drug resistance was read as the absence of wild-type band, and /or presence of mutation band.

3.7 Data Analysis

Information from the structured questionnaire was entered using Microsoft excel and validated to remove duplicates if any. Spoligotype patterns were entered in a binary format in Microsoft excel and compared with those available in the International online database MIRU-VNTRplus, useful in molecular typing of MTBCs based on a collection of 186 well-characterized reference strains (http://www.miru-vntrplus. org/). The specie, lineage and sub-lineage of an isolate was defined based on spoligotyping pattern identity to at least two stored isolates in the database. Identification of specific drug resistance using the line probe assay was based on mutations within the *katG* gene and *inhA* promoter gene for INH and *rpoB* gene for RIF respectively. Drug resistance was captured as either absence of specific wild-type band and/or presence of mutation band corresponding to specific mutations with reference to the hybridization band. Univariate and multivariable logistic regression models were fitted to assess the relationship between host adapted MTBCs and analyzed pathogen variables. All statistical analyses were performed in STATA SE. 12 (Stata Corp., College Station, TX, USA) and P-value <0.05 was considered significant given an assigned Odds ratio and confidence interval.

CHAPTER FOUR

4.0 RESULTS

4.1 Smear and Mycobacterial Culture Positivity of Collected Sputum Samples

A total of two hundred and seventy (270) sputum samples were collected and concentrated for smear microscopy to confirm the presence of acid-fast bacilli (AFB). Out of the total 270 cultured samples, 183 representing 67.78% were confirmed as AFB positives as shown in **Table 4.1** and 125 of the 183 smear confirmed positive samples (68.31%) showed macroscopic AFB growth after 8 weeks of incubation at 37°C. One hundred and twenty-five isolates were further analyzed and out of these, 117 were identified to be members of the *Mycobacterium tuberculosis* complex (MTBC) by detecting the insertion sequence IS*6110.* Confirmation of MTBC isolates was identified by gel electrophoresis with a 550 base pairs band displayed evident of the amplified insertion sequence IS*6110*. The reference strain, H37Rv was used as the positive control which is represented with 'P' and nuclease free water as negative control also represented by the letter 'N' (**Figure 4.1**). The positive isolates were then further characterized by spoligotyping and drug susceptibility pattern determined. The eight that were negative were confirmed to belong to the genus mycobacterium by the amplification of the mycobacterium genus specific heat shock protein 65 (*hsp65*) gene sequence (**Figure 4.7**).

Table 4.1: Mycobacteria Recovered from Sputum Samples

	Number	Positivity rate (%)
Samples collected	270	
AFB positivity confirmed	183	67.78
Culture positivity	125	68.31

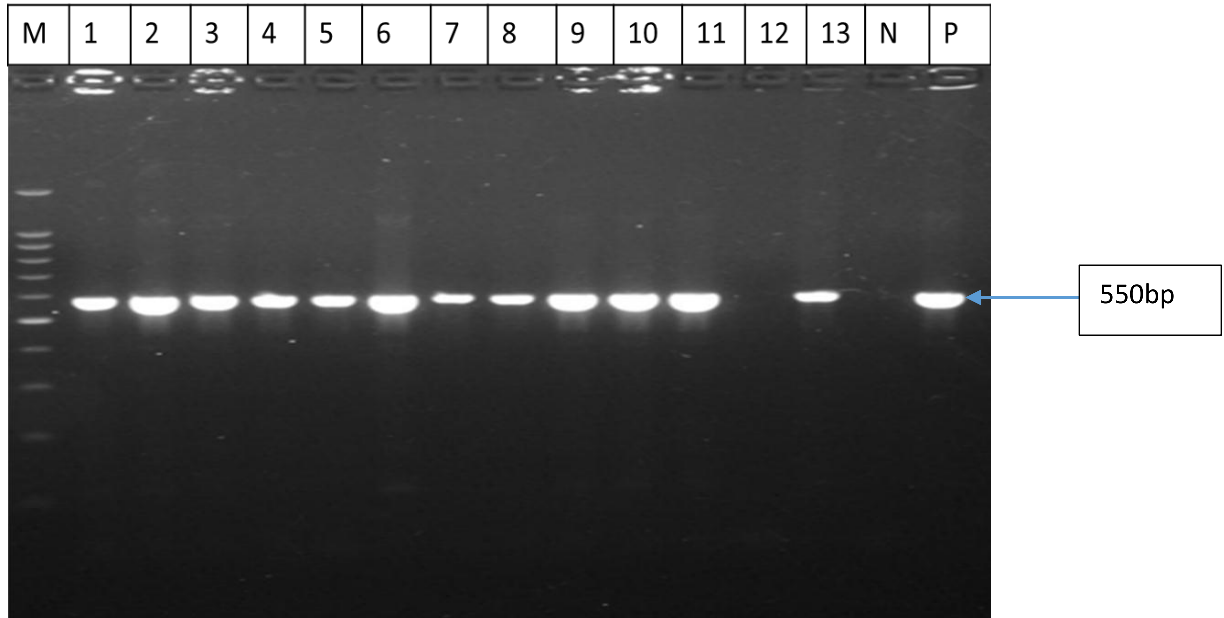


Figure 4.1: Gel electrophoresis for IS6110: Lanes 1-13 contain clinical Isolates with lanes 1-11 and 13 being positive for the insertion sequence, IS*6110* and lane 12 is IS*6110* negative. Lanes N and P are the negative and positive controls respectively

4.2 Study Population

The 125 mycobacteria isolates that were further characterized were obtained from pulmonary TB patients from 12 health facilities in the southern part of the Volta region. The study participants were recruited between January 2016 and January 2017. The highest number of isolates was obtained from the Ho municipality (N=44, 35.19%) followed by Ketu South municipality with 36 (30.77%). Participants from whom the 117 MTBC isolates were obtained included 82 (70.09%) males and 35 (29.91%) females with ages ranging from 12-86 years and an average age of 44.71 ± 14.75 years (**Table 4.2**). The participants live in 9 districts; 36 (30.77%) from Ketu South/Aflao, 6(5.13%) from Keta, 44 (37.61%) from Ho, 7 (5.98%) from Central Tongu (Adidome), 14 (11.97%) from Afadzato-South/Hohoe and 3 (2.56%) from South Tongu/Sogakofe and 1 (0.85%) from Kpando, 6 (5.13%) was from Akatsi with no isolate from Peki.

Christians were in the majority with 107 (91.45%) participants and other religions were Islam 6 (5.13%), African/Traditionalist 2 (1.71%) and 2 (1.71%) did not indicate their religious affiliation. Participants of the Ewe origin were 113 (96.58%), with 4 (3.42%) belonging to other ethnic groups. Ghanaians formed the majority; 115 (98.29%) of the participants were Ghanaians, the others being 2 (1.71%) Togolese. Majority of the participants were traders, that is, 44 (37.61%) and 35 (29.91%) were farmers. Also, the study included 3 (2.56%) teachers, 16 (13.68%) students and 6 (5.13%) commercial drivers. Most of the participants (N=113, 96.58%) were newly diagnosed TB cases and only 4 (3.42%) being treatment failures from previously treated cases. Data on TB/HIV co-infection shows that 5 (4.27%) were HIV positive, 93 (79.49%) were negative and 19

(16.24%) were not indicated. The demography of patients from whom NTMs were isolated is indicated in **Table 4.7.1**.

Table 4.2: Characteristics of participants involved in the study

Characteristics of MTBC Positive Participants		
Gender (117)	**Number**	**Percentage**
Male	82	70.09
Female	35	29.91
Occupation (117)		
Farmer	35	29.91
Pupil Teacher	2	1.71
Students	5	4.27
Traders	44	37.61
Artisans	9	8.15
Drivers	6	2.22
Hospital Orderlies	1	0.74
Unemployed	15	12.82
Religion (117)		
Christianity	107	91.45
Islam	6	5.13
Traditional	2	1.71
No Religion	2	1.71
Ethnicity (117)		
Ewes	113	96.58
Kotokoli	2	1.71
Guans	2	1.71
Nationality (117)		
Ghanaians	115	98.29
Togolese	2	1.71
HIV Status (117)		
Negative	93	79.49
Positive	5	4.27
Not Indicated	19	16.24
Age (117)		
Mean	44.71 ± 14.75YRS	
Range	12-86YRS	
Previously Treated (117)		
No	113	96.58
Yes	4	3.42

4.3 Prevalence of MTBC lineages and sub-lineages

The lineages and sub-lineages among the identified MTBC isolates were determined by spoligotyping and the patterns analyzed using the MIRU-VNTRplus database. Out of the one hundred and seventeen (117) spoligotyped isolates, 23 (19.66%) were *Mycobacterium africanum* (Maf) and 94 (80.34%) were *Mycobacterium tuberculosis* sensu stricto (MTBss). The different lineages and their corresponding spoligotyping patterns are indicated in **Table 4.3**.

In summary, the MTBss lineage 4 sub-lineages obtained were: 62 (52.99%) Cameroon, 13 (11.11%) Ghana, 8 (6.84%) Haarlem, 2 (1.71%) LAM, 1 (0.85%) X and 1 (0.85%) Uganda I. Other lineages of MTBss were identified: 1 (0.85%) EAI of lineage 1, 3 (2.56%) Beijing of lineage 2 and 1 (0.85%) Delhi/CAS of lineage 3. Among the Maf isolates, 16 (13.68%) were identified as West African 1 or lineage 5 and 7 (5.98%) were West African 2 or lineage 6 (**Table 4.3**). However, sub-lineages of 2 isolates were undefined.

Table 4.3: Prevalence of *Mycobacterium tuberculosis* complex lineages and sub-lineages

	Lineages (117, 100%)	Sub-Lineages	Number	Percentage
MTBss (94; 80.34 %)	**Lineage 1**	EAI	1	0.85%
	Lineage 2	Beijing	3	2.56%
	Lineage 3	Delhi/CAS	1	0.85%
	Lineage 4	Cameroon	62	52.99%
		Ghana	13	11.11%
		Haarlem	8	6.84%
		LAM	2	1.71%
		Uganda I	1	0.85%
		X	1	0.85%
		Unidentified	2	1.71%
Maf (23; 19.66 %)	**Lineage 5**	West Africa I	16	13.68%
	Lineage 6	West Africa II	7	5.98%

4.4 Spoligotyping Profile

In all, 39 distinct spoligotyping patterns were obtained; 26 and 13 different patterns were obtained for the MTBss and Maf lineages respectively. Ninety-six MTBC isolates were grouped into 18 spoligotype clusters of between 2 and 44 isolates and 21 singletons were obtained in this study. Fourteen (14) out of the 18 distinct clusters representing 82 (70.09%) isolates were MTBss and 4 clusters representing 14 (11.97%) isolates were Maf species. The odds of having an isolate to be in a spoligo cluster was relatively higher for MTBss than for Maf (OR= 4.39 CI= 1.56-12.35). Cameroon sub-lineage of MTBss strain produced the largest cluster with 44 isolates (37.61%) sharing a spoligotyping pattern corresponding to shared international type (SIT) number 61. In this study, Orphans were defined as singletons with no SIT number and we observed 10 (8.55%) were MTBss and 7 (5.98%) being Maf (**Table 4.4**).

Table 4.4: Spoligotype profile of *Mycobacterium tuberculosis* complex isolates analyzed

Species	Lineage	Spoligotyping profile	Sub-lineages	SIT	No.	%
MTBss	L1		EAI		1	0.85
MTBss	L2		Beijing	1	3	2.56
MTBss	L3		Delhi/CAS		1	0.85
MTBss	L4		Unidentified		1	0.85
MTBss	L4		X	200	1	0.85
MTBss	L4		Unidentified		1	0.85
MTBss	L4		Haarlem	1498	5	4.27
MTBss	L4		Ghana	472	2	1.71
MTBss	L4		Ghana	53	5	4.27
MTBss	L4		Ghana	504	2	1.71
MTBss	L4		Ghana		1	0.85
MTBss	L4		Ghana	1475	3	2.56
MTBss	L4		LAM	81	2	1.71
MTBss	L4		Cameroon		3	2.56
MTBss	L4		Cameroon		2	1.71
MTBss	L4		Cameroon		1	0.85
MTBss	L4		Cameroon		3	2.56
MTBss	L4		Cameroon		1	0.85
MTBss	L4		Cameroon	57	1	0.85
MTBss	L4		Cameroon	772	2	1.71
MTBss	L4		Cameroon		3	2.56
MTBss	L4		Cameroon	61	44	37.61
MTBss	L4		Cameroon		1	0.85
MTBss	L4		Cameroon		1	0.85
`MTBss	L4		Haarlem		3	2.56
MTBss	L5		Uganda1		1	0.85
Maf	L5		WA 1	331	3	2.56
Maf	L5		WA 1	319	3	2.56
Maf	L5		WA 1		1	0.85
Maf	L5		WA 1		1	0.85
Maf	L5		WA 1		3	2.56
Maf	L5		WA 1	438	5	4.27
Maf	L6		WA 2		1	0.85
Maf	L6		WA 2		1	0.85
Maf	L6		WA 2	181	1	0.85
Maf	L6		WA 2		1	0.85
Maf	L6		WA 2		1	0.85
MAF	L6		WA 2		1	0.85
MAF	L6		WA 2	523	1	0.85

4.5 Geospatial distribution of MTBC lineages and sub-lineages

The spoligotyping data from this study shows the circulation of different lineages in the southern part of Volta region. The Ho municipality recorded the highest number of 5 distinct lineages. As per the isolates identified, this municipality had the highest number of Maf, that is, 10/44 (22.73%) were lineage 5 and 3/44 (6.82%) lineage 6, and 29/44 (65.91%) constituted the dominant lineage 4. This municipality also had 1/44 (2.27%) isolate each belonging to lineage 1 and lineage 2 (**Table 4.5**).

The Ketu-South (Aflao) recorded the highest proportion of lineage 4 MTBC isolates thus, 32/36 (88.89%) with 1/36 (2.78%) being lineage 2, 2/36 (5.56%) lineage 5 and 1/36 (2.78%) lineage 6. The Ketu-South had 4 different lineages comprising of both MTBss and Maf. The Keta district also had 4 different lineages with 1/6 (16.67%) being lineage 3, 3/6 (50.00%) lineage 4, 1/6 (16.67%) each of the isolates lineage 5 and lineage 6 respectively. The Keta district recorded the only lineage 3 isolate in this study.

Akatsi district also had 4 different lineages with 1/6 (16.67%) each belonging to lineages 2, and 6, 2/6 (33.33%) each of lineages 4 and 5 isolates as well. However, the Adidome district had 3 different lineages, 5/7 (71.43%) lineage 4, 1/7 (14.29%) each of lineage 5 and lineage 6. Hohoe, Sogakofe and Kpando recorded only lineage 4 isolates in this study with the frequencies of occurrence; 14, 3 and 1 respectively.

The geospatial distribution of the two species as shown in **Figure 4.5**, the study showed Kpando and Hohoe which are very close to each other, had only MTBss. These two districts are located in the upper part of the study area. Ho, Adidome, Akatsi, Ketu-South and Keta had both MTBss and Maf distributed among the TB patients.

Table 4.5: Distribution of the different lineages in the districts/municipalities

District/Municipality	Lineages	Number	Maf Percentage (%)
Ho (44; 37.61 %)	Lineage 1	1	29.5
	Lineage 2	1	
	Lineage 4	29	
	Lineage 5	10	
	Lineage 6	3	
Ketu/Aflao (36; 30.77%)	Lineage 2	1	8
	Lineage 4	32	
	Lineage 5	2	
	Lineage 6	1	
Keta (6; 5.13%)	Lineage 3	1	33
	Lineage 4	3	
	Lineage 5	1	
	Lineage 6	1	
Akatsi (6; 5.13%)	Lineage 2	1	50
	Lineage 4	2	
	Lineage 5	2	
	Lineage 6	1	
Adidome (7; 5.98%)	Lineage 4	5	28.5
	Lineage 5	1	
	Lineage 6	1	
Hohoe (14; 11.97%)	Lineage 4	14	0
Sogakofe (3; 2.56%)	Lineage 4	3	0
Kpando (1; 0.85%)	Lineage 4	1	0

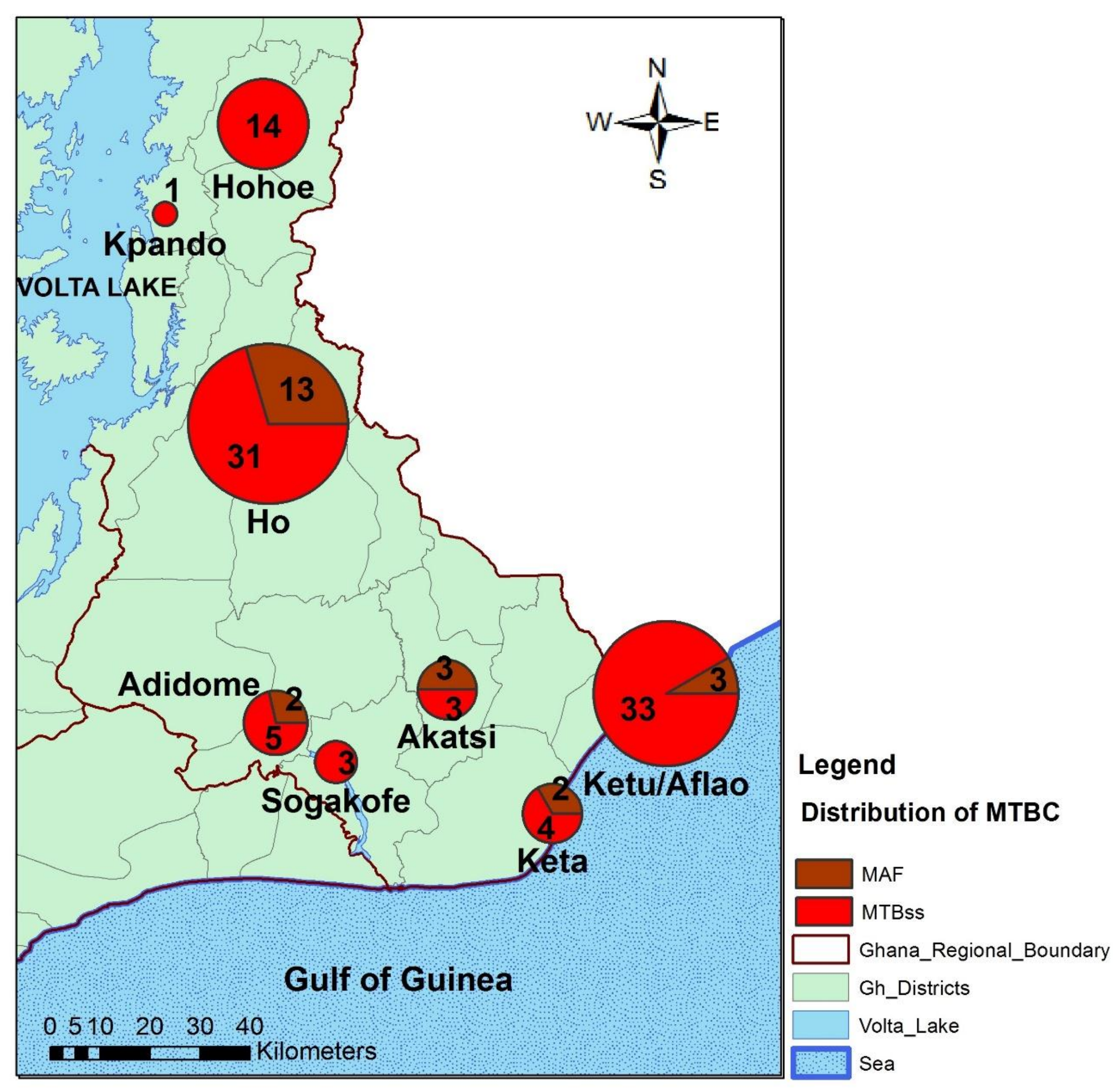


Figure 4.5: The spatial distribution and prevalence of the two human adapted MTBC species

4.6 Molecular Epidemiology

In total, one hundred and seventeen (117) MTBC isolates from TB-patients were analyzed in relation with patient demographics as shown in **Table 4.6.**

All 23 Maf isolates obtained were from Ghanaians while in the case of MTBss isolates, 97.87% (92) were from Ghanaians and the other 2.13% (2) were from Togolese. Out of the 23 Maf isolates identified, the mean age of participants was 44.06 years (range: 26-83 years) with 82.61% (19) being males and 17.39% (4) females.

Similarly, of the 94 MTBss identified, the participants had a mean age of 44.42 years (range: 12– 86 years) with 67.02% (63) being males and 32.98% (31) females.

In both the MTBss and Maf populations, the proportion of males was found to be significantly higher than that of females (**Table 4.6,** $p<0.001$).

Table 4.6: Demography of TB patients' and MTBC isolates identified

ETHNICITY(117)	Number	Percent	P-value
Ewe	114	97.44	< 0.001
Guan	1	0.85	
Kotokoli	2	1.71	
MTBss Ethnicity(94)			
Ewe	91	96.81	< 0.001
Guan	1	1.06	
Kotokoli	2	2.13	
MTBss Nationality(94)			
Ghanaian	92	97.87	< 0.001
Togolese	2	2.13	
Maf (23)			
Male	19	82.61	< 0.001
Female	4	17.39	
MTBss (94)			
Male	63	67.02	< 0.001
Female	31	32.98	
Maf age			
Mean age	44.06 years		
Range	26 - 83 years		
MTBss age			
Mean age	44.42 years		
Range	12- 86 years		

4.7 Identification of mycobacteria other than *mycobacterium tuberculosis* complex

Eight AFB positive but IS*6110* negative isolates, hence suggested to be non-tuberculous mycobacteria (NTM) were further identified by sequencing the mycobacterial specific heat shock protein 65 (*hsp65*) gene sequence, after amplification. These eight isolates after PCR produced a 439 base pairs amplicon (**Figure 4.7**) upon resolution on a 2% agarose gel. Nucleotide chromatogram sharp peaks of sequenced amplicons were then generated in Staden package for cleaned consensus sequence of amplicons (**Figure 4.7.1**).

Upon blast search of the generated consensus sequence in the NCBI database, seven isolates were identified to be *Mycobacterium abscessus* strains with only one being *M. fortuitum* (**Figure 4.7.3**). The queried sequence of one isolate produced highest identity score of 578/601 (96%) with *Mycobacterium abscessus* in the NCBI database as shown in **Figure 4.7.2.**

4.7.1 Demography of Patients with non-tuberculous mycobacteria

All seven individuals that had the *M. abscessus* and the only individual with *M. fortuitum* infection were males with mean age of 43 ± 7.45 years. The youngest was 32 years and the oldest was 56 years. Among these were 7 (87.5%) Ghanaians who were Ewes with the only Togolese being Hausa (**Table 4.7.1**). Out of the 8 patients, 4 (50%) were farmers, 3 (37.5%) Traders and 1 (12.5%) Unemployed. Seven (87.5%) were HIV negative whereas one (12.5%) was positive. One of the cases was noted as a treatment failure with the other 7 (87.5%) being new cases.

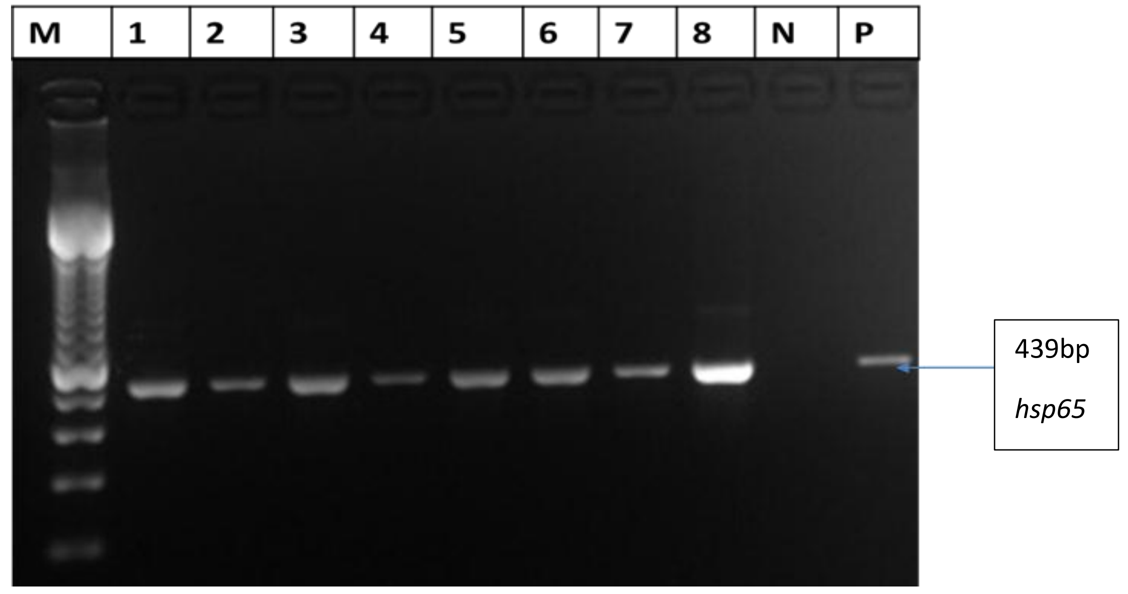


Figure 4.7: Lanes 1-8 contain IS6110 negative clinical Isolates that were positive for hsp65 gene. Lanes N and P are the negative and positive controls respectively.

Table 4.7.1: Demography of Patients with non-tuberculous mycobacteria

Gender	**Number**	**Percent**
Male	8	100
Female	0	
Ethnicity		
Ewe	7	87.5
Hausa	1	12.5
Nationality		
Ghanaian	7	87.5
Togolese	1	12.5
Occupation		
Farmer	4	50
Trader	3	37.5
Unemployed	1	12.5
HIV Status		
Negative	7	87.5
Positive	1	12.5
Age		
Mean	43.5 ± 7.45 years	
Range	32-56 years	
Previous Treatment		
Yes	1	12.5
No	7	87.5

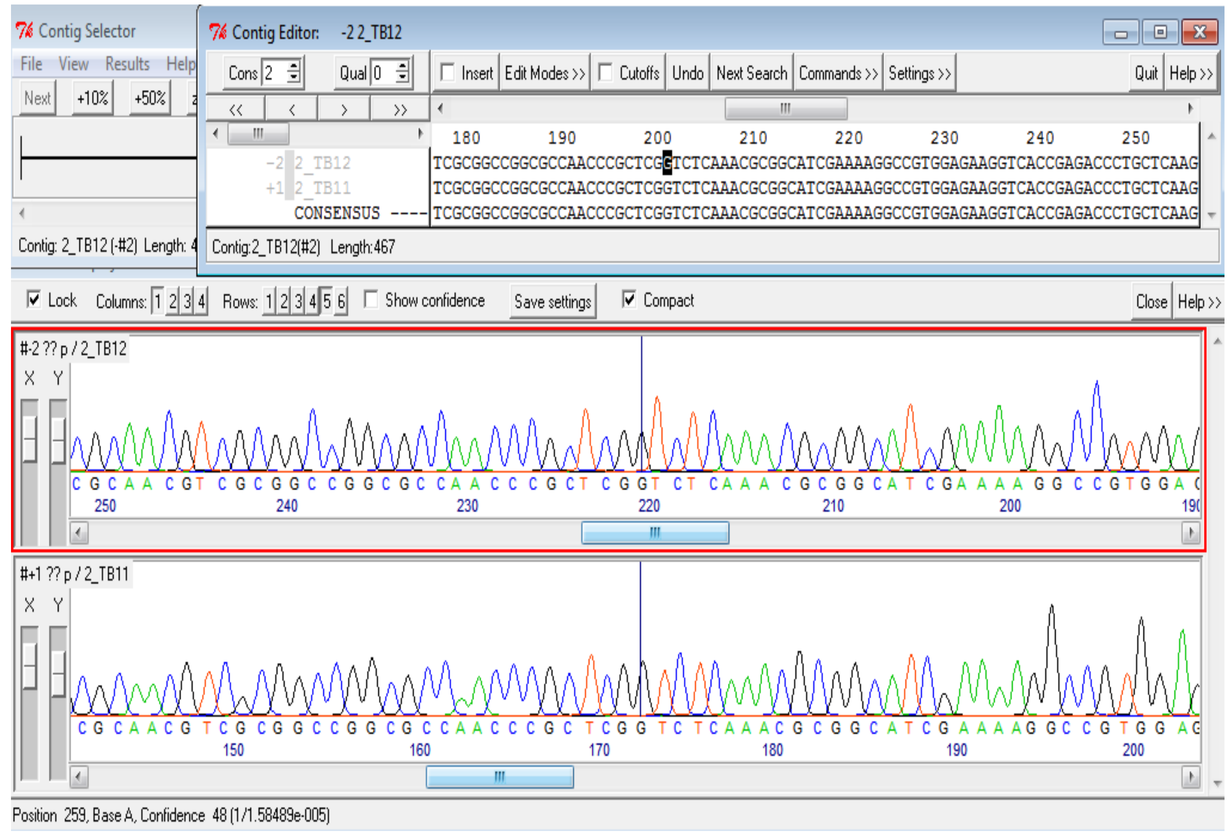


Figure 4.7.1: A chromatogram of *hsp65* nucleotide sequences

Mycobacterium abscessus subsp. bolletii strain Asan 61912 heat shock protein 65 (hsp65) gene, partial cds

Sequence ID: KX906898.1 Length: 603 Number of Matches: 1

Range 1: 1 to 601 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1020 bits(552)	0.0	578/601(96%)	0/601(0%)	Plus/Plus

```
Query  1    GAGGACCCGTACGAGATGATCGGCGCTGAGCTGGTCAAGGAAGTTGCCAAGAAGACCGAC  60
            |||||||||||||||| |||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    GAGGACCCGTACGAGAAGATCGGCGCTGAGCTGGTCAAGGAAGTTGCCAAGAAGACCGAC  60

Query  61   GACGTCGCGGGTGACGGCACCACCACCGCCACCGTGCTCGCCCAGGCTCTGGTCAAGGAA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   GACGTCGCGGGTGACGGCACCACCACCGCCACCGTGCTCGCCCAGGCTCTGGTCAAGGAA  120

Query  121  GGTCTGCGTAACGTCGCCGCCGGCGCCAACCCGCTCGGCCTGAAGCGCGGTATCGAGAAG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  GGTCTGCGTAACGTCGCCGCCGGCGCCAACCCGCTCGGCCTGAAGCGCGGTATCGAGAAG  180

Query  181  GCCGTCGAGAAGGTCACCGAGACGCTGCTGAAGAGCGCCAAGGAGGTCGAGACCAAGGAG  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  GCCGTCGAGAAGGTCACCGAGACGCTGCTGAAGAGCGCCAAGGAGGTCGAGACCAAGGAG  240

Query  241  CAGATCGCGGCCACGGCCGGTATCTCCGCGGGCGACCAGTCCATCGGCGACCTGATCGCC  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  CAGATCGCGGCCACGGCCGGTATCTCCGCGGGCGACCAGTCCATCGGCGACCTGATCGCC  300

Query  301  GAGGCCATGGACAAGGTTGGTCACGAGGCTGTCATCACCGTCGAGGAGTCCAACACCTTC  360
            ||||||||||||||||||||| |||||| |||||||||||||||||||||||||||||||
Sbjct  301  GAGGCCATGGACAAGGTTGGTAACGAGGGTGTCATCACCGTCGAGGAGTCCAACACCTTC  360

Query  361  GGCCTGCAGCTGGAGCTCACCGAGGGTATGCGCTTCGACAAGGGCTACATCTCGGGCTAC  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  GGCCTGCAGCTGGAGCTCACCGAGGGTATGCGCTTCGACAAGGGCTACATCTCGGGCTAC  420
```

Figure 4.7.2: *M .abscessus* identified after NCBI blast search

Mycobacterium fortuitum subsp. fortuitum DSM 46621 = ATCC 6841 genome

Sequence ID: CP014258.1 Length: 6257075 Number of Matches: 2

Range 1: 5397440 to 5398040 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1044 bits(565)	0.0	589/601(98%)	0/601(0%)	Plus/Minus

Features: molecular chaperone GroEL

```
Query  3        GGACCCGTACGAGAAGATCGGCGCTGAGCTCGTCAAAGAGGTCGCCAAGAAGACTGACGA  62
                |||||||||||||||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct  5398040  GGACCCGTACGAGAAGATCGGCGCTGAGCTCGTCAAAGAGGTCGCCAAGAAGACCGACGA  5397981

Query  63       CGTCGCGGGCGACGGCACCACCACCGCCACCGTTCTGGCCCAGGCCCTGGTTCGCGAAGG  122
                ||||||||||||||||||||||||||||||||||||||| |||||||||||||| |||||
Sbjct  5397980  CGTCGCGGGCGACGGCACCACCACCGCCACCGTTCTGGCACAGGCCCTGGTTCGTGAAGG  5397921

Query  123      TCTGCGCAACGTCGCTGCCGGCGCCAACCCGCTCGGCCTGAAGCGCGGCATCGAGAAGGC  182
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5397920  TCTGCGCAACGTCGCTGCCGGCGCCAACCCGCTCGGCCTGAAGCGCGGCATCGAGAAGGC  5397861

Query  183      CGTGGAAAAGGTCACCGAGACCCTCCTGAAGTCCGCCAAGGAGGTGGAGACCAAGGAGCA  242
                ||| || |||||||||||||| || ||||||  |||||||||||||||||||||||||||
Sbjct  5397860  CGTCGAGAAGGTCACCGAGACGCTGCTGAAGAGCGCCAAGGAGGTGGAGACCAAGGAGCA  5397801

Query  243      GATCGCTGCCACCGCCGGTATCTCCGCCGGTGACCAGTCCATCGGTGACCTGATCGCCGA  302
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5397800  GATCGCTGCCACCGCCGGTATCTCCGCCGGTGACCAGTCCATCGGTGACCTGATCGCCGA  5397741

Query  303      GGCCATGGACAAGGTCGGCAACGAGGGTGTCATGACCGTCGAGGAGAGCAACACCTTCGG  362
                ||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct  5397740  GGCCATGGACAAGGTCGGCAACGAGGGTGTCATCACCGTCGAGGAGAGCAACACCTTCGG  5397681
```

Figure 4.7.3: ***M. fortuitum*** **identified after NCBI blast search**

4.8 Prevalence of drug resistance among the *mycobacterium tuberculosis* complex

Drug susceptibility profile of 101 MTBC isolates comprising 78 MTBss and all 23 Maf were determined. Of these, 88 MTBCs consisting of 66 MTBss and 22 Maf were found to be susceptible to the two most potent anti-TB first-line drugs, isoniazid (INH) and rifampicin (RIF) using a line probe assay, Genotype MTBDRplus version 2.0.

Five (6.41%) of the MTBss isolates were found to be INH mono-resistant while only one (4.55%) Maf isolate was resistant to INH. Only MTBss isolates (N=5, 6.41%) were found to be RIF-mono resistant. Two other isolates were found to be resistant to RIF and INH that is, multi-drug resistant (MDR). All the two isolates were found to be MTBss (**Table 4.8**).

The likelihood of an individual being infected with a particular MTBC strain and exhibiting resistance to the first-line anti-TB drugs was estimated using odds ratio (OR). Compared to the Maf strains, MTBss had relatively higher odds of being isoniazid mono resistant (OR: 1.51, CI: 0.17-13.59), rifampicin mono resistant (OR: 3.52, CI: 0.19-66.01) as well as being multi-drug resistant (OR: 1.54, CI: 0.07-33.13).

All two MDR isolates showed the MUT3 band for *rpoB* gene which corresponds to the SNP C1592U that resulted in the locus amino acid change, S531L. One of the MDR isolates in addition to the above had the MUT2B band which is associated with the locus amino acid change, H526D. None of the RIF-mono resistant isolates showed a mutation band but rather absence of wild-type bands. Majority of the RIF-mono resistant isolates had the *rpoB wildtype band,* WT1 absent, and its absence is associated with any of these loci amino acid changes, F505L, T508A or S509T. Similarly, an absence of *rpoB*

wildtype band WT8 in three isolates corresponds with any of these loci amino acid changes, S531L, S531W or L533P.

The isoniazid resistance among the MDRs was conferred by *KatG* mutant MUT1 that corresponds to the SNP U943A that resulted in the S315T locus amino acid change. The *KatG* mutation dominated in 4 different isolates and was responsible for majority of INH resistance. However, *inhA* mutants MUT1 and MUT3B associated with the locus amino acid changes C15T, and T8A on the promoter region were also implicated in INH resistance as shown in **Table 4.8**.

Table 4.8: Drug resistance profile of the human adapted MTBC isolates amongst TB patients

Drug Resistance	MTBss (78)	Maf (23)	OR	CI	Mutations	Gene	Phenotypes
INH^{mono}	5(6.41%)	1(4.55%)	1.51	0.17-13.59	C15T	*inhA*	MUT1(1)
					T8A	*inhA*	MUT3B(2)
					S315T	*katG*	MUT1(2)
RIF^{mono}	5(6.41%)	0	3.52	0.19-66.01	F505L, T508A, S509T	*rpoB*	WT1(1)
					S531L	*rpoB*	MUT3(1)
					S531L,S531W, L533P	*rpoB*	WT8(3)
MDR	2(3.85%)	0	1.54	0.07-33.13	H526D	*rpoB*	MUT2B
					S315T	*katG*	MUT1(2)
					S531L	*rpoB*	MUT3(2)

CHAPTER FIVE

5.0 DISCUSSION

Tuberculosis (TB) remains a major public health challenge globally, despite being recognized centuries ago. The recent upsurge of cases is partly due to the interaction between TB and HIV, delays in diagnosis that perpetuate transmission in the community, the emergence of drug resistant strains of *M. tuberculosis* complex (MTBC) and the lack of effective control tools including diagnostics, vaccines and drugs. To combat this global menace, in 1993, the WHO declared TB as a global emergency and called for intensive measures, including funding of studies aimed to develop new tools. So much funding has gone into studies that aimed at understanding the biology of *M. tuberculosis* sensu stricto (MTBss), however at the neglect of *M. africanum* (Maf) due to; 1) its geographical restriction 2) the dogma that there is no significant genetic diversity within the MTBC with functional implications and, 3) the reported lower virulence of Maf and so will be eventually outcompeted by MTBss.

However, more data are accumulated indicating that 1) Maf differs from MTBss in many features including host-pathogen interactions and adaptation to anti-TB drugs which are relevant for TB control and, 2) Maf continues to be important in many West African countries including Ghana, the Gambia, Benin, Burkina Faso, Mali, and Nigeria. However, what we do not know is why Maf is restricted to West Africa (Gagneux and Small, 2007; de Jong *et al*., 2010).

The first goal of my masters' project was thus to isolate mycobacteria from smear-positive patients' sputum samples collected from the southern part of Volta Region. The second goal of my study was to genotype the recovered mycobacteria to determine the

different MTBC lineages and sub-lineages circulating in the southern part of Volta Region and finally to determine their association with drug resistance.

Prevalence of main Lineages and Sub-lineages of MTBC

Africa is the only continent that harbours all the 7 human-adapted MTBC lineages (Gagneux *et al*., 2006). Nevertheless, most of the countries harbour only 4 lineages with high prevalence of 6 out of the 7 lineages found in some West African countries including Ghana and Ivory-Coast. In Ghana, previous studies by Yeboah-Manu *et al*., (2011) and Asante-Poku *et al*., (2015) have reported the presence of the 6 lineages circulating in Greater Accra, Central and the Northern region. In this study we found that these 6 MTBC lineages also circulate in the Southern part of the Volta Region suggesting their establishment in Ghana. Our spoligotyping analysis of 117 MTBC isolates from southern Volta revealed 23 (19.66%) Maf and 94 (80.34%) MTBss. Comparing the proportion of Maf to the national prevalence of 20% (Yeboah-Manu *et al*., 2011), we observed no significant difference (p-value = 0.951) indicating that comparably, the same proportion is circulating in the Southern half of the Volta Region. The approximately 20% Maf proportion is consistent with that observed in the Greater Accra and Northern regions of Ghana and has been found in previous studies to be fairly stable over an 8-year period (Yeboah-Manu *et al.*, 2016). The probable nationwide circulation of the 6 lineages coupled with the observed stability of Maf over the past decade indicates that the pathogen has been established within the population and calls for the need to investigate the biology and the functional implication of genomic diversity in the design of control tools.

One possible reason for the stability of Maf in Ghana and West Africa irrespective of the observed lower virulence might be adaptation of this bacterial species to specific human populations. Recently, two independent molecular epidemiological studies conducted in Ghana by Asante-Poku *et al*., (2015, 2016) found a strong association of Maf with the Ewe ethnicity. Since Volta region is the home of the Ewe ethnic group, we decided to access the distribution of Maf within this region to help ascertain the observed association. Although we expected to find a greater proportion of Maf in the Volta region, more so when most of the participants were of Ewe ethnicity, we observed a similar proportion in our study across the sub-region, but interestingly we found significant difference in inter-municipality comparisons. For instance, driven by Lineage 5, the proportions of Maf in Ho Municipality, 13/44 (29.6%) showed a significantly higher proportion (p-value = 0.017) than in Ketu-South Municipality (Aflao), 3/36 (8.3%). The significantly lower prevalence of Maf in the Ketu-South Municipality may be due to the diverse human populations as a result of travelers and migrants present at every point in time crossing the border to and from the republic of Togo doing business. However, the Ho municipality is dominated by the Ewe ethnic population and again among this population we observed significantly higher Maf prevalence which goes to support the claim of association by Asante-Poku *et al*., (2015, 2016). This observation makes us suggest there could be a possible predisposing factor among this human population to Maf infection. Notably, Gagneux (2012), explained in his review that specific host–pathogen adaptation could be as a result of changes in the genome of the mycobacterium interacting with the host immune system, particularly in genes encoding antigens. Principally, in host–pathogen systems, there is a battle between pathogen and

host immune system that normally results in a modification of the pathogen antigens to evade the host immune system (Dawkins and Krebs, 1979) in order to establish infection. On the other hand the seemingly similar Maf proportion in the Southern Volta (19.66%) comparable to the national average (20.0%) could be as a result of poor mycobacterial isolation rate (< 68.3%) from some municipalities/districts hence reducing our excepted higher Maf prevalence. For instance, the Ketu-South Municipality even though contributing 30.8% (36/117) of the total mycobacterial isolates, had a very low isolation rate of 37.9% (36/95) compared to the Ho municipality where there was a similar trend as 37.6% (44/117) isolates were contributed as a result of poor isolation rate of 51.8% (44/85). The probably poor mycobacteria isolation rate could be attributed to poor storage conditions and long sample transportation distance to Noguchi Memorial Institute for Medical Research (NMIMR) for processing, all of which are likely to have influenced currently the observed trends. The difficulty in Maf isolation could not be ruled out as a contributing factor. Unlike MTBss which could be isolated on glycerol supplemented as well as on pyruvate supplemented Lowenstein Jensen (LJ) media, Maf isolation was possible only on pyruvate supplemented LJ media due to its lack of the enzyme, pyruvate kinase. Another challenge of Maf isolation is its longer generation time than MTBss which culminates in its longer incubation time to show growth on LJ media (Castets, 1979). Thus my study also supports the need of a good transport medium such as the Omingene Sputum reagent that could maintain mycobacterial viability whilst suppressing other bacterial overgrowth.

In the context of the study, we additionally confirmed the importance of a particular sub-lineage of Lineage 4 known as the Cameroon genotype as an important cause of TB in

Ghana causing about 60% of all TB cases (Yeboah-Manu *et al*., 2011; Asante-Poku *et al*, 2015)**.** This observation is consistent with findings by Gehre *et al*., (2013) and de Jong *et al*., (2010) from neighbouring countries. The reasons for its success are unknown but could be due to high fitness in the corresponding patient populations.

Prevalence of Drug resistance and Implications for TB control

Drug resistance remains a great threat to the fight against pulmonary TB. Three categories of drug resistance were recorded in this study, namely, isoniazid (INH)-mono resistance, rifampicin (RIF)-mono resistance and resistance to both INH and RIF otherwise known as multi drug resistance (MDR).

Comparing the drug resistance profile of the human adapted strains, 5 (6.4%) of MTBss isolates were each mono resistant to INH and RIF whereas 1 (4.3%) of Maf isolate was mono-resistant to only INH. The odds ratio for INH-mono resistance and RIF-mono resistance were 1.51 (CI=0.17-13.59), and 3.52 (CI=0.19-66.01) respectively. This means that, an individual infected with TB bacilli has a relatively higher risk of possessing an INH or RIF –mono resistant strain when the infecting specie is MTBss compared to Maf. Expectedly, the observed odds ratios were statistically significant ($P<0.05$). Similarly, the odds of finding an MDR MTBss infection was higher than finding an MDR Maf infection (OR: 1.54, CI=0.07-33.13).

In contrast with other studies by Asante-Poku *et al*., (2015) and Homolka *et al*., (2010) where INH mono resistance was higher, this study found a comparable INH-mono and RIF-mono resistance. This goes to suggest that these comparable INH-mono resistance would have been missed by the GeneXpert, which depends on detecting RIF-mono

resistance as a surrogate for detecting INH mono-resistance and hence MDR-TB. The implications are that the RIF-mono resistance cases would have been treated as MDR cases. However, these INH-mono-resistant cases that would have been missed by the GeneXpert would be treated as susceptible cases with first-line regimen that contains INH. The first-line regimen also contains RIF to which these isolates are susceptible thus, when administered appropriately would lead to successful treatment outcome in these individuals. In this work, we determined drug susceptibility profile of isolates by using the MTBDR*plus* based on line probe assay, and observed both INH-mono and RIF-mono resistance to each be 5 (6.4%). However, Otchere *et al*., (2016) using a phenotypic alamar blue assay and been consistent with previous studies reported an almost stable 7.5% and 0.7% occurrence for INH-mono and RIF-mono resistance respectively. Comparing this previous trend to our work, there appears to be an increase in RIF-mono resistance cases in the Volta region. The introduction of GeneXpert machine by the Ghana Health Service with support from the Global Fund must be commended. Since this real-time machine in addition to identifying MTBC bacilli, can detect resistance to RIF. It detects resistance to RIF by identifying mutations in the bacilli *rpoB* gene which codes for the β-subunit of DNA dependent RNA polymerase, the target of the drug RIF.

Previous studies have reported high level INH resistance (40-95%) to be associated with 75-90% *katG* position S315T mutation (Hazbon *et al*., 2006, Vilcheze *et al*., 2007 and Riccardi *et al*., 2009), however in this study, even though we similarly observed 40% high level INH resistance, it was associated with only 57.14% *katG* position S315T mutation. For occurrence of this resistance, this study was consistent with Otchere *et al*., (2016), as it finds the human adapted strain MTBss compared to Maf having a relatively

greater risk of possessing this position S315T mutation in *katG* ($p < 0.001$). Though both serine and threonine are polar amino acids, it is likely that the incorporation of threonine with methyl in place of serine would result in shielding hydroxyl (OH-) electrons that would have been used by the catalase peroxidase during catalysis thereby preventing INH prodrug activation and subsequently leading to resistance. In other studies, Zhang and Yew, (2009), reported, 8-43% low-level INH resistance was due to mutations in the promoter region of *inhA* gene. Falling within the range, our study recorded 42.9%.

A study by Riccardi *et al*., (2009), associated RIF resistance with the *rpoB* gene mutations, which cluster mainly in the codon region of 507-533. Inconsistent with the mutation in *rpoB* S450L reported by Otchere *et al*., (2016), our study however, in agreement with Riccardi *et al*., (2009), reports *rpoB* gene mutation distribution of 37.5% S531L and 12.5% H526D. The amino acid change from polar serine to non-polar leucine at position 531 may have contributed to conformational change in protein structure. This may have subsequently prevented proper binding of the drug RIF to the β-subunit of the DNA dependent RNA polymerase leading to drug resistance. Similarly, change in amino acids at position 526 from basic histidine to acidic aspartic acid could have led to conformational change in the binding site on the β-subunit of the DNA dependent RNA polymerase, again leading to the bacterial drug resistance to RIF.

Similar to observations by Telenti *et al*., (1997) and Coovadia *et al*., (2013), the remaining 50% constituted the absence of wild type (WT) bands, WT1 in one isolate and WT8 in 3 isolates without concomitant presence of mutant (MUT) bands. This means that the mutations leading to drug resistance are variable.

Misdiagnosis of TB and its clinical implication

The ability to differentiate between MTBCs and Non-tuberculous mycobacteria (NTMs) is very crucial for the appropriate treatment regimen to be administered (Kumar *et al*., 2016; Sinha *et al*., 2016). This is because while the standard treatment regimen for MTBC infection takes 6 months consisting of RIF, INH, pyrazinamide (PZA) and ethambutol (EMB) for a period of 2 months in the first phase, followed by the administration of RIF and INH for a period of 4 months. However, treatment of NTMs takes between 18-24 months with different drug regimen based on thorough drug susceptibility testing as the NTMs are naturally resistant to these antimicrobials (Ryu *et al*., 2016). In addition, presentation of NTMs infections typically mimics TB thereby confounding the diagnosis of TB. In this study, although MTBC constituted 93.6% of all mycobacteria isolates responsible for pulmonary infections, NTMs were isolated from 6.4% of patients presumptively diagnosed with TB using the NTP diagnostic algorithm.

This was higher than 2.5% observed by Otchere *et al*., (2017), but consistent with studies by Bertoletti *et al*., (2011). The NTMs isolated, *Mycobacterium abscessus,* and *M. fortuitum* are known to be fast growing mycobacteria that can cause pulmonary infections in both immunocompetent and immunocompromised individuals. The challenge is, microscopy, which is used for TB diagnosis in the periphery medical laboratories, lacks specificity and is unable to distinguish between MTBCs and NTMs.

This means that those individuals constituting the 6.4% could have been placed on a drug regimen for MTBC which will fail for the first-line treatment and would be considered as MDR-TB. It was mostly likely these patients would be wrongly placed on the second-line treatment regimen which also would fail. Already data from this study shows one

individual had failed the first-line treatment and had been placed on second-line drug regimen. Notably, out of 7 patients with *M. abscessus* infection, 6 which includes this treatment failure case are HIV negative patients with the other one been HIV positive. The only patient with *M. fortuitum* is HIV negative. These observations emphasize the need to pay critical attention to differential diagnosis of pulmonary infectious mycobacteria, especially among cases that do not sputum convert after two months of anti-TB treatment to allow placement of the affected in the right regimen. This could be achieved by introducing in addition to Xpert MTB/RIF rapid DNA-based such as GenoType Mycobacterium CM VER 2.0 into the national diagnostic algorithm. This diagnostic tool can be used to identify NTMs after microscopy confirms presence of acid-fast bacilli (AFB) and GeneXpert had ruled out MTBC presence. The NTMs are ubiquitous and means everyone is exposed to them. Apart from HIV infection, factors that predispose people to NTM infections are not well understood but could be due to interactions between host defense mechanisms and the load of clinical NTM exposure (Johnson and Odell, 2014). Interleukin-12 (IL-12) and interferon gamma (INF-Ɣ) are important cytokines responsible for host immune response against NTMs (Dorman and Holland, 2000). This suggests defects in pathways of these cytokines of hosts could increase susceptibility to NTM infections.

Another form of misdiagnosis noticed by this study was false AFB positives, since out of 270 positively diagnosed sputum samples collected, only 183 were confirmed to be AFB positive after bacilli concentration. This is of concern, since over 80 individuals may have been wrongly put on anti-tuberculous drugs that could lead to hepatitis as result of hepatotoxic nature of these drugs especially RIF. However, the introduction of the

GeneXpert machine which is more specific and sensitive than the microscopy could be used in MTBC case confirmation to reverse the trend.

Generally, almost all resources currently invested in the development of new TB drugs, and vaccines, have been concentrated on MTBss to the neglect of Maf. My master's thesis has contributed to the call to invest into Maf studies and the development of Maf-inclusive control tools by providing information on population structure of MTBC in the southern half of the Volta region of Ghana, confirming the importance of Maf as a TB causing pathogen in Ghana. This activity is one of the key research programs outlined by the Research Division of the Ghana Health Service.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

This study has identified false positive cases for which those individuals may have been put on Anti-tuberculous drugs with possible risk of hepatotoxicity leading to hepatitis. We have also found the need for a good sputum transport media to preserve live *Mycobacterium tuberculosis* complex (MTBC) bacilli in sputum during transportation over long distances to reference laboratories like the Noguchi Memorial Institute for Medical Research (NMIMR) for further investigation. The identification of the 6 lineages (1-6) in the Volta region which is consistent with findings from the north, Central and Greater Accra regions suggests a possible establishment of these 6 lineages in Ghana. This emphasizes the need to explore more into the genomics of these various lineages and the information gathered be factored in the design of control tools.

Lineages 5 and 6 of *Mycobacterium africanum* (Maf) constituted 19.7% of MTBC population which was found not to be significantly different from the national average. However, there was a significant presence of Maf circulating in the more Ewe dominated Ho municipality compared to the more cosmopolitan area, Ketu-South municipality (Aflao). This observation supports the association of the Ewe ethnicity with Maf as reported by Asante-Poku *et al*., (2015, 2016) and suggests there could be host predisposing factors within this human population to this pathogen.

The Cameroon sub-lineage was found to dominate *M. tuberculosis* sensu stricto (MTBss) strain and similarly, the dominance in Maf was driven by the West-African 1 sub-lineage.

It was also noted that MTBC was responsible for 93.6% of pulmonary infection in the region with the remaining 6.4% due to non-tuberculous mycobacteria (NTMs) which has implications on treatment outcome of TB in that part of the region. Generally, the odds for an MTBC infection to be a drug resistant strain was relatively higher for MTBss than for Maf. This study thus, confirms the importance of *M. africanum* lineages in Ghana and should be considered in the development of new diagnostics, drugs, and vaccines.

6.2 RECOMMENDATIONS

- Capacity building regarding sputum smear staining and microscopy is necessary.
- The rolling out of GeneXpert machine by the Ghana Health Service must be up scaled and extended to all health facilities since in addition to detecting resistance to rifampicin resistance, it is able to detect only MTBCs thereby excluding NTMs.
- The seemingly preferred localization of Maf within the Ho municipality needs further investigation in a larger population study which must include pathogen-host genomics.

REFERENCES

Agasino, C. B., Ponce de Leon, A., Jasmer, R. M., & Small, P. M. (1998). Epidemiology of Mycobacterium tuberculosis strains in San Francisco that do not contain IS6110. *International Journal of Tuberculosis and Lung Disease*, *2*(6), 518–520.

Ahmad, S. (2010). New approaches in the diagnosis and treatment of latent tuberculosis infection. *Respiratory Research*, *11*(1), 169. https://doi.org/10.1186/1465-9921-11-169

Allix-Béguec, C., Harmsen, D., Weniger, T., Supply, P., & Niemann, S. (2008). Evaluation and strategy for use of MIRU-VNTRplus, a multifunctional database for online analysis of genotyping data and phylogenetic identification of Mycobacterium tuberculosis complex isolates. *Journal of Clinical Microbiology*, *46*(8), 2692–2699. https://doi.org/10.1128/JCM.00540-08

Apers, L., Mutsvangwa, J., Magwenzi, J., Chigara, N., Butterworth, A., Mason, P., & Van der Stuyft, P. (2003). A comparison of direct microscopy, the concentration method and the Mycobacteria Growth Indicator Tube for the examination of sputum for acid-fast bacilli. *International Journal of Tuberculosis and Lung Disease*, *7*(4), 376–381.

Aranaz, A., Cousins, D., Mateos, A., & Domínguez, L. (2003). Elevation of Mycobacterium tuberculosis subsp. caprae Aranaz et al. 1999 to species rank as Mycobacterium caprae comb. nov., sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, *53*(6), 1785–1789. https://doi.org/10.1099/ijs.0.02532-0

Asante-Poku, A., Yeboah-Manu, D., Otchere, I. D., Aboagye, S. Y., Stucki, D., Hattendorf, J., Gagneux, S. (2015). Mycobacterium africanum Is Associated with Patient Ethnicity in Ghana. *PLoS Neglected Tropical Diseases*, *9*(1). https://doi.org/10.1371/journal.pntd.0003370

Asante-Poku, A., Otchere, I. D., Osei-Wusu, S., Sarpong, E., Baddoo, A., Forson, A., Yeboah-Manu, D. (2016). Molecular epidemiology of Mycobacterium africanum in Ghana. *BMC Infectious Diseases*, *16*(1), 385. https://doi.org/10.1186/s12879-016-1725-6

Ates, L. S., Ummels, R., Commandeur, S., van der Weerd, R., Sparrius, M., Weerdenburg, E., Houben, E. N. G. (2015). Essential Role of the ESX-5 Secretion System in Outer Membrane Permeability of Pathogenic Mycobacteria. *PLoS Genetics*, *11*(5). https://doi.org/10.1371/journal.pgen.1005190

Augustynowicz-Kopec, E., Jagielski, T., Kozinska, M., Zabost, A., & Zwolska, Z. (2007). [The significance of spoligotyping method in epidemiological investigations of tuberculosis]. *Pneumonologia I Alergologia Polska*, *75*(1), 22–31.

Barclay, A, D. (1988). Mycobactins and Exochelins of Mycobacterium tuberculosis, M. bovis, M. africanum and Other Related Species. *Journal of General Microbiology*, *134*(77), 1–776.

Barnard, D. L. (2001). Pegasys (Hoffmann-La Roche). *Current Opinion in Investigational Drugs*, *2*(11), 1530–1538.

Barnard, M., Gey Van Pittius, N. C., Van Helden, P. D., Bosman, M., Coetzee, G., & Warren, R. M. (2012). The diagnostic performance of the GenoType MTBDRplus version 2 line probe assay is equivalent to that of the Xpert MTB/RIF assay. *Journal of Clinical Microbiology*, *50*(11), 3712–3716. https://doi.org/10.1128/JCM.01958-12

Bates, J. H., & Stead, W. W. (1993). The history of tuberculosis as a global epidemic. *The Medical Clinics of North America*. https://doi.org/10.1016/S0025-7125(16)30188-2

Becker, C., Pohla, H., Frankenberger, B., Schüler, T., Assenmacher, M., Schendel, D. J., & Blankenstein, T. (2001). Adoptive tumor therapy with T lymphocytes enriched through an IFN-γ capture assay. *Nature Medicine*, *7*(10), 1159–1162. https://doi.org/10.1038/nm1001-1159

Beggs, M. L., Cave, M. D., Marlowe, C., Cloney, L., Duck, P., & Eisenach, K. D. (1996). Characterization of Mycobacterium tuberculosis complex direct repeat sequence for use in cycling probe reaction. *Journal of Clinical Microbiology*, *34*(12), 2985–2989.

Bentley, S. D., Comas, I., Bryant, J. M., Walker, D., Smith, N. H., Harris, S. R., de Jong, B. C. (2012). The genome of mycobacterium Africanum West African 2 reveals a lineage-specific locus and genome erosion common to the M. tuberculosis complex. *PLoS Neglected Tropical Diseases*, *6*(2). https://doi.org/10.1371/journal.pntd.0001552

Bieberly, J., & Ali, J. (2008). Treatment adherence of the latently infected tuberculosis population (Post-Katrina) at Wetmore TB Clinic, New Orleans, USA. *International*

Journal of Tuberculosis and Lung Disease, *12*(10), 1134–1138. https://doi.org/10.1371/journal.pmed.0040238

Bjerrum, S., Kenu, E., Lartey, M., Newman, M. J., Addo, K. K., Andersen, A. B., & Johansen, I. S. (2015). Diagnostic accuracy of the rapid urine lipoarabinomannan test for pulmonary tuberculosis among HIV-infected adults in Ghana-findings from the DETECT HIV-TB study. *BMC Infectious Diseases*, *15*, 407. https://doi.org/10.1186/s12879-015-1151-1

Boshoff, H. I., & Barry, C. E. (2006). Is the mycobacterial cell wall a hopeless drug target for latent tuberculosis? *Drug Discovery Today: Disease Mechanisms*. https://doi.org/10.1016/j.ddmec.2006.06.002

Brennan, P. J. (2003). Structure, function, and biogenesis of the cell wall of Mycobacterium tuberculosis. In *Tuberculosis* (Vol. 83, pp. 91–97). https://doi.org/10.1016/S1472-9792(02)00089-6

Brodin, P., Majlessi, L., Marsollier, L., De Jonge, M. I., Bottai, D., Demangel, C., Brosch, R. (2006). Dissection of ESAT-6 system 1 of Mycobacterium tuberculosis and impact on immunogenicity and virulence. *Infection and Immunity*, *74*(1), 88–98. https://doi.org/10.1128/IAI.74.1.88-98.2006

Brosch, R., Gordon, S. V, Marmiesse, M., Brodin, P., Buchrieser, C., Eiglmeier, K., Cole, S. T. (2002). A new evolutionary scenario for the Mycobacterium tuberculosis complex. *Proceedings of National Academy of Sciences USA*, *99*(6), 3684–3689. https://doi.org/10.1073/pnas.052548299

Broset, E., Mart, C., & Gonzalo-Asensio, J. (2015). Evolutionary landscape of the mycobacterium tuberculosis complex from the viewpoint of phoPR: Implications for virulence regulation and application to vaccine development. *mBio*, *6*(5). https://doi.org/10.1128/mBio.01289-15

Brudey, K., Gutierrez, M. C., Vincent, V., Parsons, L. M., Salfinger, M., Rastogi, N., & Sola, C. (2004). Mycobacterium africanum genotyping using novel spacer oligonucleotides in the direct repeat locus. *Journal of Clinical Microbiology*, *42*(11), 5053–5057. https://doi.org/10.1128/JCM.42.11.5053-5057.2004

Cambau, E., & Drancourt, M. (2014). Steps towards the discovery of Mycobacterium tuberculosis by Robert Koch, 1882. *Clinical Microbiology and Infection*. https://doi.org/10.1111/1469-0691.12555

Canetti, G., Fox, W., Khomenko, A., Mahler, H. T., Menon, N. K., Mitchison, D. A., Smelev, N. A. (1969). Advances in techniques of testing mycobacterial drug sensitivity, and the use of sensitivity tests in tuberculosis control programmes. *Bulletin of the World Health Organization*, *41*(1), 21–43.

Casal, M., Gutierrez, J., & Vaquero, M. (1997). Comparative evaluation of the mycobacteria growth indicator tube with the BACTEC 460 TB system and Lowenstein-Jensen medium for isolation of mycobacteria from clinical specimens. *International Journal of Tuberculosis and Lung Disease*, *1*(1), 81–84.

Cavanagh, R. D., Lambin, X., Ergon, T., Bennett, M., Graham, I. M., van Soolingen, D., & Begon, M. (2004). Disease dynamics in cyclic populations of field voles (Microtus agrestis): cowpox virus and vole tuberculosis (Mycobacterium microti).

Proceedings of the Royal Society B: Biological Sciences, *271*(1541), 859–867. https://doi.org/10.1098/rspb.2003.2667

Chan, B., Khadem, T. M., & Brown, J. (2013). A review of tuberculosis: Focus on bedaquiline. *American Journal of Health-System Pharmacy*. https://doi.org/10.2146/ajhp130199

Chew, W. K., Lasaitis, R. M., Schio, F. A., & Gilbert, G. L. (1998). Clinical evaluation of the mycobacteria growth indicator tube (MGIT) compared with radiometric (Bactec) and solid media for isolation of Mycobacterium species. *Journal of Medical Microbiology*, *47*(9), 821–827. https://doi.org/10.1099/00222615-47-9-821

Cho, H., Uehara, T., & Bernhardt, T. G. (2014). Beta-lactam antibiotics induce a lethal malfunctioning of the bacterial cell wall synthesis machinery. *Cell*, *159*(6), 1310–1311. https://doi.org/10.1016/j.cell.2014.11.017

Colditz, G. A., Brewer, T. F., Berkey, C. S., Burdick, E., Fineberg, H. V, & Mosteller, F. (1994). Vaccine in the Prevention of Tuberculosis Efficacy of BCG. *JAMA : The Journal of the American Medical Association*, *271*(9), 698–702. https://doi.org/10.1001/jama.1994.03510330076038.

Comas, I., & Gagneux, S. (2011). A role for systems epidemiology in tuberculosis research. *Trends in Microbiology*, *19*(10), 492–500. https://doi.org/10.1016/j.tim.2011.07.002

Coscolla, M., Lewin, A., Metzger, S., Maetz-Rennsing, K., Calvignac-Spencer, S., Nitsche, A., Leendertz, F. H. (2013). Novel Mycobacterium tuberculosis complex

isolate from a wild chimpanzee. *Emerging Infectious Diseases*, *19*(6), 969–976. https://doi.org/10.3201/eid1906.121012

Daffé, M. (2015). The cell envelope of tubercle bacilli. *Tuberculosis*, *95 Suppl 1*, S155–8. https://doi.org/10.1016/j.tube.2015.02.024

Daniel, T. M. (2006). The history of tuberculosis. *Respiratory Medicine*, *100*(11), 1862–1870. https://doi.org/10.1016/j.rmed.2006.08.006

Day, T. (2016). Interpreting phenotypic antibiotic tolerance and persister cells as evolution via epigenetic inheritance. *Molecular Ecology*, *25*(8), 1869–1882. https://doi.org/10.1111/mec.13603

De Jong, B. C., Antonio, M., Awine, T., Ogungbemi, K., De Jong, Y. P., Gagneux, S., Adegbola, R. A. (2009). Use of spoligotyping and large sequence polymorphisms to study the population structure of the Mycobacterium tuberculosis complex in a cohort study of consecutive smear-positive tuberculosis cases in the gambia. *Journal of Clinical Microbiology*, *47*(4), 994–1001. https://doi.org/10.1128/JCM.01216-08

de Jong, B. C., Antonio, M., & Gagneux, S. (2010). Mycobacterium africanum—Review of an Important Cause of Human Tuberculosis in West Africa. *PLoS Neglected Tropical Diseases*, *4*(9), e744. https://doi.org/10.1371/journal.pntd.0000744

de Jong, B. C., Hill, P. C., Aiken, A., Awine, T., Antonio, M., Adetifa, I. M., Adegbola, R. A. (2008). Progression to active tuberculosis, but not transmission, varies by Mycobacterium tuberculosis lineage in The Gambia. *The Journal of Infectious Diseases*, *198*(7), 1037–43. https://doi.org/10.1086/591504

Dharmadhikari, A. S., & Nardell, E. A. (2008). What animal models teach humans about tuberculosis. *American Journal of Respiratory Cell and Molecular Biology*. https://doi.org/10.1165/rcmb.2008-0154TR

Di Perri, G., & Bonora, S. (2004). Which agents should we use for the treatment of multidrug-resistant Mycobacterium tuberculosis? *The Journal of Antimicrobial Chemotherapy*, *54*(3), 593–602. https://doi.org/10.1093/jac/dkh377

Diacon, A. H., Pym, A., Grobusch, M. P., de Los Rios, J. M., Gotuzzo, E., Vasilyeva, I., … Dannemann, B. (2014). Multidrug-Resistant Tuberculosis and Culture Conversion with Bedaquiline. *The New England Journal of Medicine*, *371*(8), 723–732. https://doi.org/10.1056/NEJMoa1313865

Dirlikov, E., Raviglione, M., & Scano, F. (2015). Global tuberculosis control: Toward the 2015 targets and beyond. *Annals of Internal Medicine*, *163*(1), 52–58. https://doi.org/10.7326/M14-2210

Dodor, E. (2008). Health Professionals Expose TB Patients to Stigmatization in Society: Insights from Communities in an Urban District in Ghana. *Ghana Medical Journal*, *42*(4), 144–148.

Dodor, E. A. (2012). The feelings and experiences of patients with tuberculosis in the Sekondi-Takoradi Metropolitan district: implications for TB control efforts. *Ghana Medical Journal*, *46*(4), 211–8.

Dodor, E. A., & Kelly, S. (2009). "We are afraid of them": attitudes and behaviours of community members towards tuberculosis in Ghana and implications for TB control

efforts. *Psychology, Health & Medicine*, *14*(2), 170–179. https://doi.org/10.1080/13548500802199753

Driscoll, J. R. (2009). Spoligotyping for molecular epidemiology of the Mycobacterium tuberculosis complex. *Methods in Molecular Biology (Clifton, N.J.)*. https://doi.org/10.1007/978-1-60327-999-4_10

Du, Y., Qi, Y., Yu, L., Lin, J., Liu, S., Ni, H., Wang, C. (2011). Molecular characterization of Mycobacterium tuberculosis complex (MTBC) isolated from cattle in northeast and northwest China. *Research in Veterinary Science*, *90*(3), 385–391. https://doi.org/10.1016/j.rvsc.2010.07.020

Ernst, J. D. (1998). Macrophage Receptors for Mycobacterium tuberculosis. *Infection and Immunity*.

Faruque, S. M., Albert, M. J., & Mekalanos, J. J. (1998). Epidemiology, genetics, and ecology of toxigenic Vibrio cholerae. *Microbiology and Molecular Biology Reviews : MMBR*, *62*(4), 1301–14. https://doi.org/10.1073/pnas.0703993104

Ferdinand, S., Sola, C., Chanteau, S., Ramarokoto, H., Rasolonavalona, T., Rasolofo-Razanamparany, V., & Rastogi, N. (2005). A study of spoligotyping-defined Mycobacterium tuberculosis clades in relation to the origin of peopling and the demographic history in Madagascar. *Infection, Genetics and Evolution*, *5*(4), 340–348. https://doi.org/10.1016/j.meegid.2004.10.002

Finlay, B. B., & McFadden, G. (2006). Anti-immunology: Evasion of the host immune system by bacterial and viral pathogens. *Cell*.

https://doi.org/10.1016/j.cell.2006.01.034

Franzblau, S. G., Witzig, R. S., Mclaughlin, J. C., Torres, P., Madico, G., Hernandez, A., Gilman, R. H. (1998). Rapid, low-technology MIC determination with clinical Mycobacterium tuberculosis isolates by using the microplate Alamar Blue assay. *Journal of Clinical Microbiology*, *36*(2), 362–366.

Frothingham, R., Strickland, P. L., Bretzel, G., Ramaswamy, S., Musser, J. M., & Williams, D. L. (1999). Phenotypic and genotypic characterization of Mycobacterium africanum isolates from West Africa. *Journal of Clinical Microbiology*, *37*(6), 1921–1926.

Gagneux, S. (2012). Host-pathogen coevolution in human tuberculosis. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *367*(1590), 850–9. https://doi.org/10.1098/rstb.2011.0316

Gagneux, S., DeRiemer, K., Van, T., Kato-Maeda, M., de Jong, B. C., Narayanan, S., Small, P. M. (2006). Variable host-pathogen compatibility in Mycobacterium tuberculosis. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(8), 2869–2873. https://doi.org/10.1073/pnas.0511240103

Gagneux, S., & Small, P. M. (2007). Global phylogeography of Mycobacterium tuberculosis and implications for tuberculosis product development. *Lancet Infectious Diseases*. https://doi.org/10.1016/S1473-3099(07)70108-1

Gehre, F., Antonio, M., Faihun, F., Odoun, M., Uwizeye, C., de Rijk, P., Affolabi, D. (2013). The first phylogeographic population structure and analysis of transmission

dynamics of M. africanum West African 1--combining molecular data from Benin, Nigeria and Sierra Leone. *PLoS One*, *8*(10), e77000. https://doi.org/10.1371/journal.pone.0077000

Gehre, F., Otu, J., DeRiemer, K., de Sessions, P. F., Hibberd, M. L., Mulders, W., Antonio, M. (2013). Deciphering the Growth Behaviour of Mycobacterium africanum. *PLoS Neglected Tropical Diseases*, *7*(5). https://doi.org/10.1371/journal.pntd.0002220

Gey Van Pittius, N. C., Gamieldien, J., Hide, W., Brown, G. D., Siezen, R. J., & Beyers, A. D. (2001). The ESAT-6 gene cluster of Mycobacterium tuberculosis and other high G+C Gram-positive bacteria. *Genome Biology*, *2*(10), RESEARCH0044. https://doi.org/10.1186/gb-2001-2-10-research0044

Ghana Statistical Service. (2014). 2010 Population and Housing Census, District Analytical Report. *Ghana Statistial Service*, 1–109. https://doi.org/10.1371/journal.pone.0104053

Giacomini, E., Sotolongo, A., Iona, E., Severa, M., Remoli, M. E., Gafa, V., Coccia, E. M. (2006). Infection of human dendritic cells with a Mycobacterium tuberculosis sigE mutant stimulates production of high levels of interleukin-10 but low levels of CXCL10: Impact on the T-cell response. *Infection and Immunity*, *74*(6), 3296–3304. https://doi.org/10.1128/IAI.01687-05

Goh, K. S., & Rastogi, N. (2010). Simple and rapid method for detection of nitrate reductase activity of Mycobacterium tuberculosis and Mycobacterium canettii grown in the Bactec MGIT960 system. *Journal of Microbiological Methods*, *81*(2),

208–210. https://doi.org/10.1016/j.mimet.2010.03.005

Gori, A., Bandera, A., Marchetti, G., Esposti, A. D., Catozzi, L., Nardi, G. P., Franzetti, F. (2005). Spoligotyping and Mycobacterium tuberculosis. *Emerging Infectious Diseases*, *11*(8), 1242–1248. https://doi.org/10.3201/eid1108.040982

Gurung, R., Bhattacharya, S. K., Pradhan, B., Gurung, S., & Singh, Y. I. (2010). Phenotypic characterisation and drug sensitivity testing of mycobacteria isolated from extra-pulmonary tuberculosis. *Kathmandu University Medical Journal (KUMJ)*, *8*(29), 57–61.

Guth, A. M., Janssen, W. J., Bosio, C. M., Crouch, E. C., Henson, P. M., & Dow, S. W. (2009). Lung environment determines unique phenotype of alveolar macrophages. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *296*(6), L936–L946. https://doi.org/10.1152/ajplung.90625.2008

Halse, T. A., Escuyer, V. E., & Musser, K. A. (2011). Evaluation of a single-tube multiplex real-time PCR for differentiation of members of the Mycobacterium tuberculosis complex in clinical specimens. *Journal of Clinical Microbiology*, *49*(7), 2562–2567. https://doi.org/10.1128/jcm.00467-11

Halse, T. A., Escuyer, V. E., & Musser, K. A. (2011). Evaluation of a single-tube multiplex real-time PCR for differentiation of members of the Mycobacterium tuberculosis complex in clinical specimens. *Journal of Clinical Microbiology*, *49*(7), 2562–2567. https://doi.org/10.1128/JCM.00467-11

Hampshire, T., Soneji, S., Bacon, J., James, B. W., Hinds, J., Laing, K., Butcher, P. D.

(2004). Stationary phase gene expression of Mycobacterium tuberculosis following a progressive nutrient depletion: A model for persistent organisms. *Tuberculosis*, *84*(3-4), 228–238. https://doi.org/10.1016/j.tube.2003.12.010

Hermans, P. W. M., Van Soolingen, D., Dale, J. W., Schuitema, A. R. J., McAdam, R. A., Catty, D., & Van Embden, J. D. A. (1990). Insertion element IS986 from Mycobacterium tuberculosis: A useful tool for diagnosis and epidemiology of tuberculosis. *Journal of Clinical Microbiology*, *28*(9), 2051–2058.

Hershberg, R., Lipatov, M., Small, P. M., Sheffer, H., Niemann, S., Homolka, S., Gagneux, S. (2008). High functional diversity in Mycobacterium tuberculosis driven by genetic drift and human demography. *PLoS Biology*, *6*(12), 2658–2671. https://doi.org/10.1371/journal.pbio.0060311

Holmes, P., & Faulks, L. (1981). Presentation of pulmonary tuberculosis. *Australian and New Zealand Journal of Medicine*, *11*(6), 651–3.

Homolka, S., Projahn, M., Feuerriegel, S., Ubben, T., Diel, R., Nubel, U., & Niemann, S. (2012). High resolution discrimination of clinical Mycobacterium tuberculosis complex strains based on single nucleotide polymorphisms. *PLoS One*, *7*(7), e39855. https://doi.org/10.1371/journal.pone.0039855\rPONE-D-12-02200

Huang, Z. K., Luo, Q., Jiang, B. X., Li, W. T., Xu, X. M., Xiong, G. L., & Li, J. M. (2013). Performance of the microscopic observation drug susceptibility assay in pyrazinamide susceptibility testing for Mycobacterium tuberculosis. *Chinese Medical Journal*, *126*(22), 4334–4339. https://doi.org/10.3760/cma.j.issn.0366-6999.20130108

Huard, R. C., De Oliveira Lazzarini, L. C., Butler, W. R., Van Soolingen, D., & Ho, J. L. (2003). PCR-based method to differentiate the subspecies of the Mycobacterium tuberculosis complex on the basis of genomic deletions. *Journal of Clinical Microbiology*, *41*(4), 1637–1650. https://doi.org/10.1128/JCM.41.4.1637-1650.2003

Huizinga, T., Nigrovic, P., Ruderman, E., & Schulze-Koops, H. (2011). Intravenous gammaglobulin suppresses inflammation through a novel T(H)2 pathway: Commentary. *International Journal of Advances in Rheumatology*. https://doi.org/10.1038/nature10134

Isabel, O., Gomez, A. B., Kremer, K., de Haas, P., García, M. J., Martín, C., & van Soolingen, D. (2008). Mapping of IS6110 insertion sites in Mycobacterium bovis isolates in relation to adaptation from the animal to human host. *Veterinary Microbiology*, *129*(3-4), 333–341. https://doi.org/10.1016/j.vetmic.2007.11.038

Ismail, I., & Bulgiba, A. (2013). Predictors of Death during Tuberculosis Treatment in TB/HIV Co-Infected Patients in Malaysia. *PLoS ONE*, *8*(8). https://doi.org/10.1371/journal.pone.0073250

Jones-López, E. C., Ayakaka, I., Levin, J., Reilly, N., Mumbowa, F., Dryden-Peterson, S., Mugerwa, R. D. (2011). Effectiveness of the standard WHO recommended retreatment regimen (Category II) for tuberculosis in Kampala, Uganda: A prospective cohort study. *PLoS Medicine*, *8*(3). https://doi.org/10.1371/journal.pmed.1000427

Kamerbeek, J., Schouls, L., Kolk, A., van Agterveld, M., van Soolingen, D., Kuijper, S., van Embden, J. (1997). Simultaneous detection and strain differentiation of

Mycobacterium tuberculosis for diagnosis and epidemiology. *Journal of Clinical Microbiology*, *35*(4), 907–14. https://doi.org/10.1016/S0305-4403(02)00239-X

Keane, J., Balcewicz-Sablinska, M. K., Remold, H. G., Chupp, G. L., Meek, B. B., Fenton, M. J., & Kornfeld, H. (1997). Infection by Mycobacterium tuberculosis promotes human alveolar macrophage apoptosis. *Infection and Immunity*, *65*(1), 298–304. https://doi.org/8975927

Kempsell, K. E., Ji, Y. E., Estrada, I. C., Colston, M. J., & Cox, R. a. (1992). The nucleotide sequence of the promoter, 16S rRNA and spacer region of the ribosomal RNA operon of Mycobacterium tuberculosis and comparison with Mycobacterium leprae precursor rRNA. *Journal of General Microbiology*, *138 Pt 8*(November 2015), 1717–1727. https://doi.org/10.1099/00221287-138-8-1717

Kent, L., McHugh, T. D., Billington, O., Dale, J. W., & Gillespie, S. H. (1995). Demonstration of homology between IS6110 of Mycobacterium tuberculosis and DNAs of other Mycobacterium spp. *Journal of Clinical Microbiology*, *33*(9), 2290–2293.

Kieser, K. J., & Rubin, E. J. (2014). How sisters grow apart : mycobacterial growth and division. *Nature Reviews Microbiology* 12, 550–562 (2014) *12*(August). https://doi.org/10.1038/nrmicro3299

Kimerling, M. E., Kluge, H., Vezhnina, N., Iacovazzi, T., Demeulenaere, T., Portaels, F., & Matthys, F. (1999). Inadequacy of the current WHO re-treatment regimen in a central Siberian prison: Treatment failure and MDR-TB. *International Journal of Tuberculosis and Lung Disease*, *3*(5), 451–453.

Kingston, W. (2004). Streptomycin, Schatz v. Waksman, and the balance of credit for discovery. *Journal of the History of Medicine and Allied Sciences*. https://doi.org/10.1093/jhmas/59.3.441

Kleger, B., & Kantor, R. J. (1996). Diagnosis, treatment of TB: state lab is partner. *Pennsylvania Medical Society*, *99*(11), 28–29.

Koch, R. (1932). Die Aetiologie der Tuberkulose. *Klinische Wochenschrift*, *11*(12), 490–492. https://doi.org/10.1007/BF01765224

Lari, N., Rindi, L., Lami, C., & Garzelli, C. (1999). IS6110-based restriction fragment length polymorphism (RFLP) analysis of Mycobacterium tuberculosis H37Rv and H37Ra. *Microbial Pathogenesis*, *26*(5), 281–6. https://doi.org/10.1006/mpat.1998.0270

Lewandowski, C. M., Co-investigator, N., & Lewandowski, C. M. (2015). *WHO Glocal tuberculosis report 2015*. *The effects of brief mindfulness intervention on acute pain experience: An examination of individual difference* (Vol. 1). https://doi.org/10.1017/CBO9781107415324.004

Lienhardt, C., & Ogden, J. A. (2004). Tuberculosis control in resource-poor countries: Have we reached the limits of the universal paradigm? *Tropical Medicine and International Health*. https://doi.org/10.1111/j.1365-3156.2004.01273.x

Lin, P. L., & Flynn, J. L. (2010). Understanding Latent Tuberculosis: A Moving Target. *The Journal of Immunology*, *185*(12), 15–22. https://doi.org/10.4049/jimmunol.0903856

Luca, S., & Mihaescu, T. (2013). History of BCG Vaccine. *Mædica*, *8*(1), 53–8.

MacGurn, J. A., & Cox, J. S. (2007). A genetic screen for Mycobacterium tuberculosis mutants defective for phagosome maturation arrest identifies components of the ESX-1 secretion system. *Infection and Immunity*, *75*(6), 2668–2678. https://doi.org/10.1128/IAI.01872-06

Maitra, A., & Bhakta, S. (2014). TB Summit 2014: prevention, diagnosis, and treatment of tuberculosis-a meeting report of a Euroscicon conference. *Virulence*, *5*(5), 638–644. https://doi.org/10.4161/viru.29803

Malhotra, S., Cook, V. J., Wolfe, J. N., Tang, P., Elwood, K., & Sharma, M. K. (2010). A mutation in Mycobacterium tuberculosis rpoB gene confers rifampin resistance in three HIV-TB cases. *Tuberculosis*, *90*(2), 152–157. https://doi.org/10.1016/j.tube.2010.01.001

Malik, Z., Iyer, S. S., & Kusner, D. J. (2001). Mycobacterium tuberculosis phagosomes exhibit altered calmodulin-dependent signal transduction: contribution to inhibition of phagosome-lysosome fusion and intracellular survival in human macrophages. *Journal of Immunology (Baltimore, Md. : 1950)*, *166*(5), 3392–3401. https://doi.org/10.4049/jimmunol.166.5.3392

Manca, C., Reed, M. B., Freeman, S., Mathema, B., Kreiswirth, B., Barry, C. E., & Kaplan, G. (2004). Differential monocyte activation underlies strain-specific Mycobacterium tuberculosis pathogenesis. *Infection and Immunity*, *72*(9), 5511–5514. https://doi.org/10.1128/IAI.72.9.5511-5514.2004

Manca, C., Tsenova, L., Barry, C. E., Bergtold, A., Freeman, S., Haslett, P. A. J., Kaplan, G. (1999). Mycobacterium tuberculosis CDC1551 Induces a More Vigorous Host Response In Vivo and In Vitro, But Is Not More Virulent Than Other Clinical Isolates. *The Journal of Immunology*, *162*(11), 6740–6746.

Marais, B. J., Obihara, C. C., Gie, R. P., Schaaf, H. S., Hesseling, A. C., Lombard, C., Beyers, N. (2005). The prevalence of symptoms associated with pulmonary tuberculosis in randomly selected children from a high burden community. *Archives of Disease in Childhood*, *90*(11), 1166–1170. https://doi.org/10.1136/adc.2004.060640

Mathema, B., Kurepina, N. E., Bifani, P. J., & Kreiswirth, B. N. (2006). Molecular epidemiology of tuberculosis: Current insights. *Clinical Microbiology Reviews*. https://doi.org/10.1128/CMR.00061-05

Matheson, C. D., Vernon, K. K., Lahti, A., Fratpietro, R., Spigelman, M., Gibson, S., … Donoghue, H. D. (2009). Molecular exploration of the first-century tomb of the shroud in Akeldama, Jerusalem. *PLoS ONE*, *4*(12). https://doi.org/10.1371/journal.pone.0008319

Matteelli, A., Roggi, A., & Carvalho, A. C. (2014). Extensively drug-resistant tuberculosis: epidemiology and management. *Clinical Epidemiology*, *6*, 111–118. https://doi.org/10.2147/CLEP.S35839

Mazars, E., Lesjean, S., Banuls, A. L., Gilbert, M., Vincent, V., Gicquel, B., Supply, P. (2001). High-resolution minisatellite-based typing as a portable approach to global analysis of Mycobacterium tuberculosis molecular epidemiology. *Proceedings of the*

National Academy of Sciences of the United States of America, *98*(4), 1901–6. https://doi.org/10.1073/pnas.98.4.1901

Meyer, C. G., Scarisbrick, G., Niemann, S., Browne, E. N. L., Chinbuah, M. A., Gyapong, J., Horstmann, R. D. (2008). Pulmonary tuberculosis: Virulence of Mycobacterium africanum and relevance in HIV co-infection. *Tuberculosis*, *88*(5), 482–489. https://doi.org/10.1016/j.tube.2008.05.004

Mfinanga, G. S., Ngadaya, E., Mtandu, R., Mutayoba, B., Basra, D., Kimaro, G., Kitua, a Y. (2007). The quality of sputum smear microscopy diagnosis of pulmonary tuberculosis in Dar es Salaam, Tanzania. *Tanzania Health Research Bulletin*, *9*(3), 164–168. https://doi.org/10.4314/thrb.v9i3.14323

Migliori, G. B., Lange, C., Centis, R., Sotgiu, G., Mütterlein, R., Hoffmann, H., Mariandyshev, A. (2008). Resistance to second-line injectables and treatment outcomes in multidrug-resistant and extensively drug-resistant tuberculosis cases. *European Respiratory Journal*, *31*(6), 1155–1159. https://doi.org/10.1183/09031936.00028708

Mitchell, A. M., & Mitchell, T. J. (2010). Streptococcus pneumoniae: Virulence factors and variation. *Clinical Microbiology and Infection*. https://doi.org/10.1111/j.1469-0691.2010.03183.x

Mostowy, S., Onipede, A., Gagneux, S., Niemann, S., Kremer, K., Desmond, E. P., Behr, M. (2004). Genomic analysis distinguishes Mycobacterium africanum. *Journal of Clinical Microbiology*, *42*(8), 3594–3599. https://doi.org/10.1128/JCM.42.8.3594-3599.2004

Myneedu, V. P., Verma, A. K., Sharma, P. P., & Behera, D. (2011). A pilot study of same day sputum smear examination, its feasibility and usefulness in diagnosis of pulmonary TB. *Indian Journal of Tuberculosis*, *58*(4), 160–167.

Nagaraja, S. B., Satyanarayana, S., Chadha, S. S., Kalemane, S., Jaju, J., Achanta, S., Singh, S. K. (2011). How do patients who fail first-line tb treatment but who are not placed on an mdr-tb regimen fare in south india? *PLoS ONE*, *6*(10). https://doi.org/10.1371/journal.pone.0025698

Nikaido, H. (2009). Multidrug Resistance in Bacteria. *Annual Review of Biochemistry*, *78*(1), 119–146. https://doi.org/10.1146/annurev.biochem.78.082907.145923

Ntoumi, F., Kaleebu, P., Macete, E., Mfinanga, S., Chakaya, J., Yeboah-Manu, D., Zumla, A. (2016). Taking forward the World TB Day 2016 theme “Unite to End Tuberculosis” for the WHO Africa Region. *International Journal of Infectious Diseases*, *46*, 34–37. https://doi.org/10.1016/j.ijid.2016.03.003

Olaru, I. D., Heyckendorf, J., Grossmann, S., & Lange, C. (2014). Time to culture positivity and sputum smear microscopy during tuberculosis therapy. *PLoS ONE*, *9*(8). https://doi.org/10.1371/journal.pone.0106075

Otchere, I., Asante-Poku, A., Osei-Wusu, S., Aboagye, S., & Yeboah-Manu, D. (2017). Isolation and characterization of nontuberculous mycobacteria from patients with pulmonary tuberculosis in Ghana. *The International Journal of Mycobacteriology*, *6*(1), 70. https://doi.org/10.4103/2212-5531.201895

Page, B., Page, M., & Noel, C. (1993). A new fluorometric assay for cytotoxicity measurements in vitro. *International Journal of Oncology*, *3*(3), 473–476. https://doi.org/10.3892/ijo.3.3.473

Payne, D. J., Gwynn, M. N., Holmes, D. J., & Pompliano, D. L. (2007). Drugs for bad bugs: confronting the challenges of antibacterial discovery. *Nature Reviews Drug Discovery*, *6*(1), 29–40. https://doi.org/10.1038/nrd2201

Polkade, A. V., Mantri, S. S., Patwekar, U. J., & Jangid, K. (2016). Quorum sensing: An under-explored phenomenon in the phylum Actinobacteria. *Frontiers in Microbiology*. https://doi.org/10.3389/fmicb.2016.00131

Portevin, D., Gagneux, S., Comas, I., & Young, D. (2011). Human macrophage responses to clinical isolates from the Mycobacterium tuberculosis complex discriminate between ancient and modern lineages. *PLoS Pathogens*, *7*(3). https://doi.org/10.1371/journal.ppat.1001307

Prasanthi, K., & Kumari, A. R. (2005). Efficacy of fluorochrome stain in the diagnosis of pulmonary tuberculosis co-infected with HIV. *Indian Journal of Medical Microbiology*, *23*(3), 179–81. https://doi.org/10.4103/0255-0857.16591

Ramakrishnan, L. (2012). Revisiting the role of the granuloma in tuberculosis. *Nature Reviews. Immunology*, *12*(5), 352–66. https://doi.org/10.1038/nri3211

Riccardi, G., & Pasca, M. R. (2014). Trends in discovery of new drugs for tuberculosis therapy. *The Journal of Antibiotics*, *67*(July), 1–5. https://doi.org/10.1038/ja.2014.109

Richter, E., Weizenegger, M., Fahr, A. M., & Rüsch-Gerdes, S. (2004). Usefulness of the GenoType MTBC assay for differentiating species of the Mycobacterium tuberculosis complex in cultures obtained from clinical specimens. *Journal of Clinical Microbiology*, *42*(9), 4303–4306. https://doi.org/10.1128/JCM.42.9.4303-4306.2004

Richter, E., Weizenegger, M., Rüsch-Gerdes, S., & Niemann, S. (2003). Evaluation of genotype MTBC assay for differentiation of clinical Mycobacterium tuberculosis complex isolates. *Journal of Clinical Microbiology*, *41*(6), 2672–2675. https://doi.org/10.1128/JCM.41.6.2672

Riedman, M. (1990). *Pinnipeds, Seals, Sea Lions and Walruses*. *University of California Press*. https://doi.org/10.1016/0169-5347(90)90125-W

Roth, A., Garly, M. L., Jensen, H., Nielsen, J., & Aaby, P. (2006). Bacillus Calmette-Guérin vaccination and infant mortality. *Expert Review of Vaccines*, *5*(2), 277–93. https://doi.org/10.1586/14760584.5.2.277

Russell, D. G., Cardona, P.-J., Kim, M.-J., Allain, S., & Altare, F. (2009). Foamy macrophages and the progression of the human tuberculosis granuloma. *Nature Immunology.*, *10*(9), 943–948. https://doi.org/10.1038/ni.1781

Safianowska, A., Walkiewicz, R., Nejman-Gryz, P., Chazan, R., & Grubek-Jaworska, H. (2009). Diagnostic utility of the molecular assay GenoType MTBC (HAIN Lifescience, Germany) for identification of tuberculous mycobacteria. *Pneumonologia I Alergologia Polska*, *77*(6), 517–520.

Schatz, A., Bugle, E., & Waksman, S. A. (1944). Streptomycin, a substance exhibiting antibiotic activity against Gram-positive and Gram-negative bacteria. *Experimental Biology and Medicine*, *55*(1), 66–69. https://doi.org/10.3181/00379727-55-14461

Scherr, N., & Nguyen, L. (2009). Mycobacterium versus Streptomyces-we are different, we are the same. *Current Opinion in Microbiology*. https://doi.org/10.1016/j.mib.2009.10.003

Serbina, N. V., Salazar-Mather, T. P., Biron, C. A., Kuziel, W. A., & Pamer, E. G. (2003). TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection. *Immunity*, *19*(1), 59–70. https://doi.org/10.1016/S1074-7613(03)00171-7

Sharma, S. K., & Mohan, A. (2004). Extrapulmonary tuberculosis. *The Indian Journal of Medical Research*, *120*(4), 316–53. https://doi.org/10.1016/j.mpmed.2013.10.008

Sharma, S. K., Mohan, A., & Kadhiravan, T. (2005). HIV-TB co-infection: Epidemiology, diagnosis & management. *Indian Journal of Medical Research*.

Somoskovi, A., Dormandy, J., Rivenburg, J., Pedrosa, M., McBride, M., & Salfinger, M. (2008). Direct comparison of the genotype MTBC and genomic deletion assays in terms of ability to distinguish between members of the Mycobacterium tuberculosis complex in clinical isolates and in clinical specimens. *Journal of Clinical Microbiology*, *46*(5), 1854–1857. https://doi.org/10.1128/JCM.00105-07

Somoskovi, A., Song, Q., Mester, J., Tanner, C., Hale, Y. M., Parsons, L. M., & Salfinger, M. (2003). Use of molecular methods to identify the Mycobacterium

tuberculosis complex (MTBC) and other mycobacterial species and to detect rifampin resistance in MTBC isolates following growth detection with the BACTEC MGIT 960 system. *Journal of Clinical Microbiology*, *41*(7), 2822–2826.

Spellberg, B., & Edwards, J. E. (2001). Type 1/Type 2 immunity in infectious diseases. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America*, *32*, 76–102. https://doi.org/10.1086/317537

Squibb, B., Index, M., & Index, M. (2008). Isoniazid. *Tuberculosis*, *88*(2), 112–116. https://doi.org/10.1016/S1472-9792(08)70011-8

Stahl, D. A., & Urbance, J. W. (1990). The division between fast- and slow-growing species corresponds to natural relationships among the mycobacteria. *Journal of Bacteriology*, *172*(1), 116–124. https://doi.org/10.1128/JB.172.1.116-124.1990

Sullivan, Z. A., Wong, E. B., Ndung'u, T., Kasprowicz, V. O., & Bishai, W. R. (2015). Latent and Active Tuberculosis Infection Increase Immune Activation in Individuals Co-Infected with HIV. *EBioMedicine*, *2*(4), 334–340. https://doi.org/10.1016/j.ebiom.2015.03.005

Tansuphasiri, U., Boonrat, P., & Rienthong, S. (2004). Direct Identification of Mycobacterium Tuberculosis from Sputum on Ziehl-Neelsen Acid Fast Stained Slides by Use of Silica-Based Filter Combined with Polymerase Chain Reaction Assay. *Journal of the Medical Association of Thailand*, *87*(2), 180–189.

Thierry, D., Brisson-Noël, A., Vincent-Lévy-Frébault, V., Nguyen, S., Guesdon, J. L., & Gicquel, B. (1990). Characterization of a Mycobacterium tuberculosis insertion

sequence, IS6110, and its application in diagnosis. *Journal of Clinical Microbiology*, *28*(12), 2668–73.

Tsenova, L., Ellison, E., Harbacheuski, R., Moreira, A. L., Kurepina, N., Reed, M. B., Kaplan, G. (2005). Virulence of selected Mycobacterium tuberculosis clinical isolates in the rabbit model of meningitis is dependent on phenolic glycolipid produced by the bacilli. *The Journal of Infectious Diseases*, *192*(1), 98–106. https://doi.org/10.1086/430614

Uplekar, M., Weil, D., Lonnroth, K., Jaramillo, E., Lienhardt, C., Dias, H. M., Raviglione, M. (2015). WHO's new end TB strategy. *The Lancet*. https://doi.org/10.1016/S0140-6736(15)60570-0

Valway SE, Greifinger RB, M Papania, Kilburn JO, C Woodley, DiFerdinando GT, & Dooley SW. (1994). Multidrug-resistant tuberculosis in the New York State prison system, 1990-1991. *The Journal of Infectious Diseases*, *170*(1), 151–6.

van der Wel, N., Hava, D., Houben, D., Fluitsma, D., van Zon, M., Pierson, J., Peters, P. J. (2007a). M. tuberculosis and M. leprae Translocate from the Phagolysosome to the Cytosol in Myeloid Cells. *Cell*, *129*(7), 1287–1298. https://doi.org/10.1016/j.cell.2007.05.059

van der Wel, N., Hava, D., Houben, D., Fluitsma, D., van Zon, M., Pierson, J., Peters, P. J. (2007b). M-tuberculosis and M-leprae translocate from the phagolysosome to the cytosol in myeloid cells. *Cell*, *129*(7), 1287–1298. https://doi.org/10.1016/j.cell.2007.05.059

Van Embden, J. D. A., Van Gorkom, T., Kremer, K., Jansen, R., Van Der Zeijst, B. A. M., & Schouls, L. M. (2000). Genetic variation and evolutionary origin of the direct repeat locus of Mycobacterium tuberculosis complex bacteria. *Journal of Bacteriology*, *182*(9), 2393–2401. https://doi.org/10.1128/JB.182.9.2393-2401.2000

van Ingen, J., Rahim, Z., Mulder, A., Boeree, M. J., Simeone, R., Brosch, R., & van Soolingen, D. (2012). Characterization of Mycobacterium orygis as M. tuberculosis complex subspecies. *Emerging Infectious Diseases*, *18*(4), 653–655. https://doi.org/10.3201/eid1804.110888

Vasconcellos, S. E. G., Huard, R. C., Niemann, S., Kremer, K., Santos, A. R., Suffys, P. N., & Ho, J. L. (2010). Distinct genotypic profiles of the two major clades of Mycobacterium africanum. *BMC Infectious Diseases*, *10*(1), 80. https://doi.org/10.1186/1471-2334-10-80

Velayati, A. A., Masjedi, M. R., Farnia, P., Tabarsi, P., Ghanavi, J., ZiaZarifi, A. H., & Hoffner, S. E. (2009). Emergence of new forms of totally drug-resistant tuberculosis bacilli: Super extensively drug-resistant tuberculosis or totally drug-resistant strains in Iran. *Chest*, *136*(2), 420–425. https://doi.org/10.1378/chest.08-2427

Ventura, M., Canchaya, C., Tauch, A., Chandra, G., Fitzgerald, G. F., Chater, K. F., & van Sinderen, D. (2007). Genomics of Actinobacteria: tracing the evolutionary history of an ancient phylum. *Microbiology and Molecular Biology Reviews : MMBR*, *71*(3), 495–548. https://doi.org/10.1128/MMBR.00005-07

Vernon, A. (2013). Treatment of latent tuberculosis infection. *Seminars in Respiratory and Critical Care Medicine*, *34*(1), 67–86. https://doi.org/10.1055/s-0032-1333544

Viana-Niero, C., Gutierrez, C., Sola, C., Filliol, I., Boulahbal, F., Vincent, V., & Rastogi, N. (2001). Genetic diversity of Mycobacterium africanum clinical isolates based on IS6110-restriction fragment length polymorphism analysis, spoligotyping, and variable number of tandem DNA repeats. *Journal of Clinical Microbiology*, *39*(1), 57–65. https://doi.org/10.1128/JCM.39.1.57-65.2001

Vicenzi, E., Alfano, M., Ghezzi, S., & Poli, G. (2007). Immunopathogenesis of HIV infection. In *The Biology of Dendritic Cells and HIV Infection* (pp. 245–295). https://doi.org/10.1007/978-0-387-33785-2_7

Wampande, E. M., Mupere, E., Jaganath, D., Nsereko, M., Mayanja, H. K., Eisenach, K., Joloba, M. L. (2015). Distribution and transmission of Mycobacterium tuberculosis complex lineages among children in peri-urban Kampala, Uganda. *BMC Pediatrics*, *15*, 140. https://doi.org/10.1186/s12887-015-0455-z

Wanchu, A., Dong, Y., Sethi, S., Myneedu, V. P., Nadas, A., Liu, Z., Laal, S. (2008). Biomarkers for clinical and incipient tuberculosis: Performance in a TB-endemic country. *PLoS ONE*, *3*(4). https://doi.org/10.1371/journal.pone.0002071

Warren, R. M., Streicher, E. M., Sampson, S. L., van der Spuy, G. D., Richardson, M., Nguyen, D., van Helden, P. D. (2002). Microevolution of the direct repeat region of Mycobacterium tuberculosis: implications for interpretation of spoligotyping data. *Journal of Clinical Microbiology*, *40*(12), 4457–4465.

Waters, W. R., Nonnecke, B. J., Palmer, M. V, Robbe-Austermann, S., Bannantine, J. P., Stabel, J. R., Minion, F. C. (2004). Use of recombinant ESAT-6:CFP-10 fusion protein for differentiation of infections of cattle by Mycobacterium bovis and by M.

avium subsp. avium and M. avium subsp. paratuberculosis. *Clinical and Diagnostic Laboratory Immunology*, *11*(4), 729–35. https://doi.org/10.1128/CDLI.11.4.729-735.2004

Wells, C. D., Cegielski, J. P., Nelson, L. J., Laserson, K. F., Holtz, T. H., Finlay, A., Weyer, K. (2007). HIV infection and multidrug-resistant tuberculosis: the perfect storm. *The Journal of Infectious Diseases*, *196 Suppl* (Supplement 1), S86–107. https://doi.org/10.1086/518665

Who, & The World Health Organization. (2010). Treatment of tuberculosis: guidelines. In *4Th Edition* (p. 160). https://doi.org/10.1164/rccm.201012-1949OC

Wirth, T., Hildebrand, F., Allix-Béguec, C., Wölbeling, F., Kubica, T., Kremer, K., Niemann, S. (2008). Origin, spread and demography of the Mycobacterium tuberculosis complex. *PLoS Pathogens*, *4*(9). https://doi.org/10.1371/journal.ppat.1000160

Woldeyohannes, D., Kebede, N., Erku, W., & Tadesse, Z. (2011). Ten years experience of directly observed treatment short-course (dots) therapy for tuberculosis in Addis Ababa, Ethiopia. *Ethiopian Medical Journal*, *49*(3), 221–9.

World Health Organization. (2016). WHO treatment guidelines for drug-resistant tuberculosis. *Geneva, Switzerland:*

World Health Organization, (WHO). (2016). *WHO | Global tuberculosis report 2016*. *WHO*. https://doi.org/ISBN 978 92 4 156539 4

World Health Organization, W. (2016). World Health Organization (Who) Country

Office for Ghana Annual Report 2015, (May).

Yeboah-Manu, D., Asante-Poku, A., Bodmer, T., Stucki, D., Koram, K., Bonsu, F., Gagneux, S. (2011). Genotypic diversity and drug susceptibility patterns among M. tuberculosis complex isolates from South-Western Ghana. *PLoS ONE*, *6*(7). https://doi.org/10.1371/journal.pone.0021906

Zalesky, R., Abdullajev, F., Khechinashvili, G., Safarian, M., Madaras, T., Grzemska, M., Raviglione, M. (1999). Tuberculosis control in the Caucasus: Successes and constraints in DOTS implementation. *International Journal of Tuberculosis and Lung Disease*, *3*(5), 394–401.

Zink, A. R., Moln, E., Motamedi, N., Plfy, G., Marcsik, A., & Nerlich, A. G. (2007). Molecular history of tuberculosis from ancient mummies and skeletons. In *International Journal of Osteoarchaeology* (Vol. 17, pp. 380–391). https://doi.org/10.1002/oa.909

Zink, A. R., Sola, C., Reischl, U., Grabner, W., Rastogi, N., Wolf, H., & Nerlich, A. G. (2003). Characterization of Mycobacterium tuberculosis complex DNAs from Egyptian mummies by spoligotyping. *Journal of Clinical Microbiology*, *41*(1), 359–367. https://doi.org/10.1128/JCM.41.1.359-367.2003

APPENDICES

Appendix 1-0

Composition of Primary stain Carbol fuchsin

Carbol Fuchsin (0.3%)	
Basic Fuchsin	0.3g
95% Ethanol	100mL
Phenol Crystals	50g
Distilled water	900mL

Decolourizer preparation

Sulphuric acid (20%)	
Concentrated Sulphuric acid	200mL
Distilled water	800mL

Counter stain preparation

Methylene Blue (0.1%)	
Methylene blue chloride	10g
Distilled water	1000mL

Appendix 1-1

Grading of AFB smear as per W.H.O and IUATLD recommendation.

No. of Acid-Fast Bacilli (AFB)	**Fields**	**Report**
No AFB	In 100 fields	Negative
1-9 AFB	In 100 fields	Positive Scanty (Record 1-9 SC)
10-99 AFB	In 100 fields	1+
1-10 AFB	Per 50 fields	2+
More than 10 AFB	Per 20 fields	3+

Appendix 1-2

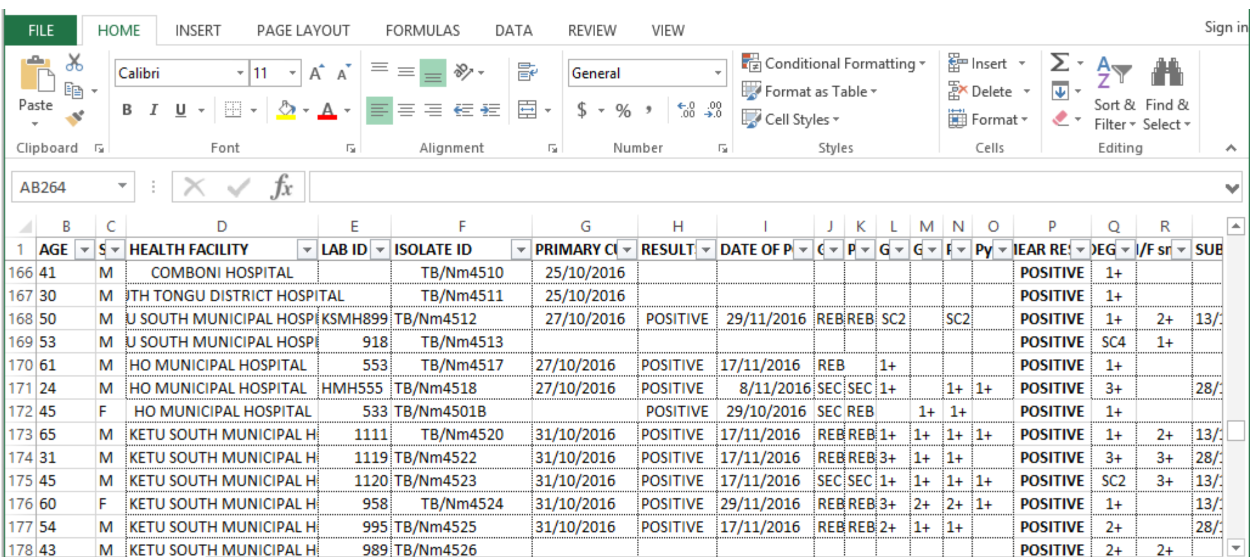
Preparation of Lowenstein Jensen (LJ) media

Constituents and their quantities used in preparing Lowenstein Jensen media

Constituent	**LJ Media Supplemented with Glycerol**	**LJ Media Supplemented with Pyruvate**
Potassium phosphate (KH_2PO_4)	0.4g	0.4g
Magnesium sulphate ($MgSO_4 . 7H_2O$)	0.04g	0.04g
Magnesium citrate	0.1g	0.1g
Asparagine	0.6g	0.6g
Glycerol/Pyruvate	2mL	1.3g
Distilled water	100mL	100mL
Egg homogenate	167mL	167mL
Malachite green (2%)	3.3mL	3.3mL
pH	7.0	7.0

Appendix 1-3

Cross-Section of patient demography linked with laboratory data



1	AGE	S	HEALTH FACILITY	LAB ID	ISOLATE ID	PRIMARY C	RESULT	DATE OF P	C	P	G	G	F	Py	IEAR RES	DEG	I/F sm	SUB
166	41	M	COMBONI HOSPITAL		TB/Nm4510	25/10/2016									POSITIVE	1+		
167	30	M	JTH TONGU DISTRICT HOSPITAL		TB/Nm4511	25/10/2016									POSITIVE	1+		
168	50	M	U SOUTH MUNICIPAL HOSPI	KSMH899	TB/Nm4512	27/10/2016	POSITIVE	29/11/2016	REB	REB	SC2		SC2		POSITIVE	1+	2+	13/1
169	53	M	U SOUTH MUNICIPAL HOSPI	918	TB/Nm4513										POSITIVE	SC4	1+	
170	61	M	HO MUNICIPAL HOSPITAL	553	TB/Nm4517	27/10/2016	POSITIVE	17/11/2016	REB		1+				POSITIVE	1+		
171	24	M	HO MUNICIPAL HOSPITAL	HMH555	TB/Nm4518	27/10/2016	POSITIVE	8/11/2016	SEC	SEC	1+		1+	1+	POSITIVE	3+		28/1
172	45	F	HO MUNICIPAL HOSPITAL	533	TB/Nm4501B		POSITIVE	29/10/2016	SEC	REB		1+	1+		POSITIVE	1+		
173	65	M	KETU SOUTH MUNICIPAL H	1111	TB/Nm4520	31/10/2016	POSITIVE	17/11/2016	REB	REB	1+	1+	1+	1+	POSITIVE	1+	2+	13/1
174	31	M	KETU SOUTH MUNICIPAL H	1119	TB/Nm4522	31/10/2016	POSITIVE	17/11/2016	REB	REB	3+	1+	1+		POSITIVE	3+	3+	28/1
175	45	M	KETU SOUTH MUNICIPAL H	1120	TB/Nm4523	31/10/2016	POSITIVE	17/11/2016	SEC	SEC	1+	1+	1+	1+	POSITIVE	SC2	3+	13/1
176	60	F	KETU SOUTH MUNICIPAL H	958	TB/Nm4524	31/10/2016	POSITIVE	29/11/2016	REB	REB	3+	2+	2+	1+	POSITIVE	1+		13/1
177	54	M	KETU SOUTH MUNICIPAL H	995	TB/Nm4525	31/10/2016	POSITIVE	17/11/2016	REB	REB	2+	1+	1+		POSITIVE	2+		28/1
178	43	M	KETU SOUTH MUNICIPAL H	989	TB/Nm4526										POSITIVE	2+	2+	

Figure 2.1: Cross-section of patient data linked with laboratory data

Appendix 1-4

Amplification and visualization of IS*6110* gene in MTBC isolates

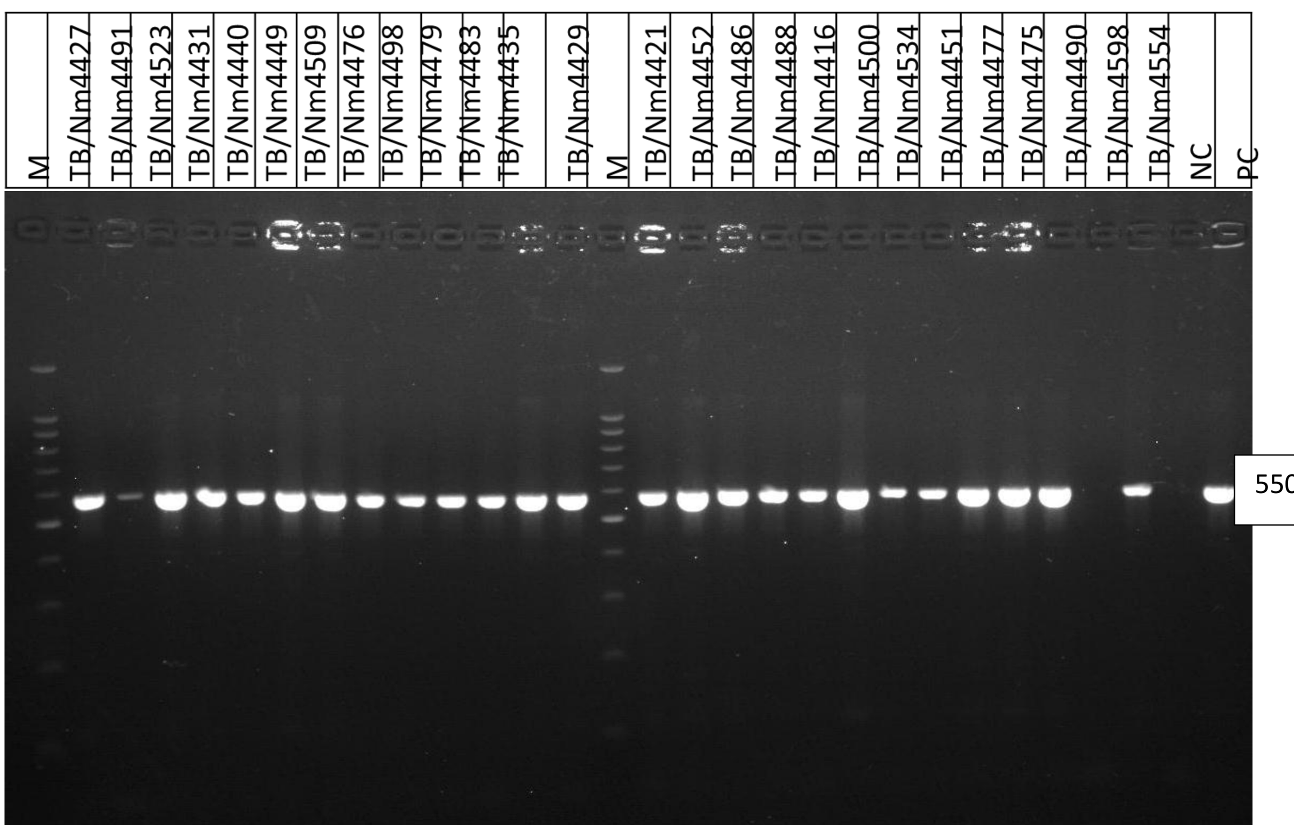


Figure 3.1: Lanes with **M** represent 100bpmolecular weight marker**,** and lanes with **TB/Nm IDs** represent mycobacteria isolates with 550bp region of IS*6110* gene amplified in MTBC isolates. Lanes **NC** (negative control) and **PC** (positive control) contain nuclease free water and H37Rv respectively.

.

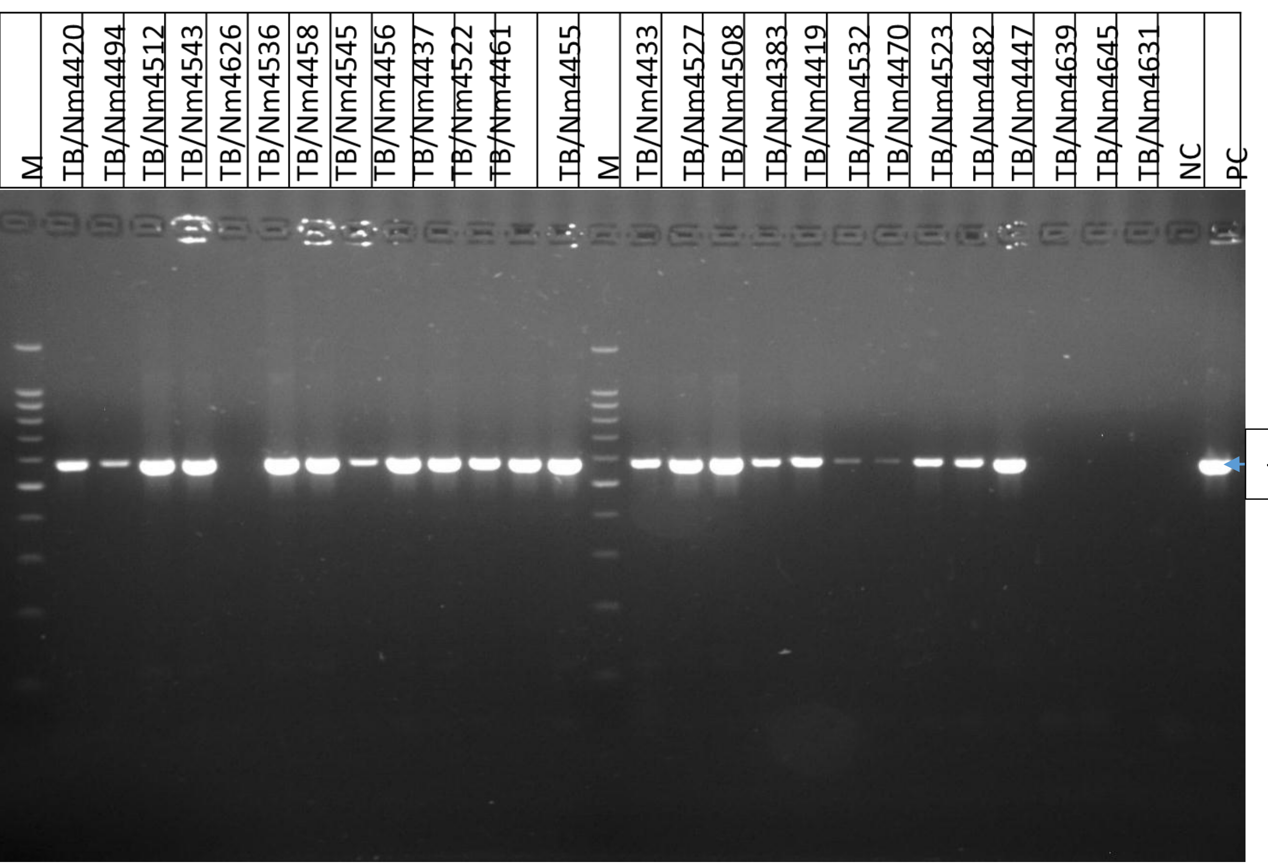


Figure 3.2: Lanes with **M** represent 100bpmolecular weight marker**,** and lanes with **TB/Nm IDs** represent mycobacteria isolates with 550bp region of IS*6110* gene amplified in MTBC isolates. Lanes **NC** (negative control) and **PC** (positive control) contain nuclease free water and H37Rv respectively.

Appendix 1-5

Amplification and visualization of spacer oligonucleotides in MTBC isolates

H37Rv

BCG

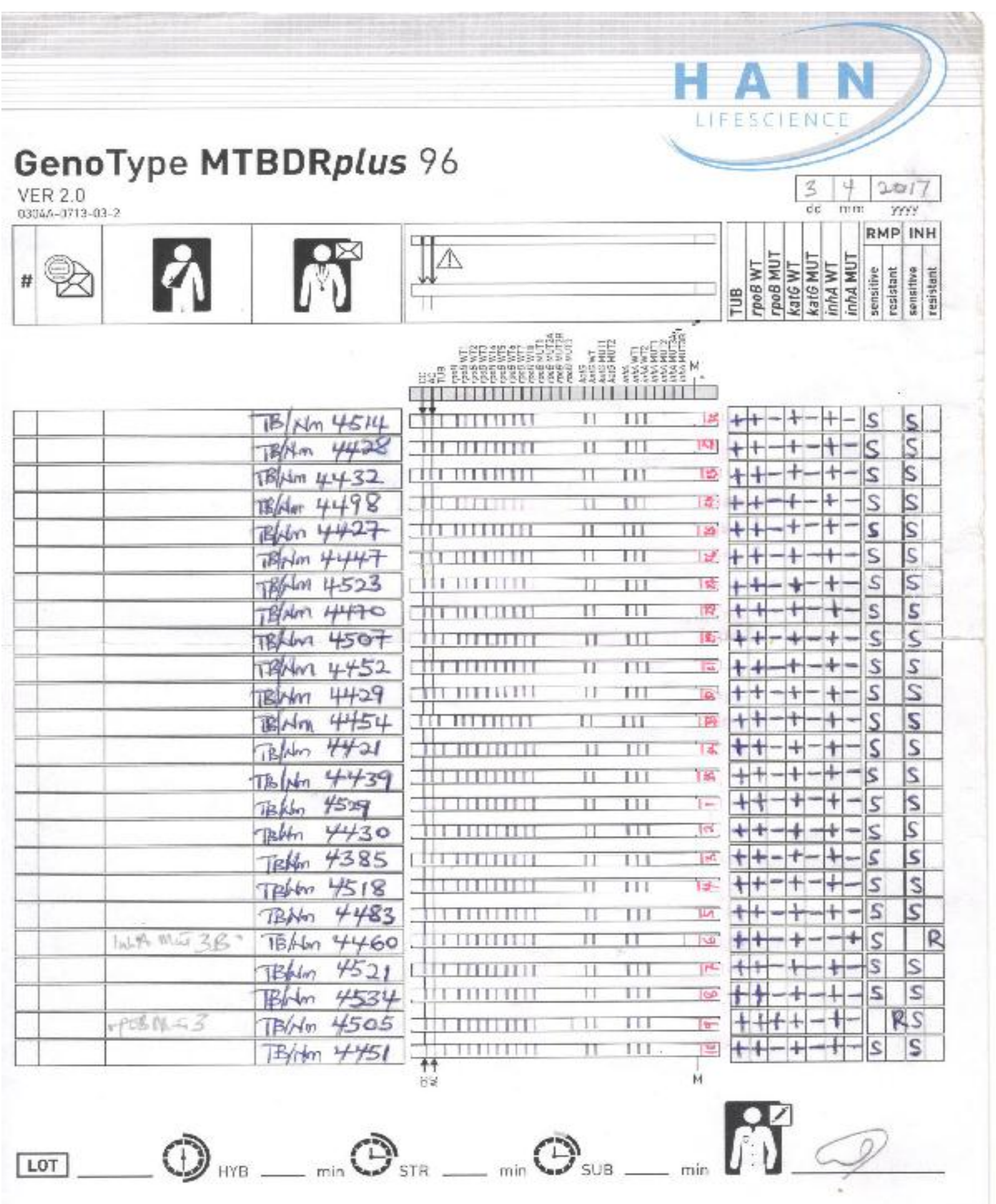
NC

MTBC isolates with TB/Nm IDs

Figure 4.1: Spoligotyping output for MTBC isolates with black squares representing amplified spacer oligonucleotides visualized after film development. Positive controls H37Rv and BCG were used and reaction reagent was used as negative control (NC).

Appendix 1-6

Genotype MTBDRplus line probe assay output and results evaluation



HAIN LIFESCIENCE

GenoType MTBDR*plus* 96

VER 2.0

03044-0713-03-2

Date: 3 / 4 / 2017 (dd mm yyyy)

#	Sample	TUB	rpoB WT	rpoB MUT	katG WT	katG MUT	inhA WT	inhA MUT	RMP sensitive	RMP resistant	INH sensitive	INH resistant
	TB/Nm 4514	+	+	−	+	−	+	−	S		S	
	TB/Nm 4428	+	+	−	+	−	+	−	S		S	
	TB/Nm 4432	+	+	−	+	−	+	−	S		S	
	TB/Nm 4498	+	+	−	+	−	+	−	S		S	
	TB/Nm 4427	+	+	−	+	−	+	−	S		S	
	TB/Nm 4447	+	+	−	+	−	+	−	S		S	
	TB/Nm 4523	+	+	−	+	−	+	−	S		S	
	TB/Nm 4470	+	+	−	+	−	+	−	S		S	
	TB/Nm 4507	+	+	−	+	−	+	−	S		S	
	TB/Nm 4452	+	+	−	+	−	+	−	S		S	
	TB/Nm 4429	+	+	−	+	−	+	−	S		S	
	TB/Nm 4454	+	+	−	+	−	+	−	S		S	
	TB/Nm 4421	+	+	−	+	−	+	−	S		S	
	TB/Nm 4439	+	+	−	+	−	+	−	S		S	
	TB/Nm 4519	+	+	−	+	−	+	−	S		S	
	TB/Nm 4430	+	+	−	+	−	+	−	S		S	
	TB/Nm 4385	+	+	−	+	−	+	−	S		S	
	TB/Nm 4518	+	+	−	+	−	+	−	S		S	
	TB/Nm 4483	+	+	−	+	−	+	−	S		S	
inhA MUT 3B	TB/Nm 4460	+	+	−	+	−	−	+	S			R
	TB/Nm 4521	+	+	−	+	−	+	−	S		S	
	TB/Nm 4534	+	+	−	+	−	+	−	S		S	
rpoB MUT 3	TB/Nm 4505	+	+	+	+	−	+	−		R	S	
	TB/Nm 4451	+	+	−	+	−	+	−	S		S	

LOT ____ HYB ____ min STR ____ min SUB ____ min

Figure 5.1: Sample of Genotype MTBDRplus line probe assay results for some MTBC isolates

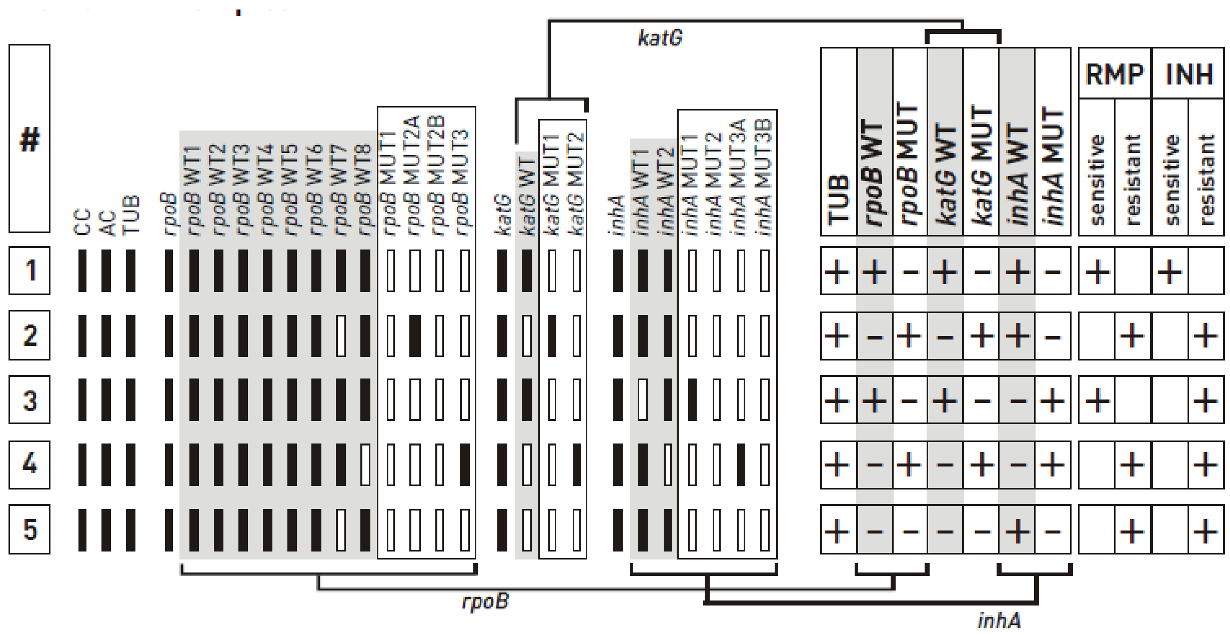


Figure 5.2: Template for evaluation of Genotype MTBDRplus line probe assay results

Appendix 1-7
Consent Form for participant

NOGUCHI MEMORIAL INSTITUTE FOR MEDICAL RESEARCH

TUBERCULOSIS MOLECULAR-EPIDEMIOLOGY

WELLCOME TRUST PROJECT

Respondent ID Number ☐☐ ☐☐☐

CONSENT FORM

Dear Participant
This is an invitation to you to participate in a study which aims to determine risk factors that may allow individuals to develop tuberculosis (TB). We will collect sputum samples from you at two different times, the same as done in the routine diagnosis of TB in Ghana. An officer will ask you a few question and this will last for about 20 minutes. He/she may contact you for additional information on your travel or social life if we find that the TB germ that infected you is similar to another person.

Possible Risks and Discomforts: Collection of sputum is considered a low-risk procedure and you will be directed by experienced laboratory technician.

Possible Benefits: You will have immediate benefit if you are found to have drug resistance TB. A resistant TB patient will have the opportunity to be transferred to a higher level for appropriate treatment.

Confidentiality: We will protect information about you and your taking part in this research to the best of our ability. You will not be named in any reports. However, the staff of the treating health facility hospital may sometimes look at your research records.

Voluntary Participation: 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 will be treated at the local hospital or clinic. If you have any problems or questions about this study, feel free to contact Dr Dorothy Yeboah-Manu – Bacteriology Department, NMIMR, Legon on 0208123882 or Dr. Audrey Forson – Chest Clinic, Korle-Bu Teaching Hospital on 0208167962

Your rights: This study has been reviewed and approved by a special body committee of NMIMR which reviews studies in order to protect participants. If you have any questions about your rights as a research participant you may Rev. Dr. Ayete-Nyampong through mobile number 0208152360.

Consent to Participant: By signing this consent indicates that you understand what will be expected of you and are willing to participate in this study.

Signatures: I hereby provide INFORMED CONSENT to take part in TB molecular epidemiology study.

________________ ________________________
Date Name and signature or mark of volunteer

witness:

________________ ________________________
Date Name and signature of witness

________________ ________________________
Date Name Signature of Person Who Obtained Consent

2

Appendix 1-8
Ethical Clearance

NOGUCHI MEMORIAL INSTITUTE FOR MEDICAL RESEARCH

Established 1979

A Constituent of the College of Health Sciences
University of Ghana

INSTITUTIONAL REVIEW BOARD

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+233-289-522574
Fax: +233-302-502182/513202
E-mail: nirb@noguchi.mimcom.org
Telex No: 2556 UGL GH

Post Office Box LG 581
Legon, Accra
Ghana

My Ref. No: DF.22
Your Ref. No:

7th March, 2012

ETHICAL CLEARANCE

FEDERALWIDE ASSURANCE FWA 00001824 **IRB 00001276**

NMIMR-IRB CPN 067/11-12 **IORG 0000908**

On 7th March, 2012, the Noguchi Memorial Institute for Medical Research (NMIMR) Institutional Review Board (IRB) at a full board meeting reviewed and approved your protocol titled:

TITLE OF PROTOCOL	:	**Understanding the genetic diversity between *Mycobacterium africanum* and *Mycobacterium tuberculosis***
PRINCIPAL INVESTIGATOR	:	**Dr. DorothyYeboah-Manu, PhD**
CO-INVESTIGATORS	:	**Dr. Audrey Forson & Dr. Frank Bonsu**

Please note that a final review report must be submitted to the Board at the completion of the study. Your research records may be audited at any time during or after the implementation.

Any modification of this research project must be submitted to the IRB for review and approval prior to implementation.

Please report all serious adverse events related to this study to NMIMR-IRB within seven days verbally and fourteen days in writing.

This certificate is valid till 6th March, 2013. You are to submit annual reports for continuing review.

Signature of Chairman: ....
Rev. Dr. Samuel Ayete-Nyampong
(NMIMR – IRB, Chairman)

cc: Professor Alexander K. Nyarko
Director, Noguchi Memorial Institute
for Medical Research, University of Ghana, Legon