Genotypic and Epidemiological Characterization of *Mycobacterium tuberculosis* Complex in Ghana

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Research Summary

Tuberculosis (TB) remains a public health challenge. In 2013, TB was estimated to have caused 9 million incident cases of which 1.1 million were coinfected with HIV and 1.5 million deaths worldwide. For the effective control of TB, the use of simplified diagnostic tools for case detection diagnosis of drug resistant TB and understanding the effects of comorbidities such as HIV on the prevalence of TB is paramount. Ghana, housing six of the seven phylogenetic lineages of *Mycobacterium tuberculosis* complex (MTBC) with high TB/HIV prevalence provides a unique opportunity to study and better understand the dynamics of TB.

In the context of TB control, we studied the level of drug resistance using phenotypic drug susceptibility testing (DST) and correlated the DST results with patient treatment outcome (Chapter 3). We found a low rate of multidrug-resistant (MDR)-TB rate (1.9%), high isoniazid (INH) mono resistance (15%) and the dependence of treatment outcome on the susceptibility to rifampicin (RIF). For the rapid diagnosis of MDR cases, we further evaluated the accuracy of a molecular base diagnostic tool (Genotype MTBDRplus) and compared it with the gold standard phenotypic DST method (Chapter4). We found 100% correlation for detection of both MDR and RIF mono resistance and 83% for INH mono resistance. The remaining 17% INH resistance detected by standard phenotypic DST but not Genotype MTBDRplus are likely due to molecular mechanisms whose targets are not interrogated by Genotype MTBDRplus. The high overall sensitivity and the relative short turn- around time of Genotype MTBDRplus makes it a valuable addition to diagnostic algorithm in Ghana.

The control of TB also depends on understanding the patterns and dynamics of TB transmission to reduce source of infection. Existing tools for studying transmission such as MIRU-15 used for routine molecular epidemiological studies have been shown to exhibit varying discriminatory power among the different human-associated MTBC lineages. We

established a robust and cost-effective PCR based reduced but lineage-specific set of MIRU-VNTR loci with high discrimination power in the main MTBC circulating in Ghana (Chapter 5). This assay will help identify risk factors that enhance transmission and patient groups at increased risk of developing TB. In addition, this assay can be used to differentiate between exogenous re-infection from true relapse cases.

SNP- based genotyping and spoligotyping established that *M. africanum* (MAF) still causes ~ 20% of all TB cases in Ghana (Chapter 6 and 7). Reasons for the restriction of MAF to West Africa have eluded researchers for many years. Using retrospective isolates, we provide for the first time plausible reason why MAF is restricted to parts of West Africa. We showed a significant association between MAF and the Ewe ethnic group. This association was confirmed using prospective isolates and supports possible host pathogen coevolution inn TB. In addition, we observed a strong association between MAF2 and HIV co-infection supporting the notion that MAF might have a lower virulence compared to other MTBC in humans.

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Abbreviations

AMK	Amikacin
BCG	Bacillus Calmette Guérin
CAP	Capreomycin
CAS	Central Asian Strain
CFP	Culture Filtrate Protein
CRISPR	Clustered Regulatory Short Palindromic Repeats
DOTS	Directly Observed Short Course Therapy
DRs	Direct Repeats
DST	Drug Susceptibility Test
EAI	East African Indian
ESAT	Early Secreted Antigen Type
ETD	Ethionamide
INH	Isoniazid
IQR	Interquartile Range
IS	Insertion Sequences
KAN	Kanamycin
LSP	large Sequence Polymorphisms
MDR	Multi- Drug Resistance
MIRU	Mycobacterial Interspersed Repetitive Unit
MTBC	*Mycobacterium tuberculosis* complex
MTBDR*sl*	*Mycobacterium tuberculosis* drug resistance second line
MTB	*Mycobacterium tuberculosis* sensu stricto
MAF	*Mycobacterium africanum*
NTMs	Non-tuberculous Mycobacteria

NTP	National Tuberculosis Control Programme
PCR	Polymerase Chain Reaction
RD	Region of Difference
RFLP	Restriction Fragment length Polymorphisms
RIF	Rifampicin
RRDR	Rifampicin Resistance Determining Region
SIT	Spoligotype International Type
SNPs	Single Nucleotide Polymorphisms
STR	Streptomycin
TDR	Totally Drug Resistance
VNTR	Variable Number of Tandem Repeats
WHO	World Health organization
XDR	Extensively Drug Resistance

Introduction

1.1. History and global burden of tuberculosis

1.1.1. Historical facts on tuberculosis

Tuberculosis (TB) is a disease of antiquity and the probability of eradicating it has been humankind's dream throughout history. Although relatively little is known about its frequency before the 19th century, its incidence in Europe and North-America is known to have peaked between the middle of the 18th century to the end of the 19th century. Over the years, the different cultures of the world gave the illness different names: *phthisis* (Greek), *consumptione* (Latin), *yaksma* (India), and *chaky oncay* (Incan), each making reference to the "drying" or "consuming" effect of the illness (**Daniel, 2006**). In the 19th century, the high mortality rate among young and middle-aged adults and the glossy dying look of the infected caused many to refer to the disease as the "romantic disease" **(Hippocrates, *Of the Epidemics*; Herzog, 1998)**.

It is presumed that the genus *Mycobacterium* originated more than 200 million years ago in East Africa, concurrently with early hominids and may have co-evolved with their host much longer than anticipated (**Daniel, 2006**; **Cave, 1939).** Initially, *Mycobacterium tuberculosis* was thought to have accompanied the Out of Africa migrations of modern humans (*Homo sapiens*) 60,000–40,000 years ago, spreading by land and sea to the rest of the world (**Hershberg *et al.*, 2008; Gutierrez *et al.*, 2005**). However, results of a recent genome study in 2014 suggested that TB is significantly younger. Using genome of the bacteria from remains of 1,000-year-old human skeletons in southern Peru, scientists found that TB was less than 6,000 years old and theorized that seals may have been the mode of transmission from Africa to South America (**Bos *et al.*, 2014**).

Understanding the disease and identification of the causative agent of TB took many years. By the end of the 19th century, several major breakthroughs by various scientists gave hope that a cause and cure might be found. One of the most important physicians frequently remembered today for his major breakthrough in understanding the disease was Rene Laennec -inventor of stethoscope in 1819 (**Daniel, 2000; Daniel, 2005**). His clear description of the pathogenesis of TB using terms still relevant today paved the way for modern understanding of TB.

In Laennec's era, the disease extended rapidly across Europe in relation with industrialization. During that period, urbanization was galloping, and poverty, poor hygienic conditions and overcrowding became the order of the day - an environment conducive for the disease to thrive. Between 1851 and 1910, the death rate due to TB soared occurring mostly in the youth; in England and Wales alone, four million aged 20 to 24 died from tuberculosis (**Bynum, 2012**), giving the disease the name '*the robber of youth'* (**Segen, 1992**).

Surrounded by so many deaths from one disease, medical practitioners and scientist sought to understand its aetiology. The history of TB was changed dramatically on March 24, 1882, with the famous Hermann Heinrich Robert Koch presentation, *Die Aetiologie der Tuberkulose,* to the Berlin Physiological Society where Dr. Koch demonstrated the infectious aetiology of the disease and presented *Mycobacterium tuberculosis* as the causative agent (**Daniel, 2005);** March 24th is recognized as the world TB day.

With the identification of TB as an infectious disease and the recognition of the illness, a new era of visualizing the disease as a public health problem began. The introduction of different anti tuberculosis agents beginning with the isolation of the first bacterial agent effective against TB*;* streptomycin first isolated from *Streptomyces griseus* in 1944 by Albert Schatz, Elizabeth Bugie and Selman Waksman **(Daniel, 2006**), followed in the 1950s and 60s by isoniazid and rifampicin came with the hope of a lesser grip of TB on humankind. However, TB never completely let go, and today, remains one of the leading infectious disease killers around the world.

1.1.2. The global burden of tuberculosis today

TB is one disease that can be found on all the continents of the world. It is the leading cause of adult mortality caused by a single infectious disease worldwide. In 2013, an estimated 9 million incident cases of TB and approximately 1.5million (including 360 000 deaths among HIV-positive people) deaths due to TB occurred worldwide (**Global TB report, 2014**). With roughly 2 billion latently infected people (one third of the world's population) providing a large reservoir for active transmission of TB that will last for decades, more stringent efforts are needed in many parts of the world to control the disease, especially Africa (**Barry *et al.*, 2009***)*.

Figure 1 depicts the global TB incidence rate in 2013.

As a poverty driven disease, the global distribution of TB cases is skewed heavily toward low-income and emerging economies. The highest prevalence of cases are in Asia, where together China, India, Bangladesh collectively make up over 56% of the global burden

(**Mathema *et al.*, 2006**). Africa, and more specifically sub-Saharan Africa alone, accounts for one quarter of the world's TB cases, with highest rates of cases and deaths relative to population (280 incident cases per 100 000, i.e. more than double the global average of 126) (**Global TB report, 2014**).

For the African continent, the rapid growth in TB cases began in the early 1980s and can been attributed to many factors, most importantly the upsurge in Human Immunodeficiency Virus (HIV) infections, emergence of strains resistant to anti-TB drugs and irregular supplies of drugs (**Harries *et al.*, 1997).**

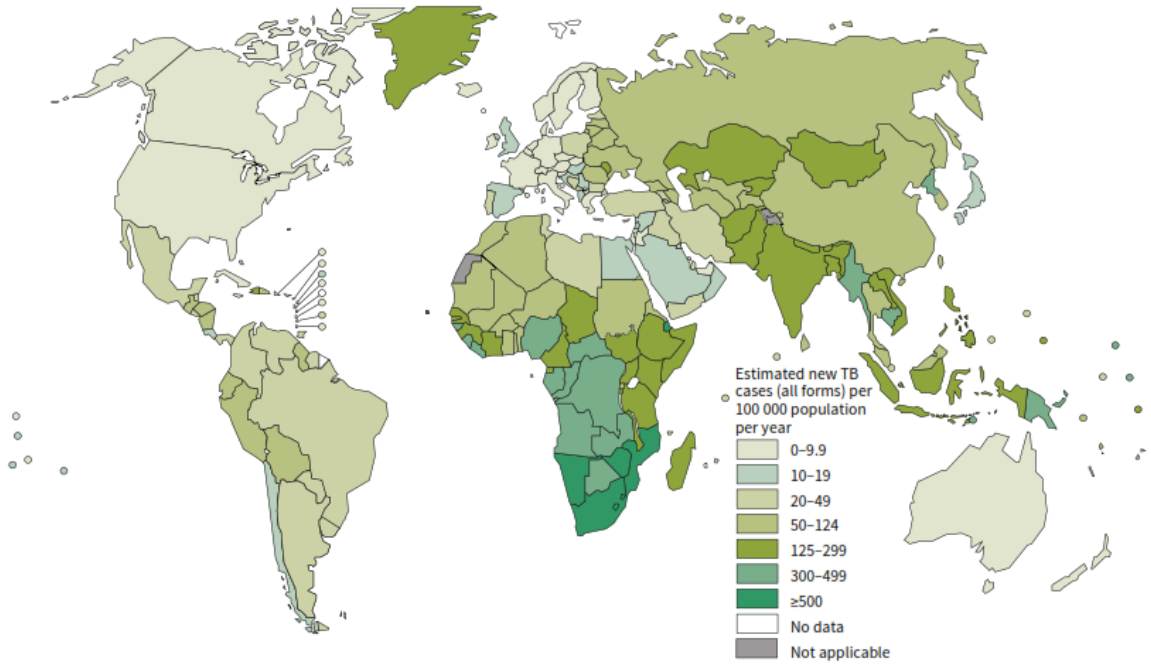

Figure 1: Global estimated TB incidence rates in 2013 **(Global TB Report, 2014).**

Currently, HIV is the most powerful known risk factor influencing *M. tuberculosis* infection and progression to active disease. In 2013, of the 1.1 million people co-infected with TB and HIV worldwide, 80% were concentrated in Africa; making Africa the

hardest hit continent of the two epidemics in the world (Figure 2) (**Global TB report, 2014**).

In terms of disease progression, not only does HIV increase the risk of reactivating latent TB, it also increases the risk of rapid TB progression. The incidence of active TB in HIV-infected patients with latent TB infection is about 10% per year compared to 10% per lifetime for an HIV-uninfected individual (**Selwyn *et al*., 1989**) creating a large pool of TB positive patients capable of spreading the disease **(Thye *et al*., 2012).**

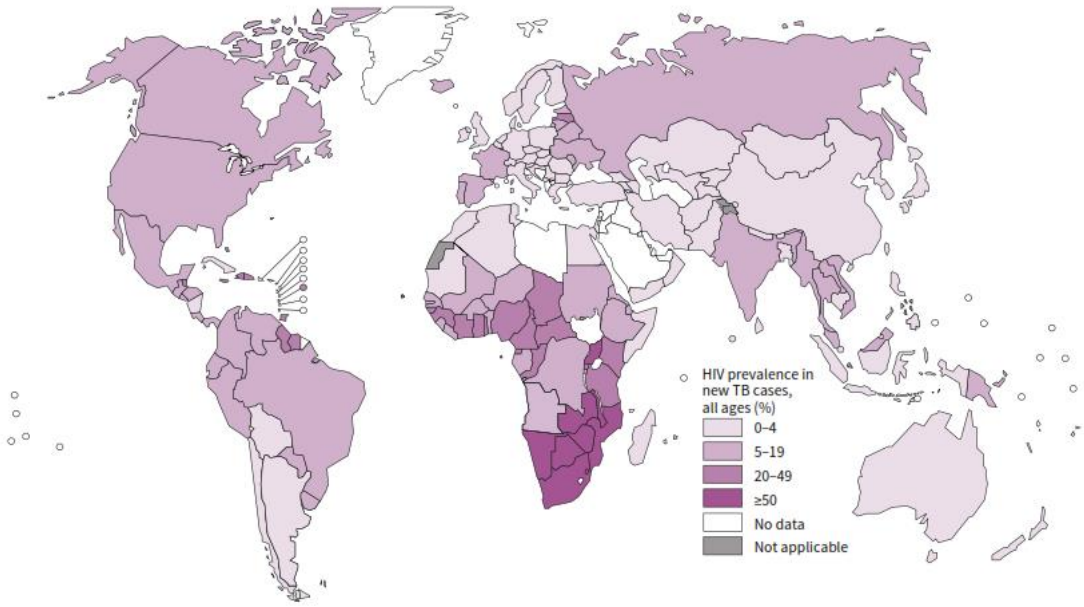

Figure 2: Estimated HIV prevalence in new TB cases in 2013 **(Global TB Report, 2014).**

In most African countries, TB is often the first manifestation of HIV infection, and it is the leading cause of death among HIV-infected patients (**Cain, 2009; Linguissi *et al*., 2014**). Approximately 78% of the 360,000 deaths attributed to TB and HIV worldwide in 2013 occurred in Africa (**Global TB report, 2014**). Studies have shown that this high case-fatality rate is likely due to a combination of the many factors, most importantly the

delay in diagnosis due to the lack of routine HIV testing in TB clinics. Until recently, in most African countries, HIV screening in TB clinics was not considered an integral part of the routine diagnosis despite the fact that it is the most important risk factor for TB **(Corbett *et al.*, 2006**).

Perhaps the most alarming aspect of the present TB epidemic in the world is the rise in multi-drug resistance (MDR)-TB cases. Globally, in 2013, 3.5% of new cases and 20.5% of previously treated cases (Figure 3) were diagnosed as MDR-TB, with the highest numbers of cases in Eastern Europe and Central Asia - 50% of all reported cases (**Global TB report, 2014)**. This figure amounts to 480,000 new cases of MDR-TB with an estimated 210,000 deaths. However, these figures could be a misrepresentation of the actual numbers as most TB burden countries, especially in Africa, lack the technical and financial resources to perform routine drug susceptibility testing (DST) on all patients.

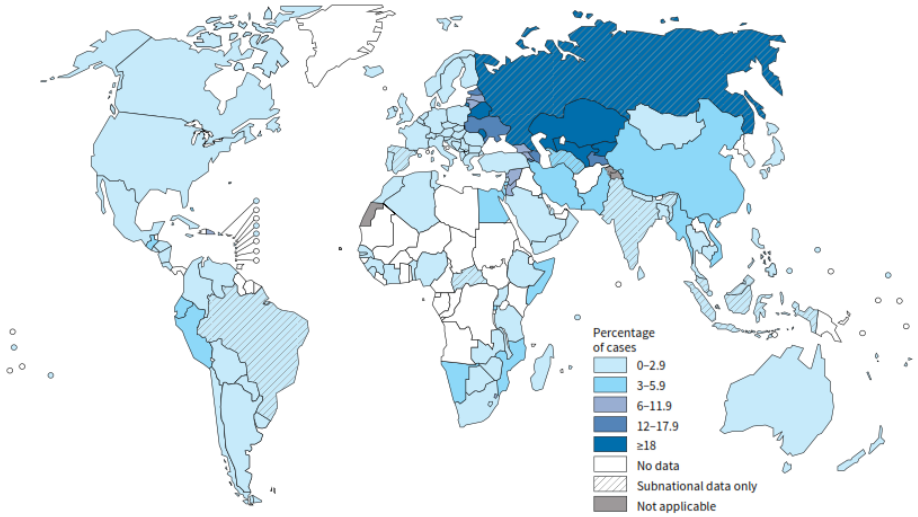

Figure 3: Estimated percentage of new TB cases with MDR-TB in 2013 **(Global TB report, 2014).**

1.1.3. Tuberculosis in Ghana

Similar to its neighbouring countries, TB is still a major public health issue in Ghana. With a population of 26 million and a TB incidence rate of 71/100,000 population per year, Ghana is ranked the 19th most TB-burdened country in Africa by WHO (**Ghana health service, 2007**).

Historically, TB treatment in Ghana began before independence, as early as 1954 with the establishment of societies and help groups. However, attempts at treating TB were sporadic and uncoordinated; targeting selected few citizens and even though the first formalised institution offering TB services was opened in 1959, (**Koch, 1960; Amo-Adjei and Awasuabo-Asare, 2013),** access to TB services was not free, disenfranchising most TB patients. TB services became accessible and free to all patients following adaptation of the Directly Observed Treatment Short Course (DOTS) strategy in 1994 (**National tuberculosis annual report, 2008**). Currently, 700 treatment centers and 1000 sub-treatment centers offer DOTS treatment in Ghana, and these are complemented by many private health facilities (**personal communication, Dr Frank Bonsu, Programme Manager, National Tuberculosis Programme, Ghana**).

Ghana, over the last two decades has recorded some successes in the control of TB; TB cases notification rose from 7,425 in 1996 to 15,207 in 2012; annual death rate (deaths/100,000 population/year) declined from 51/100,000 in 1990 to 4.4/100,000 in 2013, and treatment success rate rose from 44% in 1997 to 87% exceeding the global target of 85% **(National Tuberculosis Programme, 2012)**. Figure 4 gives an overview of the treatment success rate recorded over a 13-year period in Ghana.

Despite this concessive effort, Ghana still records very low TB detection rates; 31%, i.e. way below the African and Global targets of 50% and 70%, respectively (**personal communication, Dr Frank Bonsu, programme manager, NTP, Ghana**). This means that 69% of all TB cases in Ghana go undetected and therefore remain untreated (**Global TB report, 2014)**.

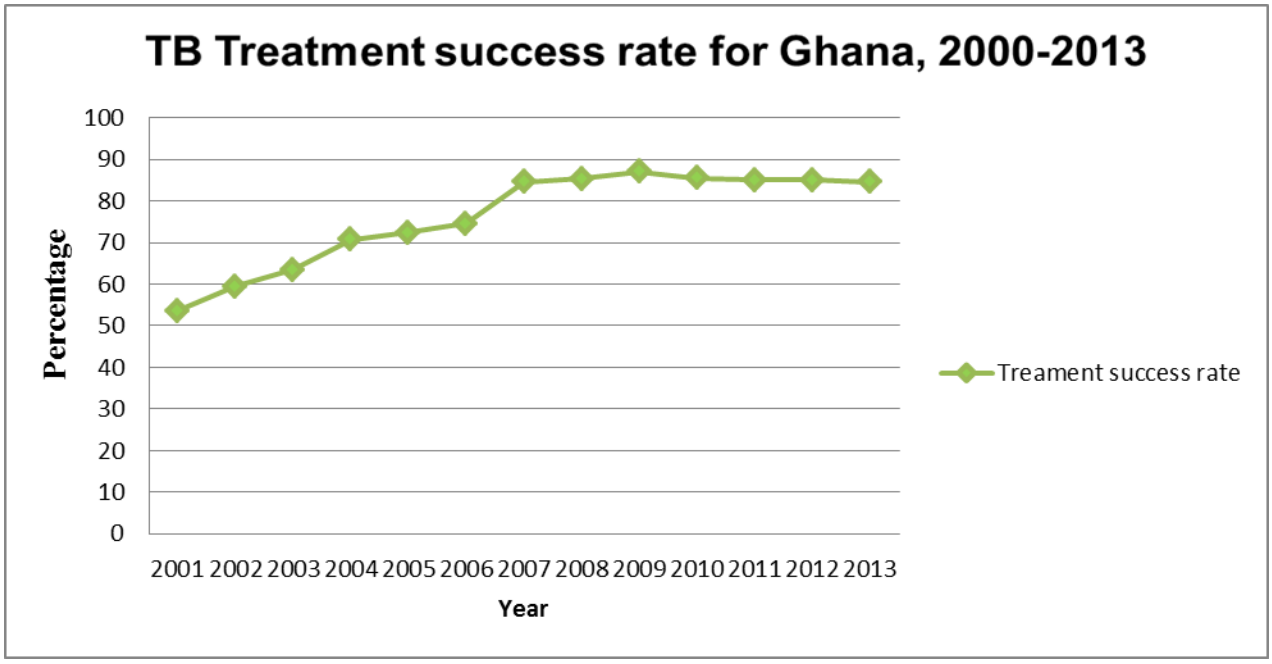

Figure 4: Treatment sucess rate in Ghana over a thirten year period (**Chart courtesy of National Tuberculosis control program, Ghana**).

In Ghana, TB most often goes hand in hand with death, and is a disease surrounded by many myths and misconceptions in most societies (**Lawn, 2000; Global TB report, 2003)**. For instance, among Akans in Ghana, it is believed that TB is an ancestral punishment for lack of care provided to family members who have died from TB, hence the name '*Nsamanwa'* (ghost cough). Likewise in the Volta Region of Ghana, TB is

known as *'Yomokpe'* (grave yard), suggesting death was unavoidable once infected (**Lawn *et al.*, 1999**). Among other biological factors, these names and the associated stigma prevent the patients from adhering to national diagnosis and treatment protocols and could account for the low detection rate recorded over the years (**National tuberculosis annual report, 2006)**.

One of the major risk factors for TB in Ghana, similar to other endemic countries, is HIV co-infection. Although HIV sero-prevalence in the general population is relatively low (1.3%-**National AIDs and STI programme report, 2013**), the influence of HIV on TB has seen an increase from 14% in 1989 to almost a quarter (24%) of all TB cases in Ghana in 2011 being HIV co-infected (**Global TB Report, 2014)**. Despite the implementation of routine HIV screening in all TB clinics, Ghana still records high TB/HIV deaths; the proportion of TB deaths attributable to HIV increased from 3.2% in 1987-88 at the beginning of the epidemic to 5.1% in 1997-98 and is currently pegged at 7%, second only to malaria with 13% (**Ansa *et al.*, 2014**). Hospital records show that approximately 25-30% of all TB patients are co infected with HIV and that as many as 50% of patients with chronic cough could be HIV-positive (**Personal communication: Dr Audrey Folson, Head Chest Clinic**).

1.2. **The causative agent of TB**

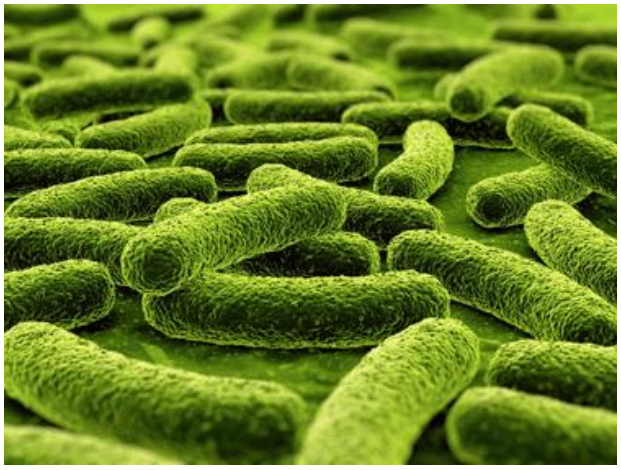

Figure 5: Rod-like structure of mycobacterium species (**www.bioquel.com**).

TB is caused by a group of closely related gram-positive bacteria, aerobic, non-motile bacilli, together referred to as the *Mycobacterium tuberculosis* complex (MTBC) (Figure 5) (**Comas *et al.*, 2011**, **Smith *et al.*, 2006; Brosch *et al.*, 2002; Gagneux *et al.*, 2008; Garnier *et al.*, 2003; Frota *et al.*, 2004; Cousins *et al.*, 2003**). Taxonomically, they belong to the phylum actinobacteria, in the order actinomycetales, suborder corynebacteriaceae and the genus mycobacterium (**Bergey's Manual of Systematic Bacteriology, 2005**).

The genus is divided into two broad taxonomic groups based on the growth rates of individual species. Those that produce colonies within seven days such as *Mycobacterium smegmatis,* are general termed fast growers or opportunistic / non-pathogenic bacteria whist the remaining group which takes more than a week for slow growers to form colonies includes slow-growing species such as the well-known pathogens *Mycobacterium tuberculosis, Mycobacterium bovis* and *Mycobacterium leprae*

(ethiological agents of human tuberculosis (TB), bovine tuberculosis (BTB) and leprosy respectively) (**Forrellad *et al.*, 2013)**.

The distinguishing characteristic of all *Mycobacterium* species is the cell wall, thicker than in other bacteria and essential for surviving and growing intracellularly (**Bhamidi, 2009**). Over 60% of the mycobacterial cell wall consists of lipids (mycolic acids, cord factor and wax-D) covalently linked to arabinogalactan and attached to peptidoglycan. Additionally, cell wall and mycomembrane contain various free lipids, such as phenolic glycolipids, phthiocerol dimycocerosates, dimycolyltrehalose or cord factor, sulpholipids and phosphatidylinositol mannosides that are intercalated with the mycolic acids (**Abdallah *et al.*, 2007).** The high concentration of lipids in the cell wall is beneficial to the bacteria. It is linked to the impermeability to normal stains and dyes used for common bacteria identification, increasing resistance to some very potent antibiotics, killing by acidic and alkaline compounds and attack by lysozymes (**Biberstein and Hirsch, 1999**). The intrinsic biosynthetic pathways of cell wall components potentially make them targets for new drugs for treating TB.

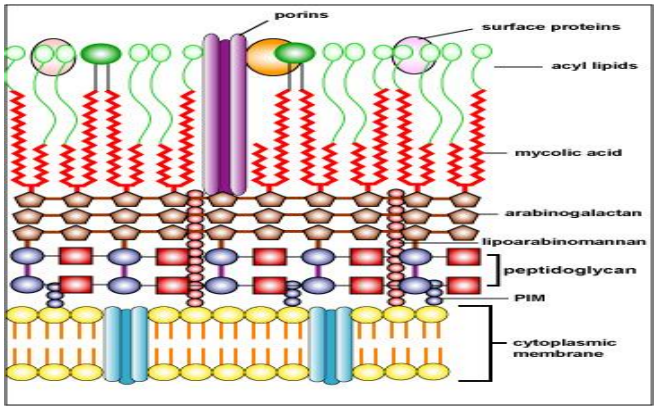

Figure 6: Schematic representation of the MTBC cell wall (**Kaiser, 2008**).

Although the organism apparently does not produce any toxins, has no classical virulence factors such as recently acquired pathogenicity islands; it possesses a huge repertoire of structural and physiological properties that aids in its survival within its host, including ability to detoxify oxygen radicals.

MTBC comprises *M. tuberculosis* sensu stricto (MTB), *Mycobacterium africanum*, *Mycobacterium microti*, *Mycobacterium bovis*, *Mycobacterium caprae*, *Mycobacterium mungi*, *Mycobacterium suricattae, Mycobacterium orygis* and *Mycobacterium pinnipedii.* Even though these species appear genetically monomorphic with a high level of DNA sequence similarity (>99.95%), with exception of *M. canettii*, they have varying host ranges: *Mycobacterium tuberculosis* sensu stricto (MTB) and *Mycobacterium africanum* (MAF) are the main causative agents of TB in humans. *Mycobacterium microti* affects voles, (**Wells 1937; Wells, 1946; Wayne *et al.*, 1986; Frota *et al.*, 2004)**, *M. caprae* a pathogen of goats and sheep (**Aranaz *et al.*, 1999**). *M. mungi:* Mangoose pathogen, *M. orygis* pathogen of antelope (**van Ingen *et al.*, 2012**), *M. pinnipedii* a pathogen of seals and sea lions (**Cousins *et al.*, 2003**). *Mycobacterium bovis* displays the broadest spectrum of host affecting humans and animals (**Garnier *et al.*, 2003**). However, although *Mycobacterium bovis* occasionally isolated from human, causing less than 1% of all human TB cases, it lacks the ability to maintain an infection cycle in human population or transmit in a sustainable way. This could be due to three mutations in the two-component regulation system PhoP/PhoR (*phoPR*) previously shown to be important regulator of virulence factors including several important lipids and proteins (ESAT-6). These mutations reduce the expression of the PhoP regulon leading to decreased ability to transmit between humans (**Berg and Smith, 2014; Smith *et al.*, 2006**).

M. canettii often considered a member of MTBC, is the most phenotypically distinct member of the complex. *M. canettii* and the other so-called "smooth tuberculosis bacilli (STBs)" are characterized by smooth glossy white colony due to the presence of lipooligosaccharides in the cell wall (**Gutierrez *et al.*, 2005**). The STBs show clear evidence of on-going horizontal gene exchange (**Gutierrez *et al.*, 2005**), but with no record of human-human transmission (**van Soolingen *et a*l., 1997; Koeck *et al.*, 2010; Fabre *et al.*, 2010)**. These places the STBs among the population of mycobacteria proposed as the originators of MTBC (**Gutierrez *et al.*, 2005).**

1.3. *Mycobacterium africanum*

Mycobacterium africanum (MAF), first identified in 1968 in Senegal was initially described biochemically as an intermediary between MTB and *M. bovis* (**Castets *et al.*, 1968**). Like MTB, MAF strains were found to be sensitive to pyrazinamide; like *M. bovis*, they tended to be a weak producer of niacin, microaerophilic, and unable to reduce nitrate to nitrite (**Pattyn *et al.*, 1970**). Furthermore, similar to *M. bovis* they are unable to use glycerol as a sole carbon source due to the lack of functional pyruvate kinase (glutamic acid is substituted by aspartic acid in the PykA gene that codes for pyruvate kinase (**Keating *et al.*, 2005**).

Initial biochemical features subdivided MAF into two separate groups, the East-African and West-African sub-species (**David *et al.*, 1978**). However, based on recent studies using regions of difference (RD) and comparative genomics (**Brosch *et al.*, 2002**; **Mostowy *et al.*, 2004**) to discriminate members of the *MTBC*, we now know that MAF West African sub-species consists of two phylogenetically distinct lineages: MAF West African 1 found in the eastern part of West-Africa, West-Africa genotype II found in the western part with few countries like Ghana and Cote d'Ivoire harboring both genetic variants ***(*de Jong *et al.*, 2010, Addo *et al.*, 2007, Yeboah-Manu *et al.*, 2011*)***. The former East-African MAF variant has been reclassified as MTB "Uganda" genotype; it shows the TbD1 deletion, a characteristic marker for a subset of MTBC lineages often referred to as evolutionarily "modern" **(Brosch *et al.*, 2002; Niemann, 2004**).

Although MAF is unique to West Africa, its prevalence varies by country. Using molecular genotyping results, the prevalence of MAF1 increases from West to East and

appears highest in Benin (39%) and Ghana (21%), while that of MAF2 increases from East to West, highest in Guinea Bissau with 51% of smear-positive TB caused by MAF2 (**de Jong *et al.*, 2010**).

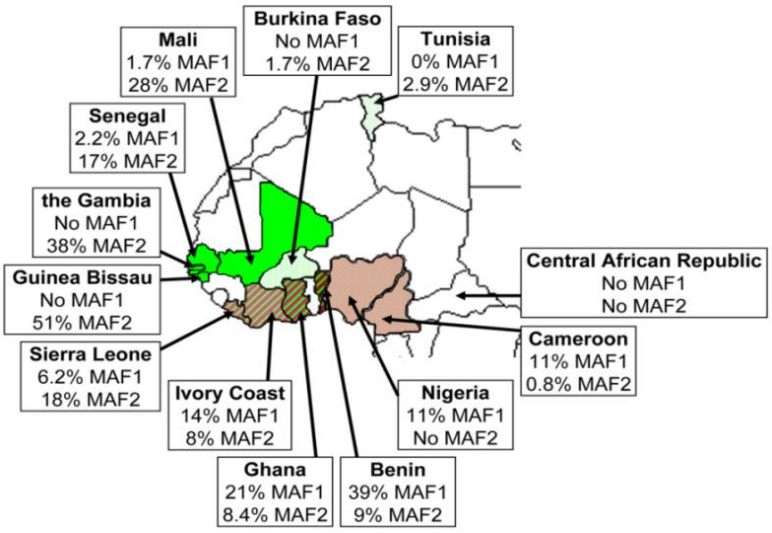

Figure 7: MAF prevalence in Western African countries (**de Jong *et al.*, 2010)**

Using traditional genotyping tools used for routine molecular epidemiological investigation such as spoligotype analysis, we know that MAF1 and 2 exhibit different genetic signatures; strains lacking spacers 8 through 12 and 37 through 39 are identified as MAF1 and those with spacers 7 through 9 and 39 as MAF2 (**de Jong *et al.*, 2009**). However, these characteristics are not always diagnostic of MAF (**de Jong *et al.*, 2010**). For example, there are some MAF strains that lack in addition to the MAF1 or MAF2-specific spacers 33–36 specific to MTB. For such strains, additional molecular tests are required for classification.

In recent time, a more robust nomenclature based on the presence or absence of particular genomic regions of difference has been developed to circumvent some of the ambiguities of spoligotyping. We know that *both* variants (MAF1 and MAF2) share one common deletion: RD9 deletion in addition to separate lineage- specific deletions: MAF1 has RD711deleted and RD702 intact (**Brosch *et al.*, 2002**; **Mostowy *et al.*, 2004)**; while MAF2 has RD702 deleted and RD711 intact (**Gagneux *et al.*, 2006**). The only limitation to the usage of these molecular markers is the need for elaborate infrastructure, and the inability of the current commercial speciation tests currently being used in endemic countries to distinguish between MAF1 and MAF2 (e.g., GenoType MTBC, Hain Lifescience, Germany).

Even though MAF is unique to West Africa, sporadically cases have been identified in areas outside the West African Region including Germany (**Meissner *et al.*, 1969**; **Jungbluth *et al.*, 1978**; **Schroder *et al.*, 1982**), England (**Grange *et al.*, 1989**), France (**Frottier *et al.*, 1990**) and Spain (**Perez-de Pedro *et al.*, 1990**). However, in all cases the TB patients carrying *MAF* were recent immigrants from West Africa. The only confirmed outbreak of *MAF* outside of West Africa occurred in France, where isolates from the first outbreak of multi-drug-resistant (MDR) TB, diagnosed during the period 1989 to 1992, were identified as MAF1 with the index case originating from Brazil, although no MAF1 or MAF2 isolates have been identified in Brazil to date (**Viana-Niero *et al.*, 2001**). Taken together, these observations suggest that MAF may have co-evolved with human populations specific to West Africa.

Although MAF is a human TB pathogen, many questions have been raised about the possibility of MAF2 as an ecotype of animal strains. These questions were based on the closeness of MAF2 to animal lineages on the phylogenetic tree of MTBC (**de Jong *et al.*, 2010**; Figure 8). However an animal reservoir for *MAF* infection has yet to be identified, even though occasionally, MAF has been isolated from animals: cattle in Nigeria (**Cadmus *et al.*, 2006; Cadmus *et al.*, 2010**), monkeys with active TB in West Africa (**Thorel, 1980**). Close interactions between humans and animals exist in West Africa, and an animal reservoir for *MAF* infection remains a possibility.

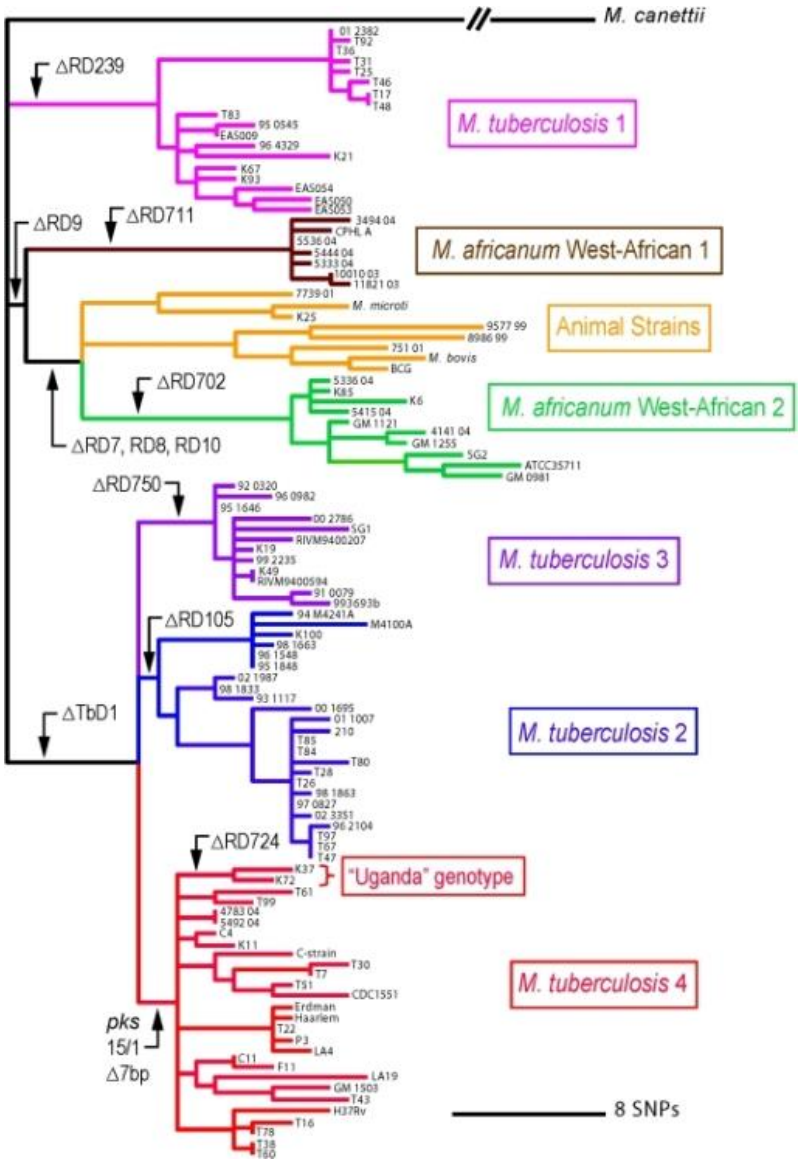

Figure 8: The position of MAF in the global phylogeny of MTBC (**de Jong *et al.*, 2010**)

1.4 Pathogenesis of TB

TB is an obligatory aerobic pathogen with a penchant for areas rich in oxygen supply (**Raja, 2004)**. For this reason, the classical TB bacillus is always found in well aerated upper lobes of the lungs. For an infection to occur, one has to inhale airborne droplet containing live tubercle bacilli generated from a person with active disease, however to be able to transmit the bacteria needs to cause active disease (**Gagneux, 2012**). The establishment of an infection is based on several factors: the droplet nuclei must be small enough in size (1 to 2 mm or less) to avoid exclusion from the lower respiratory tract by the physical barriers of the nasopharynx and upper respiratory tract, high bacteria load in the droplets, poor degree of ventilation and the longer duration of exposure between infected and uninfected persons. After inhalation, the bacterium travels down the bronchial tree into the lungs where they are engulfed by alveolar macrophages of the lungs (**Kang *et al.*, 2011)**. Upon entry into the human lung, *the bacilli* undergo a series of encounters with different host defense mechanisms and different outcomes. Hence the survival of bacilli in the lungs depends on its ability to resist elimination by the host immune system (**van Crevel *et al.*, 2002).**

From the lungs, the bacilli can spread to the lymph nodes via lymphatic system. The initial immune response against the presence of the bacteria in the lung is very complex but also quite efficient; in fact only 5-10% of these infections will lead to progressive disease for reasons unknown (**Kang *et al.*, 2011)**. TB 'infection' means the baccili are in the body but are kept under control by the host immune system. However, in the event where the initial immune response is not effective in clearing the invading pathogen, additional immune cells such as dendritic cells and lymphocytes are recruited from

neighbouring blood vessels to the focal site of infection where they engulf the invading pathogen (**Ernst, 2012)**. The attraction of host immune cells to the site of infection initiates the formation of granuloma also known as the giant wall which serves as a barrier for preventing the spread of bacteria to neighbouring cells (**Russell *et al.*, 2010)**. The granuloma makes-up changes as it matures: initially made up of disorganized cell but becomes more organized with macrophages in the centre and lymphocytes at the periphery (**Ulrichs *et al*., 2006).**

This organization reflects the complex and successful interaction between the innate and cell mediated immune cells following infection. In other words, elimination of *MTBC* infection mainly depends on the success of the interaction between infected macrophages, B and T lymphocytes. Initially thought to play no role in immune defense against MTBC, B cells and antibodies are now believed to contribute to an enhanced immune response against MTBC and together with T-cells modulate various immunological components in the infected host (**Achkar *et al.*, 2014**). However, despite the strong immune defense put up by the host, some bacilli which have evolved effective strategies to evade the immune response escape killing and enter a state of dormancy, and persist in a low replicating phase by avoiding elimination by the immune system. This asymptomatic stage otherwise known as the 'containment' phase is a hallmark of latent TB. Infected individuals at this stage are not infectious as they cannot spread the infection to other people. The dormant bacilli remain in the granuloma for decades mediated by the complex interplay between cell mediated and inflammatory cells. Nonetheless, in the event where this balance is tilted in favour of the bacilli such as in the event of systemic immune suppression as occurs in HIV co-infection, active disease develops and the center of the granuloma

undergoes necrosis and eventually becomes caseous. Live baccili are released into the alveoli and the patient becomes infectious. Viable, infectious bacilli spew into the airways resulting in productive cough spreading the infectious bacteria into the air (**Russell *et al*., 2009)**. The final outcome of infection with *MTB* largely depends on the balance between (i) outgrowth or killing of MTB and (ii) the extent of tissue necrosis, fibrosis, and regeneration.

For a pathogen like MTB, the series of immune responses triggered by exposure to the bacilli clearly defines the course of infection: be it total elimination, or containment or the inability to control the bacilli. Thus the clinical course of infection and its consequences depends largely on the interplay between host and several bacterial factors.

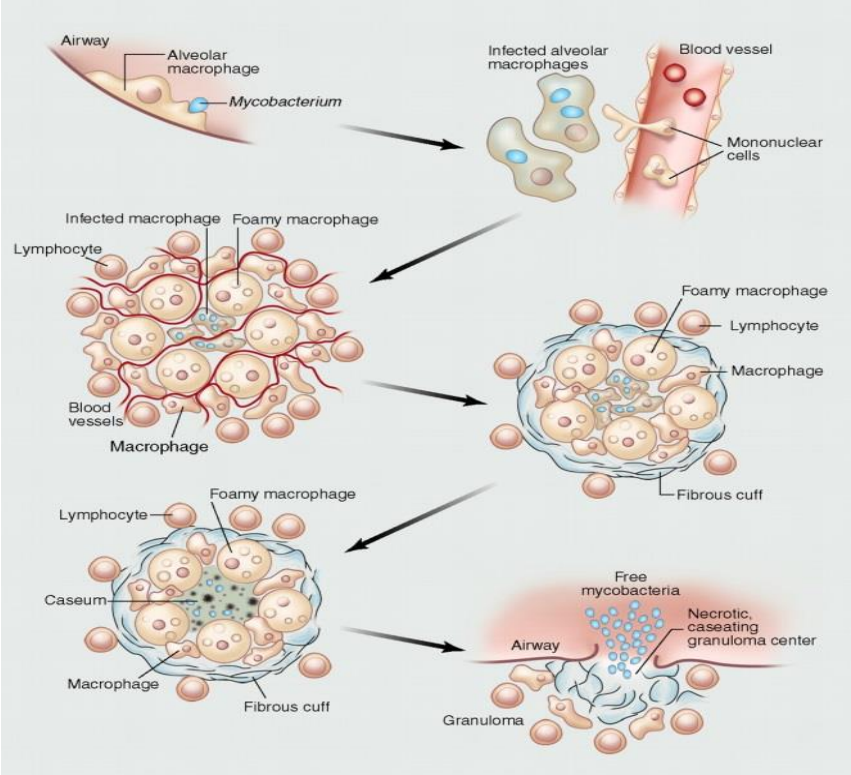

Figure 9: Pathogenesis of *M. tuberculosis* and granuloma formation (**Russell *et al*., 2010**)

1.5. Diagnosis and treatment of tuberculosis

1.5.1. Diagnosis

Primarily, TB is diagnosed by direct bacteriological identification of MTBC bacteria in a clinical specimen taken from a suspected TB patient. Pulmonary TB, the most common form of TB is diagnosed from sputum collected from a patient with an abnormal chest x-ray while for the more aggressive less common form, extra-pulmonary TB, a biopsy or fine needle aspirates from the infection site such as enlarged lymph nodes is collected and examined using histology or the microscopy. At present, methods with proven clinical utility for the diagnosis of active TB include microscopy, commercial kits to detect molecular markers, and culture.

Direct Smear Microscopy

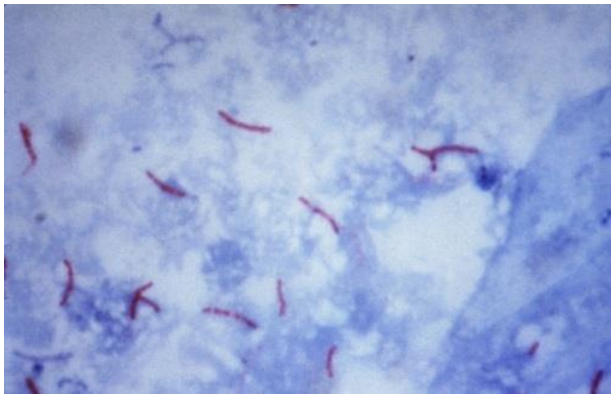

Figure 10: Ziehl Neelsen stained smear of *M. tuberculosis* from decontaminated sputum observed under oil immersion (x1000) **(http://en.wikipedia.org**)

This procedure is the most widely and routinely used diagnostic tool for active pulmonary TB in most developing countries. Smear examination by light microscopy after Ziehl Neelsen staining relies on the retention of the red carbol fuschion dye after

alcohol-acid decolourisation (**European Centre for Disease Prevention and Control, 2011; _TB_control.pdf; Forrellad *et al.*, 2013).**

Sputum smear microscopy is fast and relatively cheap. Unfortunately, its low sensitivity (~50% in average; requires a concentration of 10^4 bacilli per millilitre for positive smear test) combined with its intrinsic reliance on sputum production limits its use in some vulnerable groups such as children and HIV-positive patients who often produce little sputum with low bacillary load (**Shingadia and Novelli, 2003**; **Getahun *et al.*, 2007).**

A faster procedure based on fluorescent dyes with shorter reporting time such as auramine-rhodamine staining procedure is gradually replacing basic fuchsin Ziehl Neelsen staining procedures as an alternative staining procedure. These procedures are 10% more sensitive than light microscopy and are less time consuming, however they come with a high cost of fluorescent microscopes (**World Health Organization, 2011).**

Notwithstanding these drawbacks, sputum smear microscopy is good for its rapidity and does not require sophisticated equipment, making it suitable for endemic regions in Africa where resources are scarce.

Chest radiology

Chest X-ray is often used as a complementary tool to smear microscopy in diagnosing TB. Generally, abnormalities seen in the upper lungs (infiltration or cavities) on chest radiographs are often suggestive of but not necessarily definite for TB. Chest X-ray is often used to rule-out the possibility of pulmonary TB in a person with positive reaction to the tuberculin skin test and no symptoms of the disease (**Kumar *et al.*, 2007).**

In vitro* culturing of *Mycobacterium tuberculosis

Figure 11**:** Macroscopic mycobacterial culture on Lowenstein Jensen media (**TB reference lab, Noguchi Memorial Institute for Medical Research, Legon)**

Isolation of the causative agent provides definite evidence of the disease and is considered the gold standard for diagnosing TB. In addition, it offers the opportunity for obtaining bacterial isolates that can be used for in-depth studies. Although this technique is highly sensitive and needs only a few viable baccili to initiate growth (**Allen *et al.*, 1992**), the slow growth rate of MTBC (3-4 weeks) and the requirement of specific decontamination solution in addition to a costly biosafety level 3 laboratory prevent its usage as a first hand rapid test for the diagnosis of active TB (**Palomino *et al.*, 1998**).

Alternative diagnostic tools for the identification of MTBC

Several molecular diagnostic tests based on DNA amplification of specific markers have been developed as complementary tools to conventional microbiological diagnosis of TB. Most of these assays have the added advantage of simultaneously diagnosing TB and detecting drug resistance. Two of such assays; Xpert MTB/RIF (**Kurbatova *et al.*, 2012**)

and line probe assays from HAIN life science are currently in use in many endemic countries. Xpert MTB/RIF based on real-time polymerase chain reaction PCR amplification of *rpoB* gene detects resistance to rifampicin directly from sputum, regardless of the smear status in less than 2 hours. Furthermore, this method requires no additional reagents since all reagents are in-built, minimizing the cost. The line probe assay **Geno**Type **MTBDR***plus* on the other hand detects resistance to both isoniazid and rifampicin from pulmonary patient specimen in less than 2 hours (**Miotto *et al.*, 2008; Barnard *et al.*, 2008; Bazira *et al.*, 2010).**

Apart from molecular assays, there are several immunological based assays currently in use for diagnosing latent TB. One of the major tuberculin skin tests used around the world, largely replacing multiple-puncture tests such as the Tine test is the Mantoux test (**Mendel, 1908**). This assay is based on the measurement of delayed hypersensitive reaction, following intradermal injection of tuberculin. Regardless of its simplicity and usefulness, the Mantoux test is limited by poor specificity especially among Bacille Calmette-Guerin (BCG)–vaccinated individuals and high levels of cross-reactivity with atypical mycobacteria.

In recent times, commercial antigen specific assays measuring Interferon-γ (interferon-gamma) release from T lymphocytes by enzyme linked immunoassay (Quantiferon Gold in Tube (Cellestis, Australia) and enzyme linked immune spot (T-spot TB (Oxford, Immunotec, UK) have been developed as alternative to the Mantoux test (**Ferrara *et al.*, 2006**). Both tests are are based on the ability of MTBC antigens for early secretory

antigen target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) to stimulate host production of interferon-gamma. Because these antigens are not present in non-tuberculous mycobacteria or in any of the BCG vaccine variants (**Al-Hajoj SA, 2009**), these tests are specific for MTBC infection (**Pai *et al.*, 2004)**. Unfortunately, these tests cannot distinguish active MTB infections from latent TB (**Rangaka *et al.*, 2011**). Additional, these methods require the need for sophisticated instruments and training which limits their implementation in developing countries.

Table 1 gives a summary of the recommended TB diagnostic tool

Table 1: TB diagnostic tools approved by WHO (Dorman, 2010**)**

Method	Intended use	Main strengths	Main weakness
Sputum smear Microscopy for acid fast bacilli	Rapid , point of care test for TB case detection	Minimal infrastructure	Low sensitivity
In vivo solid culture	TB case detection	Good sensitivity	Slow growth time
Culture in liquid media	TB case detection and as a prerequisite for drug – susceptibility testing	High sensitivity	High contamination rate
Chest radiology	TB case detection (pulmonary TB)	Indicative of TB	Low specificity, low sensitivity, trained interpreter needed
Tuberculin skin test (Mantoux)	Detection of *M. tuberculosis* infection	Practical	Sensitivity decreases with immunocompromise, cross reaction with BCG vaccine
Interferon -γ release assay	Detection of *M. tuberculosis* infection	Highly specific for M.tuberculosis	Requires moderate training and equipment
Line probe assays	TB case detection and drug susceptibility testing	Short reporting time	Potential for cross contamination, requires extensive training

1.5.2. Treatment

Figure 12: Drugs for TB management (**www.indiamart.com**)

In the absence of drug resistance, TB has successfully been treated with effective chemotherapy since the discovery of streptomycin (STR) and p-aminosalicylic acid (PAS) in the 1940s, followed in the 1950s and 60s with the discovery of isoniazid (INH), ethambutol (EMB) and rifampicin (RIF: also known as rifaldazine and rifampin in the United States) (**Nguyen and Thompson, 2006**). The cell wall components of MTBC and key cellular functions are the major targets for most of the antibacterial therapeutics developed.

Table 2 lists the current first and second line drugs available for TB treatment.

Unlike most other bacterial diseases where single drug regimen is used for treatment, TB has been treated for over fifty years using combination therapy for several reasons: 1) reducing the chances of acquiring drug resistance 2) the combined modes of action of the drugs aid in effectively clearing the bacteria: rifampicin 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**). EMB inhibits the polymerization step of arabinogalactan synthesis (**Mikusova *et al.*, 1995**). 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 (**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 (defined as 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. INH/RIF are the most important drugs for TB treatment: INH is responsible for the initial killing of about 95% organisms during the first days of treatment, 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)**.

Previously treated patient are globally 5 times more likely to present with TB caused by multidrug-resistant (MDR) strains, and therefore, should be treated according to drug susceptibility test (DST) results. However, in the absence of DST results, patients are normally placed on a 8 months drug regimen comprising two months of INH/RIF/PZA/EMB (intensive phase), one month of INH/RIF/PZA/EMB (intensive phase) and five months of INH/RIF/EMB (continuation phase).

For drug resistant bacteria (MDR-TB) treatment, three groups of drugs are mainly used, including injectable aminoglycosides: STR, kanamycin (KAN), amikacin (AMK) which

inhibit protein synthesis, group 2 drugs (fluoroquinolones): ofloxacin, levofloxacin or moxifloxacin which target the DNA gyrase involved in DNA replication, and finally group 3 drugs (oral bacteriostatic drugs): ETD, cycloserine which targets cell wall biosynthesis. If any first-line drug is likely to be still effective, it should be included in the regimen, as first-line drugs are more potent and have less adverse effects than second-line drugs. The treatment regimen for MDR cases consist of at least four effective drugs: one injectable drug (group 1, preferentially AMK or KAN, since STR resistance among MDR-TB is frequent), one fluoroquinolone (group 2), and one group 3 drug.

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

Drug (year of discovery	**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

1.6. Drug resistance

Clinically, drug resistance in TB is defined as the increased capacity of the bacteria to tolerate high doses of specific antibiotics at any given time compared with drug-susceptible bacilli.

Drug resistance in MTBC is classified into two groups based on the way the resistance emerges: primary resistance and acquired resistance. Primary resistance is defined for patient infected with already drug-resistant strains. In contrast, acquired resistance is defined as the development of drug resistance in a patient during the course of treatment. Primary resistance in particular poses the biggest challenge to control because less than 20% of the estimated drug resistant cases in the world are believed to be properly diagnosed, largely due to the lack of appropriate laboratory infrastructure in low-income endemic areas (**Muller *et al.*, 2013)**.

Unlike other bacteria, drug resistance in MTBC is conferred by specific chromosomal mutations and promoted either through environmental/extrinsic effect or bacterial factors. These factors can either be 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).

Regardless of the path of emergence, drug resistant TB is more difficult to cure. The two most important forms of drug resistance are MDR-TB and XDR-TB. MDR-TB is defined for resistance to at least RIF and INH, the two most potent anti-TB drugs. XDR-TB is defined when MDR-TB cases are additionally resistant to at least one injectable drug (AMK, KAN or CAP) and one fluoroquinolone. As mentioned earlier, MDR-TB cases

are treated using more expensive and generally more toxic second line drugs. XDR emerges when control of MDR is adequate. WHO estimates that on the average 9% of all MDR cases are XDR (**Global TB report, 2014**). This means that for an effective control of drug-resistant TB, prompt identification and initiation of adequate treatment is crucial to prevent the further development or spread of resistance.

In an ideal situation, all patients should be tested for drug resistance before initiation of treatment so that the most appropriate drugs to treat the patient can be determined. This is not the case in most endemic regions where elaborate infrastructure and expertise are needed to perform DST and interpret result. Phenotypic DST is the current gold standard for the detection of drug resistance and is based on the "proportion method" which consists of monitoring mycobacterial growth on media containing the relevant drug at a critical concentration. The proportion method which hitherto was the method of choice in most low-income countries is gradually being replaced by Mycobacteria Growth Indicator Tube (MGIT) which has an added advantage of being fully automated. It detects mycobacterial growth and drug resistance reducing the delay for reporting results (**Abe *et al.*, 2001; Ardito *et al.*, 2001**).

An alternative to phenotypic DST is the use of molecular markers for the identification of chromosomal mutations (**Ramaswamy 1998**; **Sandgren *et al.*, 2009**). These techniques are designed based on earlier DNA sequencing analyses and target specific genes where mutations of interest occur. Most importantly, they 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. In 2009, the WHO approved the use of molecular line-probe assays for the detection of drug resistance in MTBC (**World Health**

Organization, 2009). Of these, the best known are Xpert MTB/RIF and GenoType MTBDR*plus* for first line drugs and GenoType MTBDRs*l* for second line drugs (Hain Lifescience, Germany). Xpert MTB/RIF is a fully automated system based on real time amplification of specific regions of the *rpoB* gene for the detection of drug resistant strains. On the other hand, GenoType MTBDR*plus* in addition to detecting resistance to RIF also detects resistance to INH. GenoType MTBDRs*l* provides a comprehensive report on second line drugs used for treating TB resistant to first line drugs. It 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).

Table 3 list genes harboring mutations associated with resistance to anti-TB drugs.

Table 3: Anti-TB drugs and their mechanism of drug resistance (Muller *et al.*, 2012)

Drugs	Genetic region involved in resistance formation	Natural function of gene	Role in resistance formation when mutated
		First Line drugs	
Isoniazid	ahpC	Alkyl hyperperoxide reductase	Compensatory mutations
	fabG	3-Oxoacyl-thioester reductases	Unknown
	fadE24	Involved in fatty acid b-oxidation n	Unknown
	inhA	Enoyl reductase	Alteration of drug target
	inhA promoter	Regulation of expression of InhA	Overexpression of drug target
	iniA	Efflux pump associated	Altered efflux pump activity
	katG	Catalase/peroxidase	Elimination of pro-drug conversion
Rifampicin	rpoA	α-Subunit of RNA polymerase	Compensatory mutations
	rpoB	β-Subunit of RNA polymerase	Alteration of drug target
	rpoC	β-Subunit of RNA polymerase	Compensatory mutations
Pyrazinamide	pncA	Nicotinamidase	Elimination of pro-drug conversion
Streptomycin	gidB	7-Methylguanosine methyltransferase	Alteration of drug target
	rpsL	S12 ribosomal protein	Alteration of drug target
	rrsb	16S rRNA	Alteration of drug target
Ethambutol	embA	Arabinosyl transferase	Alteration of drug target
	embB	Arabinosyl transferase	Alteration of drug target
	embC	Arabinosyl transferase	Alteration of drug target
	embR	Regulator of embCAB operon expression	Overexpression of drug target
	iniA	Efflux pump associated	Altered efflux pump activity
	rmlD	dTDP-4-dehydrorhamnose reductase	Unknown
		Second line drugs	
Fluoroquilones	gyrAb	DNA gyrase	Alteration of drug target
	gyrBb	DNA gyrase	Alteration of drug target
Injectables (Kanamycin/amikacin)	rrsb t	16S rRNA	Alteration of drug target
	rrs	16S rRNA	Compensatory mutations
Capreomycin/viomycin	tlyA	rRNA methyltransferase	Alteration of drug target
	rrsb t	16S rRNA	Alteration of drug target
Ethionamide	inhA	Enoyl reductase	Alteration of drug target
	inhA promoter	Regulation of expression of inhA	Overexpression of drug target
Para-amino salicylic acid	thyA	Thymidylate synthase A	Elimination of pro-drug conversion
PA-824 and OPC-67683	Rv3547	Hypothetical 16.4 kDa	Alteration of drug target
TMC207	atpE	ATP synthase	Alteration of drug target

1.7. The nature of genetic diversity in *Mycobacterium tuberculosis* complex

Similar to other monomorphic bacterial pathogens such as *M. leprae* and *Bacillus anthracis*, MTBC exhibits low DNA sequence diversity compared to other bacteria (**Achtman 2008**). On the average, monomorphic bacteria harbour a single nucleotide difference every 2-28kb (**Achtman 2008**). Because of this limited DNA sequence variation and lack of horizontal gene transfer in MTBC (**Brosch *et al.*, 2000; Supply *et al.*, 2003; Ozcaglar *et al.*, 2011**) unlike other bacteria like *Helicobacter pylori* (**Wirth *et al.*, 2004; Falush *et al.*, 2003**), it was assumed that strain diversity among individual members of MTBC had no clinical consequence (**Sreevatsan *et al.*, 1997 Comas and Gagneux, 2009)**.

For a long time it was assumed that strain diversity within individual members of MTBC played no role in terms of progression from infection to disease and propensity to develop drug resistance. This dogma was strengthened by earlier studies that were conducted using limited and often biased strain collections (**Comas and Gagneux, 2009**). Understanding the diversity of bacterial pathogens is important, both for epidemiological and biological reasons. As mentioned above, because of the low DNA sequence among MTBC, studying the impact of genetic diversity is very challenging. Standard sequence-based methods such as multilocus sequence typing (MLST) normally used for most bacteria were not applicable because of the low resolution power (**Kent and Kubica, 1985; Coscolla and Gagneux, 2010**). Nonetheless, with the advancement in molecular analyses and accessibility of more MTBC isolates from diverse geographical regions, we now know that genetic diversity within MTBC, especially the human MTBC has been underestimated.

Genetic diversity in MTBC is now known to be driven mainly by large sequence deletions in the region of difference (RD), single nucleotide polymorphisms (SNPs) and repetitive elements and insertion sequences (**Huard *et al.*, 2006**). Of the above mentioned genetic elements, deletions play a major role. The availability of the genomes of different members of the MTBC has allowed us

to compare different genomes. Comparing different genomes, Behr and colleagues identified eleven genomic regions (encompassing 91 open reading frames) deleted from BCG vaccines relative to the virulent MTBC H37Rv reference strain (**Behr *et al.*, 1999**). These regions contained important proteins which either regulated virulence or aided in immune escape. For example RD1 contains genes that belong to the ESAT6 gene cluster encoding a type 7 secretion system (**Abdullah *et al.*, 2007**; **Tekaia *et al.*, 1999**). ESAT6 has been shown to act as potent stimulator of the immune system and is an extracellular antigen recognized throughout infection (**Elhay *et al.*, 1998; Horwitz *et aI.*, 1995; Rosenkrands *et al.*, 1998**). As this 10-kb region is absent from all BCG strains tested so far, but present in virulent *M. bovis* MTB and MAF, the loss of RD1 could be associated with the attenuation of BCG.

Lineage 2, a member of 'modern' lineages of MTBC, is normally associated with hypo inflammatory and hyper virulent phenotype. This attribute is linked to the presence of an intact *pks*15/1 gene that, by contrast, contains a 7-bp deletion absent in Lineage 4 also belonging to the same group. The presence of an intact *pks*15/1 gene which encodes a polyketide synthase is associated with the production of an immunosuppressive phenolic glycolipid (PGL) (**Constant *et al.*, 2002**). The presence of intact *pks*15/1 probably partially accounts for the high transmissibility and propensity to develop multiple resistances nature of Lineage 2 in contrast to other lineages.

Apart from deletions, other genomic elements contribute to the genetic diversity among MTBC. For example, repetitive elements in the Direct Repeat (DR) region and the insertion sequence *IS6110* (**Kivi *et al.*, 2002**) have been exploited to distinguish MTBC strains during molecular epidemiological studies (**Kamerbeek *et al.*, 1997**).

Although the explanation of genetic diversity of global MTBC using molecular biological tools answers some pertinent questions, an important question is how these translates into phenotypic diversity in terms virulence, immunogenicity and drug resistance among the infecting pathogen in clinical settings **(Portevin *et al.*, 2011, Brites and Gagneux, 2012)**.

1.8 Genotyping techniques for identification of MTBC

Genotyping of MTBC offers several advantages in the context of understanding diversity among MTBC. In particular, it allows for identification of risk factors for infection and disease, distinguishes between new infection and reactivated cases, and identifies predominant strain genotypes.

Since early 1990s, several genotyping tools have been proposed to study genetic diversity among MTBC. Based on the question under investigation, the tool selected should be polymorphic enough to distinguish among unrelated strains yet still stable enough to allow for identification of closely related strains.

The classical genotyping methods to understand genetic diversity among MTBC includes insertion elements (IS) restriction fragment length polymorphism (*IS6110*-RLFP), spoligotyping, and Mycobacterial Interspersed Repetitive Units-Variable Number of Tandem Repeats (MIRU-VNTR). With the cost of high throughput DNA sequencing decreasing, whole-genome sequencing is gradually becoming the ideal tool to infer molecular epidemiology and phylogenetic aspects.

Below is an overview of different methods used in studying the diversity of MTBC.

IS*6110*-RFLP

The first genotyping method developed in the early 1990s by **van Embden *et al.*,1993** to be used for strain classification was restriction fragment length polymorphism (RFLP) based on IS*6110* insertion sequence *(*IS*6110*-RLFP). Differences in copy number and locations within the genome make this technique highly specific for MTBC. The technique involves the digestion of genomic DNA with *Pvu*II restriction enzyme that cleaves the IS*6110* sequence only once, creating several DNA fragments that are separated through gel electrophoresis. Initially, considered as the gold standard, this method has been replaced by MIRU-VNTR and spoligotyping for various reasons: 1) it is labour intensive, requires high quality DNA, and sophisticated and expensive computer

software to analyse: 2) it requires experienced personnel of high technical expertise to interpret the results, and 3) it is not discriminatory enough for strains with 6 or less IS*6110* copy numbers like e.g. *M. bovis*. Nonetheless, it paved the way for an in-depth understanding of diversity among MTBC before the development of the more recent methods.

Spoligotyping

Developed in 1997 by **Kamerbeek *et al.*, 1997** and based on polymorphisms in the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) region of MTBC is the most frequently used PCR-based approach for studying the phylogeography of MTBC in high incidence areas where infection and disease patterns are heterogeneous. Spoligotyping takes advantage of the Direct Repeat (DR) region composed of identical 36 base pair repeats interspersed by 94 unique "spacers" of 35-41 base pairs in length. Although the order of these spacers is conserved, the presence or absence of selected 43 spacers allows for discrimination among MTBC strains. The protocol entails immobilisation of amplified illuminescent PCR products on nitrocellulose membrane. The resulting pattern of presence or absence of spacer revealed by chemiluminescence can be compared with the database- SITVITWEB (formerly SpolDB4) for identification. (**Demay *et al.*, 2012; Weniger *et al.*, 2010**).

Spoligotyping is simple, cost-effective and high-throughput with accurate and reproducible results within 2 days. Its direct application in clinical samples without the need for prior culture and easy interpretation and computerized (binary (present/absent) data format makes it ideal for molecular epidemiological studies.

However, it is less discriminatory than IS*6110* RFLP analysis (it targets only a single genetic locus, covering less than 0.1% of the *M. tuberculosis* complex genome), less informative in regions with predominant or endemic strains (W-Beijing in China, Southeast Asia and Russia) and is of limited use for evolutionary studies. Furthermore, it is unable to detect contaminated isolates or multi-strain infections.

To overcome some of these limitations, two newer and improved formats of the spoligotyping method have been proposed. The first benefits from the Luminex technology: high-throughput analysis, allows 96 isolates to be assayed simultaneously, as opposed to 45 isolates in the standard spoligotyping approach. The method is based on immobilization of synthetic spacer oligonucleotide probes on microspheres by means of covalent coupling and the resulting products detected via fluorochromes. The Luminex platform (**Cowan *et al.*, 2004; Zhang *et al.*, 2009**) eliminates the membrane hybridisation step and the subjective manual data interpretation and provides greater robustness and reproducibility. The second more recent alternative is the new multiplexed primer extension-based spoligotyping assay using automated matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (**Honisch *et al.*, 2010**). Spoligotyping by MALDI-TOF MS improves the classical reverse line blot hybridization assay with respect to reproducibility, throughput, process flow, ease of use, and data analysis. However, both new innovative, technologically refined spoligotyping assay formats require advanced and expensive equipment and thus may not be applicable in resource-constrained settings.

MIRU-VNTR

The third most widely used method is Mycobacterial Interspersed Repetitive Units (MIRU-VNTR) typing. Currently, it has become the most reliable and efficient genotyping system for TB transmission studies and is the new gold standard replacing *IS6110*-RFLP. Developed in 2001 by **Supply *et al***, this technique is based on tandem repeat elements dispersed in intergenic regions of the MTBC genomes and copy number diversity. Within the MTBCs more than 40 different MIRU/VNTR loci have been identified and at least 24 of them are considered as polymorphic. Protocols based on PCR amplification of different proposed formats: 12, 15 or 24 loci using primers positioned in flanking DNA sequences have been used to study the transmission dynamics of MTBC. In general, the discriminatory power of MIRU-VNTR analysis increases with the number of loci evaluated. The variability of the number of tandem repeats from strain to strain per

locus serves as tool for differentiating among MTBC. The number of repeats (allele) is calculated after amplicon sizing by gel electrophoresis and the numerical results is matched to an online database for comparison and identification (**Allix-Béguec *et al.*, 2008; Weniger *et al.*, 2010**). MIRU-VNTR typing has been used successfully to trace on-going chains of TB transmission, differentiate relapse from re-infection cases and detect laboratory cross-contamination (**Cox *et al.*, 2008**).

Despite its usefulness in TB transmission studies, it is labour-intensive due to a high number of individual PCRs required and less informative in areas with restricted MTBC lineages (**Comas, 2009)**. To achieve the highest discriminatory power and to make the method more effective in areas with restricted lineages, in recent years, several minimal sets of loci designed to provide maximum discriminatory power and minimize genotyping costs have been developed for geographically restricted MTBC lineages (**Murase *et al.*, 2008; Shamputa *et al.*, 2010, Dong *et al.*, 2012**).

Large Sequence Polymorphisms

While discriminatory methods such as spoligotyping and MIRU-VNTR are useful in strain classification and identification, they often show ambiguities. This is because both assays are based on repetitive elements that are prone to convergent evolution thus relying only on these methods for strain classification can be misleading **(Comas *et al.*, 2009)**. Large sequence polymorphisms (LSPs) on the other hand serve as more phylogenetically robust and stable molecular markers for strain identification; they are unique irreversible events and less prone to distortion by selective pressure than other genetic markers. In addition, because these markers are unique and irreversible due to the lack of horizontal gene exchange in MTBC, they are less prone to convergent evolution and thus can be used for robust phylogenetic classification (**Gagneux and Small, 2007**). Supporting this, Comas *et al* showed that phylogenies based on LSP compared to those by DNA sequences were highly congruent (**Comas *et al.*, 2009**).

Most importantly, LSPs also known as regions of differences (RDs) (**Huard *et al.*, 2006**) have been used to define several discrete strain lineages within the human adapted members of MTBC specific for different human populations and geographical regions and unravel the evolutionary scenario of ecotypes of MTBC (**Brosch *et al.*, 2002**; **Gagneux *et al.*, 2006**:Figure 13)

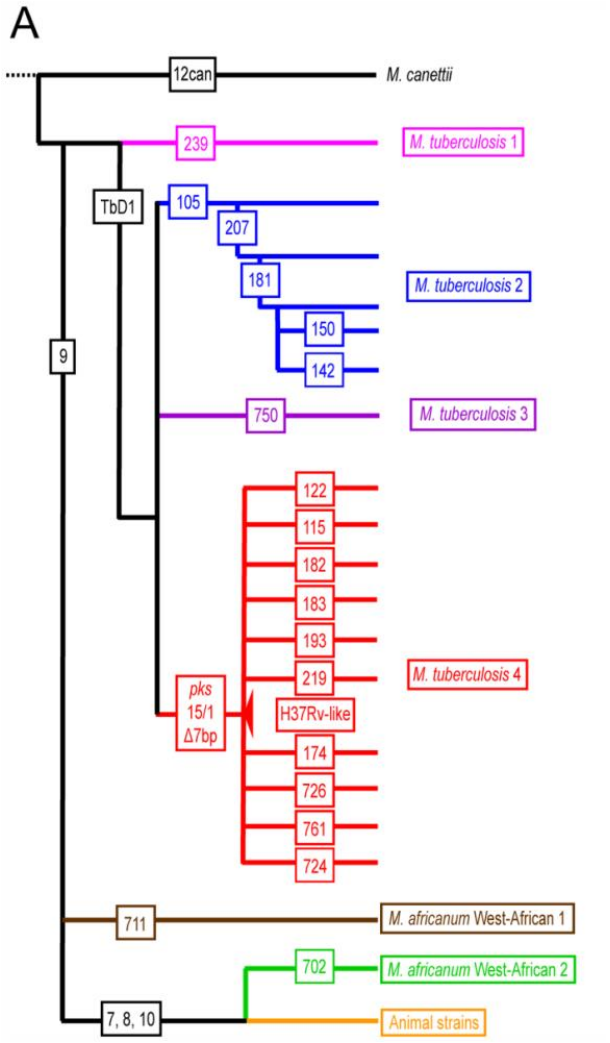

Figure 13: The global phylogeny of *Mycobacterium tuberculosis* complex (MTBC) (**Gagneux et al., 2006)**

Single Nucleotide Polymorphisms

Large deletions discussed above reflect unidirectional events and are therefore not prone to homoplasy. However, they do not allow the calculation of genetic distances and also cannot completely resolve all deep-rooting branches of the MTBC phylogeny (**Comas *et al.*, 2009**). With the availability of large-scale DNA sequencing technologies, large numbers of SNPs have been

discovered in MTBC and have been used in understanding the biology of MTBC as a pathogen with very restricted genetic diversity (**Achtman, 2008**). Because of the absence of recombination and lateral gene transfer, SNPs are ideal phylogenetic and epidemiological markers. In addition SNP-based are also unlikely to converge, as can be the case with spoligotype or MIRU markers (**Holmes *et al.*, 1995**; **Schork *et al.*, 2000)**. This was illustrated in a recent report by Fenner *et al.*, showing that strains sharing the same pattern as identified by spoligotype e.g. classical "Beijing" spoligotyping pattern can in fact belong to different lineages, as identified by specific SNPs and genomic deletion (**Fenner *et al.*, 2011**). Furthermore selectively neutral SNPs have been shown to accumulate at a uniform rate and thus can be used to measure divergence (i.e., they can act as molecular clocks). Therefore SNPs serve as the most appropriate genotyping tool for MTBC.

Table 4: List of Lineage, SNP, Primer and Probe for major Lineage typing (Stucki *et al.*, 2012)

MTBC Lineage	**SNP_Name**	**Primer**	**Primer Sequence**	**Probe**	**Probe_seq**
1	Rv3221c_0085n	RV3221c_F	TGTCAACGAAGGCGATCAGA	H37Rv_probe	6FAM-ACAAGGGCGACGTC
		RV3221c_R	GACCGTTCCGGCAGCTT	Mutant_probe	VIC-ACAAGGGCGACATC
2	Rv2952_0526n	Rv2952_F	CCTTCGATGTTGTGCTCAATGT	H37Rv_probe	6FAM-CCCAGGAGGGTAC
		Rv2952_R	CATGCGGCGATCTCATTGT	Mutant_probe	VIC-CCCAGGAAGGTACT
3	Rv3804c_0012	Rv3804c_F	GCATGGATGCGTTGAGATGA	H37Rv_probe	6VIC-AAGAATGCAGCTTGTCGA
		Rv3804c_R	CGAGTCGACGCGACATACC	Mutant_probe	6FAM-AAGAATGCAGCTTGTTGA
4	katG463	katG463_F	CCGAGATTGCCAGCCTTAAG	H37Rv_probe	6FAM-CAGATCCGGGCATC
		katG463_R	GAAACTAGCTGTGAGACAGTCAATCC	Mutant_probe	VIC-CCAGATCCTGGCATC
5	Rv1377185GC		TCCAGCAGGTGACCATCGT	H37Rv_probe	VIC-CGTGGACCTCATG-
			GGCCTGTGACCCGTTCAAC	Mutant_probe	6FAM-CGTGGACCTGATGCMGB
6	Rv378404GA		CGGCCGACAGCGAGAA	H37Rv_probe	6FAM-CTGCAAATCCCGCAGTA
			CCATCACGACCGAATGCTT	Mutant_probe	VIC-CTGCAAATCCCACAGT

Whole genome sequencing (WGS) as a typing method

Although SNPs are still used, with exponentially decreased in cost, WGS is increasing becoming the preferred technique for TB research. WGS determines the complete DNA sequence of an organism's genome at a single time and can provide several answers at a single time, making it the ideal tool for studying the pathogen. Several studies have applied large-scale WGS to different

aspects of TB research; to study chains of transmission (**Schurch *et al*., 2010**), disease outbreaks (**Bryrant *et al*., 2013**) and also to infer phylogeny (Comas et al., 2013). Furthermore, WGS have been used to identify drug-resistance associated mutations; **Comas *et al*., 2011, Casali *et al*., 2012 and Casali *et al*, 2014** found mutations compensating for the fitness defect associated with rifampicin resistance while **Köser et al., 2013** used WGS to rapidly identify drug resistance mutations of an XDR-TB patient. These studies demonstrate the potential for future routine applications of WGS in research and molecular epidemiology. However, the use of WGS for large-scale applications especially in endemic areas is limited by it cost and the need specialized expertise for analyses

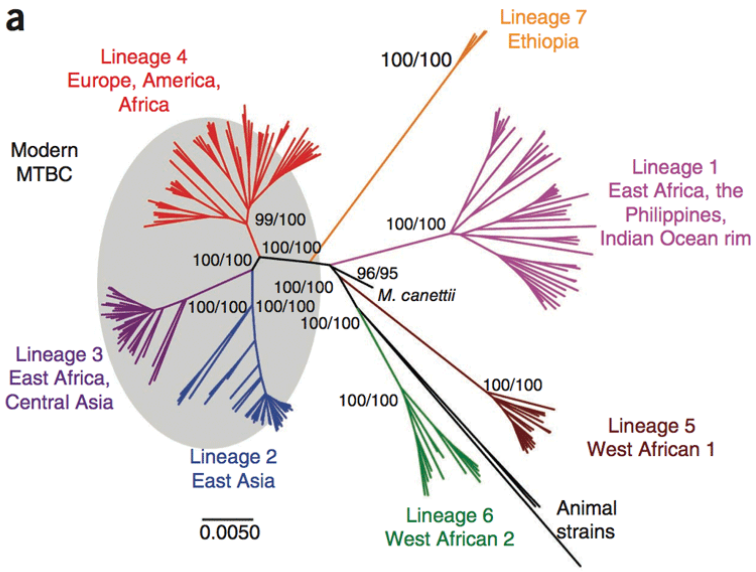

Figure 14: Whole-genome phylogeny of 220 strains of Mycobacterium tuberculosis complex (MTBC) (**Comas *et al*., 2013**)

1.9. Consequences of genetic diversity within MTBC

The impact of strain variation for human disease has been well established for a number of bacterial pathogens such as *Escherichia coli*, *Neisseria menigitidis*, *Haemophylus influenzae*, *Bordetella* and *Streptococcus* species (**Coscolla and Gagneux, 2010**). In these bacterial species, some strains are more likely to cause invasive disease than others because such virulent phenotypes possess distinct virulent markers such as genes associated with the production of toxins. However, no such canonical virulence factors have been identified in MTBC. Nevertheless, there is now mounting evidence supporting the notion that strain genetic diversity in MTBC has relevant phenotypic consequences (G**agneux and Small, 2007; Nicol *et al.*, 2008; Kato-Maeda *et al.*, 2001; Coscolla and Gagneux 2010**).

For example, initial studies carried out from the beginning of the last century using animal models gave a clear idea on the difference in virulence between MTBC strains. In one such experiment in guinea pigs comparing MTBC strains isolated from TB patients in south India to that isolated from United Kingdom, the former were shown to be of low virulence compared to the later (**Mitchison *et al.*, 1960; Naganathan *et al.*, 1986**). Similarly, early animal studies comparing *M. africanum* from Senegal to MTB showed that MAF was less virulent (**Castets and & Sarrat, 1969**). In another study conducted in Gambia, it was shown that while both MTB and MAF transmit equally, MAF seems to have a longer latency period compared to MTB (**de Jong *et al.*, 2008**). The same group further showed that MAF seems to be less virulent than MTB, as it tends to affect more HIV confected patients, and individuals infected with MAF were less likely to respond to ESAT 6 (**de Jong *et al.*, 2005; de Jong *et al.*, 2006**). Based on the phylogenetic tree of MTBC, we know that MAF strains and Lineage 1 strains from South India belong to 'ancient' MTBC strain lineages. Hence, it is possible that the low virulence phenotype exhibited by these two phenotypically distinct strains is a general characteristic of 'ancient' MTBC strains. In contrast, experiments using HN878, a strain that caused disease outbreaks in Los Angeles and

Houston, showed a different picture. HN878 belongs to the 'modern' Lineage 2 which includes the Beijing family of MTBC. Lineage 2/Beijing family has been associated with hyper virulence, epidemic spread, extra pulmonary TB and an increased likelihood of developing drug resistance due to the production of phenolic glycolipid (PGL) **(Reed *et al.*, 2007; Kato-Maeda *et al.*, 2010; Borrell and Gagneux, 2009**). PGL confers the ability to inhibit the release of key inflammatory effector such as TNF-α, IL-1β, IL-12, and IFN-γ, and an elevated production of macrophage deactivating cytokines such as IL-11 and IL-13 molecules by cells of the host innate immune response. Ultimately, this inhibition predisposes this family to various clinical outcomes as seen in mice (**Reed *et al.*, 2004; Manca *et al.*, 1999; Manca *et al.*, 2001**) and rabbits (**Tsenova *et al.*, 2005**). The lack of laboratory strains H37Rv and Erdman to produce PGL, has been found to be due to genomic deletion of 7-base-pairs in the polyketide synthase gene *pks1-15*, which gives rise to a frame shift mutation in the DNA coding sequence and a non-functional Pks15/1 gene product.

Apart from differences in virulence in animal models of infection, studies in Vietnam have shown that lung cavitations were found in higher proportion in TB patients infected with Lineage 4 (i.e. the Euro-American lineage) (**Thwaites *et al.*, 2008)** and that Lineage 4 was also less likely to cause TB meningitis **(Caws *et al.*, 2008)**. The same study showed that the shorter duration of illness among TB meningitis patients was related to infection by Lineage 2 (East Asian/Beijing lineages) **(Thwaites *et al.*, 2008)**. However, a study in Netherlands where TB cases and controls were stratified by age, previous episode of TB and ethnicity, showed that the bacterial genotypes were not associated with chest radiological presentation **(Borgdorff *et al.*, 2004)**. These differing findings on links between bacterial genotypes and disease phenotypes could also be due to factors like sample size, stage of disease, geographical differences, and patient ethnicity. On the other hand, disease outcomes could also be due to different treatment strategies, immunization, patient characteristics such as HIV, diabetes, ethnicity and age **(Dye and Williams, 2010)**. A prospective study from South Africa showed an association of late sputum smear conversion among TB

patients who smoked and who were infected with W-Beijing genotype **(Visser *et al.*, 2012)**. If the MTBC genotype affects the formation of lung cavities, then the degree of lung cavitation will have an effect on the grading of the sputum smear. Patients with larger lung cavitations tend to be positive for maximum smear grade due to a higher bacterial load. Hence, severity of disease could also be correlated with MTBC lineages.

Furthermore, there is evidence indicating that different strains show different adaptation to anti-TB drug pressure which translates into varying mutations which confer different levels of resistance. A study by Gagneux *et al* showed that drug resistance mutations are often associated with competitive fitness cost and the degree of fitness is influenced by the genetic background of the strain (**Gagneux *et al.*, 2006**). Among the six main human MTBC lineages, Lineage 2, which includes Beijing strains, has been repeatedly associated with drug resistance (**Borrell and Gagneux, 2009**). While it is well believed that the fitness demonstrated by the high transmissibility could be due to epistatic effects (the phenomenon where the phenotypic effect of one mutation differs depending on the presence of another mutation) (**Borrell and Gagneux, 2009**), the main reasons accounting for this are not well understood. Finally, distinct lineages of MTBC have preferred drug resistance conferring mutations. In a recent study across different strain lineages, drug resistance conferring mutations especially mutations conferring resistance for INH were associated with different MTBC lineages, indicating possible epistatic interactions between drug resistance mutations and other mutations linked to different lineages (**Gagneux *et al.*, 2006; Borrell and Gagneux; 2009**). For example, While MTB has been associated with of *katG* 315 mutations, MAF West-African I strains had more mutations in the *inhA* promotor region (**Homolka *et al.*, 2010; Fenner *et al.*, 2012**).

Chapter 2: Rationale, Goals and Objectives

2.1. Rationale

For many years, molecular epidemiologic studies of TB have been applied in developed countries for the control of TB. These studies have focused largely on utilising molecular techniques in conjunction with classical epidemiology study mechanism of drug resistance and to address short- and long-term epidemiologic questions such as estimates of recent-versus-reactive disease, the extent of exogenous re-infection, identify patient groups most at risk, and circulating MTBC strains (**Mathema *et al*., 2006;Crampin *et al*., 2006)**. Similar work from developing countries is limited despite the endemicity of TB. For most endemic countries including Ghana, control of TB is hampered in part by lack of knowledge about the prevalence and transmission of the disease, and on the other part on the over reliance on old control strategies based on case detection by microscopy and the use of DOTs. Exploring the genetic diversity of MTBC strains in Ghana is relevant as it not only provide answers on the level of drug resistances in the country, it also provides the evolutionary linkage between strains circulating in Ghana to the neighbouring countries and globally.

Like other West-African countries, Ghana houses six of the seven human-associated MTBC lineages with up to 20% of TB caused by the highly neglected pathogen MAF (MTBC Lineages 5 and 6) (**Addo *et al,*, 2009; Yeboah-Manu *et al.*, 2011**). Although, little is known about these organisms, one of the striking features of MAF is that it only occurs in West African with uneven distribution; Lineage 6 occurs in the western part of West Africa, Lineage 5 towards the eastern part, with few countries like Ghana harboring both lineages. Based on already published evidence, we know that this pathogen differs from *M. tuberculosis* sensu stricto in many features, including host pathogen interaction and adaptation to anti-TB drugs, which are relevant to TB control. However, what we do not know is why this pathogen is restricted to West Africa (**de Jong *et al*., 2010**). One possibility could be that MAF is associated with human genetic diversity in West Africa. Indeed it has been hypothesized that MTBC has co-evolved with modern humans for

thousands of years (**Comas *et al*., 2013**). Most research works on MAF has centered on Lineage 6, but little is known on the characteristics of Lineage 5. Ghana provides a unique opportunity to study the biology of both Lineages.

2.2 Objectives

The objectives and outline of this PhD research were:

Objective 1: To define the drug susceptibility pattern of MTBC isolates from Ghana. (Chapter 3)

Outline: we determined *in vitro* drug susceptibilities of MTBC isolates circulating in Ghana and correlated this with the clinical response of patients.

Objective 2: To establish a rapid and simple molecular diagnostic tool for detecting drug resistant (DR) TB rapid in Ghana (Chapter 4).

Outline: We established and confirmed the accuracy of a rapid diagnostic tool for the detection of drug resistant MTBC strains in Ghana. This served as a valuable addition to the conventional TB diagnostic approaches in Ghana.

Objective 3: To evaluate customized lineage-specific sets of MIRU-VNTR loci for genotyping MTBC isolates in Ghana (Chapter 5).

Outline: We expanded our focus to micro-epidemiological studies. We aimed at defining a minimal set of MIRU-VNTR loci especially for Ghana where 20% of TB cases are caused MAF. We defined a minimal set of high discriminatory and cost effective MIRU 8 loci format which can be used for characterizing MTBC strains from Ghana.

Objective 4: To study MTBC diversity, including drug resistance, as a function of HIV co-infection and other epidemiological variables (Chapters 6 and 7).

Outline: In Chapter 6, with background information on the prevalence of MAF in Ghana, we sought for associations that could explain the possible geographic restrictions of MAF to West Africa. For Chapter 7, we expanded our focus on MAF Lineages 5 and 6 and confirmed their respective association with patient ethnicity and HIV co-infection.

Chapter 3: Drug susceptibility pattern of *Mycobacterium tuberculosis* isolates from Ghana; correlation with clinical response

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Abstract

Background: The worldwide emergence of drug resistant forms of the *Mycobacterium tuberculosis* complex, the causative agents of tuberculosis (TB) has the potential to make this important human infectious disease which is generally treatable, virtually incurable. Our aim therefore was to determine the *in vitro* drug susceptibilities of *M. tuberculosis* complex isolates and to correlate this with the clinical response of patients from whom the isolates were obtained.

Methodology: Sputum samples obtained from smear positive cases were cultivated on Lowenstein Jensen (LJ) medium. The susceptibilities to isoniazid (INH), rifampicin (RIF), streptomycin (STR) and ethambutol (EMB) were determined by the indirect proportion method, following isolate identification. Drug susceptibility of the isolates was then correlated with the individual clinical outcomes.

Results: One hundred and twenty one *M. tuberculosis* complex isolates were analyzed in this study. One hundred and nine (90.08%) and 12 (9.92%) were from new and previously treated cases respectively. Thirty-eight (31.40%), 18 (14.88%), 8 (6.61%) and 4 (3.31%) were resistant to STR, INH, RIF and EMB respectively. Forty seven (38.84%) of the tested isolates was resistant to at least one drug. Thirty one (25.62%) of the isolates were mono-resistant to one of the drugs; 24 (19.83%), 3 (2.48%), 3 (2.48%) and 1 (0.82%) to STR, RIF, INH and EMB respectively. Poly-resistance to STR/RIF, STR/INH and EMB/INH was observed in 2 (1.65%), 10 (8.26%) and 1 (0.82%) isolates respectively. Three (2.48%) of the isolates were multi-drug resistant (MDR) and of these, 2 were resistant (1.65%) to all the tested drugs and one was resistant to RIF and INH. Correlating the drug susceptibility with the clinical outcome of 79 cases including 2 Multidrug resistant TB (MDRs) isolates, we found that among our study population, the clinical outcome depended on whether the isolate was sensitive or resistant to RIF ($p<0.0005$).

Conclusion: A high level of primary drug resistance was observed, particularly to STR and INH, among the *M. tuberculosis* complex isolates in our study population and that treatment outcome depends mainly on the susceptibility of RIF.

Background

Tuberculosis (TB) continues to be a major public health problem in the world. It is estimated that one person dies every 15 seconds of TB and the World Health Organization (WHO) indicates that more than 9 million cases occurs annually with a mortality of 2 million (Word Health Organization, 2008). The directly observed treatment short course (DOTS) strategy, which allows patients to take their daily drugs under observation, thereby improving treatment compliance, is known to be increasing the number of people being cured of TB(**Rodger *et al.*, 2002**). A major challenge to this strategy in TB control globally is the incidence of strains of the *Mycobacterium tuberculosis* complex, the causative agent of TB, that are resistant to the first line drugs, especially rifampicin (RIF) and isoniazid (INH) (**Ormerod, 2005**). Individuals infected by such strains are not able to be cured by the DOTS treatment strategy and also make case management more complicated and expensive**.** There are an estimated 460,000 multidrug-resistant TB (MDR-TB) cases each year and approximately 25,000 of these cases are expected to have extensively drug-resistant TB (XDR-TB). MDR TB requires 18–24 months of treatment with expensive second line drugs, some of which are injectable agents. The cure rate is much lower than for drug susceptible TB, only around 60% **(Ormerod, 2005, World Health Organization, 2006, World Health Organization, 2010)**.

A crucial strategy for reducing the spread of MDR-TB is rapid detection of drug resistance followed by prompt and effective therapy **(Ormerod, 2005, World Health Organization, 2006, World Health Organization, 2010)**. The conventional laboratory diagnosis of drug resistant TB requires a viable, pure culture of *M. tuberculosis* complex organisms, followed by further cultivation on drug containing solid medium. The slow growing nature of the species of the *M. tuberculosis* complex makes conventional drug susceptibility testing a very slow and demanding process. The time between primary isolation and final drug susceptibility testing (DST) result is usually weeks and can be more than two months **(Canetti *et al.*, 1969, Kim, 2005, Piersimoni *et al.*, 2006)**. Recent advances in the molecular detection of mutations that are associated with

resistance to certain drugs and can be performed using DNA extracts prepared directly from sputum specimens can often provide results on the same day **(Miotto *et al.*, 2009)**. However this is not possible in countries with limited resources, where TB diagnosis relies mainly on sputum smear microscopy. Thus routine surveillance of the kind and level of resistance is very important. This will help in planning treatment regimens **(Piersimoni *et al.*, 2006)**.

Ghana has an annual TB incidence rate of 203/100,000 population, and ~50,000 new TB cases occur every year **(Word Health Organization, 2008)**. Current TB control measures in Ghana (like in most other developing countries) are primarily based on sputum smear microscopy, which has the ability to detect less than 50% of all TB cases **(Muvunyi *et al.*, 2010)**. Hence the real TB burden in Ghana is likely to be substantially higher than the official WHO estimates. Importantly, because of the lack of appropriate laboratory infrastructure, DST is not routinely performed in Ghana, and the extent of drug-resistant TB is not entirely known. The two main objectives of this study were 1) to determine the *in vitro* drug susceptibilities of isolates obtained from TB patients a 2) to determine whether the clinical responses correlate with the *in-vitro* drug susceptibilities of cases, especially MDRs.

Methods

Specimen and Data Collection

This was a cross-sectional analytical study in which all consecutive individuals, diagnosed with smear-positive pulmonary TB were enrolled between October 2007 and July 2009. A total of 121 isolates from 121 TB cases attending three different health facilities in four different districts were included in this study. The National Tuberculosis programme was responsible for selecting the districts. One district has a refugee population of over 20,000. The other two districts were selected based on the capacity of the laboratory technicians to provide the specimens required and good track record of TB data documentation.

Using a designed questionnaire, data on clinical characteristics, previous illness due to TB, previous therapy received, family history of TB and standard demographic data including age, sex, and residential address were obtained from each participant. Two previously analysed smear-positive sputum samples from each participant were then kept at the diagnostic centre/laboratory after addition of an equal volume of 1% cethylpyridium chloride (CPC): 2% sodium chloride decontaminant. All collected samples were stored, tightly capped, in an enclosed container and transported to the Noguchi Memorial Institute for Medical Research (NMIMR) for in-depth analysis within one week of collection. Approval for this study was obtained from the Institutional Review board of the NMIMR.

Isolation of *Mycobacterium species*.

All collected sputum samples were inoculated into 4 Lowenstein-Jensen (LJ) slants: 2 containing glycerol and the other 2 containing pyruvate. The inoculated slants were incubated at 37°C and the culture tubes were observed for mycobacterial growth. All mycobacterial isolates was identified using biochemical methods such as susceptibility to p-nitro benzoic acid (PNB) and to thiophene carboxylic acid hydrazide (TCH), pyrazinamidase activity (PZA), nitrate reduction,

niacin production; and detection of IS*6110* and RD9 by PCR analysis **(Asiimwe *et al.*, 2008, Yeboah-Manu *et al.*, 2001).**

Anti-TB Drug Susceptibility Testing

The susceptibilities of all identified *M. tuberculosis* complex isolates to INH (0.2µg/ml), RIF (40 µg/ml), streptomycin (STR) (4 µg/ml), and ethambutol (EMB) (2µg/ml) were determined by the indirect proportion method on LJ slants, as described previously **(Canetti *et al*., 1969)**.

Briefly, 1 -2 McFarland bacterial suspensions were prepared in 5-ml screw-cap tubes containing glass beads (diameter, 3.0 mm) in sterile distilled water. The suspensions were homogenized on a vortex mixer for 1 min and left to stand for at least 15 min to allow aerosol created during vortexing to settle. Serial 10-fold dilutions up to $1/10^4$ were prepared with sterile distilled water. $1/10^2$ dilutions were then used to inoculate drug containing media in duplicate while both the $1/10^2$ and $1/10^4$ were used to inoculate drug-free controls respectively. The tubes were incubated overnight at 37°C in a slanted position with loosened caps to allow the cells to settle on the medium and residual liquid to evaporate. After overnight incubation, the screw caps were tightened and the tubes were further incubated at the same temperature in an upright position. The initial reading of the tubes was performed on day 28 of incubation, while the final reading was done after 40 days of incubation. Drug resistance was expressed as the proportion of colonies that grow on drug containing medium to drug-free medium and the critical proportion for resistance was 1% and intermediate resistance is between 1-10% for all drugs **(Canetti *et al.*, 1969)**.

Definitions

Multidrug resistance (MDR) was defined as resistance to at least INH and RIF.

Other cases were categorized as follows: **Drug sensitive**–susceptibility to all of the drugs tested, **monoresistance**–resistance to only 1 drug; **polyresistance**–resistance to two drugs excluding the

INH: RIF combination and panresistance- resistance to all four tested drugs (**World Health Organization, 2010**).

Assessment to Treatment Outcome

Cases were followed during treatment and the final outcome, as recorded by the treating facility, was compared with the drug susceptibilities of the cultured isolate in our laboratory.

Cases were defined as previously

A new patient was defined as a TB patient who either had no prior anti-TB treatment or was treated with anti-TB drugs for less than 1 month **(Manissero *et al.*, 2010).**

A defaulter was defined as a patient who interrupted his treatment for more than 2 months after having received anti-TB treatment for at least 1 month

A relapse was considered an individual who became smear positive again after having been treated for TB and declared cured after the completion of treatment

A treatment failure case was considered a patient who began treatment for smear-positive TB but who remained smear positive or became smear positive again 5 months or later during the course of treatment

Treatment was completed if the patient was converted to smear negative at month 5, completed treatment but did not produce sputum on completion to be declared cured.

A case was considered cured if the patient completed treatment and maintained smear negativity on smear microscopy examination after treatment.

Data Analysis

All collected records were entered into a Microsoft Access database and exported to Excel for analysis. Data were expressed in means ± SD and ranges. The proportions of resistance to individual drugs and to different drug combinations were tabulated. Also, resistant cases were

differentiated as primary (being treated for TB for the first time) or acquired (previously treated). Student's independent samples *t* tests for numeric variables and chi square test for categorical variables. All significant levels were based on a *p* value less than 0.05.

Results

Patients Characteristics

The majority of patients were male, 82 (67.77%) and 39 (32.23%) were female. The age range was 8-88 years, arithmetic mean 37.25, modal class is 26 and median age of 36. The median age among female subjects was 30 (SD 8.3) with a range of 18- 60 years while that of the males was 34 (SD 8.6) with a range of 18–62 years. Among them were 10 (10.26%) refugees who live in the refugee camp at Budumbura. Patients sought healthcare after one month to 5 years of productive cough, with mean diagnostic delay of 6 months. All the cases were pulmonary sputum smear positive and confirmed by culture; and of these 109 (90.08%) were recorded as new cases with no history of previous treatment, while 12 (9.92%) had received previous treatment for TB.

Resistance Profile

121 isolates comprising of 99 *M. tuberculosis* and 22 *M. africanum* West African genotype were analysed in this study. The resistant profile of tested isolates is depictured in Table 5. Of the 121 isolates, 71 (58.68%) were susceptible to STR, 12 (9.92%) were intermediately resistant and 38 (31.40%) were resistant. One hundred and two (84.30%) of the isolates, 1 (0.82%) and 18 (14.88%) were susceptible, intermediate resistant and resistant to INH respectively. From the results of the 121 isolates, 113(93.39%), and 8 (6.61%) showed susceptibility and resistance respectively to RIF. One hundred and seventeen isolates (96.69%) were susceptible and 4 (3.31%) were resistant to EMB.

Thirty one (25.62%) of the isolates were mono-resistant to either one of the drugs; 24 (19.83%), 3 (2.48%), 3 (2.48%) and 1 (0.82%) to STR, RIF, INH and EMB respectively. Poly-resistance to STR/RIF, STR/INH and EMB/INH was observed in 2 (1.65%), 10 (8.26%) and 1 (0.82%) isolates respectively. Three (2.48%) of the isolates were MDR and of these, 2 (1.65%) were pan-resistant

and one was resistant to RIF and INH. Thus in all 47 (38.84%) of the tested isolates was resistant to at least one drug.

Twelve isolates were obtained from previously treated cases; 6 (50%) were susceptible to all drugs; 1 each (8.33%) intermediate resistant and mono-resistant to RIF respectively, 2 (16.67%) were poly-resistant to STR/INH and the remaining 2 (16.67%) of the isolates were pan-resistant.

Table 5: The susceptibility patterns of tested isolates to first-line anti-TB drugs from Ghana

Anti-TB drug	**Number of isolates tested**	**Susceptible n (%)**	**Intermediate Resistant**	**Resistant**
STR	121	71(58.68%)	12 (9.92%)	38 (31.40%)
INH	121	102 (84.30%)	1(0.82%)	18 (14.88%)
RIF	121	113(93.39%)	0	8 (6.61%)
EMB	121	117(96.69%)	0	4 (3.31%)
STR/INH	121			10 (8.26%)
STR/RIF	121			2 (1.65%)
INH/EMB	121			1(0.82%)
MDR	121			3 (2.48%)

n=number of isolates

Drug susceptibility and Outcome of Patients' Treatment

All patients involved in this study were treated with the standardized 6-month short course therapy regimen with INH and RIF as the main drugs. We were able to follow 79 (65.23%) of the cases during treatment and of these, 41 (51.90%) were cured, 23 (29.11%) completed treatment, 7 (8.86%) defaulted, 4 (5.06%) died, 2 failed (2.53%) and 2 (2.53%) relapsed.

Table 6: The distribution of drug resistance phenotypes between males and females observed among study participants.

Resistance	Female n (%)	Male n (%)	P VALUE
STR	9(23.08%)	15(18.29%)	0.3065
INH	1(2.56%)	2(2.44%)	0.9447
RIF	2(5.13%)	1(1.21%)	0.1085
ETH	1(2.56%)	0(0%)	0.1422
STR/INH	4(10.26%)	6(7.32%)	0.3798
STR/RIF	1(2.56%)	1(1.21%)	0.4422
INH/ETH	1(2.56%)	0(0%)	0.1422
MDR	2(5.13%)	1(1.21%)	0.1085
TOTAL	21(53.84%)	26(31.70%)	<0.0001

Completed Cases: The *M. tuberculosis* complex isolates from 15 (65.22%) of the completed cases were susceptible to all drugs; 16 and 20 of them were susceptible to STR and INH; none of them were resistant to RIF and EMB respectively. Five (21.74%) and 1 (4.35%) were mono-resistant to STR and INH respectively and 2 (8.7%) were poly-resistant to STR and INH.

Cured Cases: The isolates from 16 (39.04%) of the 41 cured cases were susceptible to all drugs; 21 (51.22%), 33 (80.49%), 38 (92.68%) and 39 (95.12%) of them were susceptible to STR, INH, RIF and ETH respectively. Fourteen (34.15%) were resistant or intermediately mono resistant to STR; 2 (4.88%) mono resistant to INH; 2 (4.88%) mono-resistant to RIF; 1 (2.44%) mono resistant to EMB; and 5 (12.19%) poly-resistant to STR and INH.

Defaulted Cases: Five (71.43%) of the defaulted cases were susceptible to all tested drugs; 1 (14.28%) each mono-resistant to STR and poly-resistant to STR and INH respectively.

Dead Cases: There were 4 dead cases, 2 (50%) of them were pan-resistant and the remaining 2 (50%) were susceptible to all tested drugs

Failed Cases: One (50%) of the two cases was mono-resistant to RIF and the other was mono resistant to STR.

Relapsed: One was poly-resistant to STR and INH.

X^2 analysis shows that the sensitivity levels of STR (X^2 = 3.3600; P = 0.3394) and INH (X^2 = 3.4915; P = 0.3219) have no influence on treatment outcome. On the other hand, the sensitivity levels to RIF (X^2 = 17.7553; P = 0.0005) and EMB (X^2 = 13.0074; P = 0.0046) have significant associations with treatment outcome (table 7).

Table 7: The drug susceptibility of the various tested drugs was correlated to the treatment outcomes and the findings underscore the importance of rifampicin in TB treatment.

Drugs		**Cured**	**Completed**	**Defaulted**	**relapsed Failed/died**	**Total (%)**	**P. value**
STR	Sensitive	21	16	6	3	46 (59.0)	0.3394
	Resistance	19	7	2	4	32 (41.0)	
INH	Sensitive	33	20	7	4	64 (82.1)	0.3219
	Resistance	7	3	1	3	14 (17.9)	
RIF	Sensitive	38	23	8	4	73 (93.6)	0.0005
	Resistance	2	0	0	3	5 (6.4)	
EB	Sensitive	39	23	8	5	75 ((96.2)	0.0046
	Resistance	1	0	0	2	3 (3.8)	

Discussion

The two main objectives of this study were 1) to determine the *in vitro*-drug susceptibilities of isolates obtained from TB patients and 2) to determine whether the clinical responses correlate with the drug susceptibilities of the isolates from those cases, especially the MDRs. The results of *in vitro* antibiotic susceptibility testing can predict the clinical response to treatment and guide the selection of antibiotics **(Small, 2000)**. However, the relationship between DST result and clinical outcome is not always straightforward and depends on other parameters such as host factors including immune status, age and co-morbidity. It is well known that in severe bacterial infections, treatment failure can occur when the infecting organism has displayed in-vitro susceptibility to the used antibiotics **(Kim et al, 2005, Small, 2000, Bottger 2001)**. In a study on retreatment cases, it was found that cavitary disease *per se*, irrespective of drug-resistance status of the *M. tuberculosis* isolate, was associated with poor treatment outcomes (**Kritski *et al*, 1997**). On the other hand certain individuals are able to clear infections with resistance phenotypes; this may be due to the interaction of many factors, among which acquired immunity is presumably important (**Bottger 2001)**. The correlation between DST result and clinical outcome of antibiotic treatment of several mycobacterial illnesses have been documented in some countries (**Kritski et al, 1997**, **Yew et el, 2000**), however to the authors knowledge, this is the first study conducted in Ghana. Analysis confirmed that resistance to RIF and EMB as well as MDR is predictive of poor response to treatment and this draws attention to the need for a prompt response. Therefore the importance of DST of isolates from patients that do not convert to sputum negative by the third month using rapid methods like the MTBDR-plus (**Miotto *et al*, 2009**) is very crucial. Interestingly, we did not find INH resistance as important in determining the outcome of treatment in our study populations. At least two molecular mechanisms are known to be involved in INH resistance in *Mycobacterium spp*. The commonest of these are associated with mutations in the *katG* gene which encodes the catalase peroxidase, needed for activation of INH and mutations in the promoter region of the *inhA*gene encoding, NADH-dependent enoyl acyl carrier protein

reductase, the primary target for this drug. While mutations in the *KatG* gene lead to high levels of INH resistance, mutations in *inhA* promoter generally result in low level resistance (**Somoskovi *et al.*, 2001**). We hypothesised that most of the observed resistance in INH were of low-level resistance and therefore the drug might still be active *in vivo*, especially given that treatment was by combinational therapy.

Findings from this study indicated that about 31%, 15%, 7% and 3% of tested isolates were resistant to STR, INH, RIF and EMB respectively. Mono-resistance and poly-resistance were observed in about a quarter and a tenth of the isolates tested respectively. The primary MDR-TB rate among the study population was 0.9%, among those previously treated cases was 16.67 and the combined was 2.5%, which is higher than the national average of 1.9% (**World Health Organization, 2008**) as recorded, but comparable to a recent report of 2.2% (**Owusu-Dabo *et al.*, 2006**). Re-treated patients yielded more drug-resistant *M. tuberculosis*, including MDR ($P < 0.001$), than new cases. The level of resistance as observed in our study is intermediate between the two reports on drug resistant TB in Ghana, while that reported in 1989 (**van der Werf *et al.*, 1989**) is on the high side, that published in 2006 (**Owusu-Dabo *et al.*, 2006**) has some comparable figures and some lower than what we are reporting. These differences could be the result of regional variations and the commitment of regional TB programs. The isolates used in this study were from cases residing in four main health facilities including health facilities that report stigmatization by both communities and health officials (**Dodor *et al.*, 2009**). The need for more education and training on DOTs programs in the regions involved in this study is very essential. Nevertheless, the consensus of the three studies is the high rate of resistance observed to STR and INH, thus making Ghana among countries in Africa with a high rate of resistance (**Owusu-Dabo *et al.*, 2006**). These findings should serve as a clarion call to action by governments and health officials to deal with the high prevalence of drug-resistant tuberculosis. In addition the national TB program needs to look at abuse of drugs used for TB in treating other

infectious diseases. There is also the need to improve treatment compliance and early reporting. The mean duration of seeking healthcare and the high bacterial load as observed in the patients (data not shown) involved in the study suggests a probable high rate of transmission of resistant strains in the community. We are in the process of looking at the transmission of resistant genotypes in the community.

More males reported with TB than females from all the health facilities studies; all together 67% of the participants are males and this compares very well with other reports. The reasons why more men report with TB cannot be explained and this needs further investigation; is it due to health seeking behaviour and that women do not have time or they do not have the final decision when to seek help or males engage in more risk activities? We are of the opinion that the first reason may be true as shown in table 2, more females reported with difficult to treat TB than males ($p<0.0001$).

Conclusion

We found a high rate of drug resistance among the isolates we analyzed and that treatment outcome depends primarily on the susceptibility of the *M. tuberculosis* isolate to RIF. This confirms the central role of RIF in TB treatment. A conscious effort must therefore be made by the health system to restrict its use in the community. We also found that even though less females report with TB, more females had drug resistant TB compared to males ($p<0.0001$).

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Chapter 4: Establishment and Evaluation of Genotype MTBDR*plus* for Rapid Detection of Drug Resistant Tuberculosis in Ghana

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Abstract

Background: Rapid but simple diagnostic tool for detecting drug resistant (DR) tuberculosis (TB) has been acknowledged as important for effective management and control of DR-TB. Our objective was to establish a molecular line-probe assay (GenoType® MTBDR*plus*) for detecting DR-TB in Ghana.

Method: We first screened 113 *Mycobacterium tuberculosis* isolates by both standard phenotypic indirect proportion method and MTBDR*plus*. For isolates found resistant either by phenotypic DST and/or MTBDR*plus*, the *rpoB,* and *katG* genes as well as the promoter regions of *oxyR-ahpC* and *inhA* were sequenced to identify mutations. We then analyzed an additional 412 isolates by MTBDR*plus* only.

Results: Interpretable MTBDR*plus* results were obtained for all 525 isolates (100%). Overall, forty-three (8.2%) and 8 (1.5%) isolates were resistant to isoniazid (INH) and rifampicin (RIF), respectively, and 8 (1.5%) were multidrug-resistant. Among these resistant isolates, mutations in codon 450 of *rpoB* and codon 315 of *katG* conferring resistance to RIF and INH, respectively, dominated. We found two RIF resistant isolates with S450L substitution each harboring an additional mutation at S388L and Q409R, respectively. Using the phenotypic testing as gold standard, the GenoType® MTBDR*plus* assays showed a sensitivity/specificity for the detection of RIF and INH resistance and MDR of 100%/100%, 83.3%/100% and 100%/100%, respectively.

Conclusion: The high sensitivity makes GenoType® MTBDR*plus* applicable and valuable addition to the conventional TB diagnostic algorithm in Ghana.

Introduction

Tuberculosis (TB) continues to be a major public health problem globally, with an annual incidence of 9 million new cases, killing more than 1.5 million people annually, most of which occurs in low resource countries (**Global TB report, 2014**). One of the main challenges in TB control is the emergence and spread of drug resistance (DR) (**Ormerod, 2005; WHO, 2010**) Even though TB is a treatable disease, if DR is not controlled, it may eventually result in TB becoming untreatable. Multidrug resistance (MDR) is defined as resistance to at least isoniazid (INH) and rifampicin (RIF) (**WHO, 2010**). According to 2014 WHO Global Tuberculosis Control Report, there were about nine million TB cases and among these, close to 480,000 were MDR cases (**Global TB report, 2014)**. In 2005, the global Technical and Advisory Group on TB approved a new Stop TB Strategy and indicated in addition to many other things, for effective control of TB, DR-TB must be well managed (**WHO, 2005**).

Drug resistance arises due to improper use of antibiotics in chemotherapy such as inadequate treatment regimens, and failure to ensure that patients complete the whole course of treatment (**Banerjee *et al.*, 2008**). When a patient is infected with a drug-susceptible strain of *Mycobacterium tuberculosis* complex (MTBC), poor adherence to treatment will lead to a drug resistant form of the disease; this type of drug resistance is termed acquired drug-resistance. Individuals who develop active disease with a drug-resistant MTBC strain can transmit this form of TB to other individuals, if not detected early and treated appropriately. New TB patients initially infected with a drug-resistant form are termed primary resistant cases (**Blower and Supervie, 2007**). To reduce the emergence and subsequent spread of drug-resistant TB, there is the need for early diagnosis so as to put patients on appropriate drugs as soon as possible (**Blower and Supervie, 2007; Drobniewski *et al.*, 2006)**.

The conventional methods for drug susceptibility testing (DST) are labour intensive, involving sequential procedures for isolation of mycobacteria from clinical specimen in liquid or solid

media, identification of MTBC, and *in vitro* testing of susceptibility to anti-TB drugs. At the same time, MTBC is a slow growing organism taking several weeks for macroscopic growth and requiring biosafety level 3 containment. Thus standardized and optimised MTBC culture and DST procedures require well equipped and safe laboratories, as well as trained personnel operating under quality assured protocols. Because of these factors, it takes several weeks to months for laboratory results to become available, and during this time, patients may be prescribed inadequate treatment, thus fuelling the development and/or spread of drug resistance. Moreover, mycobacterial culture and DST capabilities are severely limited in resource-poor countries.

Resistance to anti-TB drugs is caused by chromosomal mutations in genes encoding drug targets, in regulatory regions of the target gene and in drug-activating genes. Several molecular diagnostic methods have been developed recently for rapid identification of MDR-TB, some of which are also suitable for resource-poor countries (**Banerjee *et al*., 2008**;

Drobniewski *et al*., 2006; GenoType MTBDR*plus,* 2007*;* Marinus *et al*., 2008). In this study, we established the line probe assay (LPA) known as MTBDR*plus* in Ghana, and compared the results to the standard phenotypic DST using the indirect proportion method (**Canetti *et al*., 1969**).

Materials and Methods

Mycobacterial Isolates

Isolates used in this study were cultivated in a previous study that aimed to genotype isolates from Ghana for phylogenetic and molecular epidemiological analysis (**Yeboah-Manu *et al*., 2012**). The procedures used for sample collection, diagnosis and treatment of TB was as routinely employed by the National Tuberculosis Programme (NTP); however the protocol was reviewed by the institutional review board of the Noguchi Memorial Institute for Medical Research (NMIMR), with federal-wide assurance number FWA00001824. The isolates which were previously stored at -80 °C were sub-cultured on Lowenstein-Jensen media slants, incubated at 37 °C until confluent growth was observed. After harvest, the pellet was heat inactivated at 95 °C in nuclease free water for 60 min and allowed to cool under room temperature. The heat-inactivated cells in 1.5 mL microfuge tubes were centrifuged at 14,000 rpm to pellet cells for DNA extraction.

Isolation of Genomic DNA

After harvest, the pellet was heat inactivated at 95 °C in nuclease free water for 60 min and allowed to cool under room temperature. The heat-inactivated cells in 1.5 mL microfuge tubes were centrifuged at 14,000 rpm to pellet cells for DNA extraction. Genomic DNA was extracted according to the protocol outlined by van Soolingen *et al*., 1993 (**van Soolingen *et al*., 1993**). Briefly, the mycobacterial cell wall was disrupted by adding lysozyme (50 µL lysozyme of 10 mg/mL) vortexed and incubated overnight, followed by addition of 75 µL of 10% SDS, 10 µL proteinase K (20 mg/mL), vortexed softly and incubated 15 min at 65 °C. After, we added 100 µL of 5M NaCl followed by 100 µL CTAB/NaCl which was pre-warmed at 65 °C. After vortexing, the extracted DNA was purified by chloroform/ isoamyl alcohol extraction. The DNA contained in the upper phase was precipitated with isopropanol and washed with ethanol. The dried DNA was then re-suspended in 100 mL of water.

Anti-TB Drug Susceptibility Testing

Phenotypic Drug Susceptibility Testing: The indirect proportion method with LJ slants using critical concentrations of INH (Sigma, I3377) (0.2 µg/mL) and RIF (Sigma, R3501) (40 µg/mL) was used to screen 113 isolates. Drug resistance was expressed as the proportion of colonies that grew on drug containing medium to drug-free medium and the critical proportion for resistance was 1% (**Canetti *et al*., 1969)**.

Molecular Drug Susceptibility Testing by Line Probe Assay: Clinical MTBC isolates were screened for their susceptibility to INH and RIF using the Genotype MTBDR*plus* (Hain lifescience), according to the manufacturer's protocol (**GenoType MTBDR*plus,* 2007)**. Drug resistance was expressed as the absence of wild-type band, presence of mutation band or both.

Mutation Analysis of Drug Targets: The isolates diagnosed as drug-resistant either by phenotypic or LPA were used for targeted DNA sequence analyses. Four resistance genes, *rpoB* (RIF), *katG* and promoter regions of *inhA* and *oxyR-ahpC* (INH), were amplified by PCR for direct DNA sequencing. The PCR reaction in all instances contained 3 µL of 10X buffer, 1.8 µL of 15 mM $MgCl_2$, 3 µL of Q solution, 0.6 µL of 10 mM dNTP mix, 1.8 µL of each primer, 0.2 µL of Hot-start Taq polymerase from Qiagen, 14.8 µL of nuclease-free water and 3 µL of template DNA. Cycling conditions were: initial denaturation at 95 °C for 5 min and 35 cycles of denaturation at 96 °C for 1 min, annealing at primer-specific Tm (Table 8) for 1 min, extension at 68 °C for 1 min and final extension at 72 °C for 10 minutes and the obtained amplicons were sequenced by outsourcing.

Table 8: The primers used for the DNA sequencing assay				
Gene	**Primer Name**	**Primer sequence (5'-3')**	**Amplicon size**	**T_m**
inhApro	Ko3	GGCACGTACACGTCTTTATGTA	478 bp	65 ºC
	Ko4	GGTGCTCTTCTACCGCCGTGAA		
katG	Ko11	CCAGCGGCCCAAGGTATC	850 bp	66 ºC
	Ko12	GCTGTGGCCGGTCAAGAAGAAGT		
rpoB	Ko1	GTAGTCCACGCCGTAAACGG	601 bp	65 ºC
	Ko2	ACGTCCATGTAGTCCACCTCAG		
oxyR-ahpC	Ko56	ACCACTGCTTTGCCGCCACC	236 bp	70 ºC
	Ko57	CCGATGAGAGCGGTGAGCTG		

Data Analysis: Data obtained from the various tests were double entered and validated to remove duplicates and data entry inconsistencies. The DNA sequence reads were screened for possible mutations by comparing the gene sequences with corresponding sequences from H37Rv genome downloaded from the Tuberculist database using the Staden software (**Staden *et al.*, 1998**). DNA sequencing was repeated for all isolates with un-reported mutation(s) for verification. The result of the phenotypic DST assay was used as the gold standard to calculate the sensitivity and specificity for detecting INH and RIF resistance by LPA.

Results

Phenotypic Susceptibility test, GenoType® MTBDR*plus* and Mutations in Drug Resistance Genes

We determined resistance profiles of 113 isolates phenotypically using the indirect proportion method. These 113 isolates form a subset of the total 525 isolates used in this study. Comparative analysis demonstrated good overall agreement between the LPA and phenotypic DST results. Ten out of the 12 (83.3%) phenotypically INH mono-resistant isolates were also found resistant by MTBDR*plus* assay (Table 9). From the DNA sequencing analyses, all the 10 INH mono-resistant isolates identified by MTBDR*plus* showed *katG* substitution S315T. The remaining 2 isolates phenotypically resistant to INH had no mutation in any of the target genes we sequenced. Both RIF mono resistant and both MDR isolates, diagnosed resistant by phenotypic DST were confirmed by MTBDR*plus*. DNA sequencing showed that both RIF mono-resistant isolates(Table 9) had H445Y *rpoB* amino acid substitution whereas one MDR isolate had *katG* S315T with *rpoB* S450L and the other *katG* S315T with *rpoB* D435V (Table 9).

Table 9: Correlation between phenotypic DST, MTBDR*plus* assay and target sequencing analyses						
	INH			**RIF**		
Isolate	**Phenotype**	**MTBDR*plus***	**Mutation**	**Phenotype**	**MTBDR*plus***	**Mutation**
TBNM008	R	R	*KatG* S315T	S	S	-
TBNM016	R	R	*KatG* S315T	S	S	-
TBNM022	R	R	*KatG* S315T	S	S	-
TBNM059	R	R	*KatG* S315T	S	S	-
TBNM072	S	S	-	R	R	*rpoB* H445Y
TBNM078	R	R	*KatG* S315T	R	R	*rpoB* S450L
TBNM082	R	R	*KatG* S315T	S	S	-
TBNM086	S	S	-	R	R	*rpoB* H445Y
TBNM114	R	R	*KatG* S315T	S	S	-
TBNM117	R	R	*KatG* S315T	S	S	-
TBNM139	R	S	-	S	S	-
TBNM147	R	R	*KatG* S315T	R	R	*rpoB* D435V
TBNM148	R	R	*KatG* S315T	S	S	-
TBNM155	R	S	-	S	S	-
TBNM169	R	R	*KatG* S315T	S	S	-
TBNM171	R	R	*KatG* S315T	S	S	-

Drug Susceptibility Testing with GenoType® MTBDR*plus*

Overall, 525 isolates were analysed by GenoType® MTBDR*plus* in this study. These came from a retrospective collection and have all been confirmed using IS*6110*-PCR and LSP as members of the MTBC (**Yeboah-Manu *et al.*, 2011**). As summarised in Table 10, MTBDR*plus* identified 43/525 (8.2%), 8/525 (1.5%) and 8/525 (1.5%) of the isolates as INH-mono-resistant, RIF mono-resistant and MDR, respectively, and 59 (11.2%) harboured at least one drug resistance mutation. Among the INH-mono resistant strains, 37/43 (86.0%) had mutation(s) within the *katG* target only; and of these, 23/37 (62.2%) and 1/37 (2.7%) had *katG* Mt1 and *katG* Mt2 mutation bands, respectively. Nine out of the 37 (24.3%) isolates had both presence of *katG* Mt1 band and absence of a wild type band, while 2 (5.4%) had both *katG* Mt2 band present and absence of a wild type band. Four out of the 43 INH resistant isolates (9.3%) had mutation(s) within the *inhA* promoter

region alone; of these, 3/4 (75%) had both *inhA* Mt1 band present and absence of *inhA* WT1 band. The remaining 1 isolate had *inhA* Mt3A present and WT2 band absent. The remaining 2 of the 43 (4.7%) INH resistant isolates had both *KatG* Mt1 and *inhA* Mt1 bands (Table 10).

Four of the 8 (50.0%) RIF mono-resistant isolates were identified by the presence of mutation bands only; 2 had *rpoB* Mt3, 1 each had *rpoB* Mt2B and both *rpoB* Mt2A and *rpoB* Mt2B bands respectively. Of the 4 (50.0%) remaining RIF resistant isolates, 2 had *rpoB* Mt3 band present as well as absence of *rpoB* WT8 band, while the other 2, had *rpoB* Mt2A band and absence of *rpoB* WT7 band (Table 3). The mutations associated with the MDRs are also indicated in Table 10.

Table 10: Summary of MTBDR*plus* Assay Results of 525 MTBC Isolates

Resistance	Isolates	Locus	WT Band	MT Band	WT and MT bands
INH Only	43 (8.2%)	*katG* (37)	2	Mt1 (23) Mt2 (1)	WT / Mt1 (9) WT / Mt2 (2)
		inhApro (4)	-	-	WT1 / Mt1 (3) WT2 / Mt3A (1)
		Both (2)	-	*katG* Mt1/ i*nhApro* Mt1 (2)	-
RIF Only	8 (1.5%)	RRDR (8)	-	Mt2A / 2B (1) Mt2B (1) Mt3 (2)	WT7 / Mt2A (2) WT8 / Mt3 (2)
MDR	8 (1.5%)	*rpoB* / *katG*.(6) *rpoB*/ *katG* /*inhApro* (2)	-	*katG* Mt1 / *rpoB* Mt1 (1) *rpoB* Mt2A / *katG* Mt1 (1) *rpoB* Mt3 / *katG* Mt1 (2) *katG* Mt1/ *inhApro* Mt3A / *rpoB* Mt2A (1)	*katG* WT / *rpoB* Mt3 (1) *rpoB* WT7 / *katG* WT /i*nhApro* WT/ *rpoB* Mt2A/*katG* Mt1/*inhApro* Mt3A (1) *rpoB* WT7/*katG* WT/*rpoB* Mt2A/ *katG* Mt1 (1)
ANY	59 (11.2%)	-	-	-	

NB: INH Only: - Isolates that had mutation (s) in the inhApro region and or in the katG gene without any mutation in the rpoB gene. RIF Only: - Isolates with mutation(s) in the rpoB gene without any in the inhApro or the katG gene. MDR: - Isolates with mutations in rpoB gene and inhApro and/or katG gene. ANY: - Total number of isolates with at least one mutation. RRDR: - Rifampicin Resistance Determining Region of the rpoB gene. WT:-Wild-type band absent. MT: - Mutation band present

Frequency of Mutations in Isoniazid and Rifampicin Resistance Associated Targets

Based on the GenoType® MTBDR*plus results*, out of the 51 INH resistant isolates, 16 (31.4%) had mutations in the promoter region of *inhA* (Table 11; Figure 1). Moreover, 42/51 (82.4%)

isolates had the S315T *katG* mutation which is generally the most prominent INH resistance associated mutation found in clinical isolates (**Homolka *et al*., 2010**). In addition to the above mentioned non-synonymous SNPs; we found several synonymous mutations (Table 11; Figure 1). All the 16 RIF resistant isolates had at least one mutation within the resistance determining region (RRDR) of the *rpoB* gene (Table 11; Figure 1). Five isolates (31.25%) each had the SNPs C1349T and C1333T translated as S450L and H445Y, respectively; 2 isolates with C1333G translated as H445D, 1 isolate each with SNP A1334G and A1304T translated as H445R and D435V, respectively, and lastly, 1 isolate each with double SNPs C1163T/C1349T and A1226G/C1349T, respectively, translated as S388L/S450L and Q409R/S450L (Table 11).

Table 11: Mutations identified from the DNA sequencing of INH and RIF resistance associated loci

Gene (Number of Isolates Screened)	Mutation	Effect of Mutation	Number of isolates with specific SNP
***inhApro* (51)**	-8T/C	-	2 (3.9%)
	-15C/T	-	4 (7.8%)
	-47G/C	-	5 (9.8%)
	-102G/C	-	5 (9.8%)
***katG* (51)**	G944C & C723G	S315T & P241P	1 (1.9%)
	G944C	S315T	39 (76.5%)
	G944A, A949G & C723G	S315N, I317V & P241P	1 (1.9%)
	G(C)944(5)C(T)	S315T	1 (1.9%)
	G944C & C1132T	S315T & L378L	1 (1.9%)
***rpoB* (16)**	C1163T & C1349T	S388L* & S450L	1 (6.3%)
	A1226G & C1349T	Q409R@ & S450L	1 (6.3%)
	C1349T	S450L	5 (31.3%)
	C1333T	H445Y	5 (31.3%)
	C1333G	H445D	2 (12.5%)
	A1334G	H445R	1 (6.3%)
	A1304T	D435V	1 (6.3%)

NB: The reference gene (rpoB) used here is the MTBC (H37Rv) and not the E.coli variant.

Discussion

We analysed 525 MTBC isolates from patients with pulmonary TB for drug resistance by the genotype MTBDR*plus* assay and identified RIF mono-resistance in 8 (1.5%), INH mono-resistance in 43 (8.2%) and MDR in 8 (1.5%) of the isolates. In all, 59 (11.2%) isolates showed any form of resistance. The proportion of INH resistance as measured by the MTBDR*plus* was found to be significantly higher than that for RIF among our clinical isolates from Ghana ($p < 0.001$); this supports our earlier findings using the proportion method (**Yeboah-Manu *et al*., 2011**). The observed proportion of MDR is similar as reported by Homolka *et al.* between 2001 and 2004 (**Homolka *et al*., 2010**), and comparable to the 1.9% reported by the National Control Programme in 2013 (**Global TB report, 2014**). These findings indicate that the MDR rate in Ghana is low and has been stable for about a decade.

It has been shown that association of RIF resistance with mutations within the RRDR varies from 78% to 100% in different countries (**Telenti *et al*., 1993; Hillemann *et al*., 2005**). Among the isolates that we worked on, all phenotypically RIF resistant strains were also detected by the MTBDR*plus*. Thus, we sequenced the RRDR of all isolates that had some form of RIF resistance and found that all the 16 RIF resistant isolates had at least one non synonymous mutation within the RRDR. The role of the new mutation Q409R we detected from the sequencing cannot at the moment be inferred from the available findings. Overall, our results strongly support the use of diagnostics that target mutations within the RRDR of the MTBC as a rapid laboratory DST to support patients care in Ghana.

Contrary to RIF resistance, MTBC acquires isoniazid (INH) resistance through mutations in multiple genes such as those involved in mycolic acid biosynthesis and cellular response to oxidative stress (**Zyang and Yew, 2009; Ozturk *et al*., 2005; Costa *et al*., 2009**). Similar to other settings, 43/51 (84.3%) of INH resistant isolates had mutations within *katG* with 42 isolates having the *katG* mutation S315T and the remaining isolate harbouring S315N,one novel amino

acid substitution I317V and an additional synonymous mutation 723C/G. Two out of the 42 *katG* S315T mutant isolates in addition had the additional silent mutations at nucleotide position 723 (C/G) and 1132 (C/T), respectively. In total, 16/51 (31.4%) of the INH resistant isolates were found to have mutations within the *inhA* promoter region; 10/16 (62.5%) of the *inhApro* mutant isolates also had the S315T *katG* mutation. Four and two out of the six *inhApro* mutant isolates without the S315T *katG* mutation respectively were -15C/T and -102G/A. These findings compare with other reports as it is known that mutations in *katG* are responsible for 50% to 95% of INH resistant strains and *inhA* promoter mutations in 10-30% of strains (**Poudel *et al*., 2012; Ramaswami and Musser, 1998; Slayden and Barry, 2000**). The role of the new mutations identified in this work were not studied further here but are worth pursuing. Within the isolates that were analysed, we did not find mutations within the promoter region of the *oxyR-ahpC* contrary to an earlier work done on some Ghanaian MTBC isolates (**Homolka *et al*., 2010**).

In summary, we found a good correlation between phenotypic RIF resistance and mutation within resistant conferring targets, making rapid diagnostic test (MTBDR*plus* line-probe assay) that explore these mutations a good tool for detection of RIF mono-resistant and MDR cases in Ghana. Nevertheless, misdiagnosis of approximately 16% of INH mono resistant isolates as susceptible by MTBDR*plus* line-probe assay requires further consideration.

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Chapter 5: Evaluation of customised lineage-specific sets of MIRU-VNTR loci for genotyping *Mycobacterium tuberculosis* complex isolates in Ghana

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Abstract

Background: Different combinations of variable number of tandem repeat (VNTR) loci have been proposed for genotyping *Mycobacterium tuberculosis* complex (MTBC). Existing VNTR schemes show different discriminatory capacity among the six human MTBC lineages. Here, we evaluated the discriminatory power of a "customized MIRU12" loci format proposed previously by Comas *et al*. based on the standard 24 loci defined by Supply *et al*. for VNTR-typing of MTBC in Ghana.

Method: One hundred and fifty-eight MTBC isolates classified into Lineage 4 and Lineage 5 were used to compare a customized lineage-specific panel of 12 MIRU-VNTR loci ("customized MIRU-12") to the standard MIRU-15 genotyping scheme. The resolution power of each typing method was determined based on the Hunter-Gaston- Discriminatory Index (HGDI). A minimal set of customized MIRU-VNTR loci for typing Lineages 4 (Euro-American) and 5 (*M. africanum* West African 1) strains from Ghana was defined based on the cumulative HGDI.

Results and Conclusion: Among the 106 Lineage 4 strains, the customized MIRU-12 identified a total of 104 distinct genotypes consisting of 2 clusters of 2 isolates each (clustering rate: 1.8%), and 102 unique strains while standard MIRU-15 yielded a total of 105 different genotypes, including 1 cluster of 2 isolates (clustering rate: 0.9%) and 104 singletons. Among, 52 Lineage 5 isolates, customized MIRU-12 genotyping defined 51 patterns with 1 cluster of 2 isolates (clustering rate: 0.9%) and 50 unique strains whereas MIRU-15 classified all 52 strains as unique. Cumulative HGDI values for customized MIRU-12 for Lineages 4 and 5 were 0.98 respectively whilst that of standard MIRU-15 was 0.99. A union of loci from the customised MIRU-12 and standard MIRU-15 revealed a set of customized eight highly discriminatory loci: 4052, 2163B, 40, 4165, 2165, 10 ,16 and 26 with a cumulative HGDI of 0.99 for genotyping Lineage 4 and 5 strains from Ghana

Introduction

Tuberculosis (TB) is a major public health problem worldwide, causing 8.8 million new cases and more than 1.4 million deaths each year (**Global Tuberculosis report, 2013**). The main strategy for controlling TB, especially in low resourced countries, is case detection and treatment using the directly observed treatment short course (DOTS) strategy (**Guidelines for the Clinical Management of TB, HIV co-Infection in Ghana, 2007**). The conventional indicators used for assessing TB control programmes focuses on the proportion of patients with new sputum smear positive pulmonary disease that are cured by the end of treatment or whose sputum microscopy becomes negative after the first 2 months of treatment (**Ghana Heath service report, 2011**). Such indicators ignore equally important aspects of TB control such as the duration of infectivity, the frequency of reactivation, and the risk of progression among the infected contacts, or the risk of transmission. Thus the control of TB also depends on understanding the patterns and dynamics of transmission which is useful for the implementation of public health measures to reduce sources of infection (**Mathema *et al*., 2006; Kato-Maeda *et al*., 2000**).

A number of molecular markers are available for differentiating members of the *Mycobacterium tuberculosis* complex (MTBC) for conventional epidemiological investigations of TB outbreaks and to assess risk factors associated with recent transmissions (**Kan *et al*., 2008; Kim *et al*., 2001**). Mycobacterial interspersed repetitive unit-variable number of tandem repeats (MIRU-VNTR) typing, have overcome most of the shortcomings of IS*6110* RFLP (**van Embden *et al*., 1993; Cowan *et al*., 2002; Mokrousov *et al*., 2004**), and have now replaced this older technique as the new gold standard for molecular epidemiological investigation of TB. MIRU-VNTR typing which uses genomic diversity at different VNTR loci can have a cumulative resolution comparable to that of IS*6110* RFLP analysis depending on the combination of loci analysed (**Savine *et al*., 2002; Sun *et al*., 2004; Blackwood *et al*., 2004; Yong-Jiang *et al*., 2004; Allix *et al*., 2006; Barlow *et al*., 2001**).

Several combinations of MIRU- VNTR loci have been published with initial methods relying on only a few loci, which turned out to have low discriminatory power among MTBC isolates (**Shamputa *et al*., 2010; Murase *et al*., 2008; Dong *et al*., 2012; Zhou *et al*., 2011**). Subsequently, a standard MIRU-12 loci set with discriminatory power close to *IS6110*-RFLP was proposed for molecular epidemiological studies in TB (**Supply *et al*., 2001; Scott *et al*., 2005; Cowan *et al*., 2005**). More recently, this initial MIRU-12 set was replaced by the standard MIRU-15 set, and currently, standard MIRU- 24 loci set (**Supply *et al*., 2006**) has been proposed for optimal discrimination of closely related strains. The standard MIRU15 set which includes six of the previous MIRU- 12 with nine additional loci has been recommended as the standard for routine molecular epidemiology of TB, including outbreak investigations and population-based transmission studies. MIRU-24 set comprises the same 15 loci plus an additional nine provide additional information aimed at phylogenetic and population genetic aspects of MTBC.

The usage of the standard MIRU-15 and MIRU-24 has helped to gain insight into the transmission dynamics of MTBC. However, the initial selection of these loci was to some extent biased towards strains belonging to Lineage 4 (Euro-American lineage) (**Supply *et al*., 2001**). The inability of the proposed loci led to new customized sets for Lineage 2 strains that include the clinically relevant Beijing family of strains (Shamputa et al., 2010). However, the human-associated MTBC includes 6 additional lineages (**Gagneux *et al*., 2006; Coscolla *et al*., 2013; Reed *et al*., 2009; Comas *et al*., 2013; Firdessa *et al*., 2013**), which show a strong phylogeographic structure (**Gagneux *et al*., 2006; Homolka *et al*., 2010; Gagneux *et al*., 2007**). As observed for Lineage 2 strains, this might suggest that the usage of high discriminatory MIRU-VNTR loci may be sub-optimal in areas such as Ghana where about 20% of all TB cases are caused by Lineages 5 and 6 of MTBC (also known as *M. africanum* West Africa 1 and 2) (**Yeboah-Manu *et al*., 2011; Addo *et al*., 2007**).

Comas *et al.* **(Comas *et al*., 2009)** using 108 global MTBC strains [30] showed that the majority of the loci included in standard MIRU-24 had a variable discriminatory power across the different

MTBC lineages. Moreover, the MIRU-VNTR loci that exhibited the highest discrimination index within one lineage were not necessarily the ones with the highest discriminatory power in other lineages. Based on the allelic diversity of individual MIRU-VNTR locus, Comas *et al*. **(Comas *et al*., 2009)** suggested different combinations of MIRU-VNTR loci that offered high resolution for the different MTBC lineages. These combinations offered two main advantages over the existing one; it maximized allelic diversity for a given MTBC lineage and allowed for cost effective analyses **(Comas *et al*., 2009)**.

Here we evaluated this concept in the Ghanaian setting and compared the standard MIRU-15 to two lineage-specific 12-loci sets (here referred to as "customized MIRU-12"), one for Lineage 4 and one for Lineage 5, which are the most frequent MTBC lineages in Ghana (**Yeboah-Manu *et al*., 2011; Addo *et al*., 2007; Goyal *et al*., 1999**).

Materials and methods

Ethics Statement

Ethical clearance for this study was obtained from the IRB of the Noguchi Memorial Institute for Medical Research, which has a Federal wide Assurance number FWA00001824. The procedure for sampling in this study was basically the same as those outlined by the National Tuberculosis Programme for the routine management of TB in Ghana. Informed consent both written (in the case of literate participants) and oral (for illiterates) was sought from all participants before their inclusion in the study. Consent was sought from their parents or guardians on behalf of children below sixteen years. As per the guidelines of the institutional review board of the Noguchi Memorial Institute for Medical Research, the objectives and benefits of the study were explained to all participants and they were assured of the confidentiality of all information collected from during the study.

Isolate selection and lineage classification

A total of 178 MTBC isolates consecutively selected from a pool of retrospective samples were included in the study. Specimens included in this study were collected consecutively over a period of 17 months (from October 2007 to March 2009) from sputum AFB-positive pulmonary TB cases attending four main government health centres covering three different regions: Central, Greater Accra and Western regions of Ghana respectively before commencement of anti-TB drug. DNA was extracted as described previously (**Abadia *et al.*, 2011**). MTBC was confirmed by *IS6110* PCR (**Yeboah-Manu *et al.*, 2001**). The isolates were then classified into lineages by analyses of various regions of difference (RDs) as previously described (**Brosch *et al.*, 2002**). Specifically, all isolates were first screened for RD9. RD9-deleted strains were screened for RD4. Isolates identified as RD9 deleted and RD4 undeleted were further sub-typed for Lineage 5 and 6 (*M. africanum* West Africa I and II) using RD711 and RD702 flanking primers, respectively.

TaqMan real time PCR was performed according to standard procedures using probes designed by Stucki *et al* for the confirmation of Lineages (**Stucki *et al*., 2012**) Although Lineage 6 strains (*M. africanum* West Africa II) are present in Ghana (**Yeboah-Manu *et al*., 2011; Addo *et al*., 2007; Goyal *et al*., 1999)**, they were removed from further analysis due to limited number (6 isolates) identified.

MIRU-VNTR Analysis

Two sets of PCRs were performed for each isolate. The first set was performed using the 12 lineage-specific MIRU-VNTR loci proposed by Comas *et al*. (**Comas *et al*., 2009**) while the second set consisted of the standard MIRU-15 as described by Supply *et al*. (**Supply *et al*., 2001**) (Table 1). Each PCR mixture contained 10X PCR buffer, 1.5 mM $MgCl_2$, 200 μM concentrations of deoxyribonuclueotide triphosphate, 5 μM concentration of each primer, 1 μl of HotstarTaq DNA polymerase enzyme, 5 μl Q solution and 10 ng of DNA template in a total volume of 25 μl. Negative (sterile water) and positive controls (H37Rv) were added to each PCR reaction to validate the assay. Locus amplification was carried out under the following conditions: initial denaturation at 95 ^oC for 15 minutes, and then 40 cycles of 95 ^oC for 1 minute, 59 ^oC for1 minute and 72 ^oC for 3 minutes, followed by a final extension at 72 ^oC for 7 minutes. Gel electrophoresis was done in 2% agarose for 5 hours at 80 constant Voltage. The amplicons were sized using a 100bp marker and the obtained size compared with allelic table as published by Supply *et al.* (**Comas *et al*., 2001**).

Table 12: List of MIRU-VNTRs used for the assay					
LOCUS	ALIAS	L5	L4	L5	L6
424	Mtub04	X	X	X	X
577	ETRC	X	X	X	
580	MIRU04	X			X
802	MIRU40	X	X	X	X
960	MIRU10	X	X	X	X
1644	MIRU16	X			X
1955	Mtub21	X	X	X	X
2163b	QUB11b	X	X	X	X
2165	ETRA	X	X	X	X
2401	Mtub30	X	X		
2461	ETRB				X
2531	MIRU23		X	X	
2996	MIRU26	X			X
3007	MIRU27			X	
3192	MIRU31	X		X	X
3690	Mtub39	X	X		
4052	QUB26	X	X	X	X
4156	QUB4156	X	X	X	

SNP typing

TaqMan real time PCR was performed as published by Stucki *et al*. (**Stucki et al., 2012**). Briefly, in a 200 µl sterile PCR tube, 2 µl of DNA was added to a 5 µl sterile water containing 0.21 µM each reverse and forward primers for the targeted regions, 0.83 µM each probe A for ancestral allele and probe B for mutant allele (each labelled with different dyes); and 5 µl Taqman Universal MasterMix II (Applied Biosystem). The reaction was performed in Applied Biosystems 7300 real time PCR system under the following conditions: 60 ^{o}C for 30 seconds, 95 ^{o}C 10 minutes, 95 ^{o}C 15 seconds and 60 ^{o}C 1 minute for 40 cycles; 60 ^{o}C for 30 seconds. The fluorescence intensity in the dyes (VIC and FAM) channels were measured at the end of each cycle.

Data analysis

The number of repeats for each locus was determined based on the allelic table by Supply *et al.* (**Supply *et al*., 2001**) and clustering analysis was done using the online tool at http://www.MIRU-VNTR*plus*.org. MIRU-VNTR clusters were defined as isolates sharing identical patterns. The

clustering rate was defined as (*n*c - *c*)/*n*, where *n*c is the total number of clustered cases, *c* is the number of clusters, and *n* is the total number of cases in the sample (**Kremer *et al*., 2005**)

The Hunter-Gaston Discriminatory Index (HGDI) was used to calculate the discriminatory power of each locus as well as that of each method (**Hunter and Gaston, 1988**).

Determination of a minimal set of MIRU-VNTR loci

Stepwise analysis was performed to identify a set of loci needed to achieve maximum discrimination. Firstly, we combined loci from the customised sets and standard MIRU-15 for each lineage under investigation. Twelve loci were shared between the customised Lineage 4 set and standard MIRU-15, addition of the remaining 4 non-shared loci from standard MIRU-15 gave a total of 16 loci for analysis. For Lineage 5, addition of 6 non-shared loci to the 9 shared loci gave a total of 17 loci. Subsequently, we calculated individual locus HGDI. The results obtained were arranged in a descending order. Starting with the highest HGDI, cumulative HGDI was then calculated by successively adding one locus after the other. Finally, the clustering rate was calculated in a similar manner by successively adding one locus after the other. The result (cumulative HGDI and percentage clustering) obtained for each lineage was plotted on a graph and the cut-off point for selection of the minimal set of loci was set at where graph plateaued meaning further addition of loci resulted in the same cumulative HGDI. The customized minimal loci-set was then extracted from the graph.

Results

MTBC isolates and lineage determination

All 178 isolates included in this study were classified into Lineage 4 (N=126) or Lineage 5 (N=52) based on the RD and SNP typing analysis (**Brosch *et al.*, 2002; Stucki *et al.*, 2012**). Discordant samples were excluded from the study. A full set of MIRU allelic data was obtained for 158/178 (88.8%), comprising 106 Lineage 4 and 52 Lineage 5 isolates, respectively. The remaining 20 of the 178 (11.2%) isolates were excluded from the analysis for various reasons. 90% (18/20) of excluded isolates had no PCR amplicon at one or several loci whilst the remaining 10% (2/20) had double alleles at one or more MIRU-VNTR loci, indicative of the possible presence of two independent strains (**Yesilkaya *et al.*, 2006**).

Evaluation of customized MIRU-12 for Lineage 4

One hundred and four distinct patterns comprising 102 singleton and 2 clusters (2 isolate per cluster) was obtained using customized MIRU-12 (clustering rate: 1.8%). Discriminatory power was calculated separately for each locus and classified into highly (HGDI ≥0.6), moderately (0.3 to 0.6), and poorly (<0.3) discriminatory based on the HGDI scores as previously reported (**Cowan *et al.*, 2005**). Based on this definition, the discriminatory power of 10 loci (MIRU-VNTR loci 10, 40, 2163b, 2165, 3690, 4052, 4165 2401, 0424, and 0577) was higher than 0.6, supporting their designation as highly discriminatory loci with the remaining 2 MIRU/VNTR loci (VNTR 1955, and 23) being "moderately discriminatory"(DI: 0.3–0.59) (Table 13). Using the same set of isolates, standard MIRU-15 identified 105 distinct patterns with only one cluster of two isolates (clustering rate: 0.9%). Ten loci (66.6%; MIRU-VNTR loci 4052, 2163b, 40, 2165, 10, 4165, 3690, 2401, 26 and 0424) were highly discriminatory, 4 (26.6%) (VNTR 1955, 0577 and 23) moderately discriminatory and the remaining 1 (MIRU 4: 6.7%) poorly discriminated among the isolates.

Table 13: Cumulative HGI and clustering rate for Lineage 4 successive addition of individual MIRU-VNTR Loci. (N=106).

Locus	VNTR Locus	VNTR alias	No. of Clusters	No. of clustered isolates	No. of isolates in individual cluster	Clustering Rate (%)	Individual HGI	Cumulative HGI
1	QUB26	VNTR 4052	9	96	2-34	84.9	0.829	0.829
2	QUB11b	VNTR 2163B	23	88	2-9	61.3	0.804	0.966
3	MIRU 40	VNTR802	20	53	2-8	31.1	0.752	0.988
4	ETRA	VNTR 2165	13	28	2-4	14.2	0.722	0.996
5	MIRU 10	VNTR960	8	19	2-4	10.4	0.714	0.997
6	QUB4156	VNTR 4156	4	11	2-4	6.6	0.689	0.999
7	Mtu39	VNTR 3690	3	6	2	2.8	0.66	0.999
8	Mtub30	VNTR 2401	3	6	2	2.8	0.64	0.999
9	2996	MIRU 26	2	4	2	1.8	0.628	0.999
10	Mtub04	VNTR 0424	2	4	2	1.8	0.623	0.999
11	ETRC	VNTR 0577	2	4	2	1.8	0.612	0.999
12	Mtub21	VNTR 1955	2	4	2	1.8	0.579	0.999
13	2531	VNTR 23	2	4	2	1.8	0.555	0.999
14	1644	MIRU 16	2	4	2	1.8	0.452	0.999
15	3192	MIRU 31	1	2	2	0.9	0.37	0.999
16	580	MIRU 4	1	2	2	0.9	0.092	0.999

Evaluation of customized MIRU-12 for Lineage 5

Among 52 isolates analyzed, we obtained 51 unique patterns with 1 cluster comprising 2 isolates and a clustering rate of 0.9%. Five MIRU-VNTR loci (2163B, 4156, 4052, 40, 27) were highly discriminatory and the remaining 7 (23, 0577, 2165, 10, 0424, 31, 1955) being moderately discriminatory (Table 14). Standard MIRU-15 on the other hand identified 52 unique patterns. Six of the 15 loci (VNTR 2163B, 4156, 4052, 26, 16 and 40) had HGDI above 0.6 with the remaining nine MIRU-VNTR loci (0424, 10, 1955, 0577, 4, 2401, 3690, 2165 and 31) showing moderate discrimination (HGDI: 0.3–0.59).

Table 14: Cumulative HGI and clustering rate for Lineage 5 with successive addition of individual MIRU-VNTR Loci. N=52

Locus	VNTR Locus	VNTR alias	No. of Clusters	No. of Clustered isolates	No. of isolates in individual cluster	Clustering Rate (%)	Individual HGI	Cumulative HGI
1	VNTR 2163B	QUB11B	6	52	2-13	88.5	0.797	0.798
2	VNTR 4156	QUB4156	12	45	2-9	63.5	0.731	0.935
3	802	MIRU 40	2	4	2	40.4	0.7	0.971
4	2996	MIRU 26	7	16	2-3	17.3	0.7	0.992
5	VNTR 4052	QUB26	10	31	2-5	5.8	0.69	0.997
6	1644	MIRU 16	3	6	2	3.8	0.689	0.998
7	3007	MIRU 27	2	4	2	3.8	0.6	0.998
8	2531	MIRU 23	2	4	2	3.8	0.58	0.998
9	VNTR 0424	Mtub04	2	4	2	3.8	0.572	0.998
10	960	MIRU 10	2	4	2	3.8	0.526	0.998
11	VNTR 1955	Mtub21	2	4	2	3.8	0.489	0.998
12	VNTR 0577	ETRC	2	4	2	3.8	0.487	0.998
13	VNTR 2401	Mtub30	1	2	2	1.9	0.473	0.998
14	580	MIRU 4	2	4	2	3.8	0.472	0.999
15	VNTR 3690	Mtub39	0	0	0	0	0.428	0.999
16	VNTR 2165	ETRA	0	0	0	0	0.387	0.999
17	3192	MIRU 31	0	0	0	0	0.352	0.999

Determination of a minimal set of MIRU-VNTR loci for genotyping main MTBC lineages from Ghana

Customized MIRU-12 for Lineage 4 shared 11 loci with standard MIRU-15 whilst 9 loci were shared between customized MIRU-12 for Lineage 5 and standard MIRU-15. A union of both sets of typing schemes gave a total of 16 and 17 loci for Lineage 4 and 5, respectively. For Lineage 4, we identified six top most discriminatory loci (4052, 2163B, 40, 2165, 10 and 4165) with a cumulative HGDI of 0.99 (Table 13; Figure 1 a). Similarly, for Lineage 5, six loci: 2163B, 4165, 40, 26, 4052 and 16 (Table 14) with a cumulative HGDI of 0.99 were identified. Further addition of loci gave no significant change in cumulative HGDI as shown in Figure 1b. Note that 4 loci (4052, 2163B, 4162 and 40) were

among the 6 most discriminatory in both lineage-specific sets. Hence, based on this, we propose the usage of a new set of customised typing system comprising 8 loci showing the highest discriminatory power for genotyping strains from the two most common lineages circulating in Ghana.

Discussion and Conclusion

Different combinations of MIRU and other VNTR loci have been proposed to complement the standard MIRU-15 scheme to achieve higher discrimination. Results accumulated from such studies clearly revealed that due to the strong phylogeographic structure exhibited by MTBC, the most relevant MIRU-VNTR typing schemes will likely differ depending on the specific geographical setting. For example, Shamputa *et al.* (**Shamputa *et al.*, 2010**) successfully identified a reduced set of 8 loci from standard MIRU-24, which could be used to discriminate isolates from the Republic of Korea. Similarly, Musare *et al.* (**Musare *et al.*, 2008**) Dong *et al.* (**Dong *et al.*, 2012**) and Zhou *et al.* (**Zhou *et al.*, 2011**)successfully defined a minimal set of 12 loci for genotyping Beijing strains which made up more than 90% of the isolates investigated from Asia. Most of the studies have been focused on Lineage 2 including the clinically important Beijing family based on its association with drug resistance (**Borrell and Gagneux, 2009**). However, no study has been carried out in most resource-limited settings like Ghana, where *M. africanum* is an important pathogen (**Yeboah-Manu *et al.*, 2011; Addo *et al.*, 2007; Goyal *et al.*, 1999**). If customized lineage-specific sets of MIRU-VNTR loci could be implemented in such settings, this will have an impact in terms of reducing work load and saving resources. In the present study, we evaluated such an approach for genotyping MTBC strains from Ghana, (**Yeboah-Manu *et al.*, 2011; Addo *et al.*, 2007)** and compared our results with the current gold standard typing method; standard MIRU 15 as proposed by Supply *et al.* **(Supply *et al.*, 2001**)].

Although standard MIRU-15 showed higher discrimination in its ability to accurately identify clusters among these two lineages in our study when compared to customised lineage-specific MIRU-12 proposed previously (**Comas *et al.*, 2009**), we found that not all the 15 loci were as informative for typing MTBC strains in Ghana. Even with the customized MIRU-12, based on our data, not all 12 loci were needed to achieve maximum discrimination (Figure 1 and 2). Specifically, our analysis showed

that 10 of a total of 16 loci tested for Lineage 4 strains added no or only limited additional information in terms of discriminatory power. Similarly, 11 of a total of 17 loci screened for Lineage 5 strains showed limited discriminatory power. We thus explored the possibility of a minimal set of loci HGDI selected by combining the standard MIRU-15 and the customized MIRU-12 data set. Based on individual and cumulative HGDIs, and clustering rate, we defined the six top discriminatory loci for Lineage 4 (4052, 2163B, 40, 2165, 10 and 4165) (Figure 1 and 2) and similarly for Lineage 5 (2163B, 4165, 40, 26, 4052 and 16) (Figure 3 and 4) with 4 loci shared among the two sets (4052, 2163B, 40 and 4165). A combination of loci from Lineage 4 and 5 gave a unique customized set of 8 loci with HGDIs similar to that of standard MIRU-15.

Figure 14: Individual and cumulative HGI of all MIRU-VNTR locus analysed _Lineage 4. The pink bars are the cumulative HGI values while blue bars are for the individual locus values

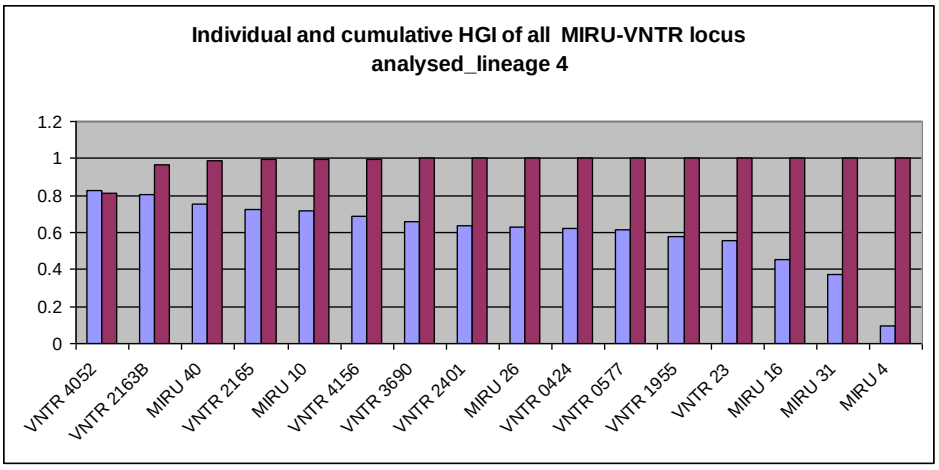


Figure 15: Individual and cumulative HGI of all MIRU-VNTR locus analysed _Lineage 5. The pink bars are the cumulative HGI values while blue bars are for the individual locus values

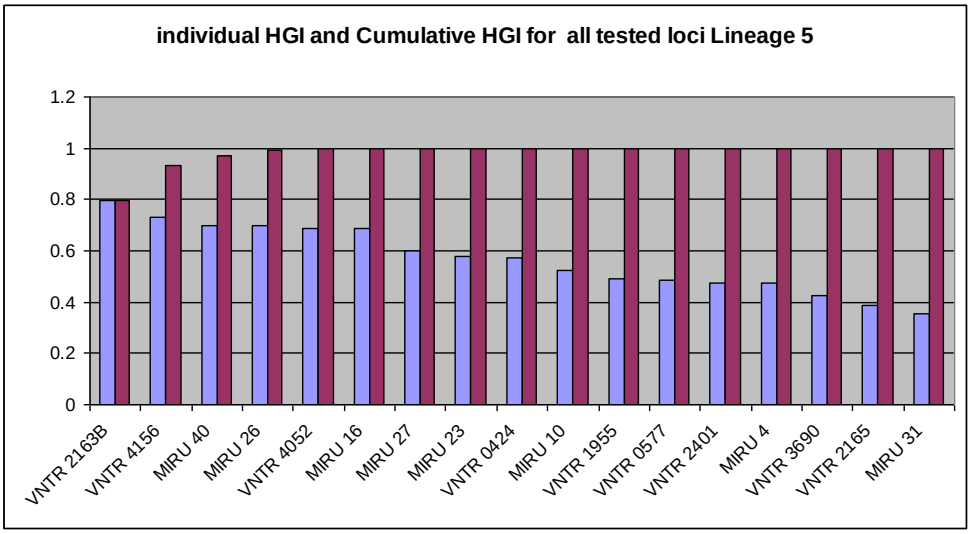


Figure 16: Clustering rate for lineages 4 calculated using after successive addition of analysed loci

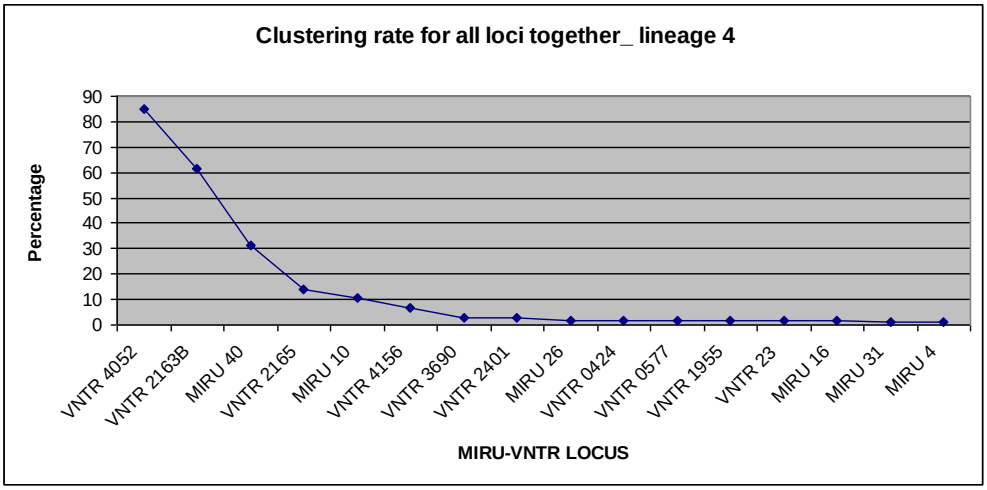
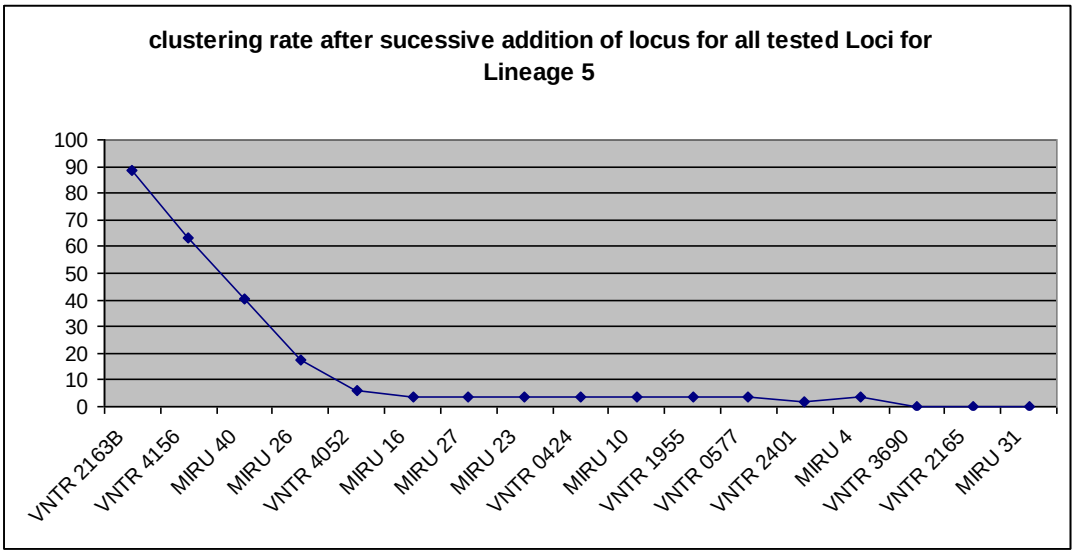


Figure 17: Clustering rate for lineages 5 calculated using after successive addition of analysed loci



We now plan to apply these minimal MIRU-VNTR set for molecular epidemiological investigation of MTBC transmission in population based study in Ghana. We anticipate that this approach will save a significant amount of time. In addition we perform cost analysis on the different VNTR schemes analysed in this study. Cost was calculated based on the direct cost of reagents, materials and equipment. We compared the cost of genotyping using standard MIRU-15 and our proposed customized set of MIRU-8. With a unit cost of $11.24, the cost of performing standard MIRU-15 on one sample was $168.60, with the total material costs of analyses using our proposed customized MIRU-8 set for one sample being $89.2. Hence, by screening for only the relevant loci, we not only maximize discriminatory power but also minimize genotyping costs.

Currently, human-associated MTBC is known to comprise a total of seven main phylogenetic lineages (**Coscolla *et al.*, 2013; Comas *et al.*, 2013; Firdessa *et al.*, 2013**). We propose that additional lineage-specific sets of MIRU-VNTR could be identified for molecular epidemiological investigation of TB transmission in resource-limited settings. Moreover, each MTBC lineage consists of a number of sub-lineages, some of which also show strong geographical associations (**Gagneux *et al.*, 2006; Gagneux, 2012; Reed *et al*, 2009**). For example, the "Uganda" sub-lineage of Lineage 4 causes up to 60% of TB

in Kampala, Uganda (**Wampande *et al,* 2013**), suggesting that a similar customized Lineage 4 set for Uganda could be developed, which possibly would include other loci considering that most of Lineage 4 in Ghana consists of the "Cameroon" sub-lineage (**Niobe-Eyangoh *et al*., 2003; Assam *et al*., 2011; Assam *et al*., 2013**).

This study set out to define a set of loci for genotyping MTBC strains from Ghana. We acknowledge the high prevalence of *M. africanum* strains in Ghana, however, this prevalence is driven by Lineage 5 (*M. africanum* West Africa I) with limited number of Lineage 6 (*M. africanum* West Africa II). We acknowledge the fact that this makes our proposed customized MIRU-8 country-specific, and thus suggest that countries within West African where the high prevalence of *M. africanum* is driven by Lineage 6 (*M. africanum* West Africa II) could equally determine the minimal set of loci which gives the highest discrimination. Nevertheless, the strength of our study is the ability to genotype an unknown strain in Ghana with the proposed customized MIRU-8 loci in the most cost-efficient way.

In conclusion, this study identified a reduced set which can be applied for strain differentiation of the main MTBC lineages from Ghana.

Chapter 6*: Mycobacterium africanum* is associated with patient ethnicity in Ghana

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Abstract

Mycobacterium africanum is a member of the *Mycobacterium tuberculosis* complex (MTBC) and an important cause of human tuberculosis in West Africa rarely observed elsewhere. Here we genotyped 613 MTBC clinical isolates from Ghana and searched for associations between the different phylogenetic lineages of MTBC and patient variables. We found that 17.1% (105/613) of the MTBC isolates belonged to *M. africanum*, with the remaining belonging to *M. tuberculosis* sensu stricto. No *M. bovis* was identified in this sample. *M. africanum* was significantly more common in tuberculosis patients belonging to the Ewe ethnic group (adjusted odds ratio: 3.02; 95% confidence interval**:** 1.67-5.47, $p<0.001$). Stratifying our analysis by the two phylogenetic lineages of *M. africanum* (i.e. MTBC Lineages 5 and 6) revealed that this association was mainly driven by Lineage 5 (also known as *M. africanum* West Africa 1). Our findings suggest interactions between the genetic diversity of MTBC and human diversity, and offer a possible explanation for the geographical restriction of *M. africanum* to parts of West Africa.

Authors Summary

Tuberculosis remains one of the main global public health problems. Human tuberculosis is caused by bacteria known as the *Mycobacterium tuberculo*sis complex (MTBC). The MTBC includes a variant called *Mycobacterium africanum*, which causes up to half of all tuberculosis cases in West Africa. For reasons unknown, *M. africanum* does not occur in other parts of the world. To explore the possible reasons for this geographic restriction of *M. africanum*, we analysed a large collection of bacterial strains isolated from tuberculosis patients in Ghana. We genetically characterized these bacterial isolates and collected relevant socio-demographic and epidemiological data. We found tuberculosis patients infected with *M. africanum* were more likely to belong to the Ewe ethnic group compared to patients carrying other MTBC bacteria. The Ewes are indigenous inhabitants of coastal regions in West Africa that have previously been shown to harbour a high prevalence of *M. africanum*. Our findings support the hypothesis that different variants of MTBC have adapted to different human populations, and offer a possible explanation for the geographical restriction of *M. africanum* to West Africa.

Introduction

Tuberculosis (TB) remains the leading cause of adult death by a single infectious disease world-wide (**Global TB report, 2013**). Despite the high mortality caused by TB, only 5% to 10% of infected immunocompetent individuals progress from initial infection to active disease (**Global TB report, 2013)**. In 2012, an estimated 8.6 million new cases and 1.3 million deaths due to TB occurred; with 30% of the global burden of TB occurring in Africa, an indication of the strong association with HIV/AIDS (**Global TB report, 2013)**.

TB is caused by a group of closely related bacteria referred to as the *Mycobacterium tuberculosis* complex (MTBC). MTBC comprises *M. tuberculosis* sensu stricto and *M. africanum* which are the main agents of TB in humans, and several variants adapted to various domestic and wild mammal species, including *M. bovis, M. caprae, M. microti* and *M. pinnipedii* (**Brosch *et al*., 2002**). MTBC relevant to human disease has been classified into seven main phylogenetic lineages (**Firedessa *et al*., 2013;Gagneux and Small, 2007**): Lineages 1 to 4 together with Lineage 7 are collectively known as *M. tuberculosis* sensu stricto, whereas Lineage 5 and 6 are also known as *M. africanum* West Africa I and II, respectively (**de Jong *et al*., 2010**).

Because MTBC harbours limited genetic diversity compared to other bacteria (**Achtman, 2008**), for a long time the assumption was that host and environmental factors were the only relevant determinants driving the course of TB infection. However, recent studies have challenged this dogma. Indeed, experimental infection models have shown that MTBC strains differ in virulence and immunogenicity (**Coscolla and Gagneux, 2010**), and epidemiological studies have demonstrated that in addition to host and environmental factors, strain diversity contributes to the variable outcome of TB infection and disease in clinical settings (**Nicol *et al*., 2008**)

The MTBC lineages adapted to humans exhibit a strong phylogeographic population structure (**Gagneux and Small, 2007**). This together with the finding that the MTBC most likely originated in Africa and accompanied human migrations over millennia (**Comas *et al*., 2013**) has led to the

proposal that the different lineages of human-associated MTBC might have locally adapted to different human populations (**Gagneux, 2012**). Support for this notion comes from the observation that in metropolitan settings, MTBC lineages tend to transmit preferentially among sympatric (as opposed to allopatric) host populations (**Gagneux *et al*., 2006**), and that this sympatric host-pathogen association is perturbed by HIV co-infection (**Fenner *et al*., 2013**).

Previous work showed that in Ghana, human TB is caused by six out of the seven MTBC lineages, with 20% of all cases attributed to Lineages 5 and 6 (**Yeboah-Manu *et al*., 2011**) (i.e. *M. africanum* West-Africa I and West-Africa II, respectively). *M. africanum* is highly restricted to West-Africa for reasons unknown (**de Jong *et al*., 2010**; **Gagneux, 2012**). One possibility could be that *M. africanum* has adapted to particular human populations in that region of the world. To address this possibility, we performed a retrospective molecular epidemiological study of MTBC in Southern Ghana. We combined bacterial genotyping with detailed demographic and epidemiological patient data and sought for associations between host factors and the main MTBC lineages prevailing in Ghana.

Methods

Ethics statement

All study protocols including oral and written consent format used for this study were approved by the Institutional Review Board (IRB) of the Noguchi Memorial Institute for Medical Research, Legon-Ghana (NMIMR; Federal wide Assurance number FWA00001824) and from the Ethikkommission Beider Basel (EKBB) in Basel, Switzerland. The standard procedure for sampling as outlined by the National Tuberculosis Program (NTP) for the routine management of TB in Ghana was used in the study. Written (in the case of literate participants) or oral (for illiterates) informed consent was sought from all participants before inclusion in the study. For minors (below sixteen years of age) consent was sought from their parents/guardians before enrolment into the study. In the case of minors between sixteen and eighteen years, in addition to parental consent, assent was sought from them before enrolment into the study. As per the guidelines of the IRB of NMIMR, information confidentiality was strictly adhered to. In addition, objectives and benefits of the study were explained to all participants.

Study setting and patients characteristics

The study was conducted from July 2007 to August 2011. All patients were diagnosed as sputum AFB-positive pulmonary TB cases by microscopy. The patients were recruited before treatment initiation from five main health facilities; Korle-Bu Teaching Hospital in the Greater Accra region, Agona Swedru Government Municipal Hospital, Winneba Government Hospital, St Gregory Catholic Clinic from the Central Region and Effia-Nkwanta Regional Hospital from Western Region of Ghana. Information on age, sex, nationality, ethnicity, employment status, previous history of TB, crowding, substance abuse and duration of symptoms were obtained from the patients with a structured questionnaire. Patients with missing information or culture-negative status were excluded from analysis. Ethnicity was classified in line with Ghana demographic data 2010 (**Ghana demographic and Health survey, 2010**)

Isolation of mycobacterial species and genotyping

Sputum samples obtained were decontaminated using 5% oxalic acid (**Yeboah-Manu *et al.*, 2004) a**nd inoculated on two pairs of Lowenstein Jensen (LJ) slants; one supplemented with 0.4% sodium pyruvate to enhance the isolation of *M. africanum* and *M. bovis,* and the other with glycerol for the growth of *M. tuberculosis* sensu stricto. The cultures were incubated at 37˚C and were read weekly for growth for a maximal duration of 16 weeks. MTBC strains were identified by detection of insertion sequence *IS6110* as previously described (**Yeboah-Manu *et al.*, 2001)**Classification into the main phylogenetic lineages of MTBC was achieved by large sequence polymorphism typing identifying regions of difference (RD) (**Brosch et al., 2002**) in a stepwise manner. Firstly, all isolates were screened for RD9. RD9-undeleted strains were further sub-typed for the "Cameroon" strain family (known to be most dominant Lineage 4 sub-lineage in Ghana) by screening for deletion of RD726 (**Gagneux *et al.*, 2006**). Isolates identified as RD9-deleted were subsequently sub-typed for Lineage 5 and 6 using RD711 and RD702 flanking primers, respectively. All lineages identified were confirmed by TaqMan real time PCR (TaqMan, Applied Bio systems, USA) using probes targeting lineage-specific SNPs as reported (**Stucki *et al.*, 2012**)

Spoligotyping

All MTBC isolates were typed by spoligotyping (**Kamerbeek *et al.*, 1997**). This was performed according to the manufacturer's instructions, using commercially available kits (Isogen Bioscience BV Maarssen, The Netherlands). Spoligotyping patterns were defined according to SITVITWEB database (**Demay *et al.*, 2012**) (http://www.pasteur-guadeloupe.fr:8081/ SITVIT_ONLINE). SITVITWEB assigned shared types numbers were used whenever a spoligotyping pattern was found in the database while families and subfamilies were assigned based on the MIRU-VNTRplus database (http: //www. miru-vntrplus.org) (**Weniger *et al.*, 2010**). Shared types were

defined as patterns common to at least two or more isolates. All patterns that could not be assigned were considered orphan spoligotypes.

Data entry, management and analysis

Information from the structured questionnaire was double entered using Microsoft© Access and validated to remove duplicates and data entry inconsistencies. Multivariable logistic regression models were used to compare patient characteristics associated with *M. africanum* compared to *M. tuberculosis* sensu stricto. All statistical analyses were performed in STATA 10.1 (Stata Corp., College Station, TX, USA).

Results

Tuberculosis patients and their characteristics

A total of 622 TB patients were included in this study. Age of patients ranged from 8 to 77 years with a median age of 35 years (Table 1). Overall, 208/622 (33.4 %) were females with median age of 33 years; the remaining 414 (66.6%) were males with a median age of 36. Twenty-nine out of the 622 patients (4.6%) were children (age<16 years). Most patients originated from Greater Accra Region (325 cases, 52.3%), followed by 268 cases (43.1%) from Central Region with the remaining twenty-nine patients (4.6%) from Western Region of Ghana. Out of the 622 patients, 596 (95.8%) were Ghanaians, 21 (3.3%) were Liberians, 2 Togolese (0.3%) and 1 (0.2%) each of Nigerian, Ivorian and Gambian origin, respectively. Most of the patients were of Akan ethnicity (N=427, 68.7%), followed by Ga (N=104, 16.7%), Ewe (N=71, 11.4%) with the remaining ethnicities accounting for 3.2 % (N=20). In terms of education, 436 patients (70.1%) were illiterates, 44 (7.1%) primary education 132 (21.2%) had up to secondary education, and the remaining 10 (1.6%) tertiary education. More than half of the study population (N=324, 52%) consumed alcohol on a regular basis, while only 44 (7%) smoked.

Table 15: Characteristics of patients included in the study		
Variable	N=622	**%**
Sex		
Male	414	**66.6**
Female	208	**33.4**
Age		
Yrs 08-25	124	**20.0**
Yrs 26-40	282	**45.3**
Yrs 41-77	216	**34.7**
Residency		
Rural	117	**18.8**
Urban	505	**81.2**
Region		
Greater Accra	325	**52.3**
Central	268	**43.1**
Western	29	**4.6**
Ethnicity		
Akan	427	**68.7**
Ewe	71	**11.4**
Ga	104	**16.7**
Other	20	**3.2**
Religion		
Christian	564	**90.7**
Muslim	37	**5.9**
Pagan	21	**3.4**
Level of Education		
No education	436	**70.1**
Primary school	44	**7.1**
Secondary	132	**21.2**
Tertiary	10	**1.6**
Alcohol intake		
Yes	324	**52.1**
No	298	**47.9**
Smoking Status		
Smokers	44	**7.1**
Non smokers	578	**92.9**
Crowding(1-4 pers)	195	**31.4**
(>5 pers)	427	**68.6**
Occupation		
Farmer	45	**7.2**
Others	**577**	**92.8**

Prevalence of MTBC Lineages and Sub-Lineages

MTBC isolates were obtained from all 622 TB patients. Upon genotyping, 9 of these (1.4%) produced ambiguous results, and were thus excluded from further analysis. Hence a total of 613

isolates were used for further analysis. Based on LSP and SNP typing, we identified six out of the seven human-associated MTBC lineages in our study sample. The most dominant lineages were Lineage 4 with 483 cases (78.8%), Lineage 5 (N=86, 14.0%) and Lineage 6 (N=19, 3.1%). Eleven isolates (1.8%) belonged to Lineage 1, 10 to Lineage 2 (includes Beijing; 1.6%), and the remaining 4 isolates to Lineage 3 (0.7%). Among the 483 Lineage 4 isolates, 313/483 (65.0%) belonged to the sub-lineage of Lineage 4 known as the 'Cameroon family'. No *M. bovis* was identified in our sample.

All isolates were further sub-typed using spoligotyping (Table S 2). We detected a total of 117 spoligotypes, 485/613 isolates (79%) had previously defined shared type number (SIT). The remaining 128 isolates could not be defined by the SITVIT database and thus were defined as 'orphan'. In addition to Cameroon sub-lineage, seven additional sub-lineages isolates were identified among Lineage 4 based on spoligotyping; Ghana (N=75, 15.5%), Haarlem (N=37, 7.7%), Uganda I (N=15, 3.1%), Uganda II (N=7, 1.4%), LAM (N=5, 1.0%), S (N=4 (0.8%), and X (N=2, 0.4%).

Association between MTBC Lineages and Patient Characteristics

Table 3 illustrates the association of socio demographic and behavioural factors with the main MTBC lineages present in our study sample. Using multivariable logistic regression model analysis, we found that individuals of Ewe ethnicity were significantly more likely to present with TB caused by *M. africanum* as opposed to *M. tuberculosis* sensu stricto irrespective of their place of residence (adjusted odds ratio (adjOR) =3.02; 95% confidence interval (CI)**:** 1.67-5.47, P<0.001) (Table 16; Figure S1). This association was independent from other risk factors. Moreover, we found TB caused by *M. africanum* to be associated with smoking (adjOR=2.02; 95% CI**:** 0.95-4.27) when compared to *M. tuberculosis* sensu stricto. However, this association was only borderline statistically significant (P=0.07). No significant associations between MTBC lineages and other patient variables were found. Because *M. africanum* comprises two

phylogenetic distinct lineages (i.e. MTBC Lineages 5 and 6), we performed a stratified analysis by lineage. Using multivariate logistic regression model analysis, we observed a significant association between Ewe ethnicity and Lineage 5 (adjOR) =2.79; 95% CI**:** 1.47-5.29, P<0.001) (Table 17). This association was independent from other risk factors (Table 5). Interestingly, based on univariate analysis, we also saw an association between Ewe ethnicity and Lineage 6 (adjOR=4.03; 95% CI**:** 1.33-10.85); however, because of the limited number of Lineage 6 isolates (n=18) multivariate analyses could not be performed to confirm the independence of this association.

Table 16: Risk factors for TB caused by *M. africanum* compared to *M. tuberculosis* sensu stricto

Risk factor	***%(n) Mafr***	***%(n) MTBss***	***OR (95%CI)***	*adjOR (95%CI)*[a]
	(n = 102)	(n = 511)		
Sex (male)	68% (69)	66% (338)	0.93 (0.59-1.47)	
Age category				
yr 08-25	17% (17)	21% (105)	ref	
yr 26-40	53% (54)	44% (223)	1.50 (0.83-2.70)	
yr 41-77	30% (31)	36% (183)	1.05 (0.55-1.98)	
Rural residency	20% (20)	18% (93)	1.10 (0.64-1.88)	
Region				
Accra	55% (56)	52% (267)	ref	**ref**
Central	42% (43)	43% (218)	0.94 (0.61-1.45)	**0.97 (0.60-1.56)**
Western	3% (3)	5% (26)	0.55 (0.16-1.88)	**0.44 (0.12-1.63)**
Ethnicity				
Akan	58% (59)	71% (359)	ref	**ref**
Ewe	23% (23)	9% (48)	2.91 (1.65-5.14)*	**3.02 (1.67-5.47)***
Ga	15% (15)	17% (89)	1.03 (0.56-1.89)	**0.97 (0.51-1.83)**
other	5% (5)	3% (15)	2.03 (0.71-5.79)	**2.35 (0.77-7.13)**
Religion				
Christian	92% (94)	90% (462)	ref	
Muslim	7% (7)	6% (29)	1.18 (0.50-2.79)	
Pagan	1% (1)	4% (20)	0.25 (0.03-1.85)	
Educational level				
No education	74% (75)	70% (356)	ref	
Primary school	6% (6)	7% (38)	0.75 (0.30-1.83)	
Secondary	21% (21)	23% (117)	0.85 (0.50-1.44)	
Alcohol	57% (58)	52% (263)	1.23 (0.81-1.90)	
Smoking	11% (11)	6% (32)	1.81 (0.88-3.72)	**2.02 (0.95-4.27)†**
Crowding (>5 pers)[b]	63% (64)	70% (359)	0.71 (0.45-1.10)	
Occupation farmer[c]	**9% (9)**	**7% (35)**	**1.32 (0.61-2.83)**	

[a]All variables with an OR above 1.5 or below 2/3 were included in the multivariable model, *P<0.001, †P=0.07, Mafr=*Mycobacterium africanum*, MTBss=*Mycobacterium tuberculosis* sensu stricto [b]5 or more persons per room vs 1 to 4

Risk factor for TB caused by Lineage 5 compared to *M. tuberculosis* sensu stricto				
Risk factor	**%(n) Lineage 5 (**n = 84)	**%(n) MTBss (**n = 511	**OR (95%CI)**	**adjOR (95%CI)[a]**
Sex (male)	59% (58)	66% (338)	1.41 (0.69-1.88)	
Age category				
yr 08-25	18% (15)	21% (105)	ref	
yr 26-40	51% (43)	43% (223)	1.35 (0.72-2.54)	
yr 41-77	31% (26)	36% (183)	0.99 (0.5-1.96)	
Rural residency	19% (16)	18% (93)	1.06 (0.59-1.91)	
Region				
Accra	54% (45)	52% (267)	ref	
Central	42% (36)	43% (218)	0.98 (0.61-1.57)	
Western	4% (3)	5% (26)	0.68 (0.2-2.36)	
Ethnicity				
Akan	61% (51)	70% (359)	ref	ref
Ewe	20% (17)	9% (48)	2.49 (1.33-4.66)**	2.79 (1.47-5.29) **
Ga	14% (12)	17% (89)	0.95 (0.49-1.86)	0.85 (0.43-1.69)
other	5% (4)	3% (15)	1.88 (0.6-5.88)	1.64 (0.53-5.34)
Religion				
Christian	93% (78)	90% (462)	ref	
Muslim	6% (5)	6% (29)	1.02 (0.38-2.72)	
Pagan	1% (1)	4% (20)	0.29 (0.04-2.24)	
Educational level				
No education	70% (59)	70% (356)	ref	
Primary school	7% (6)	7% (38)	0.95 (0.39-2.35)	
Secondary +	23% (19)	23% (117)	0.98 (0.56-1.71)	
Alcohol	62% (52)	52% (263)	1.53 (0.95-2.45)ǂ	1.62 (0.99-2.68)ǂ
Smoking	11% (9)	6% (32)	1.8 (0.82-3.91)	1.54 (0.68-3.50)
Crowding (>5 pers)[c]	63% (53)	70% (359)	0.72 (0.44-1.16)	
Occupation farmer	11% (9)	7% (35)	0.61 (0.28-1.32)	0.64 (0.29-1.45)

Discussion

Our retrospective molecular epidemiological investigation of MTBC clinical isolates from Southern Ghana revealed that i) the Cameroon sub-lineage of Lineage 4 is the dominant cause of human TB in this region, ii) 17.1% of human TB is caused by *M. africanum*, iii) TB patients infected with *M. africanum* were more likely to smoke, and iv) to belong to the Ewe ethnic group.

Our finding that the Cameroon sub-lineage causes 65% of human TB in Ghana confirms our previous report from Ghana (**Yeboah-Manu *et al.*, 2011**), and is in agreement with findings from neighbouring countries. In particular, the Cameroon sublineage was previously found to cause 40% of human TB in Cameroon (**Niobe-Eyangoh *et al.*, 2003**), 45% in Nigeria (**Lawson *et al.*, 2012**) and 33% in Chad (**Diguimbaye *et al.*, 2006**). The reasons for the success of this sub-lineage in this sub-region of Africa are unclear but could be due to a founder effect or particularly high fitness in the corresponding patient populations. Similarly, other successful sub-lineages of Lineage 4 have been observed in other regions of Africa, including Uganda (**Wampande *et al.*, 2013**) and Zimbabwe (**Easterbrook *et al.*, 2004**).

We found that in Ghana, *M. africanum* still accounts for 17.0% of all human TB, which is similar to the prevalence we reported several years ago (**Yeboah-Manu *et al.*, 2011**). This is in contrast to a study in Cameroon (**Niobe-Eyangoh *et al.*, 2003)** that indicated a sharp decrease in TB caused by *M. africanum* during the last decades. A potential explanation for the decline of *M. africanum* in some West African countries includes possible out-competition by *M. tuberculosis*, as *M. africanum* has been associated with reduced virulence in animal models (**Castets *et al.*, 1969; Bold *et al.*,2012**); and a longer latency and a slower rate of progression to active disease in humans (**de Jong *et al.*, 2008**). Of note, our finding that smoking was associated with infection by *M. africanum* as opposed to *M. tuberculosis* sensu stricto is consistent with the notion that *M. africanum* might be less virulent in immunocompetent hosts (**Coscolla and Gagneux, 2010**). This notion is also supported by a

previous study in the Gambia reporting a significant association between *M. africanum* West Africa II and HIV co-infection (**de Jong *et al.*, 2005**). However, no such association was found between *M. africanum* West Africa I and II in Ghana (**Meyer *et al.*, 2008**). Because information on HIV status was not available for the present study, we could not explore this question here. Taken together, there is a need for further investigation to ascertain why *M. africanum* is declining in some regions of West Africa, but not in Ghana, and whether this phenomenon can be attributed to differences in virulence and/or other factors.

One reason for why the prevalence of *M. africanum* might be more stable in Ghana than in some other countries is that this bacterial lineage might be particularly well adapted to (some) human populations in Ghana. Our finding that *M. africanum* was independently associated with Ewe ethnicity supports this possibility. Moreover, this association was largely driven by Lineage 5, and not the result of a single outbreak as the spoligotyping patterns among *M. africanum* isolates from Ewe patients were diverse (Table 18).

From available data, we know that *M. africanum*, in particular Lineage 5 is prevalent in countries around the Gulf of Guinea (**Yeboah-Manu *et al.*, 2011, Gehre *et al.*, 2013**), and particularly frequent in Benin and Ghana (**Affolabi *et al.*, 2009;Yeboah-Manu *et al.*, 2011**), two countries with large Ewe populations (**The Ewe people, 2014**). The Ewe speaking ethnic group traditionally forms part of the Gbe language family which includes the Fons of Benin, the Aja of Togo and the Phla-phera of western Nigeria (**Anyidoho, 2003**) Although the Ewe, Fons, Aja and phla-phera are different dialects of the same Gbe language family, members of theses individual groups are interrelated (**The Ewe people, 2014**). Together they constitute the indigenous inhabitants of coastal West Africa.

Associations between particular MTBC lineages and human ethnicities have been observed before. For example, in San Francisco, Lineage 1, 2 and 4 were strongly associated with Filipino, Chinese, and "white" ethnicities, respectively (**Gagneux *et al.*, 2011**) More recently, Hui ethnicity was found to be associated with the Beijing family of MTBC in China (**Pang *et***

***al.*, 2012)**. While social "cohesion" is likely to restrict intermingling between individuals belonging to different ethnic groups and thus transmission of MTBC between these groups, biological factors could also play a role in the association between different MTBC genotypes and human populations. Self-defined ethnicity has been shown to be a reliable proxy for human ancestry (**Rosenberg *et al.*, 2002**) and human genetic diversity has been linked to an increased or reduced susceptibility to TB (**Abel *et al.*, 2014**). Importantly, recent studies indicate that human genetic susceptibility to TB is further influenced by the MTBC genotype (**Gagneux, 2012**). In particular, studies have reported human genetic polymorphisms that influence the susceptibility to TB caused by *M. africanum* but not *M. tuberculosis* sensu stricto or *vice versa* (**Intemann *et al.*, 2009**). For example, a study performed in Ghana reported a human polymorphism in 5-lipoxygenase (ALOX5) associated with increased TB risk (**Herb *et al.*, 2008**). Stratification by MTBC lineage revealed that this association was mainly driven by *M. africanum* indicating that this human polymorphism increases the risk of TB in a MTBC lineage-specific matter. ALOX5 is involved in the synthesis of leukotrienes and lipoxins, which are important mediators of the inflammatory response (**Herb *et al.*, 2008**). Conversely, a human polymorphism reported recently in the Mannose Binding Lectin (MBL) was associated with protection against TB caused by *M. africanum* but not *M. tuberculosis* sensu stricto (**Thye *et al.*, 2011**). Moreover, this latter study also found that *M. africanum* bound human recombinant MBL more efficiently, perhaps leading to an improved uptake of *M. africanum* by macrophages and selection of deficient MBL variants among human populations exposed to *M. africanum* (**Thye *et al.*, 2011)**.

Our study has several limitations. First, data on HIV co-infection was not available. This might have influenced our results on the patient characteristics associated with *M. africanum*. Secondly, this study was not population-based as patients were recruited only at three government hospitals. Hence, some degree of selection bias cannot be excluded.

In conclusion, our study provides novel insights into the interaction between environmental, host and pathogen variability in human TB. In particular, the observed association between *M. africanum* and Ewe patient ethnicity suggests a possible explanation for the geographical restriction of *M. africanum* to parts of West Africa. Our findings also highlight the need to consider this variability in the development of new tools and strategies to control TB.

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Chapter 7: *Mycobacterium africanum* is associated with HIV and Ethnicity in Ghana

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Abstract

Background: Emerging evidence suggests that apart from the old selective pressure acting on TB, new pressures are changing the dynamics of TB. Here, we analysed 1211 MTBC isolates from TB patients in Ghana and searched for association between the different phylogenetic lineages of MTBC and patient variables.

Method: MTBC isolates were identified by PCR amplification of *IS6110*, and genotyped by large sequence polymorphism analysis, TaqMan-based SNP-typing and spoligotyping. Associations between the different phylogenetic lineages of MTBC and epidemiological variables were assessed using univariate and multivariate logistic regression.

Results: Eight hundred and eighty-three isolates (71.9%) belonged to Lineage 4 (Euro-American), 152 (12.6.0%) to Lineage 5 (*M. africanum* West Africa I) and 112 (9.2%) to Lineage 6 (*M. africanum* West Africa II). Fifteen isolates (1.2%) belonged to Lineage 1 (includes EAI), 42 (3.5%) to Lineage 2 (includes 'Beijing'), 12 (1%) to Lineage 3 (includes CAS) with the remaining 7 (0.6%) isolates identified as *M. bovis*. With respect to socio-demographic and behavioral characteristics, stratifying by lineage, we found *M. africanum* West Africa I significantly more common in the Ewe patient ethnic group (adjusted odds ratio (aOR): 3.02; 95% confidence interval (CI)**:** 1.5-4.7, p<0.001) and *M. africanum* West Africa II more likely to be found among HIV positive TB patients (adjusted odds ratio (adjOR) =2.4; 95% confidence interval (CI)**:** 1.4-3.9) P<0.000). We found no significant association between MTBC lineage and patient age, gender, prior BCG vaccination, or bacterial burden estimated by the degree of sputum smear positivity.

Introduction

Tuberculosis (TB) still remains one of the main global public health problems, particularly in resource limited settings (**Global TB report, 2014**). The global TB epidemic is further exacerbated by a strong synergy with HIV/AIDS, which is particularly a big challenge in sub-Saharan Africa. The Word Health Organization (WHO) estimates that in 2013, of the 1.1 million TB cases co-infected with HIV, 80% occurred in Africa, making Africa the hardest hit of the two epidemics (**Global TB report, 2014)**.

Human TB is caused mainly by 2 species (*Mycobacterium africanum* (MAF) and *Mycobacterium tuberculosis*) of the group of bacteria known as the *Mycobacterium tuberculo*sis complex (MTBC) consisting of nine different species (**Brosch *et al.*, 2002; Gagneux and Small, 2007; Niemann *et al.*, 2000**). Genetic strain typing has subdivided the human TB species into seven major lineages which epidemiologically co-associate with distinct geographical regions (**Firdessa *et al.*, 2013**). Genetically MAF is closely related to MTB, but can be differentiated from MTB by deletion in genomic region 9 in MAF genome and the presence of Tb1 genomic element by spoligotyping the absence of spacers 8, 9 and 39 (**de Jong *et al.*, 2010;Streicher *et al.*, 2007;de Jong *et al.*, 2010**). Using both whole genome analysis, Lineage 6 was found to be closer to the animal adapted species including *M. bovis* while Lineage 5 is closer to the four lineages of *Mycobacterium tuberculosis* (MTB) (**Hershberg *et al.*, 2008**). Phenotypically MAF either does not or weakly produce niacin, weakly to negative nitrate reductase, dysgonic colonial morphology or prefers microaerophilic growing conditions (**Castets *et al.*, 1968**).

Of particular interest to West Africa is MAF subdivided into 2 lineages (Lineage 5 and 6), which can cause up to half of all tuberculosis cases in West Africa (**de Jong *et al.*, 2010**). Reasons why MAF has not established itself outside of the West Africa region still remains unknown even though earlier paleopathological investigation using spoligotype analysis of

human remains from Egypt's Middle Kingdom (c. 2000–1600 B.C.) identified MAF2 alongside *M. tuberculosis* (**Nerlish *et al.*, 2009; Zink *et al.*, 2003**). The strong phylogeography of MAF suggest possible adaptation to distinct human population(s) in West-Africa (**Herb *et al.*, 2008**). Indeed, a study performed in Ghana found a variant of 5-lipoxygenase (ALOX5) gene to be associated with protection against the globally distributed Euro-American MTB lineage but not against MAF; perhaps this polymorphism provides a selective advantage for MAF in West African populations (**Herb *et al.*, 2008**).

Using a limited retrospective collection of MTBC isolates from South western Ghana, we recently observed an association between MAF and the Ewe ethnic group found only in areas with high prevalence of Lineage 5. Here, we followed-up on this observation with a larger population-based prospective study involving cases from both the southern and northern part of Ghana. Our findings confirm the establishment of MAF as an important cause of TB in Ghana, and validate the strong association between Lineage 5 with the Ewe ethnic population. Moreover, we found MAF/Lineage 6 associated with HIV co-infection, supporting previous findings from the Gambia.

Methods

In the context of a prospective TB study in the Ghana, consecutive sputum smear-positive TB cases were enrolled after informed consent. The standard procedure for sputum sample collection as outlined by the National Tuberculosis Program (NTP) for routine diagnosis of TB in Ghana was used in this study. The study and its protocols were approved by the internal review board of the Noguchi Memorial Institute for Medical Research (NMIMR) and Basel. Participants provided written consent unless the participant was illiterate; in which case witnessed oral consent was used. In accordance with ethical review board regulation in Ghana consent was sought from guardians of children below the age of 18 before enrolment into the study and in some cases child assent was also sought. Eligible patients were smear-positive, pulmonary tuberculosis cases presenting to health centers before initiation of treatment or less than 4 weeks of treatment. All eligible TB patients were encouraged to undergo HIV tests before initiation of anti TB drugs according to national guidelines. All patients and staff involved with the study were blinded to the final data obtained.

The sputum samples were re-examined for the presence of AFBs at NMIMR and cultured on solid agar, after which DNA was extracted and used for genotyping analysis. Briefly, we took a 10 μL loop from a Lowenstein-Jensen medium slope and, after heat killing, extracted DNA first by digestion with lysozyme and proteinase K, solubilized by detergents sodium dodecyl sulphate and cetrimonium bromide, followed by chloroform isopropanol extraction (**van Soolingen *et al*., 1993**).

Classifications into main lineages within MTBC were by TaqMan real time PCR (TaqMan, Applied Bio systems, USA) using probes targeting lineage-specific SNPs as reported by Stucki *et al.*(**Stucki *et al*., 2012**) Spoligotyping was performed to define the sub-lineages and strain families within each of the main lineages circulating in Ghana (**Kamerbeek *et al*., 1997**). Spoligotyping patterns were defined according to SITVITWEB database (**Demay *et al*., 2012**) (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE). SITVITWEB

assigned shared types numbers were used whenever a spoligotyping pattern was found in the database while families and subfamilies were assigned based on the MIRU-VNTRplus database (http: //www. miru-vntrplus.org) (**Weniger *et al*., 2010**). Shared types were defined as patterns common to at least two or more isolates. Data collected from enrolled patients, including age, sex, immunosuppressive co-morbidity with HIV, place of work, ethnicity, status of smoking, level of education, status of smoking , income, presence of BCG scar were also recoded.

Data entry, management and analysis

Information from the structured questionnaire was double entered using Microsoft© Access and validated to remove duplicates and data entry inconsistencies. Spoligotype patterns were entered in a binary format. A series of univariate and multivariable logistic regression models were fitted to assess the relationship between MTBC lineage(s) (primary independent variable) and host variables.

Results

Study population

During the study period July 2012 to February 2014, a total of 1230 smear-positive TB cases were identified and 1211 (98.5%) included in the study. Age of patients ranged from 3 to 91 years with a median age of 39 years (Table 19). Overall, 373/1211 (31%) were females with median age of 33 years; the remaining 838 (69%) were males with a median age of 36. Twenty-eight out of the 1211 patients (2.3%) were children (age<16 years). Most of the patients (N=1112; 91.8%) originated from the southern part of Ghana with the remaining 99 (8.2%) from northern Ghana. Out of the 1211 patients, 1160 (95.8%) were Ghanaians and the remaining 51 (4.2%) were other West African nationals. Most of the patients were of Akan ethnicity (N=604, 49.9%), followed by Ga (N=280, 23.1%), Ewe (N=184, 15.2%) with the remaining ethnicities accounting for 11.8 % (N=143). In terms of education, 346 patients (28.6%) had no formal education, 134 (11.1%) primary education, 632 (52.1%) had up to secondary education, and the remaining 99 (8.2%) tertiary education. About half of the study population (N=591, 48.8%) were unskilled labourers, 314 skilled (26%) with the remaining 306 (25.2%) unemployed. Most of the study population had high bacteria burden as measured by sputum smear microscopy; 3+ (N=534, 44.1%), followed by 2+ (N=295, 24.4%), 1+ (N=266, 22%) and scanty (N=115, 9.5%)

Table 19: General Characteristics of patients included in the study

Variable	N(1211)	(%)
	n(%	n(%
Sex (male)	838	69.0
female	373	31.0
Age category		
yr 08-25	227	18.7
yr 26-40	496	41.0
yr 41-77	488	40.3
Residency		
North	99	8.2
South	1112	91.8
Occupation		
unskilled	591	48.8
Skilled	314	26.0
Unemployed	306	25.2
Nationality		
Ghanaian	1160	95.8
West Africans	51	4.2
Income(GHC)		
None	449	37.1
<100	184	15.2
100-500	505	41.7
>500	73	6.0
Smear positivity		
Scanty 1-9	115	9.5
+1	266	22.0
+2	295	24.4
+3	534	44.1
Ethnicity		
Akan	604	49.9
Ewe	184	15.2
Ga	280	23.1
other	143	11.8
HIV status		
Positive	160	13.2
Education		
No Education	346	28.6
Primary	134	11.1
Secondary	632	52.6
Tertiary	99	8.2
Presence of BCG Scar		
yes	505	41.7
Smoking status		
yes	248	20.5

Prevalence of MTBC Lineages and Sub-Lineages

Of 1230 study cases, 1224 (99.5%) had TB DNA available for genotyping, of which 1211 (98%) gave interpretable spoligotype results. Ten isolates repeatedly failed to amplify and 3 isolates had results suggestive of mixed infection. SNP typing identified 152 isolates (12.6%) as MAF West-African type 1 (MAF1; Lineage 5), 112 isolates (9.2%) as MAF West-African type 2 (MAF2; Lineage 6), 871 (71.9%) isolates as the Euro American lineage (Lineage 4), 15

isolates (1.2%) as lineage 1, 42 isolates (3.5%) as Lineage 2, 12 isolates (1%) as Lineage 3 and 7 (0.6%) as *M. bovis*. All isolates were further sub-typed using spoligotyping.

Among the 871 Lineage 4 isolates, 503/871 (58%) belonged to 'Cameroon family', the most dominant sub family of Lineage 4 with the most prevalent spoligotype 61 accounting for 349 isolates. Seventy –three percent of strains isolated from West African nationals (N=41) belonged to the 'Cameroon family' defined by spoligotyping pattern 61.

In addition to Cameroon sub-lineage, seven additional sub-lineages isolates were identified among Lineage 4 based on spoligotyping; Ghana (N=198, 22.7%), Haarlem (N=83, 9.5%), Uganda I (N=27, 3.0%), Uganda II (N=2, 0.2%), LAM (N=26, 2.9%), S (N=2 (0.2%), New_1 (N=1, 0.1%) and H37RV_like (N=1,0.1%).

Spoligotyping of the 264 MAF isolates revealed 92 distinct spoligotypes patterns. Fifty-two unique patterns (singletons) were observed with the remaining 40 patterns grouped into clusters of 2-26 isolates respectively. Within the 940 MTBss isolates, we had 101 patterns, consisting of 90 distinct patterns and with the remaining 11isolates identified as singletons. We compared the 193 spoligotypes found in this study with those contained in an international spoligotype database, 130 of our spoligotypes were already described in SITVIT database. The other 63 spoligotypes were novel. Of the 63 novel spoligotypes, 52 clustered already clustered and 13 were unique.

Association between MTBC lineages and Patient Characteristics

Using univariate analysis comparing the host variables sex, age, BMI, maximum smear grade, and presence of a BCG scar and the different MTBC lineages, no significant differences were identified (Table 20). Since the number of isolates belonging to the Lineages 1-3 were small, we combined all the *M. tuberculosis* sensu stricto lineages, therefore, for the remainder of the analysis we compared Lineage 5 (n=152) and Lineage 6 (n=112) with the all the other MTBC

lineages (n=940, Lineage 1-4). Based on logistic regression model after adjusting for age and gender, Lineage 5 was significantly found more common in patients of Ewe ethnicity (adjusted odds ratio (adjOR) =3.0; 95% confidence interval (CI)**:** 1.5-4.7) compared to other ethnic groups.

All 1211 patients consented to HIV testing and among them, 160 (13.2%) were HIV sero-positive. After adjusting for age and gender, TB patient infected with Lineage 6 were significantly more likely to be co-infected with HIV(adjusted odds ratio (adjOR) =2.4; 95% confidence interval (CI)**:** 1.4-3.9)P<0.001 (Table 21). No other significant lineage association was found with other patient variables, including age, sex, the presence of a BCG scar, or the degree of smear positivity.

Table 20: Distribution of patient variables across the six MTBC lineages in Ghana

Risk factor	Lineage 1 (n=15)	Lineage 2 (n=42)	Lineage 3 (n=12)	Lineage 4 (n=871)	Lineage 5 (n=152)	Lineage 6 (n=112)	*M. bovis(n=7)*
	n(%)	n(%	n(%	n(%	n(%	n(%	n(%
***Sex* (male)**	12(80)	28(66.7)	7(58.3)	615(70.6)	96(63.2)	73(65.2)	7(100)
female	3(20)	14(33.3)	5(41.7)	256(29.4)	56(36.8)	39(34.8)	0
Age category							
yr 08-25	2(13.3)	8(19.1)	2(16.7)	169(19.4)	28(18)	17(15.2)	1(14.3)
yr 26-40	6(40)	19(45.2)	4(33.3)	354(40.6)	62(41)	49(43.8)	2(28.6)
yr 41-77	7(46.7)	15(35.7)	6(50)	348(40)	62(41)	46(41)	4(57.1)
Residency							
North	0	3(7.1)	0	79(9)	8(5.3)	8(7.1)	1(14.3)
South	15(100%)	39(92.9)	12(100%)	792(91)	144(94.7)	104(92.2)	6(85.7)
Occupation							
Skilled	3(20)	10(23.8)	3(25)	237(27.2)	35(23)	24(21.4)	2(28.6)
Unskilled	12(80)	32(76.2)	9(75)	634(72.8)	117(77)	88(78.6)	5(71.4)
others							
Income(GHC)							
None	5(33.3)	18(42.9)	3(25.0)	412(47.3)	65(42.7)	43(38.4	3(42.8)
<100	3(20.0)	7(16.7)	1(8.3)	125(14.5)	32(21.1	16(14.3)	0
100-500	6(40.0)	14(33.3)	6(50.0)	282(32.3)	51(33.5	45(40.2)	1(14.4)
>500	1(6.7)	3(7.1)	2(16.7)	52(5.9)	4(2.6)	8(7.1)	3(42.8)
Smear positivity							
Scanty 1-9	2(13.3)	5(11.9)	0	80(9.2)	2(1.3)	0	
+1	3(20.0)	9(21.4)	3(25.0)	199(22.8)	34(22.6)	18(16.1)	
+2	4(26.7)	9(21.4)	4(33.3)	213(24.6)	37(24.3)	28(25)	
+3	6(40.0)	19(45.2)	5(41.7)	352(40.4)	79(52.0)	66(58.9)	7(100%)
Ethnicity							
Akan	10(66.7)	18(42.8)	6(50)	448(51.4)	39(25.7)	78(69.7)	5(71.4)
Ewe	1(6.7)	4(9.5)	1(8.3)	110(12.6)	58(38.2)	10(8.9)	0
Ga	4(26.7)	15(35.7)	5(41.7)	197(22.6)	47(30.9)	11(9.8)	1(14.3)
Others	0	5(11.9)	0	116(13.3)	8(5.3)	13(11.6)	1(14.3)
HIV status							
Positive	2(28.6)	1(7.7)	3(42.9.0)	96(18.5)	28(29.5)	29(34.9)	1(25.0)
Education							
No Education	5(33.3)	15(35.7)	4(33.3)	231(26.5)	50(32.9)	40(35.7)	1(14.3)
Primary	1(6.7)	5(11.9)	6(50)	91(10.4)	21(13.8)	15(13.4)	0
Secondary	9(60.0)	18(42.9)	1(8.3)	477(54.8)	70(46.1)	47(42.0)	5(71.4)
Tertiary	0	4(9.5)	1(8.3)	72(8.3)	11(7.2)	10(8.9)	1(14.3)
Crowding							
<10 per house	2(13.3)	9(21.4)	6(50.0)	238(27.3)	44(28.9)	33(29.5)	2(28.6)
>10 per house	13(86.7)	33(78.6)	6(50.0)	633(72.6)	108(71.1)	79(70.5)	5(71.4)
BCG Scar							
yes	9(60.0)	17(40.5)	4(33.3)	361(41.4)	64(42.1)	46(41.1)	4(57.1)
no	6(40.0)	25(59.5)	8(66.7)	510(58.6)	88(57.9)	66(58.9)	3(42.9)
Smoking status							
yes	4(26.7)	7(16.7)	2(16.7)	179(20.6)	25(16.4)	28(25.0)	2(28.6)
no	11(73.3)	35(83.3)	10(83.3)	692(79.4)	127(83.6)	84(75.0)	5(71.4)

Table 21:Distribution of patient variables across the three main MTBC lineages in Ghana

Risk factor	Lineage 5 (n=152)	Lineage 6 (n=112)	MTBss (n=940)	OR (95%CI)	adjOR (95%CI)[a]
	n(%	n(%	n(%		
Sex (male)	96(63.2%)	73(65.2%)	662(70.6%)	1.3 (0.97-1.98)	
female	56(36.8%)	39(34.8%)	278(29.4%)		
Age category					
yr 08-25	28(18%)	17(15.2%)	181(19.4%)	1.42 (0.82-2.64)	
yr 26-40	62(41%)	49(43.8%)	383(40.6%)	0.96 (0.4-1.96)	
yr 41-77	62(41%)	46(41%)	376(40%)	1.06 (0.59-1.91)	
Residency					
North	8(5.3%)	8(7.1%)	82(9%)	0.58(0.27-1.22)	
South	144(94.7%)	104(92.2%)	858(91%)	ref	
Occupation					
Skilled	35(23%)	24(21.4%)	171(27.2%)	0.9(0.81-1.0)	
Unskilled	117(77%)	88(78.6%)	769(72.8%)	ref	
Income(GHC)					
None	65(42.7%)	43(38.4%)	260(47.3%)	0.8(0.62-0.98)	
<100	32(21.1%)	16(14.3%)	136(14.5%)	0.91(0.76-1.0)	
100-500	51(33.5%)	45(40.2%)	408(32.3%)	ref	
>500	4(2.6%)	8(7.1%)	58(5.9%)	1.3(0.97-1.98)	
Smear positivity					
Scanty 1-9	2(1.3%)		80(9.2%)	0.6(0.45-0.72)	
+1	34(22.6%)	18(16.1%)	199(22.8%)	0.78(0.65-1.8)	
+2	37(24.3%)	28(25%)	213(24.6%)	0.98(0.42-1.84)	
+3	79(52.0%)	66(58.9%)	352(40.4%)	ref	
Ethnicity					
Akan	39(25.7%)	78(69.7%)	482(51.4%)	ref	
Ewe	58(38.2%)	10(8.9%)	116(12.6%)	3.0(1.5-4.7)	2.79 (1.47-5.29)*
Ga	47(30.9%)	11(9.8%)	221(22.6%)		0.85 (0.43-1.69)
other	8(5.3%)	13(11.6%)	118(13.3%)		1.64 (0.53-5.34)
HIV status					
Positive	28(29.5%)	29(34.9%)	96(18.5%)	2.4(1.4-3.9	2.2(1.32-3.7)
Education					
No Education	50(32.9%)	40(35.7%)	231(26.5%)	0.83(0.75-1.0)	
Primary	21(13.8%)	15(13.4%)	91(10.4%)	0.72(0.65-1.11)	
Secondary	70(46.1%)	47(42.0%)	477(54.8%)	0.96(0.83-1.3)	
Tertiary	11(7.2%)	10(8.9%)	72(8.3%)		
Presence of BCG Scar					
yes	64(42.1%)	46(41.1%)	430(41.4%)	1.1(0.69-1.7)	
Smoking status					
yes	25(16.4%)	28(25.0%)	248(20.6%)	0.6(0.7-1.8)	

Discussion

In this study, we observed a strong association between MAF and HIV; stratifying by Lineage we found this association to be driven by MAF2. HIV infection is known to impair cellular immunity thereby increasing the risk of reactivation of latent TB thus making TB the most common co-infection in subjects infected with HIV (**Pawlowski *et al.*, 2012)**. Furthermore HIV infection has emerged as by far the most important of all the predisposing factors for TB: in persons co-infected with the tubercle bacillus and HIV, the overall annual risk of developing active tuberculosis is 20 times the risk of immunocompetent individuals (**Pawlowski *et al.*, 2012**). Our finding and that of a previous study conducted in the Gambia that showed an association between HIV and MAF/Lineage 6 (**de Jong *et al.*, 2005**); suggest that this lineage may not be as virulent as MTB in immunocompetent individuals.

Early animal models and macrophage infection assays indicated differences in virulence of tubercle bacilli isolated from different geographical regions (**de jong *et al.*, 2005**; **Castets *et al.*, 1979**). Early animal studies comparing MAF from Senegal to MTB showed that MAF was less virulent (**Bold *et al.*, 2012; Korsak *et al.*, 1979**). Similarly, de Jong *et al* found that the rate of progression to active disease in TB exposed household contacts is longer for those infected with MAF2 compared to MTB (**de Jong *et al.*, 2008; Smith, 2003**). The MTBC have evolved multiple mechanisms to interfere with the host immune system, allowing the state of latency (**Portevin *et al.*, 2011**). Many of these mechanisms are mediated through specific components of the mycobacterial cell wall; different components of this cell wall, as well as other mycobacterial molecules modulate various aspects of the innate and the adaptive immune response (**Portevin *et al.*, 2011**). These mechanisms include interfering with phagocytosis and phagosome-lysosome fusion, the production of anti-inflammatory cytokines such as IL-10, and interfere with the IFN-γ signaling (**Portevin *et al.*, 2011**). MTBC has also the capacity to inhibit cytokine production, antigen presentation, and MHC II expression in

antigen presenting. To test whether MAF and MTB elicit different host innate immune responses, it was found by the group in the Gambia that host-pathogen interaction after infection depends on the infecting MTBC lineage using infected human monocyte-derived macrophages. Hours of infection, MAF elicited in average a statistically significantly higher pro-inflammatory response as measured by IL-12, TNF, and IL-6 when compared to representatives of MTB lineages (**de jong *et al*., 2005**).

A previous paper from Ghana did not detect any association between MAF and HIV co-infection (**Meyer *et al*., 2008**). The prevalence of MAF is reported to be going down in some West-African countries whereas in Ghana, the prevalence of MAF overall has been stable over the last 10 years around 20%. Yet, the present distribution inferred from this study indicates that the proportion of MAF1 among all MAF is going down while that of MAF2 is increasing. In our current analysis we found the proportion of MAF2 among all MAF (42%) to be significantly higher (odds ratio (3.52; 95% confidence interval (CI)**:** 2-6.1) P<0.001 18% compared to the 18% reported in our earlier studies of total MAF isolates (**Yeboah-Manu *et al*., 2011**). This difference could account for the discrepancies between the previous study by Yeboah-Manu *et al* and our study. We speculate from our study that increasing prevalence of TB/HIV in Ghana may drive the increase in prevalence of MAF2, even though the overall prevalence of MAF has not changed over the last 10 years.

Additionally, de jong *et al* reported an association between MAF and patient of old age consistent with the long latency period associated with this lineage (**de Jong *et al*., 2010**). Longer latency in MAF might be an adaptation to low host densities, whereas a reduced latency period (i.e. increased "virulence") in MTB infections might be an adaptation reflective of the crowded conditions leading to higher high rates of TB in urban areas. However, we did not find any differences between the two species with regard to age.

In our previous study (Chapter 6), we demonstrated a plausible reason for the restriction of MAF to West Africa. Our analysis revealed that patients infected by MAF1 as opposed to MTB were more likely to belong to the Ewe ethnic group. This association was independent of other variables and was not due to a single outbreak as the Ewe isolates differed in their spoligotypes. In the present study, we were able to replicate this finding with an independent and much larger dataset. Although social cohesion interlace with less intermingling could account for the geographical restrictiveness of MAF, biological factors could equally account. In our previous study, the samples were collected mainly from the central, western and Greater-Accra regions of Ghana. The present study which is population based collected samples from the Greater Accra and Northern regions of Ghana.

Given the current efforts in the development of new TB vaccine, strain and host diversity should be considered when evaluating new vaccine candidates especially in areas where MAF is prevalent.

Chapter 8: General Discussion and Conclusion

8.1. General Discussion

TB remains a major public health challenge globally, despite 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 and the emergence of drug resistant strains of MTBC. To overcome these challenges, the STOP-TB strategy was developed by the World Health Organization (WHO) with the aim to dramatically reduce the global burden of TB by 2050 by ensuring that all TB patients benefit from universal access to high-quality diagnosis and patient-centered treatment. To eliminate TB as a public health problem by 2050, STOP-TB was tasked to identify and intensify research into areas of importance which might provide answers needed for the elimination. These areas include: 1) development and evaluation of simplified diagnostic tools, 2) development of new drugs, 3) understanding drug resistant TB in the context of TB control, and 4) the effects of commodities such as HIV on the prevalence of TB.

The present PhD work contributed to the STOP TB research agenda by: 1) analyzing the level of drug resistant in Ghana and implication on treatment outcome 2) established for the first time a rapid molecular tool for diagnosing drug resistant TB in Ghana 3) established molecular methods in an endemic country and analyzed the phylogenetic diversity of MTBC isolates in-country and 4) analyzed patient variables associated with MTBC diversity.

8.1.1 Drug resistance in the context of TB control in Ghana.

One of the goals of STOP-TB is to drastically reduce the burden of TB. However, this goal is undermined by the development of drug resistance which threatens to make TB untreatable. In low-resource TB-endemic countries including Ghana, current control strategies rely mainly on case detection by microscopy and treatment by the DOTS strategy, monitoring of

treatment complemented with microbiological support at 2 months and 5 months of drug treatment. While microscopy is good as it is simple and cheap and therefore applicable at peripheral centers of low-resource countries, it is not very sensitive. More importantly, it has a major limitation of not being able to detect the resistance status of the infecting pathogen. Thus, in such countries including Ghana, policies guiding re-treatment regimens rely solely on periodic drug resistance surveys.

One of the main contributions of the present PhD work was to analyze the level of drug resistance in Ghana and for the first time correlating clinical response to *in vitro* drug susceptibility. We found a high rate of drug resistance among the isolates analyzed and for the first time in Ghana reported that indeed good clinical treatment outcome depends primarily on the susceptibility of the *M. tuberculosis* isolate to RIF (Chapter 3). RIF and related rifamycins are the most important drugs used for the treatment of TB (**American Thoracic Society/Centers for Disease Control and Prevention/Infectious Diseases Society of America, 2003***)*. Rifamycins interfere with bacterial DNA-dependent RNA polymerase and are potent bactericidal agents (**Kohanski *et al*., 2010; Chairsson, 2003**). In addition, RIF and its analogues actively kill multiplying extracellular and intracellular mycobacteria (**Chairsson, 2003**), and hence is an important drug for successful sputum smear-conversion at 2 months of drug treatment. The addition of RIF to treatment regimens for TB reduced the duration of therapy needed for active disease from 12 to 6 months. Because of their potencies and sterilizing activities, rifamycins are the cornerstone of modern therapy for active TB (**Chaisson, 2003**) and are extremely effective in the treatment of *mycobacterial diseases* (**Bishai and Chaisson, 1997).**

For effective case management and control of drug resistance, the National TB Program of Ghana is currently rolling out the one of WHO rapid diagnostic tools (WRD); Xpect MTB/RIF at regional centers for treatment support of risk groups which include previously

treated cases, TB patients with previous contact with a DR case, and smear-positive persistent cases.

Our finding also confirms the need for restricted use of RIF to guard against incidence of DR. Currently RIF is also important for the treatment of leprosy and Buruli ulcer (BU) (**Dega *et al.*, 2000; Dega *et al.*, 2002).** BU is the third most common mycobacterial disease in the world and second after TB in Ghana (**Amofah *et al.*, 2002**). Similar to other African BU endemic countries, treatment of BU usually relies on clinical judgment without microbiological confirmation. This is due to the reported low sensitivity of microscopy; extremely slow growth of *M. ulcerans* and the high infrastructural and expertise demand of PCR. This inevitably has reduced microbiological confirmation to a quality control tool for clinical diagnosis of BU. Although, the general perception is that diagnosis based on clinical judgment alone can be adequate, incidents of misdiagnosis have been reported. BU cases have been missed initially and, on the other hand, presumed BU lesions proved to be due to other conditions including cutaneous TB caused by MTBC (**Bratschi *et al.*, 2012**). If this approach of BU treatment is not checked, the development of RIF resistance will be inevitable, threatening TB treatment. There is urgent need for collaborative efforts between the TB and BU programs in Ghana to curb the misuse of RIF in the communities.

Using Genotype MTBDRplus (Chapter 4), this study confirmed high INH resistance in Ghana. In previous reported studies and that from this thesis (Chapter 3) using phenotypic methods, Ghana has been shown to be among the countries in Africa with a comparably high rate of resistance of INH (**van der Werf *et al.*, 1989; Owusu-Dabo, 2006**). An effective TB treatment is based on at least threefold objective; 1) to reduce bacilli load rapidly 2) prevent emergence of drug resistance 3) prevent relapse. Objectives 1 and 2 are achieved by the multi-therapy while that of objective 3 is through the extension of treatment in the continuous phase. In patients with INH resistance, the continuous phase regimen of combined INH and RIF becomes RIF mono-therapy, risking the incidence of RIF resistance. This effect is more

worrying in HIV co-infected cases. RIF has an effect on the activity of the CYP3A4 enzyme (oxidizes small foreign organic molecules such as toxins or drugs, so that they can be removed from the body) (**Yamashita *et al*., 2103**) and potentially reducing the concentration of RIF and lowering blood levels of other drugs that utilize this pathway of metabolism, including many antiretroviral medications (**Burman *et al*, 1999**). These interactions mean that RIF cannot be maximally used by most HIV-infected people taking antiretroviral therapy leaving INH as the main sterilizing drug. The high INH resistance of more than 10%, probably, calls for a third drug such as ethambutol to be added in the continuous phase for countries like Ghana to guard against relapse and drug resistance. Although Ghana is yet to introduce INH preventive therapy (IPT) for TB/HIV patients, considering the high level of INH resistance in Ghana, this idea may not be prudent.

8.1.2 Phylogeography of MTBC and implications for TB Control in Ghana

Human adapted MTBC lineages are known to exhibit a strong phylogeographic population structure (**Gagneux and Small, 2007).** One main achievement of this study was to confirm the importance of Lineage 5 and 6 in Ghana; spanning over 8 years, these pathogens still accounts for approximately 20% of pulmonary TB cases (Chapter 5 and 6) despite reported decline in some West African countries. Although the prevalence of MAF overall was consistent over the years, we observed a gradual decline in the prevalence of Lineage 5 and whilst there was an increase in Lineage 6 cases. A study conducted in the Gambia by de Jong *et al* showed a strong association between Lineage 6 and HIV (**de Jong *et al*., 2005**). This same group in a further population based study showed that while MTB infected cases and MAF cases equally transmit infection among close contacts, progression to active disease was much slower in contacts of MAF infected individuals. (**de Jong *et al*., 2008**). Probably one can speculate that the high HIV-TB co-infection is one possible factor driving the up-surge of Linage 6 causing TB among the Ghanaian population.

Possibly, one reason for the stability of MAF in Ghana in contrast to other west African countries might be adaptation of this bacterial lineage to (some) human populations. Indeed associations between different MTBC lineages and human ethnicities have been observed before; in San Francisco, Lineage 1, 2 and 4 were strongly associated with Filipino, Chinese, and "white" ethnicities, respectively (**Gagneux *et al.*, 2006**) and in more recently, Lineage 2 with Hui ethnicity in China (**Pang *et al.*, 2012**). Our reproducible findings of an association between Lineage 5 and Ewe patient ethnicity together with those discussed above are consistent with host-specific adaptation of MTBC lineage and provide plausible reasons for the establishment of MAF in West Africa. This provides the bases for in-depth host genetic factors given that that host polymorphism associated with protection against Euro-American *M. tuberculosis and not MAF* have been identified in Ghana. Whether the Ewe population in Ghana harbors this polymorphism that makes them more susceptible to TB caused by MAF especially Lineage 5 is unknown. Results from such studies will help inform the development of effective control strategies.

Five of the seven main human-adapted MTBC lineages belong to MTB sensu stricto and among these various sub-lineages and clades exist (**Gagneux *et al*, 2006**). Within the MTB sub-groups, sub-lineages and clades seems to be well adapted within certain populations. This observation informed a study that looked at the evolutionally history of the pathogen by analyzing the whole genomes of 259 MTBC strains compared with that of human genomes from the same geographic regions and concluded that based on the long standing association between MTBC and its human host, distinct MTBC genotypes/clades might have adapted to different human population, perhaps as a consequence of long co- evolutionary process between MTBC and its host (**Comas *et al.*, 2013**). For example more than 60% of TB in Uganda is caused by one particular sub strain-Uganda genotype and this genotype is mainly found in Uganda and neighboring countries, and rarely found elsewhere (**Wampande *et al.*,**

2013). Similarly the Beijing family of Lineage 2 associated with Asia causes more than 90% of all TB cases in that region (**Parwati *et al*., 2010**)

In the context of the PhD work, we confirmed the importance of a one particular sub-lineage of Lineage 4 known as the Cameroon (CAM) 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*, 2014 unpublished data)** This genotype first described in Cameroon as having a typical spoligotype, SIT61 signature (spacers 23–25 and 33–36 missing) and representing 34% of the *MTBC* isolates in 2003 in Cameroon (**Niobe-Eyangoh *et al*., 2003**; **Koko *et al*., 2013**) has been identified as dominant in other West-African states. The CAM genotype belongs to the principal genetic group 2 (i.e. *modern* strains) and lacks the TbD1 region (**Niobe-Eyangoh *et al*., 2004**). Following its initial identification, several CAM spoligotype variants (SIT403, SIT852, SIT808, and SIT852) have been reported in different West-African and some central African states (**Niobe-Eyangoh *et al*., 2004**). Whether these different variants exhibit different levels of virulence is still unknown. The virulence of this lineage has been established by various reports and as an important predisposing factor for occurrence of DR-TB has been published (**Koro *et al*., 2013; Assam *et al*., 2013; Lawson *et al*., 2012**). In our work (Chapter 3), we found a strong association between the occurrence of STR and any form of resistance was significantly higher compared to the MAF1 lineage (**Yeboah-Manu *et al*., 2011**). Probably the virulence and transmissibility of this sub-lineage may have accounted for the observed decline of the MAF1 in some West-African states, which was previously identified as an importance cause of TB, accounting for about 50% of TB. While in 1971 Huet *et al*. showed that 56% of human TB cases in Cameroon were caused by *MAF (***Huet *et al*., 1971**), in 2003 Niobe-Eyangoh *et al*. reported a rate of 9% and more recent data indicated an even lower prevalence of MAF1 in that country (**Sarah Niobe-Eyangoh, personal communication**). In addition, most studies in endemic setting in including Ghana and Nigeria using number of molecular genotyping analyses using spoligotyping and MIRU/VNTR, found

that MTB isolates were more likely than the *M. africanum* isolates to be part of a spoligotyping cluster, implicating the high transmissibility or being involved in cases of recent TB transmission. This phenomenon suggests an emergence of this *MTBC* sub- family in the countries of West Africa. This observation calls for a critical effort for case detection, diagnosis to reduce transmission of TB in Ghana. The Ghanaian National TB Program with support of the Global Fund and the WHO just finished a National TB prevalence survey and preliminary results indicate a high prevalence of TB (3 times the WHO estimated figure; personal communication with NTP Ghana). This is worrying and could consequences for the control of TB in Ghana bearing in mind that Cameroon has a high virulence nature and is important factor for occurrence of DR-TB. This calls for more education of the communities and rigorous case finding activities to reduce transmission.

Geographic confinement and socio-economic factors could also account for the predominance of the Cameroon family within this sub-region of Africa. Data on the genetic diversity of MTBC in sub-Saharan Africa suggested that this family is found mainly within countries in Central and West African (**Koro *et al.*, 2013**) where substantial amount of intermingling occurs due to the free passage of goods and services. Hence, cross-border transmissibility could accounts for its high prevalence across West Africa. Active case detection and treatment especially at the country's borders will probably slow down the transmission of this very successful lineage.

In addition to the Cameroon genotype, this study showed for the first time that more than 3% of strains in Ghana belong to Lineage 2 which includes the Beijing stains. The Beijing genotype, normally found in Asia is one of the most successful clades in the present worldwide TB epidemic and is often associated with drug resistance. With an increasing

influx of Chinese migrants to Ghana, it would not be surprising to find a higher prevalence of the Beijing strains in the near future. The control program should actively screen for active cases especially in areas where these migrates are located to prevent spread of drug resistance associated with Beijing strains in Ghana.

8.1.3 TB/HIV Comorbidity and its impact on control strategies in Ghana

HIV and TB co-infection is acknowledged globally and each infection alters the natural course of the other infection. HIV co-infection increases the risk of both primary and reactivation of TB by 20-fold (**Selwyn *et al*., 1989**), whilst the development of active TB increases viral load and decreases cytokines that could suppress HIV growth (**Goletti *et al*., 1996; Toosi, 2003**). Thus *M. tuberculosis* and HIV act in synergy, hastening the decline of important immune functions and leading to subsequent death if untreated, making HIV the single most important risk factor for progression to active TB with high mortality rate. Although the mechanisms behind the breakdown of the immune defense of the co-infected individual are not well known, , we do know that HIV impairs CD4 T cell mediated immunity which is essential for control of *M. tuberculosis* infection (**Pawlowski *et al*., 2012**)

Within the frame work of this study, 13% of sputum smear positive patients analyzed were HIV positive in a country where the general population HIV sero-prevalence is below 2%, confirming the synergy between TB and HIV. The prevalence reported in this study is far lower than the estimated 24% reported by the NTP in 2013.This is likely because this study focused mainly on sputum smear positive TB patients while that of the NTP included sputum negative- and extrapulmonary TB patients. HIV patients usually present with sputum negative smear and extrapulmonary TB due to the reduced immune status requiring low bacterial load to stimulate progression of infection to disease state in contrast to low sensitivity of microscopy which requires a minimum bacilli load of 5,000/ml (**Shingadia *et al*., 2003**;

Getahun *et al.*, 2007). Current case detection and bacteriological monitoring of treatment policy in Ghana, similar to other endemic low-resourced countries relies on sputum smear microscopy, meaning many TB/HIV cases go undetected. In response, the WHO has provided several key guidance for national TB control programs such as the use of light-emitting diode (LED) microscopes to improve the sensitivity and turnaround time of sputum smear microscopy (**World Health Organization, 2011**). Furthermore, to improve the accuracy of TB detection and drug susceptibility testing through the use of liquid culture and molecular line probe assays (LPAs) such as Xpect MTB/RIF *(***Kurbatova *et al.*, 2012; Barnard *et al.*, 2008)**. The Ghana NTP with the aid of developing partners has implemented some of these recommended tools at selected regional and tertiary facilities to help evaluate alternative algorithms for case confirmation. Progress in the implementation of these initiatives will be dependent on key partnerships with the international laboratory community and ensuring that quality assurance procedures are followed by the country's national laboratory network.

One reason for the high prevalence of TB/HIV in Ghana could be due to the reported association between HIV and Lineage 6. MAF has been associated with reduced virulence even in early animal models (**Castets, 1969**; **Bold *et al.*, 2012**), a longer latency and a slower rate of progression to active disease in humans. All these characteristics are consistent with an opportunistic pathogen. Indeed, MAF exhibits some characteristics of opportunistic pathogens in that they become pathogenic following an immune alteration of their host e.g. prior infection, immunodeficiency, and ageing. For example studying 19 HIV-positive and 228 HIV-negative patients TB patients from The Gambia, de Jong and co-workers reported an association between *MAF* and HIV, ageing and with severe malnourishment (**de jong *et al.*, 2005**). These data considered MAF to have properties of an opportunistic pathogen. In contrast, **Meyer *et al*** reported no differences in the rates of HIV co-infection in Ghana between *MAF* and MTB infections.

Within the framework of this PhD work, Lineage 6 infected patients were more likely to be HIV positive than other MTBC lineages (Chapter 7). This confirms earlier reports by de Jong *et al* (**de Jong *et al*., 2005**). If indeed this association is not by chance, then one will expect to be MTB out-competed by MAF where rates of HIV are low, as was likely to be the case in Cameroon in the 1970s and 1980s, while increasing HIV prevalence would be associated with an increase in lower virulence strains such as MAF. In Ghana, over the last couple of year the rate of TB-HIV co infection has risen from 14% to 24% in 2014, a phenomenon which is consistent with confirms the above hypothesis.

On the other hand, TB/HIV co-infection is associated with disseminated TB which in turn is associated with lower transmission. One could assume that because of their immune-suppression and impaired CD4 T cell mediated immunity which is essential for control of *M. tuberculosis* infection, HIV-infected patients will be more likely to get infected by any genotype including geographically restricted genotypes compared to HIV negative patients. In this case ‘‘Any MTBC genotype can make irrespective of their virulence **(Fenner *et al*., 2013; Brites and Gagneux, 2012**). This assumption is supported by an observation from a study in San Francisco (**Gagneux *et al.*, 2006**) where HIV positive individuals had a higher likelihood of being infected with allopatric MTBC lineages than HIV negative patients. This is further supported by the assumption that HIV infected patients harbor the same or more MTBC genotypes than HIV negative, thus it is possible that in HIV negative individuals, the less virulent strains may not easily transmit in the presence of a more virulent genotype. Considering the fact that Lineage 4 is the most dominant lineage in both Gambia and Ghana, based on our assumption above, in the presence of Lineage 4 in immunocompetent individual, Lineage 6 will have a lower progression to disease. This is the opposite in immunocompromised individual where Lineage 6 is expected to have equal opportunity to progress to disease as Lineage 4 hence its higher prevalence in HIV infected individual.

8.1.4 Micro-epidemiological studies and TB control in Ghana

The conventional indicators for assessing performance of the national program in Ghana similar to all TB endemic countries depend on a number of factors. This includes the total number of TB cases detected, treated and either cured, completed, failed, relapsed and died. While these indicators are useful, they ignore important information such as transmission within the community. The use of molecular biology tools together with conventional epidemiology termed molecular epidemiology/micro-epidemiology has been acknowledged globally as an additional tool for effective TB control. As well as enabling researchers to identify circulating strains and highlight particularly virulent strains, molecular epidemiological techniques have been used successfully to detect recent transmission, identify populations at risk for TB and environmental risk factors.

One of the major contributions of the present PhD studies to the control of TB in Ghana was to establish a robust PCR based reduced set of MIRU-VNTR loci for strain differentiation in a TB endemic country (Chapter 5). This assay is specific to the main lineages found in Ghana (Chapter 5) and especially useful for a country like Ghana where ~ 20% of all TB cases are caused by *M.africanum* (**Yeboah-Manu *et al*., 2011**) (Chapter 6 and 7).

In addition, cost analysis performed on this assay found it to be more cost effective than the traditional genotyping schemes used for TB transmission studies. For a low resourced country like Ghana, this assay is beneficial as it not only defines the main lineages circulating, combined with classical epidemiological methods, it also identify groups most at risk and risk factors for transmission, differentiate relapse from re-infection cases and detect laboratory cross-contamination. In a country like Ghana where TB control is mainly based on case detection and treatment, and largely ignoring potential hot spots for TB transmission, this assay can provide important information for the development of country-specific TB control action plan. We are now using these minimal MIRU-VNTR set for molecular epidemiological investigation of MTBC transmission in population based study in Ghana However, although

this method has been adopted by the NTP to trace on going transmission, this assay is limited by the need for elaborate equipment like the PCR machine.

Since 1999, the Bacteriology Department of the Noguchi Memorial Institute for Medical Research (NMIMR) has assumed the role of the National TB Reference Laboratory in Ghana and has been actively involved in national control activities. Some of the activities that have been carried out include, conducting quality assurance, development of diagnostic manuals for peripheral and regional laboratories, drug susceptibility and case prevalence surveys. Findings from these activities have been used to improve control program activities, especially in the areas of case detection and treatment. An important question that the reference laboratory needs to help the program to address is: what are the risk factors that enhance transmission and what are the patient groups that are at increased risk of developing TB. The establishment of these genotyping methods at the reference laboratory will facilitate the identification of local characteristics that enhance TB transmission, and will be able to direct the control program to use its limited resources to establish measures that will limit the transmission of TB in high-risk populations. In addition, TB cases that do not clinically respond to treatment are classified as failures/relapse; these genotyping methods will be useful to differentiate between exogenous re-infection from true relapse cases.

8.1.5 Conclusions

This PhD work was conducted partly in Ghana a country where ~ 20% of TB is caused by *MAF*. Several research stays at the Swiss Tropical and Public Health Institute in Basel helped with confirmation of results obtained and facilitated technology transfer to the laboratories of the Noguchi Memorial Research Institute in Ghana. The most important scientific achievements were:

1. We determined the level of drug resistance in Ghana and for the first time confirmed the importance of RIF in the management of TB in Ghana.
2. We evaluated the sensitivity of the WHO recommended commercial rapid DNA-based drug susceptibility testing kits -Genotype MTBDR-*plus* for the identification of MDR strains in Ghana and confirmed its correlation with phenotypic DST. This provided the platform for the usage of Genotype MTBDR-*plus* in all regional hospitals as a means of controlling the spread of MDR strains in Ghana.
3. DNA fingerprinting of MTBC isolates from Ghana by SNP genotyping, spoligotyping and large sequence analysis provided for the first time, information on different genotypes within an endemic country of Africa. Two of the most dominant genotypes-Lineage 5 and 6 confirms the establishment of MAF in Ghana.
4. We provided for the first time a plausible explanation for why MAF/Lineage 5 might be restricted to West Africa and also confirmed the possible opportunistic nature of TB caused by Lineage 6.
5. Analysis of samples collected from pulmonary TB patients revealed a low prevalence of *M. bovis*, confirming that infection by *M. bovis* is not a public health (as opposed to economic) problem in Ghana.
6. We defined a cost effective genotyping assay for TB transmission studies. Complemented with classical epidemiological studies, this assay identifies clusters and gives clear indication of on-going TB transmission in Ghana and also identifies

groups most at risk of infection. Screening of Ghanaian MTBC isolates with this method provides useful information for the control program.

8.1.6 Future outlook

This PhD established the Cameroon sublineage as the most dominant MTBC genotype in Ghana. The Cameroon family is one of the most successful sublineages of Lineage 4, exclusive to West Africa. Based on the "Out-of-and-back to Africa" scenario of MTBC evolution (**Hershberg *et al.*, 2008**), it is likely that the Cameroon sublineage established itself before the reintroduction of the other sublineages. This suggests it has a selective advantage over other sublineages. However, unlike the globally distributed Beijing sublineage, where detailed epidemiological and animal model studies have established its association with drug resistance (**Mokrousov *et al.*, 2006**), hyper-virulence (**Chen *et al.*, 2012**) and higher clustering rate (**Hanekom *et al.*, 2007**), not much work has been done on the Cameroon sublineage.

In order to understand and control the spread of the Cameroon sublineage, a detailed population based study using our proposed reduced cost effective MIRU-VNTR assay and spoligotyping is essential. These tool combined with epidemiological data can provide an overhead view including local risk factors contributing to spread of the pathogen. Results from this study will inform us on the clustering ability of this pathogen. Clustering is a proxy for on-going transmission and several studies have shown the propensity of this pathogen to form clusters indicating enhanced transmission.

Secondly, a series of experimental studies, including e.g. animal models, experimental evolution studies comparing the rate of progression to disease caused by the Cameroon sublineage, *M. tuberculosis* clinical isolates; CDC1551, a highly immunogenic strain (**Tsenova *et al.*, 2005**), and HN878; member of the W/Beijing family of strains known for high virulence (**Bishai *et al.*, 1999)** can be performed to establish the virulence of the

Cameroon sublineage. The outcome of such studies could inform us on the virulence nature of this sublineage. While some members of the modern MTBC especially the Beijing sublineage lineage have been shown to exhibit lower early inflammatory response including cytokine induction (**Sarkar *et al.*, 2012**) this has not been established for the Cameroon sublineage. This phenotypic property might aid the pathogen to escape early detection by the immune system and promote more rapid progression to disease. Immunological studies could take advantage of the Cameroon sublineage to study its ability induce production of inflammatory cytokines in e.g. human monocyte-derived macrophages.

Within the frame work of this PhD project, we established the importance of MAF in Ghana and also showed an intriguing interaction between host- and pathogen genotype; i.e. MAF/Lineage 5 was associated with Ewe patient ethnicity. This is the first time an association between MAF and an ethnic group found only in West Africa has been observed. To explore this association further, we need to conduct an in-depth host genetic study and look for candidate genes/signatures that make this ethnic group susceptible to TB caused by MAF. Most new TB drugs and vaccines being developed are based on the most globally distributed lineage: Lineage 4 and might not be effective in West Africa where MAF is prevalent. Host genetic studies will help identify deficiencies in important cytokines such as IFN-ϒ which might make such groups of populations more susceptible to TB by MAF. Building on earlier studies which showed host polymorphism that offers protective immunity against infection by other lineages (**Intemann *et al.*, 2009; Thye *et al.*, 2011; Herb *et al.*, 2008**), an added advantage will be to study the different metabolites produced from the different metabolic pathways of MAF. The expression of the different levels of these metabolites at different time points during infection can inform targets for development of new TB drugs and vaccines.

M. africanum is made up of two distinct lineages: Lineage 5 and 6 (**de Jong *et al.*, 2010; Yeboah-Manu *et al.*, 2010**) However, most research works have concentrated on Lineage 6

showing its attenuated virulence and its association with immunocompromised (i.e. HIV co-infected) patients (**de Jong *et al*., 2005**). However, very little is known about the phenotypic characteristics of Lineage 5. With both lineages occurring in substantial numbers in Ghana, comparative genomic analysis can be done to look for candidate genes/signatures that help us understand biological differences between the two lineages. Some reports indicate that intra-species genetic diversity between the *M. africanum* and MTB is reflected in pathogenesis and epidemiology (**Coscolla and Gagneux, 2010**). Perhaps the same could be true for the two *M. africanum* lineages. This is of importance as it will affect the choice of drug targets, vaccine antigens and development of diagnostic tools. RNA-Seq, a recently developed approach to study transcriptomic profiles (**Wang *et al*., 2009**) can be use to compare gene expression differences between the two *M. africanum* lineages (**Rose *et al*., 2013**).

Several studies have shown that that the proportion of *M. africanum* causing TB varies by region (**Diop *et al*., 1976; Goyal *et al*., 1999; Addo *et al*., 2006**). Indeed it was speculated that due to the low virulence of *M. africanum*, its prevalence will be high in rural areas with minimal number of people as opposed to MTB. Perhaps the same can be true for the two lineages. Phylogenetically Lineage 6 is closer to the animal strains than Lineage 5 (**de Jong *et al*., 2010**), and may have the tendency to be abundant in areas with more animal activities. A more detailed spatial and population-based study over a longer period of time could inform us on the prevalence of the Lineage 5 and 6 in different parts of Ghana.

In most African countries, diabetes is an increasing public health problem due to changing lifestyles. Studies have shown that countries with increasing diabetes prevalence also see a significant increase in TB. Ghana has seen an increase in diabetes prevalence from 5% to over 10 % in the last 10 years (**personal communication; Prof Amoah**). Similar to HIV, diabetes also compromises the host immune system by effecting macrophage and lymphocyte function, essential for the control of TB. Nothing is known with respect to the impact of

diabetes on the transmission dynamics and population genomics of MTBC. These prevailing new conditions for TB could account for the increase in prevalence of low virulence genotypes like Lineage 6 as observed in Ghana. To confirm this notion, an in-depth population study to search for association between diabetes and MAF can be done in Ghana. This would give better insight into our understanding of the difference in virulence, if any, between Lineage 5 and 6.

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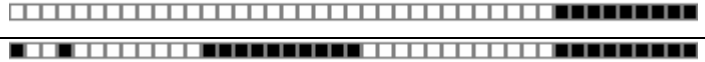
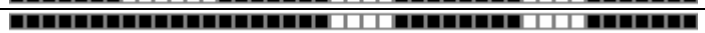
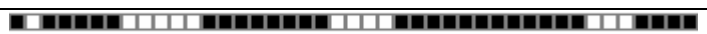
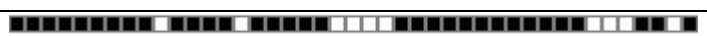
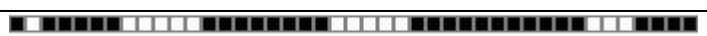
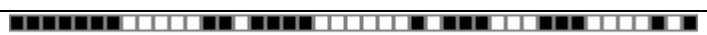
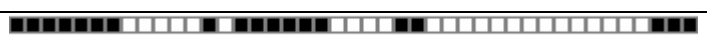
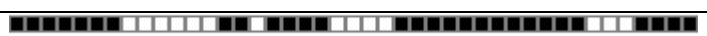
Appendix to Chapter 6 (M. africanum is associated with ethnicity in Ghana)

Table 2: Genotyping profiles of 613 *M. tuberculosis* complex isolates from Ghana

Species	SNP	RD9	RD726	RD711	RD702	Spoligotyping profile	Sub-lineage[a]	SIT	No	%
MTBss	L1	Undel	Undel	ND	ND		*EAI*	340	8	1.3
MTBss	L1	Undel	Undel	ND	ND		*EAI*		1	0.2
MTBss	L1	Undel	Undel	ND	ND		*EAI*	342	1	0.2
MTBss	L1	Undel	Undel	ND	ND		*EAI*	236	1	0.2
MTBss	L2	Undel	Undel	ND	ND		*Beijing*	1	10	1.6
MTBss	L3	Undel	Undel	ND	ND		*DEHLI/CAS*		2	0.3
MTBss	L3	Undel	Undel	ND	ND		*DEHLI/CAS*		1	0.2
MTBss	L3	Undel	Undel	ND	ND		*DEHLI/CAS*	1092	1	0.2
MTBss	L4	Undel	Del	ND	ND		*Cameroon*	61	226	36.8
MTBss	L4	Undel	Del	ND	ND		*Cameroon*	772	20	3.2
MTBss	L4	Undel	Del	ND	ND		*Cameroon*	115	7	1.1
MTBss	L4	Undel	Del	ND	ND		*Cameroon*	838	3	0.4
MTBss	L4	Undel	Del	ND	ND		*Cameroon*		26	4.2
MTBss	L4	Undel	Del	ND	ND		*Cameroon*		1	0.2
MTBss	L4	Undel	Del	ND	ND		*Cameroon*		1	0.2
MTBss	L4	Undel	Del	ND	ND		*Cameroon*		2	0.3
MTBss	L4	Undel	Del	ND	ND		*Cameroon*	1141	1	0.2
MTBss	L4	Undel	Del	ND	ND		*Cameroon*	403	1	0.2
MTBss	L4	Undel	Del	ND	ND		*Cameroon*		2	0.3
MTBss	L4	Undel	Del	ND	ND		*Cameroon*		2	0.3
MTBss	L4	Undel	Del	ND	ND		*Cameroon*		3	0.4
MTBss	L4	Undel	Del	ND	ND		*Cameroon*		2	0.3
MTBss	L4	Undel	Del	ND	ND		*Cameroon*		1	0.2
MTBss	L4	Undel	Del	ND	ND		*Cameroon*		1	0.2

Species	SNP	RD9	RD726	RD711	RD702	Spoligotyping profile	Sub-lineage[a]	SIT	No	%
MTBss	L4	Undel	Del	ND	ND		*Cameroon*		1	0.2
MTBss	L4	Undel	Del	ND	ND		*Cameroon*		2	0.3
MTBss	L4	Undel	Del	ND	ND		*Cameroon*		3	0.4
MTBss	L4	Undel	Del	ND	ND		*Cameroon*		1	0.2
MTBss	L4	Undel	Del	ND	ND		*Cameroon*		2	0.3
MTBss	L4	Undel	Del	ND	ND		*Cameroon*		3	0.4
MTBss	L4	Undel	Del	ND	ND		*Cameroon*		1	0.2
MTBss	L4	Undel	Del	ND	ND		*Cameroon*		1	0.2
MTBss	L4	Undel	Undel	ND	ND		*Ghana*	53	26	4.2
MTBss	L4	Undel	Undel	ND	ND		*Ghana*	65	4	0.7
MTBss	L4	Undel	Undel	ND	ND		*Ghana*	504	7	1.1
MTBss	L4	Undel	Undel	ND	ND		*Ghana*	118	12	1.9
MTBss	L4	Undel	Undel	ND	ND		*Ghana*	804	1	0.2
MTBss	L4	Undel	Undel	ND	ND		*Ghana*	462	4	0.7
MTBss	L4	Undel	Undel	ND	ND		*Ghana*	44	1	0.2
MTBss	L4	Undel	Undel	ND	ND		*Ghana*	86	12	1.9
MTBss	L4	Undel	Undel	ND	ND		*Ghana*	167	1	0.2
MTBss	L4	Undel	Undel	ND	ND		*Ghana*	373	1	0.2
MTBss	L4	Undel	Undel	ND	ND		*Ghana*	393	1	0.2
MTBss	L4	Undel	Undel	ND	ND		*Ghana*	272	1	0.2
MTBss	L4	Undel	Undel	ND	ND		*Ghana*		4	0.7
MTBss	L4	Undel	Undel	ND	ND		*Harlem*	1652	4	0.7
MTBss	L4	Undel	Undel	ND	ND		*Harlem*	1498	6	0.9
MTBss	L4	Undel	Undel	ND	ND		*Harlem*	50	15	2.4
MTBss	L4	Undel	Undel	ND	ND		*Harlem*	45	2	0.3

Species	SNP	RD9	RD726	RD711	RD702	Spoligotyping profile	*Sub-lineage*	SIT	No	%
MTBss	L4	Undel	Undel	ND	ND		*Harlem*	655	3	0.4
MTBss	L4	Undel	Undel	ND	ND		*Harlem*	47	2	0.3
MTBss	L4	Undel	Undel	ND	ND		*Harlem*	62	2	0.3
MTBss	L4	Undel	Undel	ND	ND		*Harlem*		2	0.3
MTBss	L4	Undel	Undel	ND	ND		*Harlem*		1	0.2
MTBss	L4	Undel	Undel	ND	ND		*LAM*	306	1	0.2
MTBss	L4	Undel	Undel	ND	ND		*LAM*		1	0.2
MTBss	L4	Undel	Undel	ND	ND		*LAM*	42	2	0.3
MTBss	L4	Undel	Undel	ND	ND		*LAM*	33	1	0.2
MTBss	L4	Undel	Undel	ND	ND			70	7	1.1
MTBss	L4	Undel	Undel	ND	ND		*Uganda I*		2	0.3
MTBss	L4	Undel	Undel	ND	ND		*Uganda I*	52	4	0.7
MTBss	L4	Undel	Undel	ND	ND		*Uganda I*	244	1	0.2
MTBss	L4	Undel	Undel	ND	ND		*Uganda I*	848	3	0.4
MTBss	L4	Undel	Undel	ND	ND		*Uganda I*		2	0.2
MTBss	L4	Undel	Undel	ND	ND		*Uganda I*	78	1	0.2
MTBss	L4	Undel	Undel	ND	ND		*Uganda I*		1	0.2
MTBss	L4	Undel	Undel	ND	ND		*Uganda I*	125	1	0.2
MTBss	L4	Undel	Undel	ND	ND		*Uganda II*	51	2	0.3
MTBss	L4	Undel	Undel	ND	ND		*Uganda II*		2	0.3
MTBss	L4	Undel	Undel	ND	ND		*Uganda II*		3	0.4
MTBss	L4	Undel	Undel	ND	ND		*S*	1223	2	0.3
MTBss	L4	Undel	Undel	ND	ND		*S*	1211	2	0.3
MTBss	L4	Undel	Undel	ND	ND		*X*	119	2	0.3
MTBss	L4	Undel	Undel	ND	ND			200	7	1.1
MTBss	L4	Undel	Undel	ND	ND				2	0.3

Species	SNP	RD9	RD726	RD711	RD702	Spoligotyping profile	*Sub-lineage*	SIT	No	%
MTBss	L4	Undel	Undel	ND	ND				2	0.3
MTBss	L4	Undel	Undel	ND	ND				1	0.2
MTBss	L4	Undel	Undel	ND	ND				1	0.2
MTBss	L4	Undel	Undel	ND	ND				4	0.7
MTBss	L4	Undel	Undel	ND	ND				1	0.2
Mafric	L5	Del	ND	del	Undel		*WA I*	331	17	2.8
Mafric	L5	Del	ND	del	Undel		*WA I*		1	0.2
Mafric	L5	Del	ND	del	Undel		*WA I*		1	0.2
Mafric	L5	Del	ND	del	Undel		*WA I*		1	0.2
Mafric	L5	Del	ND	del	Undel		*WA I*	319	16	2.6
Mafric	L5	Del	ND	del	Undel		*WA I*	438	9	1.5
Mafric	L5	Del	ND	del	Undel		*WA I*	860	1	0.2
Mafric	L5	Del	ND	del	Undel		*WA I*	1592	2	0.3
Mafric	L5	Del	ND	del	Undel		*WA I*		1	0.2
Mafric	L5	Del	ND	del	Undel		*WA I*		1	0.2
Mafric	L5	Del	ND	del	Undel		*WA I*		1	0.2
Mafric	L5	Del	ND	del	Undel		*WA I*		3	0.4
Mafric	L5	Del	ND	del	Undel		*WA I*		1	0.2
Mafric	L5	Del	ND	del	Undel		*WA I*	330	7	1.1
Mafric	L5	Del	ND	del	Undel		*WA I*		1	0.2
Mafric	L5	Del	ND	del	Undel		*WA I*		1	0.2
Mafric	L5	Del	ND	del	Undel		*WA I*		1	0.2
Mafric	L5	Del	ND	del	Undel		*WA I*		1	0.2

Species	SNP	RD9	RD726	RD711	RD702	Spoligotyping profile	Sub-lineage	SIT	No	%
Mafric	L5	Del	ND	del	Undel	■■■■■■■□□□□□■■■■■■■□□□□□■■■■■■■■■■■■□□□■■■■	*WA I*		1	0.2
Mafric	L5	Del	ND	del	Undel	■□■■■■■□□□□□■■■■■■■■□□□□■■■■■■■■■■■■□□□■■■■	*WA I*		9	1.5
Mafric	L5	Del	ND	del	Undel	■■□■■■■□□□□□■■■■■■■■□□□□■■■■■■■■■■■■□□□■■■■	*WA I*		3	0.4
Mafric	L5	Del	ND	del	Undel	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□□■■■■	*WA I*		1	0.2
Mafric	L5	Del	ND	del	Undel	■■■■■■■□□□□□■■■■■■■■□□□□□□□□□□□□□□□□□□□□□□□	*WA I*		1	0.2
Mafric	L5	Del	ND	del	Undel	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□□□□■■■■	*WA I*		1	0.2
Mafric	L5	Del	ND	del	Undel	■■■■■■□□□□□□■■■■■■■■□□□□□□□□□□□□□□□□□□□■■■■	*WA I*		1	0.2
Mafric	L5	Del	ND	del	Undel	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■	*WA I*		1	0.2
Mafric	L5	Del	ND	del	Undel	□□□□□□□□□□□□■■■■■□□□□□□□■■■■■■■■■■■■□□□■■■■	*WA I*		1	0.2
Mafric	L5	Del	ND	del	Undel	■□■■■■■□□□□□■■■■■■■■□□□□■■■■■■■■■■■■□□□■■■■	*WA I*		1	0.2
Mafric	L6	Del	ND	Undel	del	■■■■■■□□□□□■■□□■■■■■■■■■■■■■■■■■■■■■■□■■■■	*WA II*		2	0.3
Mafric	L6	Del	ND	Undel	del	■■■■■■□□□■■■■■□■■■■■■■■■■■■■■■■■■■■■■□■■■■	*WA II*	324	3	0.4
Mafric	L6	Del	ND	Undel	del	■■■■■■■■■□■■■□□■■■■■■■■■■■■■■■■■■■■■■□■■■■	*WA II*		2	0.3
Mafric	L6	Del	ND	Undel	del	■■■■■■□□□■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■■■■	*WA II*	181	5	0.8
Mafric	L6	Del	ND	Undel	del	■□■■■■□□□■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■■■■	*WA II*	318	3	0.4
Mafric	L6	Del	ND	Undel	del	■■■■■■□□□■■■■■■■■■■■■□□□■■■■■■■■■■■■■■□■□■■	*WA II*	186 7	1	0.2
Mafric	L6	Del	ND	Undel	del	■■■■■■□□□■■■■■■■■■■■■□□□■■■■■■■■■■■■■■□■■■■	*WA II*	326	1	0.2
Mafric	L6	Del	ND	Undel	del	■□□■■■□□□■■■■■■■■■■■■■■■■■■■■■■■■■■■□□■■■■	*WA II*		1	0.2
Mafric	L6	Del	ND	Undel	del	■■■■■■□□□■■■■■■■■■■■■■■■■■■■■■■□□□□□□□■■■■	*WA II*		1	0.2

[a]Sublineage as defined by the MIRU-VNTRplus database, ND=Not done

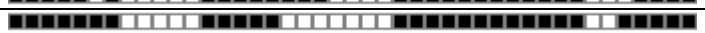
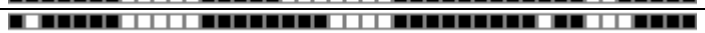
Appendix to Chapter 7 (*Mycobacterium africanum* is associated with HIV and Ethnicity in Ghana)

Table 4: Genotyping profile of 1211 MTBC isolates from Ghana

Specie	SNP	Spoligotyping profile	Sub lineage	SIT	No	%
MTBss	L1		EAI	236	1	0.08
MTBss	L1		EAI	340	3	0.24
MTBss	L1		EAI	342	7	0.60
MTBss	L1		EAI	380	1	0.08
MTBss	L1		EAI	Orphan	1	0.08
MTBss	L1		EAI		2	0.16
MTBss	L2		Beijing	1	36	3.00
MTBss	L2		Beijing	1184	3	0.24
MTBss	L2		Beijing	941	1	0.08
MTBss	L2		Beijing		2	0.17
MTBss	L3		Dehli_CAS	25	1	0.08
MTBss	L3		Dehli_CAS	129	1	0.08
MTBss	L3		Dehli_CAS	702	1	0.08
MTBss	L3		Dehli_CAS	1199	2	0.17
MTBss	L3		Dehli_CAS		2	0.17
MTBss	L3		Dehli_CAS		3	0.24
MTBss	L3		Dehli_CAS	Orphan	1	0.08
MTBss	L3		Dehli_CAS	Orphan	1	0.08
MTBss	L4		Cameroon	57	5	0.41
MTBss	L4		Cameroon	61	349	29.0
MTBss	L4		Cameroon	114	1	0.08
MTBss	L4		Cameroon	403	2	0.17
MTBss	L4		Cameroon	772	39	3.22
MTBss	L4		Cameroon	838	12	1.00
MTBss	L4		Cameroon	1141	8	0.67
MTBss	L4		Cameroon	1580	1	0.08
MTBss	L4		Cameroon		10	0.82
MTBss	L4		Cameroon		15	1.25
MTBss	L4		Cameroon		5	0.41
MTBss	L4		Cameroon		15	1.25
MTBss	L4		Cameroon		7	0.60
MTBss	L4		Cameroon		15	1.25
MTBss	L4		Cameroon		7	0.60
MTBss	L4		Cameroon		4	0.33
MTBss	L4		Ghana	37	1	0.08
MTBss	L4		Ghana	44	1	0.08
MTBss	L4		Ghana	53	138	11.40
MTBss	L4		Ghana	54	3	0.24
MTBss	L4		Ghana	58	1	0.08

Specie	SNP	Spoligotyping profile	Sub lineage	SIT	No	%
MTBss	L4	[redacted]	Ghana	278	1	0.08
MTBss	L4	[redacted]	Ghana	373	1	0.08
MTBss	L4	[redacted]	Ghana	462	3	0.24
MTBss	L4	[redacted]	Ghana	504	25	2.10
MTBss	L4	[redacted]	Ghana	804	1	0.08
MTBss	L4	[redacted]	Ghana	926	1	0.08
MTBss	L4	[redacted]	Ghana	1105	1	0.08
MTBss	L4	[redacted]	Ghana	1196	2	0.17
MTBss	L4	[redacted]	Ghana	1227	2	0.17
MTBss	L4	[redacted]	Ghana	1547	1	0.08
MTBss	L4	[redacted]	Ghana		4	0.33
MTBss	L4	[redacted]	Ghana		3	0.24
MTBss	L4	[redacted]	Ghana	orphan	1	0.08
MTBss	L4	[redacted]	H37Rv	orphan	1	0.08
MTBss	L4	[redacted]	Haarlem	36	1	0.08
MTBss	L4	[redacted]	Haarlem	45	3	0.24
MTBss	L4	[redacted]	Haarlem	47	1	0.08
MTBss	L4	[redacted]	Haarlem	50	26	2.14
MTBss	L4	[redacted]	Haarlem	62	1	0.08
MTBss	L4	[redacted]	Haarlem	124	1	0.08
MTBss	L4	[redacted]	Haarlem	144	2	0.17
MTBss	L4	[redacted]	Haarlem	316	8	0.67
MTBss	L4	[redacted]	Haarlem	655	11	0.90
MTBss	L4	[redacted]	Haarlem	775	3	0.24
MTBss	L4	[redacted]	Haarlem	1159	1	0.08
MTBss	L4	[redacted]	Haarlem	1498	11	0.90
MTBss	L4	[redacted]	Haarlem		4	0.33
MTBss	L4	[redacted]	Haarlem		3	0.24
MTBss	L4	[redacted]	Haarlem		3	0.24
MTBss	L4	[redacted]	Haarlem		3	0.24
MTBss	L4	[redacted]	LAM	20	1	0.08
MTBss	L4	[redacted]	LAM	42	13	1.10
MTBss	L4	[redacted]	LAM	535	2	0.17
MTBss	L4	[redacted]	LAM	765	1	0.08
MTBss	L4	[redacted]	LAM		2	0.17
MTBss	L4	[redacted]	LAM	orphan	1	0.08
MTBss	L4	[redacted]	LAM		2	0.17
MTBss	L4	[redacted]	LAM		2	0.17
MTBss	L4	[redacted]	NEW	orphan	1	0.08
MTBss	L4	[redacted]	S		2	0.17
MTBss	L4	[redacted]	Uganda I	49	2	0.17
MTBss	L4	[redacted]	Uganda I	52	2	0.17

Specie	SNP	Spoligotyping profile	Sub lineage	SIT	No	%
MTBss	L4		Uganda I	78	1	0.08
MTBss	L4		Uganda I	848	1	0.08
MTBss	L4		Uganda I	524	1	0.08
MTBss	L4		Uganda I	712	1	0.08
MTBss	L4		Uganda I	1056	1	0.08
MTBss	L4		Uganda I		10	0.82
MTBss	L4		Uganda I		3	0.24
MTBss	L4		Uganda I		4	0.33
MTBss	L4		Uganda II	237	2	0.17
MTBss	L4		Uganda II	92	11	0.90
MTBss	L4		Uganda II	200	5	0.41
MTBss	L4		Uganda II	1178	4	0.33
MTBss	L4		Uganda II		5	0.41
MTBss	L4		Uganda II	orphan	1	0.08
MTBss	L4		Uganda II		6	0.50
MTBss	L4		Uganda II		2	0.17
MTBss	L4		Uganda II		4	0.33
MTBss	L4		Uganda II	orphan	1	0.08
MTBss	L4		Uganda II		3	0.24
MTBss	L4		Uganda II	orphan	1	0.08
Mafric	L5		West Africa I	319	26	2.14
Mafric	L5		West Africa I	320	3	0.24
Mafric	L5		West Africa I	330	4	0.33
Mafric	L5		West Africa I	331	26	2.14
Mafric	L5		West Africa I	438	5	0.41
Mafric	L5		West Africa I	1592	2	0.17
Mafric	L5		West Africa I		15	1.24
Mafric	L5		West Africa I	orphan	1	0.08
Mafric	L5		West Africa I	orphan	1	0.08
Mafric	L5		West Africa I	orphan	1	0.08
Mafric	L5		West Africa I	orphan	1	0.08
Mafric	L5		West Africa I		3	0.24
Mafric	L5		West Africa I	orphan	1	0.08
Mafric	L5		West Africa I		2	0.17
Mafric	L5		West Africa I		2	0.17
Mafric	L5		West Africa I	orphan	1	0.08

Specie	SNP	Spoligotyping profile	Sub lineage	SIT	No	%
Mafric	L5		West Africa I	orphan	1	0.08
Mafric	L5		West Africa I	orphan	1	0.08
Mafric	L5		West Africa I		2	0.17
Mafric	L5		West Africa I	orphan	1	0.08
Mafric	L5		West Africa I	orphan	1	0.08
Mafric	L5		West Africa I	orphan	1	0.08
Mafric	L5		West Africa I	orphan	1	0.08
Mafric	L5		West Africa I		3	0.24
Mafric	L5		West Africa I	orphan	1	0.08
Mafric	L5		West Africa I	orphan	1	0.08
Mafric	L5		West Africa I		2	0.17
Mafric	L5		West Africa I		2	0.17
Mafric	L5		West Africa I		2	0.17
Mafric	L5		West Africa I		2	0.17
Mafric	L5		West Africa I	orphan	1	0.08
Mafric	L5		West Africa I	orphan	1	0.08
Mafric	L5		West Africa I	orphan	1	0.08
Mafric	L5		West Africa I		5	0.41
Mafric	L5		West Africa I		2	0.17
Mafric	L5		West Africa I	orphan	1	0.08
Mafric	L5		West Africa I	orphan	1	0.08
Mafric	L5		West Africa I		2	0.17
Mafric	L5		West Africa I	orphan	1	0.08
Mafric	L5		West Africa I	orphan	1	0.08
Mafric	L5		West Africa I	orphan	1	0.08
Mafric	L5		West Africa I	orphan	1	0.08
Mafric	L5		West Africa I		2	0.17

Specie	SNP	Spoligotyping profile	Sub lineage	SIT	No	%
Mafric	L5	■■■■■■□□□■■■■■■■■■■■■□□□□■■□□□□□□□□□■□□■■■■	West Africa I	orphan	1	0.08
Mafric	L5	■■□■■■■□□□□□□■■■■■■■□□□□■■■■■■■■■■■■□□□□■■■	West Africa I		2	0.17
Mafric	L5	■■□■■■■□□□□□■■■■■■■■■□□■■■■■■■■■■■■■□□□■■■■	West Africa I	orphan	1	0.08
Mafric	L5	■□■■■■■□□□□□■■■■■■■□□□□■■■■■■■■■■■■■□□□■■■■	West Africa I		4	0.33
Mafric	L5	■■■■■■■□□□□□■■■■■□□□□□□□■■□□□□□■■■■■□□□■■■■	West Africa I	orphan	1	0.08
Mafric	L5	■■■■■■□□□□□■□■■□■■■□□□□□□□□□□□□□□□■■□□□■■■■	West Africa I	orphan	1	0.08
Mafric	L5	■■■■■■■□□□□□■■■■□□□□□□□□■■□□□□□■■■■■□□□■■■■	West Africa I	orphan	1	0.08
Mafric	L5	■■■■■■■□□□□□□■■■■■■■□□□□■■■■■■■■■■■■□□□■■■■	West Africa I	orphan	1	0.08
Mafric	L5	■□■■■■■□□□□□■□■■■■■■□□□□■■■■■■■■■■■■□□□■■■■	West Africa I	orphan	1	0.08
Mafric	L5	■□■■■■■□□□□□■■■■■■■■□□□□■■■□□■■■■■■■□□□■■■■	West Africa I	orphan	1	0.08
Mafric	L5	■■■■■■■□□□□□■■■■□□□□□□□□□□□□□□□■■■■■□□□■■■■	West Africa I	orphan	1	0.08
Mafric	L5	■■■■■■□□□□□□■■■■■■■■■□□□■■■■■■■■■■■■□□□■■■■	West Africa I	orphan	1	0.08
Mafric	L6	■■■■■■□□□■■■■■■■■■■■■■■■■■■■■■■■■■■■□■■■■	West Africa II	181	23	1.90
Mafric	L6	■■■■■■□□□■■■■■■■■■■■■□□□■■■■■■■■■■■■■■□■■■■	West Africa II	326	24	2.00
Mafric	L6	■■■□□□□■■■■■■■■■■■■■■■□□■■■■■■■■■■■■■■■■■■■	West Africa II	1200	1	0.08
Mafric	L6	■■■■■■□□□■■■■■■■■■■■■□□□■■■■■■■■■■■■■■□■□■■	West Africa II	1867	1	0.08
Mafric	L6	■■■■■■□□□■■■■■■■■■■■■□□□■■■■■■■■■■■■■■□□■■■	West Africa II		4	0.33
Mafric	L6	■■■■■■□□□■■■□□■■■■■■■■■■■■■■■■■■■■■■■■□■■■■	West Africa II		4	0.33
Mafric	L6	■■■■■■■■■□■■■□□■■■■■■■■■■■■■■■■■■■■■■■□■■■■	West Africa II	orphan	1	0.08
Mafric	L6	■■■■■■■■■□■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■	West Africa II	orphan	1	0.08
Mafric	L6	■■■■■■■□■□□□■□□■■■■■■■■■■■■■■■■■■■■■■■□■■■■	West Africa II	orphan	1	0.08
Mafric	L6	■■■■■■□□□□■■■■■■■■■■■■■■■■■■■■■■■■■■□■■■■	West Africa II		19	1.57
Mafric	L6	■■■■■■□□■□■■■■■■■■■■■■■■■■■■■■■■■■■■□■■■■	West Africa II		3	0.24
Mafric	L6	■■■■■■□□□■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□	West Africa II		3	0.24
Mafric	L6	■■■■■■■■■□■■■□■■■■■■■■■■■■■■■■■■■■■■■■□■■■■	West Africa II	orphan	1	0.08
Mafric	L6	■■■■■■□□□■■■■□■■■■■■■□□□■■□□■■■■■■■■■■□■■■■	West Africa II		3	0.24
Mafric	L6	■■■■■■□□■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■	West Africa II		3	0.24

Specie	SNP	Spoligotyping profile	Sub lineage	SIT	No	%
Mafric	L6	■■■■■■□□□■■■■■■■■■■■■□□□■■■■■■■■■■■■■■□■■□□	West Africa II	orphan	1	0.08
Mafric	L6	■■■■■■□□□□■■■□■■■■■■■□□□■■■■■■■■■■■■■■□■■■■	West Africa II		5	0.41
Mafric	L6	■■■■■■■■■□■■■■■■■■■■■■■■■■■■■■■■■■■□■■□■■■■	West Africa II	orphan	1	0.08
Mafric	L6	■■■■■■□□□□□□■■■■■■■■■□□□■■■■■■■■■■■■■■□■■■■	West Africa II	orphan	1	0.08
Mafric	L6	■■■■■■□□□■■■■■■■■■■■■■■■■■■□■■■■■■■■■■□■■■■	West Africa II		4	0.33
Mafric	L6	■■■□□□□□□□□■■■■■■■■■■■■■■■■■■■■■■■■□□□■■■■	West Africa II	orphan	1	0.08
Mafric	L6	■■■■■■□□□□■■■□■■■■■■■■■■■■■■■■■■■■■■■■□■■■■	West Africa II	orphan	1	0.08
Mafric	L6	■■■■■■□□□■■■■■■■■■■■■□□□■□□■■■■■■■■■■■□□■■■	West Africa II	orphan	1	0.08
Mafric	L6	■■■■■■□□□□■■■■■■■■■■■■■■■■■■■■■□□■■■■■□■■■■	West Africa II	orphan	1	0.08
Mafric	L6	■■■■■■□□■■■■■■■■■■■■■□□□■■■■■■■■■■■■■■■■■■■	West Africa II	orphan	1	0.08
Mafric	L6	■■■■■■□□□□■■■■■■■■■■■■■■■■■■■■■■■■■□■■□■■■■	West Africa II	orphan	1	0.08
Mafric	L6	■■■■■■□□□■■■■■■■■■■■■■■■■■■□□□■■■■■■■■□■■■■	West Africa II	orphan	1	0.08
Mafric	L6	■■■■□■□□□■■■■■■■■■■■■■□□□■■■■■■■■■■■■■□■■■■	West Africa II	orphan	1	0.08
M. bovis		■■□■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■■□□□□□	BOVIS 1_BCG	482	1	0.08
M. bovis		■■□■■■■■□■■■■■■□■■■■■■■■■■■■■□■■■■■■■■□□□□□	BOVIS 1	1037	2	0.17
M. bovis		■■□■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■■□□□□□	BOVIS	orphan	1	0.08
M. bovis		■■□■■□■■□■■■■■■□■■■□■■■■■■■■■□■■■■■■■■□□□□□	BOVIS	orphan	1	0.08
M. bovis		■■□■■■■□□□□□■□□□■■■■■■■■■■■■■□□□■■■■■■□□□□□	BOVIS	orphan	1	0.08
M. bovis		■■□■■□■■□■■■■■■□■■■■■■■■■■□■■■■■■■■■■■■□□□□	BOVIS	orphan	1	0.08

Curriculum Vitae

A: PERSONAL DETAILS

Surname: ASANTE-POKU WIREDU

Other Names: ADWOA

Date of Birth: 7th October 1976

Nationality: Ghanaian

Marital Status: Married

B: EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE	YY	FIELD OF STUDY
University of Ghana	BSc.	2000	Animal Biology and Conservation Science
University of Ghana	MPHIL	2009	Animal Science
Swiss Tropical Institute, University of Basel	PhD	2014	Microbiology

C: HONOURS

- 2014: 1st runner –up (Noguchi Memorial Institute for Medical Research Best Research Assistant)
- 2011-2014: PHD fellowship from amt fuer ausbildkungsbeitraege des kantons Basel

- 2011 PhD Research Funding from Leverhulme Royal Society , Africa

D: JOB SUMMARY

- October 2007 to date: Principal Research Assistant, Bacteriology Department (NMIMR)

- February 2003-Sept 2007: Senior Research Assistant, Bacteriology Department (NMIMR)

- August 2001-Jan 2003: Senior Research Assistant, Parasitology Unit, NMIMR

- January 2001-July 2001: National service personnel-Bacteriology unit, NMIMR

E: RESEARCH INTEREST

Mycobacterial diseases of public health importance in Ghana

F: COURSES ATTENDED

- 2010: Scientific writing Organized by Regional Institute for Population Studies
- 2004: Research Proposal Writing: Organized by College of health sciences
- University of Ghana
- 2004: Research ethics Training Curriculum. Organized by Family Health
- International
- 2003: Quality Assurance in TB Microscopy. Organized by NMIMR and

Ghana Health Service

- 2003: Good Laboratory Practice for clinical Research laboratories, Organized by USA Naval Medical Research Center Malaria Programme training,

G: WORKSHOPS /CONFERENCES ATTENDED

- 5th Ghana Biomedical Conference, Accra, Ghana, 2013
- 43rd Union World Conference on Lung Health, Paris, France, 2012
- Swiss Society of Tropical Medicine and Parasitology Conference, Basel, Switzerland, 2011
- 42nd Union World Conference on Lung Health, Lille, France, 2011
- Biovalley Science week, Basel, Switzerland, 2011
- 3rd Ghana Biomedical Conference, Accra, Ghana, 2011
- 2nd Ghana Biomedical Conference, Accra, Ghana, 2010
- 30TH All African Health Science conference. Accra, Ghana, 2010
- 1st Ghana Biomedical Conference, Accra, Ghana, 2009

H: INSTITUTIONAL SEMINARS

- Genotypic and epidemiological characterization of *Mycobacterium tuberculosis* complex in Ghana, December 2014, Swiss Tropical and Public Health Institute, Switzerland
- Molecular Epidemiology of Tuberculosis, June 2013, NMIMR

I: PROPOSAL WRITING

- Genotypic characterization of Mycobacterium tuberculosis in Ghana, project 2012-2017, Funded by Wellcome trust
- Stop Buruli Project, 2011-2014, Funded by UBS, Switzerland

- Genotypic and Phenotypic Characterization of *Mycobacterium africanum*, 2010-2012, Funded by Leverhulme-Royal Society Africa

- Molecular Epidemiology of Tuberculosis in Ghana: genotyping of *Mycobacterium tuberculosis* strains in refugee (Budumbura camp) and non-refugee populations, 2007-2009. Re-entry Grant, WHO

J: TEACHING

- February, 2014 — Molecular Epidemiology ,MPhil, Department of Biochemistry, and Molecular biology, University of Ghana
- December, 2014 — Molecular biology ,Bachelor students, University of Basel
- February, 2013 — Bachelor students, Pharmacy department , University of Ghana
- December, 2012 — Molecular biology, Bachelor students, University of Basel
- December, 2011 — Molecular biology, Bachelor students, University of Basel

K: STUDENT SUPERVISION

- 2014-date: Stephen Osei-Wusu, Department Biochemistry, Cell and Molecular Biology, university of Ghana (MPHIL, candidate).
- 2014-date: Prince Asare, Department Biochemistry, Cell and Molecular Biology, University of Ghana (MPHIL, candidate)
- 2013-date: Isaac Darko Otchere, Department Biochemistry, Cell and Molecular Biology, university of Ghana (PHD, candidate)
- 2012-2014: Zuliehatu Nakobu, MPHIL, Department of Animal Biology and Conservation Science. University of Ghana.
- 2012-2013: Santus Adadzie. BSC, Department Biochemistry, Cell and Molecular Biology, University of Ghana.
- 2011-2012: Gifty Dankwah, BSC, Department Biochemistry, Cell and
- Molecular Biology, University of Ghana.
- 2010-2011 Michael Selasi Nyaho, MPHIL, Department Biochemistry, Cell and Molecular Biology, University of Ghana.

L: PROJECT COORDINATION

- Molecular Epidemiology of Tuberculosis in Ghana: genotyping of *M. tuberculosis* strains in refugee (Budumbura camp) and non-refugee populations, 2007-2009. Re-entry Grant, WHO
- Genotypic and Phenotypic Characterization of *Mycobacterium africanum*, 2010-2012, Funded by Leverhulme-Royal Society Africa
- Genotypic characterization of Mycobacterium tuberculosis in Ghana, project 2012-2017, Funded by Wellcome

M: OTHER SERVICES RENDERED

- Standard operating procedure development; coordinator, Bacteriology department
- Laboratory confirmation and monitoring of treatment of Buruli Ulcer patients for Ghana health service.
- Confirmation of TB drug resistance strains For 37 military hospital.
- Training of field workers for collection of TB specimen and epidemiological data.
- NMIMR annual research week, protocol member.

N: MEMBERSHIP OF PROFESSIONAL SOCIETIES

- Ghana Science Association
- Ghana Biomedical Association
- American Society for Microbiology

O: PRESENTATIONS AT SCIENTIFIC CONFERENCES

Oral presentations

- **Asante-Poku A.** Genotypic and Epidemiological Characterization of Mycobacterium tuberculosis Complex in Ghana. Oral presentation, Swiss Tropical and Public Health Institute. 2014

- **Asante-Poku A**. Oral Presentation: Swiss Society of Tropical Medicine and Parasitology Conference, Basel, Switzerland, 2011

- **Asante-Poku A**, Aning KG, Boi Kikimota B, Yeboah-Manu D. Use of Molecular techniques for the diagnosis of Bovine tuberculosis in Ghana. Oral Presentation: 30TH All African Health Science conference. Accra, Ghana, 2010

Poster presentations

- Yeboah-Manu D, **Asante-Poku A**, Otchere ID , Osei-Wusu S, Ganiyu HA , Baddoo A, Forson A , Bonsu F, Gagneux S. TB Lineages and association with patients' demography and co-morbidities in Ghana. Poster presentation, 45th Union World Conference on Lung Health, Barcelona, Spain, 2014

- **Asante-Poku A**, Otchere ID, Danso E, Borrell S, Stucki D, Gagneux S, and Yeboah-Manu D. Characterization of rural versus urban *Mycobacterium tuberculosis* Complex (MTBC) strains circulating in rural versus urban environments of southern Ghana. Poster presentation: 5th Ghana Biomedical Conference, Accra, Ghana, 2013

- Yeboah-Manu D, Otchere ID, **Asante-Poku A**, Mensah DD, Borrell S, Gagneux S. Characterization of drug-resistant *Mycobacterium tuberculosis* complex isolates from Ghana. Keystone symposia(Tuberculosis : Understanding the enemy), Canada, 2013

- Yeboah-Manu D, Otchere ID, **Asante-Poku A**, Borrell S, Gagneux S. Low Rate of Non-synonymous gyrA Mutation *in Mycobacterium tuberculosis* Complex Isolates from Ghana. 43rd Union World Conference on Lung Health, Paris, France, 2012

- **Asante-Poku A**, Nyaho S, Borrell S, Comas I, Gagneux S, Yeboah-Manu D. Characterization of *Mycobacterium tuberculosis* strains from Ghana using a customized MIRU-typing Method . Poster presentation: 42nd Union World Conference on Lung Health, Lille, France, 2011

- Yeboah-Manu D, **Asante-Poku A**, Bodmer T, Stucki D, Koram K, Bonsu F, Pluschke G, Gagneux S. Genotypic Diversity & Drug Susceptibility Pattern Among *M. tuberculosis* Complex Isolates From Ghana. Poster presentation: 42nd Union World Conference on Lung Health, Lille, France, 2011

P: PUBLICATIONS

1. **Asante-Poku A**, Darko Otchere I, Danso E, Mensah DD, Bonsu F, Gagneux S, Yeboah-Manu D. Establishment and evaluation of the genotype MTBDR*plus* for rapid detection of drug resistant tuberculosis in Ghana. *The International Journal of Tuberculosis and Lung Disease* (*Accepted for publication*); 2015
2. **Asante-Poku A**, Yeboah-Manu D, Otchere Darko I, Aboagye SY, Stucki D, Hattendorf J, Borrell S, Feldmann J, Danso E, and Gagneux S. *Mycobacterium africanum* is associated with patient ethnicity in Ghana. *PLoS Neglected Tropical Diseases* 9(1): e3370, 2014
3. **Asante-Poku A**, Nyaho MS, Borrell S, Comas I, Gagneux s, *et al.* evaluation of customized lineage-specific sets of MIRU-VNTR loci for genotyping *Mycobacterium tuberculosis* complex isolates in Ghana. *PLoS ONE* 9(3): e92675, 2014.
4. **Asante-Poku A**, Aning KG, Boi-Kikimoto B, Yeboah-Manu D. Prevalence of Bovine tuberculosis in a dairy cattle farm and a research farm in Ghana. *Onderstepoort Journal of Veterinary Research* 23; 81(2):e1-6, 2014.
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