UNIVERSITY OF GHANA
COLLEGE OF BASIC AND APPLIED SCIENCES

GENOTYPING AND DRUG SUSCEPTIBILITY TESTING OF
MYCOBACTERIAL ISOLATES FROM A POPULATION-BASED
TUBERCULOSIS PREVALENCE SURVEY IN GHANA

BY
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DECLARATION

This is to certify that this thesis is the result of research undertaken by Samuel Ofori Addo towards the award of the Master of Philosophy in Molecular Cell Biology of Infectious Diseases in the Department of Biochemistry, Cell and Molecular Biology, University of Ghana. References to the works of other investigators have been duly acknowledged.

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ABSTRACT

Sub-Saharan Africa accounted for about 28% of the estimated 9.6 million of all notified tuberculosis (TB) cases in 2014. Due to high incidence of TB caused by *Mycobacterium tuberculosis* complex (MTBC) in developing countries, TB control activities have centered on MTBC to the neglect of non-tuberculous mycobacterial (NTM) infections. MTBC and NTM infections differ clinically, however, microscopy which is the mainstay of TB diagnosis in Ghana cannot distinguish between them. The inability to characterize acid fast bacilli (AFB) has led to improper treatment resulting in susceptible MTBC strains having an increased risk of developing resistance to first-line anti-TB drugs. In Ghana, very few studies have reported on the incidence and prevalence of TB due to specific MTBC species and their drug susceptibility test (DST) to first-line anti-TB drugs in Ghana. However, these studies did not have nationwide coverage. On the other hand, there is lack of national data on the species of NTM causing pulmonary infections as well as their DST patterns. This study sought to use World Health Organization (WHO) approved line probe assay (LPA) to differentiate MTBC and NTM isolates obtained from population-based TB prevalence survey in Ghana and to determine their DST patterns to isoniazid (INH) and rifampicin (RIF); and macrolides and aminoglycosides, respectively.

A retrospective study was conducted whereby a total of 361 mycobacterial isolates were differentiated and their drug susceptibility patterns determined using GenoType Mycobacterium Assays: MTBC and CM/AS for differentiating MTBC and NTM as well MTBDRplus and NTM-DR for DST patterns of MTBC and NTM, respectively.

All the isolates were obtained from sputum of participants aged between 15-100 years comprising 159 males and 202 females. Out of 361 isolates, 165 (45.7%) were MTBC
while 196 (54.3%) were NTM. The MTBC comprised 161 (97.6%) *Mycobacterium tuberculosis* and 4 (2.4%) *Mycobacterium africanum*. Eighteen (10.9%) and 2 (1.2%) of the MTBC isolates were INH and RIF monoresistant respectively while 11 (6.7%) were multi-drug resistant (MDR). RIF resistance was associated with D516V (46.7%), H526Y (23.1%) and S531L (15.4%) mutations in *rpoB* whiles INH resistance was frequently associated with S315T (82.8%) mutation in *katG*. Mutations in the promoter region of *inhA* (34.4%) also resulted in isoniazid resistance. Fourteen different NTM species were identified, majority (21.4%) being *M. fortuitum*. NTM-DR enabled the DST of thirty six NTM isolates belonging to the *M. avium* complex and *M. abscessus* complex. These isolates were all susceptible to macrolides (clarithromycin, azithromycin) and aminoglycosides (kanamycin, amikacin, and gentamicin).

In conclusion, *M. tuberculosis* is still the dominant species causing TB in Ghana whiles *M. fortuitum* is the most frequently isolated NTM species from sputum. Also, resistance to isoniazid is high compared to rifampicin. The study showed the usefulness of the GenoType Mycobacteria assay series as appropriate tools for simple and rapid differentiation and DST of mycobacterial isolates to enhance adequate and prompt treatment of mycobacterial infections in Ghana.
DEDICATION

I dedicate this work to Almighty God for His abundant blessings upon me. To Him be all the Glory and Honour.
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LIST OF ABBREVIATIONS

AFB – Acid Fast Bacilli

AM-A – Amplification Mix A

AM-B – Amplification Mix B

ATS/IDSA – American Thoracic Society/ Infectious Diseases Society of America

BCG – Bacillus Calmette-Guerin

BD – Becton Dickenson

CFP-10 – Culture Filtrate Protein 10-kDa

CO₂ – Carbon (IV) Oxide

CON-C – Conjugate Solution C

CON-D – Conjugate Solution D

DANIDA – Danish International Development Agency

DC – Dendritic Cells

DNA – Deoxyribonucleic Acid

DST – Drug Susceptibility Test

ESAT-6 – Early Secreted Antigenic Target 6-kDa

GC – Genus Control

HBCs – High Burden Countries

HIV/AIDS – Human Immunodeficiency Virus/ Acquired Immune Deficiency Syndrome

http://ugspace.ug.edu.gh/
HYB – Hybridization Reagent

IFN-γ – Interferon Gamma

IMR – Isoniazid Monoresistant

INH – Isoniazid

IRB – Institutional Review Board

IUALTD – International Union against Lung and Throat Diseases

KNCV – Koninklijke Nederlandse Centrale Vereniging tot bestrijding der Tuberculose (Dutch Tuberculosis Foundation)

L-J – Lowenstein-Jensen

LPA – Line Probe Assay

LTBI – Latent Tuberculosis Infection

MAC – Mycobacterium avium complex

MAF 1 – Mycobacterium africanum (West-African genotype I)

MAF 2 – Mycobacterium africanum (West-African genotype II)

M. africanum – Mycobacterium africanum

M. bovis – Mycobacterium bovis

M. canettii – Mycobacterium canettii

M. caprae – Mycobacterium caprae

MD – Madison

MDR-TB – Multi-Drug Resistant Tuberculosis
MGIT – Mycobacterium Growth Indicator Tube

*M. microti* – *Mycobacterium microti*

MOTT – Mycobacterium Other Than Tuberculosis

*M. pinnipidii* – *Mycobacterium pinnipidii*

MTBC – *Mycobacterium tuberculosis* complex

*M. tuberculosis* – *Mycobacterium tuberculosis*

MUT – Mutant Probe

NaOH-NALC – Sodium hydroxide-N- Acetyl-L-Cysteine

NK – Natural Killer

NMIMR – Noguchi Memorial Institute for Medical Research

NTM – Non-tuberculous mycobacterium

NTP – National Tuberculosis Programme

OR – Odds Ratio

PBS – Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

PNM – Primer Nucleotide Mix

PPD – Purified Protein Derivative

PTB – Pulmonary Tuberculosis

QFTGIT – QuantiFERON-TB Gold In-Tube test

RD – Region of Difference
RIF – Rifampicin

RIN – Rinsing Reagent

RMR – Rifampicin Monoresistance

RRDR – Rifampicin Resistance Determining Region

rRNA – Ribosomal Ribonucleic Acid

SNPs – Single Nucleotide Polymorphisms

STR – Stringent reagent

SUB-C – Substrate Solution C

SUB-D – Substrate Solution D

TB – Tuberculosis

TNF-α – Tumor Necrosis Factor Alpha

USAID – United States Agency for International Development

WHO – World Health Organization

WT – Wild-type Probe

XDR-TB – Extensively-Drug Resistant Tuberculosis

ZN – Ziehl Nielsen
CHAPTER ONE

1.0 BACKGROUND

1.1 Introduction

Tuberculosis (TB) is a global menace and one of the common cause of high mortality in adults from any bacterial agent (Michel et al., 2006). The World Health Organization (WHO) issued a global health alert on TB in 1993 as a result of increasing burden of the disease especially in low income countries (WHO, 1997). Twenty two countries with high TB burden also known as TB high burden countries (HBCs) have been given a special global attention since the last decade. In 2011, these countries accounted for about 82% of all reported cases of TB worldwide (WHO, 2012). Ghana is not among the WHO TB HBCs, however TB still poses a major public health concern with a total of 14,668 cases (Pulmonary, bacteriologically confirmed-7,682; Pulmonary, clinically diagnosed -5,364; Extrapulmonary-1,181; Relapsed- 441) reported in 2014 (WHO, 2015). Women formed about one third of all notified TB cases with children under 15 years constituting almost 5% of recorded cases. More than 76,000 people were estimated to have TB in Ghana with about 9,700 deaths occurring each year (WHO, 2015). These notification data from Ghana like in most developing countries often does not reflect the actual number of cases in the country due to incomplete coverage and absence of appropriate surveillance systems. Thus, direct measurement of the burden of disease through TB prevalence surveys remains key for understanding the spread and extent of the disease and aid in developing appropriate control measures in these settings. The Ghana National TB prevalence survey was conducted from March – December, 2013 to obtain useful estimates of TB prevalence within the time period. The adjusted prevalence of smear positive pulmonary TB and bacteriologically confirmed TB among adults aged 15 years and above were 111 (95% CI
76-145) per 100,000 population and 356 (95% CI 288-425) per 100,000 population respectively (personal communication with the programme manager for the National TB Programme, Ghana, unpublished data). To effectively control TB there is the need to identify the Mycobacterium species causing TB, as well as their drug susceptibility test (DST) patterns. The causative agent of TB is a group of species and sub-species closely related at the gene level generally referred to as Mycobacterium tuberculosis complex (MTBC) (Yeboah-Manu et al., 2011). These include M. tuberculosis, M. africanum, M. bovis, M. microti, M. caprae, M. pinnipidii and M. canettii (Van Soolingen et al., 1997). M. tuberculosis and M. africanum are the main cause of human TB (de Jong et al., 2009a). Species within MTBC have almost 100% similarity at the gene sequence level (Brosch et al., 2002). However, they exhibit differences in their host spectrum, geographic region, pathogenicity, drug susceptibility and in certain phenotypic parameters (Parsons et al., 2002). Therefore it may be clinically helpful to identify members of the M. tuberculosis complex to the species level. For instance, M. bovis has an innate resistance to the first-line drug pyrazinamide, and disseminated M. bovis BCG may be found as a complication following vaccination or intravesical instillation as treatment for bladder cancer (Redelman-Sidi, Glickman, & Bochner, 2014). Apart from MTBC, other mycobacteria collectively called non-tuberculous mycobacteria (NTM), mycobacteria other than tuberculosis (MOTT) or atypical mycobacteria have been implicated in TB infection. NTM, though generally not pathogenic compared to MTBC, increasingly are being associated with human diseases as causative agents (Griffith et al., 2007; Kendall & Winthrop, 2013). NTM diseases in humans can be clinically classified as pulmonary, lymphadenitis, skin and soft tissue infections and disseminated disease (Martin-Casabona et al., 2004; Griffith et al., 2007; Kendall & Winthrop, 2013).
MTBC and NTM are different clinically, so rapid detection, isolation, identification and drug susceptibility testing of these mycobacteria species are very critical for disease management, infection control as well as the right treatment options. About 90% of TB patients can be cured with first-line anti-TB drugs (isoniazid, rifampicin, pyrazinamide and ethambutol) if the TB was caused by a drug susceptible strain. However, treatment of TB caused by a drug resistant strain is very complicated. It is therefore important to establish the DST patterns of the mycobacterium species. Currently, novel approaches have been developed for rapid and specific identification of TB as well as resistance patterns to some common anti-TB drugs using molecular methods such as WHO endorsed line probe assays (LPA). Some examples of LPA are the GenoType Mycobacterium Assays: GenoType MTBC, GenoType CM/AS, GenoType MTBDRplus and GenoType NTM-DR (Hain Lifescience, Nehren Germany); INNO LiPa Mycobacteria (Innogenetics, Ghent, Belgium) and Nipro Assay (Nipro Corporation and Home Diagnostics, Inc., Japan).

1.2 Problem Statement

Due to high incidence of TB caused by *Mycobacterium tuberculosis* complex (MTBC) in developing countries including Ghana, TB control policies have centered on MTBC to the neglect of non-tuberculous mycobacterial (NTM) infections. Thus information on the extent of the burden of pulmonary disease from NTM is lacking due to limitations in tools for mycobacterial species identification and DST patterns. Microscopy which is the mainstay of TB diagnosis in Ghana cannot distinguish between MTBC and NTM. The inability to characterize acid fast bacilli (AFB) has led to improper treatment resulting in susceptible MTBC strains having an increased risk of developing resistance to first-line anti-TB drugs. Meanwhile, the first-line drug therapeutic regimen for TB are ineffective for treating NTM pulmonary infections. The inappropriate use of standard anti-TB drug treatment regimen for NTM infections have adverse effects including worsening of the
disease condition of the patient and the possible risk of the infecting strain developing resistance to the drugs (Pokam & Asuquo, 2012). In Ghana, very few studies have reported on the incidence and prevalence of TB due to specific MTBC species and their resistance to first-line anti-TB drugs in Ghana. However, these studies did not have nationwide representations. On the other hand, there is lack of national data on the species of NTM causing pulmonary infections as well as determination of DST patterns. As such, the current study sought to bridge the knowledge gap by providing a nationally representative data on mycobacterial strains and their DST patterns in Ghana.

1.3 Aim
To differentiate and determine drug susceptibility test patterns of mycobacterial isolates obtained from population-based TB prevalence survey using LPA.

1.4 Specific objectives
(i) To differentiate genetically species within MTBC using GenoType MTBC (Hain Lifescience GmbH, Nehren, Germany).

(ii) To differentiate genetically NTM species using GenoType CM/ AS (Hain Lifescience GmbH, Nehren, Germany).

(iii) To determine rifampicin and isoniazid resistance patterns and associated mutations in MTBC isolates using GenoType MTBDRplus version 2.0 (Hain Lifescience GmbH, Nehren, Germany).

(iv) To determine aminoglycosides and macrolides resistance patterns and associated mutations in NTM isolates using GenoType NTM-DR (Hain Lifescience GmbH, Nehren, Germany).
1.5 Significance of the study

The study was a follow-up study to the national TB prevalence survey which showed the usefulness of the GenoType Mycobacteria Assay series as appropriate tools for simple and rapid differentiation and DST of mycobacterial isolates to enhance adequate and prompt treatment of mycobacterial infections in Ghana. The study provided data that suggests that *M. tuberculosis* continues to be the dominant MTBC species causing TB in Ghana and that TB in humans due to *M. bovis* is rarely identified. Of significant note is that, it was observed from this study that quite a number of people within the various communities had MDR-TB without being identified. Such data are very useful for the TB control program in the country in terms of disease management and treatment options. Currently, in Ghana patients with NTM infections are treated with broad spectrum antibiotics which often result in treatment failure. To the best of my knowledge, this is the first study in Ghana to have used a line probe assay for the simultaneous determination of *M. avium* complex and *M. abscessus* subspecies as well as resistance to macrolides (clarithromycin, azithromycin) and aminoglycosides (kanamycin, amikacin, and gentamicin). Therefore, results from the study can serve as a guide to policy makers, clinicians and other stakeholders in the choice of drugs for treatment of pulmonary NTM infections.
CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The Global burden of Tuberculosis

Tuberculosis (TB) is an infectious disease that predates to several thousands of years ago. It is a global health menace with very high morbidity and mortality. More than two billion people in the world have TB infection. However, only a small proportion (5-10%) of these infections progress to active TB (WHO, 2007). About 90-95% of the remaining infected people will be asymptomatic but still have the bacilli due to the competence of their immune system. This condition is known as Latent TB infection (LTBI). About 5-10% of LTBI people will sometime in their lifetime develop active TB due to low health status, HIV/ AIDS infection and use of immunosuppressant. Globally, 9.6 million cases of TB were estimated in 2014 (WHO, 2015). However, only 6 million (63%) of the estimated number were reported to WHO. The remaining number of TB cases (37%) that were either not detected or reported presents an uncertainty in the kind of care they were given. South-East Asia and Western Pacific regions (58%) had the highest number of cases worldwide with India (23%), Indonesia (10%) and China (10%) forming a greater proportion of the global total. The African Region (28%) is the second in the lead of reported TB cases but the most severe in terms of high burden to population ratio: 281 cases per 100 000 population, more than double the global average of 133. More men (890 000) died from TB than women (480 000) and children (140 000) (WHO, 2015). The high prevalence of HIV/ AIDS has contributed to a steady increase in TB incidence in Africa. Over 60 % of TB patients in Botswana, South Africa, Zambia and Zimbabwe are also infected with HIV (Alimuddin et al., 2013; Frieden et al., 2003). In 2014, Ghana screened 49 343 HIV-positive people for TB with 2 858 HIV-positive TB patients reported (WHO, 2015). One
hundred and twenty three thousand MDR-TB cases were detected in 2014 which is about a quarter of the WHO estimated figure of 480 000. About 9.7% of people with MDR-TB have extensively drug-resistant TB (XDR-TB) (WHO, 2015). XDR-TB is an MDR-TB with additional resistance to fluoroquinolones and at least an injectable second line drug such as amikacin, capreomycin and kanamycin ("CDC | TB | Fact sheets | Extensively Drug-Resistant Tuberculosis (XDR TB)", 2016). As at the end of 2015, about 105 countries had reported XDR-TB cases (WHO, 2015).

2.2 TB Prevalence Survey

In order to know the true burden of TB in countries with reported cases of TB of 100 or more cases per 100,000 population, a direct measure can be made through a nationwide population-based survey using a sample size of about 50 000 people (WHO, 2013). Bacteriologically-confirmed pulmonary TB cases can be obtained through prevalence surveys which can yield useful estimates TB (all forms) prevalence in a whole country over a specific period of time (WHO, 2013). WHO and other partners in the ‘Stop TB’ partnership including donor agencies play key roles in conducting prevalence surveys through technical, financial and logistics support to the National Tuberculosis Programmes (NTPs). A budget estimate of between US$ 1 and 4 million is required for a successful conduct of prevalence surveys. Funding is usually by multi- donor agencies which include among others Global Fund (for fighting HIV/AIDS, TB and Malaria), DANIDA and USAID (WHO, 2011). Certain criteria including TB epidemiology of a particular country must be met to qualify for the conduct of a prevalence survey. As a result, in 2007, WHO through its Global Task Force on TB Impact Measurement considered 53 countries out of which 22 were selected to receive special global support from 2008-2015 to conduct prevalence surveys. These countries were mainly from the Africa (Ethiopia, Ghana, Kenya, Malawi, Mali, Mozambique, Nigeria, Rwanda, Sierra Leone, South Africa, Uganda, the
United Republic of Tanzania and Zambia) and Asia (Bangladesh, Cambodia, China, Indonesia, Myanmar, Pakistan, the Philippines, Thailand and Viet Nam) regions (WHO, 2013). Ghana conducted its nationwide TB prevalence survey from March – December 2013 in 98 clusters from all the ten regions (Appendix A). The survey was sponsored by the Global Fund, WHO, Italian government and KNCV Tuberculosis Foundation, Netherlands. The final report is in an advanced stage of preparation.

**Figure 2.1:** Countries in which national population-based surveys of the prevalence of TB disease have been implemented using currently recommended screening and diagnostic methods\(^a\) since 1990 or are planned in the near future: status in July 2013.

Source: WHO TB Report, 2013

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\(^a\) Screening methods include field Chest X-ray; Culture is used to confirm diagnosis

\(^b\) “National survey planned” means that a country has submitted at least a draft survey protocol and a budget to the WHO Global Task Force on TB Impact Measurement.
2.3 Epidemiology of Tuberculosis

Tuberculosis (TB) is an infectious disease which is transmitted through aerosols. More than 95% of deaths due to TB occur in developing countries where there are major economic and social challenges (WHO, 2015). WHO estimates that in 2014, cases of TB in men, women and children were 5.4, 3.2 and 1.0 million respectively (WHO, 2015). Though most TB cases and death (12.1 %) generally occur in men, women have a high disease burden. One of the reasons for this observation in women could be attributed to cultural and financial barriers that act as major obstacles for women seeking care resulting in delayed presentation and more severe illness (WHO, 2015). Globally, young people in their productive years (25-45 years) are the age group most infected with TB (WHO, 2013). About 5-10% of latently infected TB individuals especially those whose immune system has been compromised will have TB disease in their lifetime. HIV positive individuals have higher risk of TB reactivation than non-HIV infected people (Girardi et al., 2000). HIV infection is a major cofounder to the continual rise in the global incidence of TB (Raviglione et al., 1992).

2.4 Mycobacterium tuberculosis complex (MTBC)

Mycobacterium tuberculosis complex (MTBC) which is a group of highly related, weakly gram positive/acid fast bacteria is the causative agent for tuberculosis (Yeboah-Manu et al., 2011). The complex consists of M. tuberculosis and M. africanum which are obligate TB pathogens in humans. Other members include species which generally cause disease in animals: M. bovis (cattle), M. caprae (sheep and goats), M. microti (rodents) and M. pinnipedi (seals and sea lions). M. canettii which was widely thought to be a subspecies of M. tuberculosis has recently been classified though arguably as an out group of MTBC (Gagneux & Small, 2007; Wirth et al., 2008; Smith et al., 2009). Some of the animal adapted species can cause human zoonotic diseases (Lewerin, 2015). Speciation in MTBC
will allow for discrimination between obligate human and zoonotic pathogens which is very critical for treatment initiation (Djelouadji et al., 2008). All members of MTBC have similar genetic make-up, but differ broadly in their host preference, certain phenotypes, pathogenicities and drug resistance profile (Sreevatsan et al., 1997a). The presence of large sequence deletions in the region of difference (RD), repetitive elements and insertion sequences, and single nucleotide polymorphisms (SNPs) have contributed to diversity in MTBC (Yeboah-Manu, 2013). Bioinformatic analysis of Mycobacterium genome have revealed seven lineages of MTBC strains which are specific to a geographic location: lineage 1 (Indo-Oceanic); lineage 2 (East Asian- Beijing); lineage 3 (India/East Africa); lineage 4 (Euro-American, Latin); lineage 5 (West African I); lineage 6 (West African II); lineage 7 (Ethiopia and among Ethiopian immigrants in Djibouti) (Yimer et al., 2015).

2.4.1 Mycobacterium tuberculosis (M. tuberculosis)

Mycobacterium tuberculosis is the dominant species that causes TB in humans (Gagneux & Small, 2007), and the frequently isolated mycobacterial species from respiratory specimen worldwide (Zivanovic et al., 2014). M. tuberculosis can be sub-grouped genetically to reflect their virulence levels, immunogenicities, and geographical distributions (Gagneux & Small, 2007). Findings from several studies (Addo et al., 2007; Neonakis et al., 2007; Yeboah-Manu et al., 2011) on differentiation between MTBC species shows M. tuberculosis to be the dominant species isolated.

2.4.2 Mycobacterium africanum (M. africanum)

Mycobacterium africanum causes TB in humans but its infection is generally restricted to the West Africa sub-region. However, there have been some reported cases of M. africanum infections in Europe (Perez-de Pedro et al., 2008), the United States of America (Desmond
et al., 2004) and South America (Gomes et al., 2012). M. africanum was first discovered in 1968 in Senegal was initially classified biochemically into two groups: M. africanum I and M. africanum II (Castets et al., 1968). This form of classification was revised based on recent advances in molecular genetics which has revealed that M. africanum II, normally found in East-Africa is a variant of M. tuberculosis. The old M. africanum I biochemical sub group has been divided further into separate groups by genetic analysis: West-African genotype 1 (MAF 1) and West-African genotype 2 (MAF 2) (Yeboah-Manu et al., 2011). MAF 1 and MAF 2 are genetically similar to M. tuberculosis and M. bovis, respectively (Comas et al., 2010; Firdessa et al., 2013; Vasconcellos et al., 2010). Earlier studies in Ghana by Addo et al. (2007) and Yeboah-Manu et al. (2011) reported the prevalence of M. africanum in Ghana to be around 23% and 20%, respectively. In Gambia about 39% of reported TB cases are caused by M. africanum (de Jong et al., 2009a). The symptoms and infectivity of M. africanum infection resemble those of M. tuberculosis. M. africanum is an important opportunistic pathogen in the immunosuppression due to HIV (de Jong, Antonio, & Gagneux, 2010).

2.4.3 Mycobacterium bovis (M. bovis)

Mycobacterium bovis is primarily a pathogen of cattle. However, it can cause human disease known as bovine TB. According to Addo et al. (2007) about 3% of Mycobacterium species isolated from sputum samples from TB patients at the Korle-bu Teaching Hospital in Accra, Ghana were M. bovis. The isolation of M. bovis from sputum and tissue specimen particularly among some Fulani herdsmen in Nigeria has been reported in a study (Abubakar et al., 2011). M. bovis has an innate resistance to pyrazinamide which is included in the first line anti TB drug treatment regimen.
2.4.4 *Mycobacterium microti* (*M. microti*)

*Mycobacterium microti* naturally causes diseases in voles and other small rodents. However, some cases have been reported in other animals (van Soolingen *et al.*, 1998) and humans (de Jong *et al.*, 2009b; Emmanuel *et al.*, 2007; van Soolingen *et al.*, 1998).

2.5 Immune responses to *Mycobacterium tuberculosis*

*Mycobacterium tuberculosis* uses the natural immune system of its host for its own advantage. TB is transmitted through aerosols which can contain about 1 to 400 bacilli per droplet. Up to around 200 bacilli in an aerosol droplet is enough for infection when inhaled (Balasubramanian *et al.*, 1994). When the TB bacilli are inhaled, they are ingested by resident alveolar macrophages and tissue dendritic cells (DC) upon reaching the lungs through a process known as phagocytosis. *M. tuberculosis* can subdue the killing mechanisms of the alveolar macrophages and DCs which enables their replication. Pro-inflammatory cytokines are released by infected cells which results in the recruitment of more DC, monocytes and neutrophils from the blood stream. Specific T cells are activated in the local lymph node where the activated DCs have migrated to. Natural Killer (NK) cells are induced by cytokines interleukin 12 and interleukin 18 from infected cells to produce interferon gamma (IFN-γ). IFN-γ activates the macrophages to produce tissue necrosis factor alpha (TNF-α) and other microbicidal substances (Korbel, Schneider, & Schaible, 2008; North & Jung, 2004). By a series of cytokine and chemokine signaling, other immune cells are recruited to form a very hard structure called granuloma which is the main pathological marker for TB. In advance form of active disease the granuloma is calcified which forms cavities in the lungs with the help of necrotic macrophages. As the disease progresses, the calcified structure bursts out releasing infectious bacilli into the airways to allow for spread to other people (de Chastellier, 2009; Russell, 2007).
2.6 Clinical manifestation of tuberculosis

Pulmonary TB is the major clinical manifestation of TB infection though every part of the body can be infected such as the circulatory/lymphatic systems (disseminated TB) or other organs of the body other than the lungs (extra pulmonary TB) (Ducati et al., 2006; Donoghue, 2009). The release of cytokines like TNF-α during active TB causes symptoms including chronic bloody coughs, night sweats and weight loss (Ernst 1998; Orme & Cooper, 1999). Conversely, in latent TB infection, granuloma or other tissue associated with immunity contains the bacilli and maintains them in a state of no or slow replication for a very long time. Replication starts when the hosts’ immune system becomes compromised (Ehlers, 2009).

2.7 Diagnosis of tuberculosis

TB diagnosis is somehow difficult which may require several tests usually depending on the type of TB suspected.

2.7.1 Medical History

Information on the medical history of the TB suspect can be gathered through symptoms based approach. Exhibiting any of the following symptoms like continuous cough for more than two or more weeks with or without blood, production of mucopurulent sputum, and chest pains can be a very good step in diagnosis. Other symptoms are fatigue, loss of appetite, loss of weight, night sweating and fever. HIV status and history of conditions that expose an individual to risk for TB are assessed for TB diagnosis (Kumar et al., 2007).

2.7.2 Microscopy

The primary diagnostic tool for TB in most developing countries with limited resources is microscopy. Microscopy can be done using light microscope (Ziehl-Neelsen or Kinyoun
stain) or a fluorescent microscope (Auramine-O). About 1 x 10^4 bacilli must be present in a sputum smear in order to be seen by microscopic examination (Toman, 2004). To enhance sensitivity, the type of microscopy used, workload and sampling time are very critical considerations. Ziehl-Neelsen microscopy has a very high specificity particularly in TB endemic areas but low sensitivity ranging from 20% to 80% (WHO, 2010). It is widely considered that early morning sputum samples contain more organisms than spot samples. A study by Ulukanligil and colleagues demonstrated a high sensitivity for both ZN and fluorescence microscopy with repeat sampling. Thus when a single specimen was used sensitivity of ZN (61%) and FM (83%) increased to 80% and 92%, respectively, when three or more samples were used (Ulukanligil, Aslan, & Tasçi, 2000).

2.7.3 Bacteriological technique

The gold standard for diagnosing TB is culture which is relatively expensive, sensitive and labour intensive compared to microscopy. In the analyses of a study on 40 sputum samples, TB detection rate for ZN microscopy and bacterial culture was 77.5% and 85%, respectively (Darwish & Abd Elwadood, 2013). Conventional culture involves growth on solid media such as Lowenstein-Jensen (L-J) which is egg based or agar based (Middlebrook 7H10 and 7H11). Automated liquid culture systems like MGIT 960 (Becton Dickinson, New Jersey, USA) and MB/BacT system (bioMerieux, France) have been shown to be more sensitive as well as have rapid detection time as against L-J (Muyoyeta et al., 2009; Somoskovi et al., 2000; Sorlozano et al., 2009). The MB/BacT system is a modified Middlebrook 7H9 broth which detects the presence of replicating organisms by the CO₂ they produce which changes the colour of the pH-dependent indicator as a result of fall in pH. In the MGIT 960 detection system, the presence of an organism is detected by the fluorescing of a flourophore bound to oxygen in the bottom of the tube. This is as a
result of the utilization of oxygen by dividing organisms for metabolic processes. MGIT 960 has a higher recovery rate than conventional culture in extrapulmonary specimen (Hillemann, Richter & Rüsch-Gerdes, 2006).

2.7.4 Molecular test (Nucleic Acid Amplification)

Despite the advantages of the recent advances in the bacteriological techniques, there still could take some days to achieve results. Molecular techniques provide more rapid detection and has higher sensitivities. Many molecular tests available focus on nucleic acid amplification techniques to identify specific organisms as well as techniques such as gene sequencing or nucleic acid hybridization to show mutations that are responsible for drug resistance. Line probe assays (Barnard et al., 2012) and Genexpert MTB/RIF (Cepheid, Sunnyvale, CA) (Helb et al., 2010) are commercially available molecular tools for rapidly detecting INH and RIF resistance associated mutations and the detection of Mycobacterium tuberculosis as well as its resistance to RIF, respectively. Several studies (Barnard et al., 2012; Lawn et al., 2013; Theron et al., 2011) have reported on the usefulness of the Genexpert MTB/RIF.

2.7.5 Immunological Assays

The tuberculin skin test was the first immunoassay used in diagnosing TB infection. It has been in use since 1910 and it remains one of the oldest medical diagnostic tool in the world (Richeldi, 2006). A major limitation in the use of PPD test is its inability to safely distinguish BCG vaccinated individuals from those exposed to non-tuberculous mycobacteria or have M. tuberculosis infection (Fine et al., 1994). In order to overcome this limitation, M. tuberculosis antigen specific tests have been developed. These antigen specific tests involve the use of blood samples for testing for latent TB infection. They are
based on how the body reacts to the bacilli such as the presence of early secreted antigenic target 6-kDa (ESAT-6) and culture filtrate protein 10-kDa (CFP-10) in *M. tuberculosis* which are lacking in *M. bovis* (Andersen *et al.*, 2000). Examples of these tests include QuantiFERON®–TB Gold In-Tube test (QFTGIT) and T-SPOT® TB test (T-Spot).

### 2.8 Multi-Drug Resistant TB (MDR-TB)

Resistance to at least both isoniazid and rifampicin (MDR-TB) can be primary (infected by someone else who has the resistant strain) or acquired (results when susceptible TB is inadequately treated, which causes bacteria to develop resistance to the drugs used) (Musser, 1995; Victor, van Helden & Warren, 2002). About 5% of TB cases worldwide were MDR-TB in 2014. In Ghana out of the 1 799 (328 new; 1 471 retreatment) cases tested for MDR, 93 (5.2%) were laboratory confirmed to be MDR in 2014 (WHO, 2015). Globally, drug resistance testing for TB patients increased from 17% and 8.5% for previously treated patients and new cases respectively in 2013 to 58% and 12% in 2014. MDR-TB treatment enrollment in 2014 was 110 000. Treatment outcomes for all detected cases in 2012 were 50%, 16%, 24% and 10% for successfully treated, death, unavailable documentation or treatment interruption and failed treatment, respectively. The proportion of new cases of TB with MDR has remained fairly constant at about 3.3% from 2008 to 2014 (WHO, 2015). When an MDR-TB case develops resistance to any flouroquinolone and any of the injectable TB drugs-amikacin, capreomycin and kanamycin the condition is known as extensively drug-resistant TB (XDR-TB) (Barnard *et al.*, 2012).

### 2.9 Tuberculosis Drug Therapy

Tuberculosis can be cured if standardized treatment is administered to patients. Failure to follow the treatment regimen may result in developing drug resistance TB (www.who.int).
There are three main types of treatment regimen based on the category of patient as described in Table 2.1 below.

**Table 2.1: Recommended Treatment Regimens for Each Treatment Category**

<table>
<thead>
<tr>
<th>Patient Category</th>
<th>Definition</th>
<th>Initial Phase</th>
<th>Continuation Phase</th>
</tr>
</thead>
</table>
| I                | All New Cases  
- New smear-positive  
- New smear negative PTB  
- Concomitant HIV disease  
- Extra-pulmonary TB | 2 months (HRZE)\(^c\) = 56 doses of HRZE | 4 months (HR) = 112 doses of HR |
| II               | Previously treated sputum smear-positive PTB  
- Relapse  
- Treatment after interruption  
- Treatment failure | 2 months (HRZE)\(^S\) + 1 month (HRZE) = 84 doses of HRZE + 56 doses of S | 5 months (HRE) = 140 doses of HRE |
| III\(^d\)        | Children under 12 years | 2 months (HRZ) = 56 doses of HRZ | 4 months (HR) = 112 doses of HR |

\(H=\) Isoniazid; \(R=\) Rifampicin; \(Z=\) Pyrazinamide; \(E=\) Ethambutol; \(S=\) Streptomycin

\(^a\)Adapted from TB-HIV Clinical Guidelines, Chapter 7: Standardized TB Case Definitions and Treatment Categories, and Staging for HIV.

\(^b\)Direct observation of treatment intake is required, and always in regimens including rifampicin.

\(^c\)Streptomycin may be used instead of ethambutol. In meningitis, ethambutol should be replaced by streptomycin.

\(^d\)In children with meningitis, add streptomycin in the initial phase.
2.9.1 Isoniazid (INH)

Isoniazid is one of the most effective drug for treating *M. tuberculosis* infections. It has a very high activity at MICs of 0.02 to 0.2 μg/ml (Zhang Min *et al*., 2005). It is a prodrug and relies on the *katG* encoded catalase-peroxidase enzyme in the TB bacilli to be activated (Zhang *et al*., 1992). As it becomes activated, INH terminates the synthesis of mycolic acids—very important components of the cell wall. The termination process is made possible through the inhibition of the NADH-dependent enoyl-ACP reductase coded by the *inhA* gene. As a result, the bacteria losses its cell wall integrity. On average 80-85% of INH resistance are due to mutations in the *katG* gene (codon 315) and the *inhA* regulatory region of the TB bacilli (Mokrousov *et al*., 2002; Piatek *et al*., 2000; Seifert *et al*., 2015; Telenti *et al*., 1997) (Appendix B [I] & [II]). The most frequent katG mutation (S315T) of lowers catalase-peroxidase activity by half leading to a relatively high level of resistance. Other mutations responsible for INH resistance are found in the ahpC-oxyR intergenic region (Piatek *et al*., 2000; Telenti *et al*., 1997) often associated with *katG* mutations at codons other than codon 315 (Sreevatsan *et al*., 1997b). Positions -24, -16, -8 and -15 in the promoter region is where most frequent mutations of *inhA* gene occur (Barnard *et al*., 2012).

2.9.2 Rifampicin (RIF)

Rifampicin, a derivative of rifamycin has been in use as anti-TB drug since 1966. It has a wide spectrum activity and acts by binding to the β subunit of RNA polymerase (*rpoB*) found in the cells of the bacilli leading to interruption of mRNA synthesis. Rifampicin has a unique characteristic of high activity against both actively growing and non-growing bacilli (Mitchison, 1979). Majority (95%) of RIF resistance are due to mutations occurring in the 81 base pair hot spot (codon 507-533) –rifampicin resistance determining region
(RRDR) of the \textit{rpoB} gene (Zhang Ying & Wallace Jr, 2005) (Appendix B [III]). Two independent studies (Mani \textit{et al.}, 2001; Riska, Wallace & Alland, 2000) showed that a small number (<5\%) of rifampicin resistant strains do not show mutations in RRDR. Rifampicin mono-resistance (RMR) is very rare and this suggests that resistance to other drugs particularly isoniazid may always be associated with rifampicin resistant strains. Therefore detection of rifampicin resistance may be a useful marker for MDR detection (Traore \textit{et al.}, 2000).

\textbf{2.9.3 Ethambutol (EMB)}

Ethambutol acts by interfering in the biosynthesis of cell wall arabinogalactan of actively growing bacilli (Takayama \textit{et al.}, 1979). A study by Sreevatsan \textit{et al.} (1997c) demonstrated that ethambutol-resistant strains (50\%) had mutations in \textit{embB} which is part of the \textit{embCAB} operon which encodes mycobacterial arabinosyl transferase. Mutations which results in amino acid change may have varying effects on ethambutol resistance (Safi \textit{et al.}, 2008).

\textbf{2.9.4 Streptomycin (STR)}

Streptomycin was the first anti TB drug and was isolated from \textit{Streptomyces griseus}, a soil microorganism. It belongs to the aminoglycoside group of antibiotics. It acts by inhibiting translation through binding to the 16S rRNA (Moazed & Nollar, 1987). Emergence of resistance to streptomycin was quick due to its single use in treatment in the early stages of usage. Streptomycin resistance occurs when the binding site is altered due to mutations in \textit{rrs} or \textit{rpsL} genes. The most frequent point mutations (K43R) associated with streptomycin resistance occur in \textit{rpsL}, (Zhang & Telenti, 2000). Mutations in \textit{gidB} also contribute to streptomycin resistance but on a low level (Silva \textit{et al.}, 2001; Okamoto \textit{et al.}, 2007).
2.9.5 Pyrazinamide (PZA)

Pyrazinamide can only be effective in its active form. As a pro drug, it is converted into pyrazinoic acid by the \textit{pncA} gene encoded enzyme pyrazinamidase (Scorpio & Zhang, 1996). Frequent mutations in the 561 bp region of the open reading frame or in the 82 bp region of the putative promoter of the \textit{pncA} gene confers resistance to pyrazinamide (Scorpio \textit{et al.}, 1997; Jure´en \textit{et al.}, 2008).

2.10 Prevalence of Non-tuberculous mycobacteria (NTM)

Non-tuberculous mycobacteria species can cause disease but with varying levels of pathogenicity (van Ingen \textit{et al.}, 2009). At least 42 out of more than 125 species of NTM that are related with diseases have been catalogued and are available (Tortoli, 2003). NTM are mostly found in environmental sources such as soil, water bodies, dust and some animals and birds (Jamison \textit{et al.}, 2006). Specific environmental factors linked to water and soil exposure are high risk factors for pulmonary NTM infection (Mirsaeidi \textit{et al.}, 2014). The American Thoracic Society and Infectious Diseases Society of America (ATS/ IDSA) have developed protocols for NTM disease determination, including clinical symptoms, radiographic finding, and microbiologic criteria (ATS/ISDA, 2007). In Sub-Saharan Africa, data on the true burden of diseases due to non- tuberculous mycobacteria is lacking or very scanty as a result of limited infrastructure and tools for mycobacterial species identification (Aliyu \textit{et al.}, 2013). However, few studies (Asiimwe \textit{et al.}, 2013; Chanda-Kapata \textit{et al.}, 2015; Limo \textit{et al.}, 2015) have recently reported on NTM disease prevalence in some African countries. These studies covered only certain regions in these countries and the findings could hardly be generalized. The prevalence of NTM species causing pulmonary disease differs by geographic location. Two independent research groups (Aliyu \textit{et al.}, 2013; Limo \textit{et al.}, 2015) in Nigeria and Kenya, respectively reported
M. intracellulare as the most NTM causing disease in patients. Other researchers (Maurya et al., 2015; Zivanovic et al., 2014) reported a relatively high isolation of M. fortuitum and M. xenopi in India and Serbia, respectively. A collaborative study on NTM prevalence in some 30 countries showed that M. avium complex (MAC), M. gordonae and M. xenopi were the most dominant species isolated (Hoefsloot et al., 2013). MAC is the leading cause of pulmonary infections in the United States of America; following in the order is M. kansasii (Griffiths et al., 2007).

2.10.1 Diagnosis of NTM

The isolation of NTM in pulmonary specimens does not necessarily indicate active infection or disease. Therefore factors such as chest X-ray, clinical investigations and microbiological tests are required for definite diagnosis (Johnson & Odell, 2014). Diagnostic criteria of non-tuberculous mycobacterial lung disease is as specified by the American Thoracic Society (ATS) and the Infectious Diseases Society of America (IDSA) guidelines (ATS/IDSA, 2007).

2.10.2 Treatment and drug resistance

Majority of NTM excluding M. kansasii have natural resistance or moderate susceptibility to the standard anti-TB drugs. A prolonged course of multi drug therapy is needed. NTM normally form biofilm (Schulze-Robbecke, Janning, & Fischeder, 1992) which contributes to resistance to antibiotics and disinfectants (Falkinham, 2007; Jarlier & Nikaido, 1994). The ATS/IDSA has recommended antibiotic therapy for some NTM infections in their consensus statement (ATS/IDSA, 2007). The type of treatment and infection management differ according to the causative species (Mwikuma et al., 2015). Antibiotics such as streptomycin, amikacin, fluoroquinones, tetracyclines, clarithromycin, rifabutin and
tigecycline are effective drug available in treating most NTM infections (Li et al., 2013). Multi-drug therapy consisting of a macrolide (azithromycin or clarithromycin), rifampin or rifabutin, and ethambutol with or without an intravenous aminoglycoside has been suggested as treatment regimen for lung infections with MAC. Therapy continues for a period of not less than a year even after sputum conversion (Griffith et al., 2007). Pulmonary infections caused by *M. abscessus* are extremely difficult to treat and often require chemotherapy and surgical resection. Treatment with drug therapy alone will be unsuccessful (Johnson & Odell, 2014).

### 2.11 Line Probe Assays (LPAs)

Line probe assays (LPAs) are reverse hybridization-based assays available commercially for rapid differentiation and detection of mutations responsible for drug resistance. LPAs are based on hybridization of amplicons to specific probes for conserved regions of a particular gene (Barnard et al., 2012). The INNO LiPa Mycobacteria (Innogenetics, Ghent, Belgium) was the first commercially available LPA. The GenoType Mycobacterium Assays (Hain Lifescience, Nehren Germany) and Nipro Assay (Nipro Corporation and Home Diagnostics, Inc., Japan) are two new LPAs commercially available for use.

#### 2.11.1 GenoType MTBC

The GenoType MTBC is used for differentiation of MTBC. It consists of DNA probes immobilized on nitrocellulose membranes that target polymorphisms in the *gyrB* DNA sequence and the RD1 deletion of MTBC and the *M. bovis* BCG, respectively (Richter et al., 2003). Several evaluation studies on the performance of the GenoType MTBC assay (Neonakis et al., 2007; Safianowska et al., 2009; Somoskovi et al., 2008) have demonstrated 100% sensitivity. In contrast, another study showed a sensitivity of 94% (Richter et al., 2003).
2.11.2 GenoType Mycobacterium CM (Common Mycobacteria)

The GenoType CM assay differentiates and identify different species of NTM most frequently isolated from cultures. It involves reverse hybridization of amplified 23S rRNA gene region to specific oligonucleotide probes immobilized on nitrocellulose membrane strips. It detects 15 most frequently isolated NTMs and also MTBC (Singh et al., 2013). Separate studies to evaluate the GenoType Mycobacterium CM assay both reported 100% sensitivity and specificity (Mäkinen et al., 2006; Richter, Rüscher-Gerdes, & Hillemann, 2006). However, another study showed 98.9% and 88.9% for sensitivity and specificity, respectively (Russo, Tortoli, & Menichella, 2006).

2.11.3 GenoType Mycobacterium AS (Additional Species)

The GenoType AS is used to identify 16 less frequently isolated NTM species which are not included in the GenoType Mycobacterium CM. In evaluating the performance of the GenoType Mycobacterium AS, Russo et al. (2006) reported 99.4% and 100% sensitivity and specificity, respectively.

2.11.4 GenoType MTBDRplus version 2.0

The GenoType MTBDRplus is a qualitative assay for simultaneous identification of MTBC and mutations associated with INH and RIF drug resistance from fresh sputum specimen or cultivated samples (Hain Lifescience, 2014). The assay screening involves probing for the presence and or absence of wild type and mutant alleles of the rpoB (associated with RIF resistance), katG and inhA (associated with high-level and low-level INH resistance, respectively) (Barnard et al., 2012). An evaluation study on the performance of GenoType MTBDRplus assay detected a very high rate of rifampicin resistance using clinical isolates (98.7 %) and sputum specimens (96.8%). Likewise the detection rate for isoniazid
resistance was 92% and 90% respectively (Hillemann et al., 2006). Another evaluation study of GenoType MTBDRplus assay by Asante-Poku et al. (2015a) using phenotypic testing as gold standard showed sensitivity/ specificity for the detection of rifampicin monoresistance, isoniazid monoresistance and MDR of 100%/100%, 83.3%/100% and 100%/100%, respectively.

2.11.5 GenoType NTM-DR

Some NTM are genetically related but exhibit different epidemiological and clinical significance, for instance, *M. chimaera* and *Mycobacterium intracellulare* and the subspecies of *M. abscessus*. To date, most assays available for identification cannot distinguish among them. GenoType NTM-DR (Hain Lifescience, Nehren, Germany) is a novel line probe assay that allows the detection of several clinically relevant NTMs including the differentiation between *M. intracellulare* and *M. chimaera*. Furthermore, the mycobacteria resistance to macrolides and aminoglycosides are also detected within the same step. It also enables identification of *M. avium* complex and *M. abscessus* subspecies and the simultaneous determination of antibiotic resistance to macrolides and aminoglycosides (Kehrmann et al., 2016). A recent study in Germany found out that the GenoType NTM-DR showed 98% correlation with molecular and phenotypic results for determining clarithromycin and aminoglycoside resistance (Kehrmann et al., 2016).
CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The prevalence survey from which the mycobacterial isolates used for this current study was conducted in 98 selected clusters from all 10 regions in Ghana (Figure 3.1).

Figure 3. 1: Map showing study area.
3.2 Study Design

This current study was a follow-up on the Ghana TB prevalence survey conducted in 2013. The prevalence survey was cross sectional and population based carried out randomly in a sample of the general adult population in Ghana in order to determine the number of people with TB disease. All the mycobacterial isolates obtained from the survey were genotyped and their DST profiles determined. Information such as sex, age, occupation and region of residence of participants whose specimen were culture positive were culled from the database of the TB prevalence survey through the assistance of the National Tuberculosis Programme (NTP).

3.3 Study Population

The study population were all persons who met the survey eligibility criteria based on symptoms and chest X-ray screening for the production of sputum for bacteriological examination in the study area.

3.4 Inclusion criteria

Positive cultures of mycobacteria obtained from adults aged 15 years and above who participated in the nationwide TB prevalence survey were included in the study.

3.5 Exclusion criteria

Smear positive but culture negative mycobacteria obtained from adults aged 15 years and above who participated in the nationwide TB prevalence survey as well as children less than 15 years were excluded from the study.

3.6 Sample size

The sample size was all the 361 mycobacterial isolates available from the TB prevalence survey. Therefore, there was no need for sample size calculation for the current study.
3.7 Laboratory Procedures

Both commercially available kits and in-house prepared reagents were used.

Commercial Kits (Appendix C)

GenoType MTBC (Hain Lifescience, Nehren Germany)
GenoType Mycobacterium CM (Hain Lifescience, Nehren Germany)
GenoType Mycobacterium AS (Hain Lifescience, Nehren Germany)
GenoType MTBDR plus Version 2.0 (Hain Lifescience, Nehren Germany)
GenoType NTM-DR (Hain Lifescience, Nehren Germany)

3.7.1 Sputum processing for microscopy and culture

A combination of symptom screening using standardized questionnaire and chest X-ray was used in order to selectively target the survey participants who provided sputum for bacteriological tests. Sputum were first processed using Mycoprep (BD Diagnostic System, Sparks, MD, USA) which is a commercially available Sodium hydroxide-N-acetyl L-cysteine (NaOH-NALC) formulation for sputum digestion and decontamination. This initial process was done according to manufacturer’s instructions. In brief, equal volumes of specimen (3-5 ml) and NaOH-NALC solution were mixed together and the preparation allowed to stand for 15 minutes at room temperature (25°C). After that about 40-45 ml of phosphate buffer saline (PBS) solution with pH 6.8 was added to the decontaminated specimen and allowed to stand for 20 minutes to neutralize the reaction. The whole preparation was spun in a refrigerated centrifuge (Eppendorf, Germany) at 3000g for 15 minutes to concentrate the specimen and also wash the NaOH solution. The supernatant was then discarded to obtain sediment. A small volume (2 ml) of PBS (pH 6.8) was added to the sediment to make the inoculum for the smears (Ziehl-Nielsen and Auramine O) and cultures (Lowenstein Jensen and Mycobacteria Growth Indicator Tube (MGIT)). The
remaining sediments after smear preparation and inoculation were stored in -20°C for future use. Briefly, two smears were prepared from each inoculum and respectively stained for microscopy using Ziehl-Nielsen (ZN) and Auramine O staining methods. These smears were air dried after staining and examined microscopically. The ZN stained smear was examined using bright field microscope with oil immersion at 1000x and the Auramine O stained smear examined with LED microscope without oil immersion at 400x. Smears were graded using the IUAlTD/WHO standards (Appendix D). For inoculation, 0.5 ml of decontaminated specimen were each inoculated onto LJ slants and MGIT medium and incubated at 37 0C in an incubator (maximum of 8 weeks) and BACTEC MGIT 960 instrument (6 weeks) respectively. Growth of mycobacteria and other bacteria in the MGIT was indicated by increasing fluorescence. Once a tube flagged positive, smear was prepared and ZN stained for the presence or absence of acid fast bacilli (AFB). Additionally, a portion of the positive culture was inoculated onto blood agar plates by streaking to check for contamination.

All the positive mycobacterial isolates obtained were differentiated generally as MTBC and NTM using BD MGIT TBc ID test kit (BD Diagnostic System, Sparks, MD). The test was done according to manual provided by the manufacturer. Briefly, each TBc ID cassette was inoculated with 100 µl of a positive MGIT culture or in the case of LJ culture, one loopful of colonies suspended in 200 µl of extraction buffer (phosphate buffer with 0.05 % Tween 20 and 0.02 % sodium azide) and 100 µl of the suspension used in the assay. The results were interpreted 15 min after application of the sample. A positive result was indicated by the development of two pink bands, one in the control zone (C) and another in the test zone (T) (Appendix E).

The isolates were preserved in an enriched storage media (reconstituted Middlebrook 7H9 with glycerol) at -80°C until they were used.
3.7.2 Molecular testing

The GenoType assay platform (GenoType MTBC, GenoType Mycobacterium CM/AS and GenoType MTBDRplus) involves three main steps: DNA extraction, multiplex PCR amplification with biotinylated primers and reverse hybridization (Appendix F).

3.7.2.1 DNA extraction

An in-house DNA extraction method was used. About 0.5 ml of thawed suspension of isolates was dispensed into a 1.5 ml screw capped micro centrifuge tubes. To heat-kill the isolates, the tubes were placed in a heating block at 90°C for about one hour to disrupt the cell wall and release DNA into solution. Then, the supernatant containing the mycobacterial DNA was carefully transferred into different tubes using Pasteur pippette and kept at -20°C until used for PCR.

3.7.2.2 Multiplex amplification with biotinylated primers

The tests were performed according to instructions provided for by the manufacturer. Briefly, 10μl of AM-A (5μl 10x buffer, 2μl MgCl2, 3μl of molecular grade water, 0.2μl (1U) Taq DNA polymerase) was mixed with 35μl of AM-B (nucleotides, biotinylated primers, dye). Then, 5μl of extracted DNA sample was added to the master-mix. This was followed by PCR using a thermocycler (AB Systems) at the following cycling conditions: one cycle at 95°C for 15 minutes (denaturation); ten cycles at 95°C and 58°C for 30 seconds and two minutes, respectively (annealing); twenty cycles at 95°C, 53°C and 70°C for 25, 40 and 40 seconds, respectively (elongation/extension); single cycle at 70°C for eight minutes (final elongation/extension).

3.7.2.3 Pre-hybridization

The following pre-hybridization processes were carried out: Hybridization (HYB) and Stringent (STR) solutions were pre-heated to 45°C with intermittent shaking. All other
reagents excluding Conjugate C/D (CON-C/ CON-D) and Substrate C/D (SUB-C/ SUB-D) were pre-warmed to room temperature. CON-C and SUB-C were diluted 1:100 with their respective buffer (CON-C with CON-D, SUB-C with SUB-D) in the required volumes. The solutions were mixed well and brought to room temperature. The membrane strips were marked with a water proof marker pen respectively according to sample identification numbers.

3.7.2.4 Reverse hybridization

Hybridization was carried out with the GT-Blot 48® automated hybridizer (Hain Lifescience Nehren, Germany) in accordance with manufacturer’s instructions. Pre-heated hybridization and stringent buffers, diluted conjugate and substrate solutions, rinsing and sterile distilled water were placed into their respective colour-coded slots in the GT-Blot 48®. Then suction heads were placed into corresponding colour-coded solutions. Equal volumes (20μl) of denaturing solution and amplicons were mixed together in individuals wells of a tray placed in the GT-Blot 48® and allowed to stand for five minutes at 25°C. During the period of denaturation sample codes are written on the strips and placed into respective sample wells. The automated process of dispensing and aspirating the various solutions is set in a sequential order: (hybridization, stringent wash, rinsing, conjugate, rinsing, sterile distilled water, substrate, sterile distilled water). The strips were then dried using the heating systems in the GT-Blot 48®.

3.7.2.5 Evaluation and Interpretation of results

The fully dried strips were scanned using GenoScan® which generated an automated read-out of the banding patterns. The strips were then pasted on an evaluation sheet which was included in the kit and kept away from light source (Appendix G). The final results on the read-out were verified manually with the eye.
3.8 Statistical Analyses
All the data collected were entered into Microsoft Excel 2013 (Microsoft Corporation, USA) for analysis. Results were presented in tables and graphs showing frequencies and percentages. For objectives 1 and 2, Fisher exact test was used to interpret the results of risk associated with MTBC and NTM infection/disease respectively and socio-demographic characteristics of study population. The risk associated with developing multi-drug resistant TB (MDR-TB) and socio-demographic characteristics of study population was determined with Fisher exact test for objective 3 and 4. The statistical significance level was \( p \leq 0.05 \) (Appendix H).

3.9 Ethical Considerations
The parent study (Assessing tuberculosis disease prevalence in Ghana through a population based survey) obtained ethical approval from the Institutional Review Board (IRB) of Noguchi Memorial Institute for Medical Research (FWA 00001824; IRB 00001276) (Appendix J).
CHAPTER FOUR

4.0 RESULTS

4.1 Characteristics of patients from whom study isolates were obtained

Out of 67,757 (males-28,185; females-39,572) individuals eligible for participation in the prevalence survey, 61,726 (males-24,688; females-37,038) actually participated. After symptoms and chest X-ray screening 8,298 were eligible for sputum production for microscopy and culture with their ages ranging from 15 to 100 years. However, 8,126 individuals produced at least one sputum for bacteriological examination. Of the 361 mycobacterial isolates obtained, 165 (45.7%) were MTBC and 196 (54.3%) were NTM. Ninety-one MTBC isolates (55.2%) were obtained from males and 74 (44.8%) from females. On the other hand, the number of NTM isolates from males and females were 68 (34.7%) and 128 (65.3%), respectively (Figure 4.1A). Based on the 361 isolates, the age group from 65 years and above had the highest number 42 (25.5%) of MTBC isolates with the lowest 22 (13.3%) being in two different age groups; 25-34 and 35-44 years. The highest 55 (28.1%) and lowest 20 (10.2%) number of NTM isolates were within the 65 years and above and 55-64 years group, respectively (Figure 4.1B). About 41.6 % of the isolates were from farmers while 8.9 % were from people who were unemployed (Figure 4.1C). Both MTBC and NTM isolates were obtained from all the ten regions. In both cases respectively, Ashanti region had the highest (18.2 % and 28.6 %) while the Upper West region had the lowest (4.2 % and 2.6 %) (Figure 4.1D).
Figure 4.1: Characteristics of patients from whom mycobacterial isolates used in the study were obtained (N = 361)

A: Gender distribution of patients

B: Age distribution of patients

C: Distribution of patients according to their occupation

D: Distribution of patients according to their region of residence
4.2 Differentiation of MTBC using GenoType MTBC

Out of one hundred and sixty five (165) MTBC isolates genotyped, 161 (97.6 %) were identified as *M. tuberculosis* while 4 (2.4 %) were *M. africanum*. Other members of MTBC such as *M. bovis* and *M. microti* that has been reported to cause disease in humans were not identified (Table 4.1).

**Table 4.1: MTBC species differentiated by GenoType MTBC Assay (N= 165)**

<table>
<thead>
<tr>
<th>MTBC Species</th>
<th>Number</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. tuberculosis</em></td>
<td>161</td>
<td>97.6</td>
</tr>
<tr>
<td><em>M. africanum</em></td>
<td>4</td>
<td>2.4</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td><em>M. microti</em></td>
<td>0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

MTBC = *Mycobacterium tuberculosis* complex; *M. tuberculosis* = *Mycobacterium tuberculosis*; *M. africanum* = *Mycobacterium africanum*; *M. bovis* = *Mycobacterium bovis*; *M. microti* = *Mycobacterium microti*

4.3 Differentiation of NTM using GenoType Mycobacterium CM/AS

Out of 196 NTM isolates, 120 (61.2%) could be speciated completely with the GenoType CM/AS while 76 (38.8%) could not due to the limited ability of the kit for speciation. The 120 speciated NTM consisted of diverse species including *M. fortuitum* 42 (21.4 %), *M. intracellulare/ M. chimaera* 27 (13.8 %), *M. mageritense* 9 (4.6 %), *M. abscessus* 8 (4.1 %), *M. gordonae* 7 (3.6 %), *M. lentiflavum* 7 (3.6 %), *M. scrofulaceum* 6 (3.1 %), *M. asiaticum* 4 (2.0 %), *M. goodii* 4 (2.0 %), *M. interjectum* 2 (1.0 %), *M. perigrinum* 2 (1.0 %), *M. avium* 1 (0.5 %) and *M. smegmatis* 1 (0.5 %). Of the 76 unspeciated NTM, 24 (12.2%) were only identified as *Mycobacterium* species, 37 (18.9%) as high G+C Gram positive bacterium and the remaining 15 (7.7 %) as other bacteria (Figure 4.2)
Figure 4.2: Differentiation of NTM using GenoType Mycobacterium CM/AS

*M. fortuitum* = *Mycobacterium fortuitum*; *M. mageritense* = *Mycobacterium mageritense*

*M. abscessus* = *Mycobacterium abscessus*; *M. perigrinum* = *Mycobacterium perigrinum*

*M. smegmatis* = *Mycobacterium smegmatis*; *M. intracellulare* = *Mycobacterium intracellulare*

*M. chimaera* = *Mycobacterium chimaera*; *M. gordonae* = *Mycobacterium gordonae*

*M. lentiflavum* = *Mycobacterium lentiflavum*; *M. scrofulaceum* = *Mycobacterium scrofulaceum*

*M. asiaticum* = *Mycobacterium asiaticum*; *M. goodii* = *Mycobacterium goodii*

*M. interjectum* = *Mycobacterium interjectum*; *M. avium* = *Mycobacterium avium*

*Unidentified Mycobacterium species*

† Members of a group of gram-positive bacterium with high guanine and cytosine content

‡ Group of bacteria that were neither mycobacteria nor high G+C gram-positive bacteria
4.4 Drug Susceptibility Testing of MTBC Isolates

Out of the 165 MTBC isolates, 133 (80.6 %) were susceptible to both INH and RIF, 31 (18.8 %) were resistant, while the DST status of the remaining 1 (0.6 %) could not be determined (assay yielded an indeterminate result) (Figure 4.3).

![Proportion of MTBC isolates based on DST](http://ugspace.ug.edu.gh/)

**Figure 4. 3:** Drug Susceptibility Test (DST) Patterns of MTBC isolates (N= 165).

- **Susceptible:** isolates that are susceptible to both isoniazid and rifampicin.
- **Resistant:** isolates that are resistant to at least isoniazid and rifampicin.
- **Indeterminate:** isolate whose resistance profile to isoniazid or rifampicin could not be determined by the GenoType MTBDRplus.
In summary, eighteen (10.9 %) isolates were isoniazid monoresistant (IMR), 2 (1.2 %) were rifampicin monoresistant (RMR) while 11 (6.7 %) were multidrug resistant (MDR) (Figure 4.4).

**Figure 4.4:** Drug Susceptible and resistant patterns of MTBC isolates

INH = isoniazid; RIF = rifampicin; MDR = Multi-drug resistant; N = Total number of isolates used

INH monoresistant: Resistant to isoniazid only

RIF monoresistant: Resistant to rifampicin only

MDR: Resistant to both isoniazid and rifampicin

Indeterminate: Resistance to either of the drugs or both could not be determined

Susceptible: Susceptible to both isoniazid and rifampicin

The frequency of mutations in the genes responsible for isoniazid resistance including MDR were as follows: *katG* only 18 (62.1 %), *inhA* only 5 (17.2 %) and *katG + inhA* 6 (20.7 %) (Figure 4.5).
Figure 4.5: Frequency of gene mutations responsible for isoniazid resistance.

*katG*: number of isolates whose resistance to isoniazid is due to mutations in the katG gene only

*inhA*: number of isolates whose resistance to isoniazid is due to mutations in the inhA gene promoter region only

*katG + inhA*: number of isolates whose resistance to isoniazid is due to mutations in both katG gene and inhA gene promoter region
One of the two rifampicin monoresistant isolates had mutations in codon 530-533 resulting in the absence of wild type band (WT8) without the presence of the corresponding mutation band whiles the other showed no mutation in the wild type but MUT 3 (S531L) mutation (Table 4.2). For isoniazid monoresistant isolates (IMR), mutations in \textit{katG} MUT1 (S315T1) only was the most frequent, making up 14/18 (77.8 %); of these 11/ 14 (78.6 %) had both the presence of \textit{katG} MUT1 and the absence of corresponding WT band. The remaining four IMR out of 18 (22.2 %) had mutations in \textit{inhA} MUT1 (C15T) with the absence of corresponding WT1 (-15/-16). None of the IMR had mutations in both \textit{katG} and \textit{inhA} (Table 4.2). Of the 11 MDR isolates, the most frequent mutations were 6/11 D516V (54.5 %), 10/11 S315T1 (90.9 %) and 6/11 T8C (54.5 %) in \textit{rpoB}, \textit{katG} and \textit{inhA} respectively. One out of the 6 (16.7 %) MDR isolates with D516V mutation in \textit{rpoB} also had absence of corresponding WT3 band. Other mutations include 1/11 (9.1 %) S531L, 3/11(27.3 %) H526Y with absence of corresponding WT7 and 1/11 (9.1 %) absence of WT8. Ten out of the 11 (90.9 %) MDR isolates had \textit{katG} MUT1 S315T1 with 6/10 (60 %) having additional mutations in \textit{inhA} MUT3A T8C. Four out of these 10 (40 %) isolates showed both presence of \textit{katG} MUT1 band and absence of corresponding wild type (WT) band. Only 1/11(9.1 %) of MDR isolate had mutation in \textit{inhA} alone. This mutation was indicated by the presence of MUT1 (C15T) with the absence of corresponding WT1 (-15/-16). Furthermore 1 out of the 6 isolates with mutations in both \textit{katG} MUT1 S315T1 and \textit{inhA} MUT3A T8C also showed the absence of corresponding wild type (WT2) band in the \textit{inhA} region (Table 4.2).
Table 4.2: Specific gene mutations in resistant strains

<table>
<thead>
<tr>
<th>Gene locus</th>
<th>Band</th>
<th>Gene region/mutation</th>
<th>*RIF monoresistant</th>
<th>†INH monoresistant</th>
<th>‡MDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>rpoB</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT1</td>
<td>506-509</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT2</td>
<td>510-513</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT3</td>
<td>513-517</td>
<td></td>
<td></td>
<td></td>
<td>$1</td>
</tr>
<tr>
<td>WT4</td>
<td>516-519</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT5</td>
<td>518-522</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT6</td>
<td>521-525</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>WT7</td>
<td>526-529</td>
<td></td>
<td></td>
<td></td>
<td>$3</td>
</tr>
<tr>
<td>WT8</td>
<td>530-533</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MUT1</td>
<td>D516V</td>
<td></td>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>MUT2A</td>
<td>H526Y</td>
<td></td>
<td></td>
<td></td>
<td>$3</td>
</tr>
<tr>
<td>MUT2B</td>
<td>H526D</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MUT3</td>
<td>S531L</td>
<td></td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>katG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>315</td>
<td></td>
<td>$11</td>
<td>$4</td>
<td></td>
</tr>
<tr>
<td>MUT1</td>
<td>S315T1</td>
<td></td>
<td>14</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>MUT2</td>
<td>S315T2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>inhA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT1</td>
<td>-15/-16</td>
<td></td>
<td>$4</td>
<td>$1</td>
<td></td>
</tr>
<tr>
<td>WT2</td>
<td>-8</td>
<td></td>
<td></td>
<td></td>
<td>$1</td>
</tr>
<tr>
<td>MUT1</td>
<td>C15T</td>
<td></td>
<td>$4</td>
<td>$1</td>
<td></td>
</tr>
<tr>
<td>MUT2</td>
<td>A16G</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MUT3A</td>
<td>T8C</td>
<td></td>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>MUT3B</td>
<td>T8A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

WT- wild type band; MUT- mutant band; RIF- rifampicin; INH- isoniazid; MDR- multi-drug resistance

*Isolates with mutation(s) in the rpoB gene and none in the inhA/pro or katG gene
†Isolates with mutation(s) in the inhA/pro region and/or in the katG gene, with no mutation in the rpoB gene
‡Isolates with mutations in the rpoB gene and inhA/pro and/or katG gene
§Number of isolates that had presence of mutation band and the absence of corresponding wild type band
4.5 Drug Susceptibility Testing of NTM Isolates

All thirty six isolates from the *M. avium* complex (*M. avium*-1; *M. intracellulare*/ *M. chimaera*-27) and *M. abscessus* complex (*M. abscessus* subsp. *abscessus* -2; *M. abscessus* subsp. *massiliense*) were susceptible to both macrolides (clarithromycin, azithromycin) and aminoglycosides (kanamycin, amikacin, gentamicin) (Table 4.3).

**Table 4.3: Gene mutations in NTM species**

<table>
<thead>
<tr>
<th>Species/Subspecies</th>
<th>erm(41)a</th>
<th>rrlb</th>
<th>rrsb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C28b</td>
<td>T28c</td>
<td>WT</td>
</tr>
<tr>
<td><em>M. avium</em></td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>M. intracellulare</em></td>
<td>26</td>
<td></td>
<td>26</td>
</tr>
<tr>
<td><em>M. chimaera</em></td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>M. abscessus</em> subsp. <em>abscessus</em></td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><em>M. abscessus</em> subsp. <em>massiliense</em></td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td><em>M. abscessus</em> subsp. <em>bolletii</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**WT** = wild type probe comprises the most important resistance region of the rrl and rrs genes

**MUT** = mutation probes detect the most common resistance-mediating mutations in rrl and rrs genes

The erm(41) gene is examined for detection of resistance to macrolides (Clarithromycin or azithromycin) and is only present in members of the *M. abscessus* complex.

The erm(41) C28 probe detects a genotype that carries a C at position 28 of the erm(41) gene. When the erm(41) C28 probe stains positive, this indicates that the tested strain is sensitive to macrolides (except for strains with an additional rrl mutation).

The erm(41) T28 probe detects a genotype that carries a T instead of a C at position 28 of the erm(41) gene. When the erm(41) T28 probe stains positive, this indicates that the tested strain is resistant to macrolides.

**Note:** The probes erm(41) C28 and erm(41) T28 are only relevant for *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii*, but not for *M. abscessus* subsp. *massiliense*. Due to deletions in the erm(41) gene of *M. abscessus* subsp. *massiliense* the gene is nonfunctional, leading to macrolide sensitivity in spite of a developed erm(41) T28 band (except for strains with an additional rrl mutation).
The rrl gene is examined for detection of resistance to macrolides (clarithromycin or azithromycin).

The rrs gene is examined for detection of resistance to aminoglycosides (kanamycin, amikacin, gentamicin).
CHAPTER FIVE

5.0 DISCUSSION

This study was primarily set out to use line probe assay (LPA) to differentiate species of mycobacterial isolates obtained from a population based TB prevalence survey in Ghana and to determine their drug susceptibility patterns.

The survey participation rate was higher in females (93.6%) than in males (87.6%). Some of the possible explanations may include: male tend to be more mobile than females, more males go to work during survey time. However, based on the MTBC isolates identified, there was an indication that more males 91 (55.2 %) [OR= 2.3; p <0.001] than females 74 (44.8 %) had TB which is consistent with a WHO report that most TB cases generally occur in males (WHO, 2015). A study in Ghana also reported a higher TB prevalence for males (Ghanaians- 67.4%; foreigners- 68.8%) as compared to females (Yeboah-Manu et al., 2011). Other studies have reported similar results (Kurniawati, Sulaiman, & Gillani, 2012; Sharma et al., 2011). Socioeconomic and cultural factors may account for the relatively high prevalence of TB in men compared to women especially in developing countries. It has also been suggested that the differences in susceptibility to TB between males and females could be attributed to some biological factors such as sex hormones, genetic background and some metabolic activities (Neyrolles & Quintana-Murci, 2009). It was also observed from this study that the older age group (65 years and above) were the most infected with MTBC. Although some studies have shown that old age is a major risk factor for TB infection and progression to active disease, the risk was not statistically significant (p = 0.63) for this study (Bates et al., 2007; Donald, Marais, & Barry, 2010). This observation, however, correlates with results from Tanzania which showed a high number of people with tuberculosis aged 65 and above (Tanzania National TB Prevalence Survey.
Report, 2013). However, it contradicts an earlier report in Ghana which stated that majority (60-70%) of TB cases occur among persons in the reproductive age groups of 15-49 years (Bonsu et al., 2007). The high prevalence of TB in the very old adult population may be ascribed to compromised immune responses due to old age or as a result of reactivation of long held latent infection. Majority of the MTBC isolates were obtained from participants whose occupation is farming. The reason for this observation was not clearly identified in this study. However, a possible explanation may be due to sample proportion; thus farming being the major occupation reported by majority (29%) of the survey participants. The highest and lowest number of MTBC was isolated from participants from the Ashanti and Upper West regions, respectively. Occurrence of MDR was not significantly associated with gender, occupation and region of residence (p > 0.05). However, it was significantly associated with age group with the 55-64 year age category having the greater likelihood of MDR (p = 0.04).

Unlike MTBC, more females than males had NTM infections based on the positive cultures. These results are in line with other published reports (Griffith et al., 2007; Cassidy et al., 2009; Limo et al., 2015). It could also be seen that most of the NTM infections were from relatively older adults and people who engage in farming compared to younger aged participants and those associated with an occupation other than farming. Similar finding have been reported by other researchers (Chetchotisakd et al., 2007; Aliyu et al., 2013; Negin, Abimbola, & Marais, 2015). Compromised immune response and other age-related chronic diseases could account for the high numbers of NTM infections in the older aged individuals. It has been suggested that broken skin as a result of farming activities could allow tissue invasion and infection of NTM which are very ubiquitous in soil, water and dust (Chetchotisakd et al., 2007). This could explain why in this study, there were more species of NTM isolated from the participants originating from the Ashanti, Brong Ahafo,
Eastern, Volta (middle and upper parts) and Northern regions which are the major farming areas in Ghana. Majority 161 (97.6%) of MTBC isolates were \textit{M. tuberculosis} which is consistent with results from other studies. For instance, a study conducted in Greece showed that more than 99% of the isolates tested were \textit{M. tuberculosis} (Neonakis \textit{et al.}, 2007). In the current study, only four isolates (2.4%) were identified as \textit{M. africanum}. Although this percentage is consistent with what exists in many parts of the world, a higher percentage of \textit{M. africanum} was expected to be isolated from this study as \textit{M. africanum} is very prevalent in people of West Africa origin. Previous studies in Ghana reported about 23% and 20% \textit{M. africanum} isolation, respectively (Addo \textit{et al.}, 2007; Yeboah-Manu \textit{et al.}, 2011). The former study used biochemical methods only while the latter used both biochemical and molecular methods for identification and genotyping. Both previous studies did not cover the whole country- one region in the case of Addo \textit{et al.} (2007) and two regions in Yeboah-Manu \textit{et al.} (2011). A study in Senegal observed that there is regional variation in the proportion of TB caused by \textit{M. africanum} (Diop \textit{et al.}, 1976). This observation may be same in Ghana as can be seen in varied proportions of \textit{M. africanum} isolated in this current study compared to earlier studies in Ghana. Another study (Asante-Poku \textit{et al.}, 2015b) using both biochemical and molecular methods (spoligotyping) reported that \textit{M. africanum} accounted for about 17.1% of human TB in Ghana, a study that also demonstrated that these individuals were likely to be of the Ewe ethnic group. However, none of the four \textit{M. africanum} species identified in this study, were from individuals of Ewe ethnicity or lived in areas dominated by Ewes. Isolation rates of \textit{M. africanum} in many West African countries ranges from 9% to 28% and could go as high as 50% of all TB cases (de Jong \textit{et al.}, 2010). \textit{M. africanum} has been infrequently isolated in Europe, America and other parts of the world mostly from immigrants of West African origin (de Jong \textit{et al.}, 2010). Other species of the MTBC reported to cause TB in humans were not
isolated in this study. Some researchers have reported the lack of or very low isolation of *M. bovis* (Addo et al., 2007; Neonakis et al., 2007; Zivanovic et al., 2014). *M. bovis* BCG was not isolated confirming the notion that its isolation is more common in children than in adults who were the participants for this study. *M. microti* has low pathogenicity and thus for it to progressively cause disease in animals and humans, very large doses of the bacilli are required (O’Reilly & Daborn, 1995). Hence, *M. microti* is rarely isolated from human specimen.

Data on the different species of non-tuberculous mycobacteria (NTM) and their contribution to the cause of pulmonary diseases is very limited in Ghana. This study therefore identified some of the species of NTM circulating in Ghana. About one hundred and ninety six (196) mycobacterial isolates other than MTBC were isolated from sputum specimens and characterized. This high number of NTM isolated from sputum of participants exhibiting symptoms suggestive of TB in the nationwide survey poses a health challenge that must be addressed. The most frequently used method for the identification of NTM species is the GenoType Mycobacterium CM/AS assays (van der Werf et al., 2014). In this study, more than half (61 %) of the NTMs were correctly identified to the species level by the combined use of both the GenoType Mycobacterium CM and GenoType Mycobacterium AS assays. However, other studies have reported as high as over 80 % species level identification using these same assays (Ruiz et al., 2002; Makinen et al., 2006; Singh et al., 2013). Fourteen different species of NTM were identified with *M. fortuitum* 42 (21.4 %), *M. intracellulare* 27 (13.8 %) and *M. mageritense* 9 (4.6 %) being the top three most frequent species identified. These findings are consistent with results from similar studies conducted in Nigeria (Daniel et al., 2011; Pokam & Asuquo, 2012), Uganda (Asiimwe et al., 2013), India (Maurya et al., 2014; Singh et al., 2013) and Papua New Guinea (Ley et al., 2015). The higher frequencies of *M. fortuitum* and *M.
*intracellulare* reported in this study and others may be due to their wide distribution in the environment, especially in potable water (Whiley *et al*., 2012). *M. avium* complex (MAC) is a group of closely related NTM species and one of the most frequently isolated (Kim & Rheem, 2013). However the GenoType Mycobacterium CM identifies the individual species within the group separately rather than together as MAC. *M. intracellulare* which is part of the *M. avium complex* (MAC) is one of the most frequently isolated NTM in pulmonary specimens and has been associated with HIV infection (Kankya *et al*., 2011; Shao *et al*., 2015). However, in this study the HIV status of participants were not determined hence it was not possible to associate the isolation of *M. intracellulare* to HIV infection in the individuals concerned. On the other hand, *M. avium* also a member of MAC, is more common in disseminated disease (Griffiths *et al*., 2007). It was therefore not surprising that only one species of *M. avium* was isolated in this study because only sputum specimens were tested. The GenoType Mycobacterium CM is limited to the identification of only 14 frequently isolated NTM, therefore it is recommended that, the GenoType Mycobacterium AS be used for further differentiation when it was not possible to identify a species with the former. Three of the identified species from the current study (*M. asiaticum, M. goodii* and *M. lentiflavum*) resulted from the use of GenoType Mycobacterium AS. The remaining unidentified species (39 %) may be other acid fast gram positive bacteria whose correct identification is beyond the scope of the two assays used for this study. Therefore these organisms were inconclusively identified as *Mycobacterium* species, high G+C gram positive bacterium or other bacteria. Similar results were obtained in other studies (Russo *et al*., 2006; Aliyu *et al*., 2013; Shao *et al*., 2015). Sequencing may be helpful in the exact identification of these uncommon and unidentifiable NTM species. The clinical relevance of the identified NTM species based on the American Thoracic Society (ATS) and the Infectious Diseases Society of America (IDSA) guidelines
(ATS/IDSA, 2007) was not exploited in this study. The possibility of some of the NTMs identified in this study colonizing the individuals concerned without causing any infection could not be ruled out.

Determining drug resistance profile of mycobacterial isolates is very critical for effective management of the diseases they cause. This study considered resistance to INH and RIF as well as associated mutations in MTBC isolates. As expected, majority of the isolates (80.6%) were susceptible to both drugs while about one fifth of the isolates were resistant to at least one of the two drugs tested. Isoniazid monoresistance (10.9%) was relatively higher than rifampicin monoresistance (1.2%). This finding corroborates that of earlier studies in Ghana (Lawn et al., 2001; Owusu-Dabo et al., 2006) but contradicts a study in India (Rufai et al., 2014) where 29 (10.4%) showed INH monoresistance, and 62 (22.2%) showed RIF monoresistance out of a total number of 279 smear positive samples. The low frequency of RIF monoresistance could be attributed to its high potency and limited use as compared to INH. Resistance to RIF usually occurs alongside resistance to INH, leading to the development of MDR. The observed proportion of MDR (6.7%) from this study is higher than the 1.9% reported by the National Tuberculosis Programme (NTP) in 2013 (WHO, 2014) as well as previous studies in Ghana which reported MDR rates of between 2.2 -2.5% (Asante-Poku et al., 2015a; Homolka et al., 2010; Owusu-Dabo et al., 2006; Yeboah-Manu et al., 2012). Contrary to the relatively low MDR rate previously reported by these researchers in Ghana, findings from this study suggest that many people within communities may have MDR-TB without being detected. It is interesting to note that only 6 (19.2%) resistant isolates (excluding the MDR) from this study were obtained from people who have been previously diagnosed of TB. Four of these people had been on anti-TB treatment prior to the survey whilst two were still on anti-TB therapy at the time of the survey interview. Majority of these people could therefore be assumed to have primary
resistance to INH and/or RIF. The drug resistance status of one of the isolates was indeterminate because the bands generated after running the assay did not match any of the patterns on the interpretation chart provided. Of the 13 RIF resistant isolates, 6 (46.2%) showed a mutation (specifically D516V) in the 513-517 region of the *rpoB*. There were mutations in the 526-529 (H526Y) and 530-533 (S531L) regions in 3 (23.1%) and 2 (15.4%) isolates respectively. Two isolates each had a mutation other than the S531L in the 530-533 region of *rpoB*. These findings are consistent with that of other studies in terms of common mutations in the *rpoB*, however there are contradictions in the order of most frequently encountered mutations (Barnard *et al.*, 2008; Lacoma *et al.*, 2008; Tolani, D’souza, & Mistry, 2012). Resistance to isoniazid generally is associated with mutations in *katG* (50-95%) and *inhA* (10-30%) promoter region (Abal, Ahmad, & Mokaddas, 2002). In this study, it was frequently associated with mutations in codon 315 in *katG* (82.8%) which compares with reports from other settings (Abbadi *et al.*, 2009; Haas *et al.*, 1997; Musser *et al.*, 1996). The most common amino acid substitution is Ser (AGC) → Thr (ACC), but substitution of Ser with Arg, Asn, Ile, or Gly has also been reported (Bostanabad *et al.*, 2006; Marttila *et al.*, 1998). Other mutations in INH resistant isolates were seen in *inhA* (34.4%). Mutations in *katG* are more common and frequent than in *inhA* (Barnard *et al.*, 2008; Causse *et al.*, 2008). It is possible that other amino acid substitution and/or mutations in other codons other than 315 in *katG* as well as mutations in other genes associated with INH resistance may be present in the isolates used in this study but were not detectable by GenoType MTBDRplus version 2.0.

Drug susceptibility testing was done for species within the *M. avium* complex (*M. avium, M. intracellulare, M. chimaera*) and *M. abscessus* complex (*M. abscessus subsp. abscessus, M. abscessus subsp. massiliense, and M. abscessus subsp. bolletii*) because the NTM-DR was intended to be used for these group of NTM only. Infections caused by these NTM are
difficult to treat and the various species and subspecies within the complexes differ in drug resistance and treatment outcomes (Kehrmann et al., 2016). In addition to determination of resistance to macrolides and aminoglycosides, the use of this novel NTM-DR assay enabled the differentiation between *M. intracellulare* and *M. chimaera* as well as *M. abscessus* subspecies identification.

5.1 Limitations of study

The study had some limitations. This include the use of only one DST method as well as the inability to test drug resistance of other first line drugs, and the possible underestimated resistance to isoniazid because the GenoType MTBDR plus had been designed to detect mutations in a limited number of genes associated with isoniazid resistance. Again, differentiation to the species level as well as DST for all NTMs could not be done because the GenoType Mycobacterium CM/AS and NTM-DR assays were restricted to identification and detection of gene mutations in few NTM out of the many available. That notwithstanding the limitations did not compromise the quality of the study.
CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Based on the specific objectives and the findings obtained from the study, the following conclusions are made:

1. The predominant mycobacterial species causing TB in Ghana is *M. tuberculosis* followed by *M. africanum. M. bovis* rarely cause human pulmonary TB in Ghana.

2. Diverse species of NTM were found in sputum specimens from presumptive TB cases in Ghana with *M. fortuitum* being the most frequently isolated species.

3. An MDR rate of 6.7% with resistance to isoniazid and rifampicin commonly associated with mutations in the *katG* (Ser315Thr) and *rpoB* (Asp516Val) respectively.

4. All species and subspecies of the *M. avium* complex and *M. abscessus* complex respectively are susceptible to macrolides (clarithromycin, azithromycin) and aminoglycosides (kanamycin, amikacin and gentamicin).

5. GenoType Mycobacteria assay series are appropriate tools for rapid speciation of mycobacterial isolates and drug susceptibility testing in Ghana.

6.2 Recommendations from this study

The following are recommended:

1. Mycobacterial speciation and DST must be incorporated into algorithm for TB diagnosis at least at the district level of healthcare delivery.

2. Active TB case finding must be intensified all over the country in order to capture almost every TB patient in our communities and also identify MDR-TB strains for prompt and adequate treatment.
3. Drugs such as macrolides (clarithromycin, azithromycin) and aminoglycosides (kanamycin, amikacin and gentamicin) must be made available by government and other stakeholders in healthcare for the treatment of NTM infections caused by *M. avium* complex and *M. abscessus* complex

### 6.3 Recommendations for future studies/research

The following recommendations are made for further/ future studies:

1. Population-based TB prevalence survey should be conducted at least every five years in order to know the true TB burden at every point in time. The HIV status of survey participants must be determined in subsequent surveys.

2. In addition to the use of the GenoType Mycobacteria assays and/or other molecular DST methods, phenotypic DST (involving all first-line TB drugs) must be done.

3. Again, research efforts should be directed towards areas that will lead to understanding of NTM disease and their pathogenesis for prevention and treatment. This should include among others a baseline study to determine the drug resistance profile of commonly isolated NTMs of clinical relevance. Just as exists for TB, there should be a national policy guideline on diagnosis and treatment of NTM disease.
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Future Research Directions

- Identify new antigens for tuberculosis
- Develop new vaccines
- Improve diagnostic tools
- Study the role of Mycobacterium africanum in the context of human tuberculosis

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Zivanovic, I., Vukovic, D., Dakic, I., & Savic, B. (2014). Species of mycobacterium tuberculosis complex and nontuberculous mycobacteria in respiratory specimens from Serbia. *Archives of Biological Sciences*, 66(2), 553-561. [http://dx.doi.org/10.2298/abs1402553z](http://dx.doi.org/10.2298/abs1402553z)
### APPENDICES

### APPENDIX A: NATIONAL TUBERCULOSIS PREVALENCE SURVEY

#### CLUSTERS

<table>
<thead>
<tr>
<th>REGION</th>
<th>CLUSTERS</th>
</tr>
</thead>
<tbody>
<tr>
<td>GREATER ACCRA</td>
<td>ACHIMOTA, DOME, OLD NINGO, TEMA NEW TOWN, ASHAIMAN, ACCRA NEW TOWN, KORLE GONNO, NEW APLAKU, OSU, RUSSIA, NEW ACHIMOTA, ADENTAN EAST, GBAWE, AVENOR</td>
</tr>
<tr>
<td>CENTRAL</td>
<td>ABODOM, AGONA NKUM, ASSIN ANYINABREM, BONTROASE, NYANYANO KAKRABA, SOLOMON ABRESA, CAPE COAST, SALTPOND</td>
</tr>
<tr>
<td>WESTERN</td>
<td>ASUOPORI, AWASO, ENYINABREM, HUNI VALLEY, KWATENGKROM, EAST TANOKROM, ESSIKADO, MELENU, SAMREBOI</td>
</tr>
<tr>
<td>VOLTA</td>
<td>AGBEDRAFOR, HO BANKOE, HOHOE, LOLITO, VAKPO, JUAPONG, KPASSA, DWADWE, POASE CEMENT, AVOEME</td>
</tr>
<tr>
<td>EASTERN</td>
<td>ADAKOPE, ADARKWA, ADIIKPO, AKIM ATIANKAMA, AMANKWA TORNU, ASAMANKESE, DODI ASANTEKROM</td>
</tr>
<tr>
<td>Region</td>
<td>Districts</td>
</tr>
<tr>
<td>-----------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td><strong>ASHANTI</strong></td>
<td>KOTOKUOM&lt;br&gt;NKAWKAW&lt;br&gt;NYENSI AWUKUGUA&lt;br&gt;TAKROWASE&lt;br&gt;APUTUOGYA&lt;br&gt;KENYASE&lt;br&gt;KUMASI ANLOGA&lt;br&gt;KUMASI MOSHIE ZONGO&lt;br&gt;MAMPONG WORAKESE&lt;br&gt;OFFINSO AGYEMFRA&lt;br&gt;SAMSO&lt;br&gt;AGOGO&lt;br&gt;AKORABUOKROM&lt;br&gt;AMAKOM&lt;br.DOMPOASE&lt;br&gt;FUFUO&lt;br&gt;KUMASI TANOSO&lt;br&gt;NIMERESO&lt;br&gt;NKENKAASU&lt;br&gt;TOKWAI&lt;br&gt;SEPE WUSUANSA&lt;br&gt;TUTUKA&lt;br&gt;OLD SUAME</td>
</tr>
<tr>
<td><strong>BRONG AHAFO</strong></td>
<td>BONSU&lt;br&gt;KOKOA&lt;br&gt;NKOMI&lt;br&gt;AMASU&lt;br&gt;BIADAN&lt;br&gt;CHIEF AKURA&lt;br&gt;DUAYAW NKWANTA&lt;br&gt;TANOSO&lt;br&gt;FIANKO</td>
</tr>
<tr>
<td><strong>NORTHERN</strong></td>
<td>CHEREPONI&lt;br&gt;CHIBE&lt;br&gt;GAMBAGA&lt;br&gt;KPALGBINI&lt;br&gt;NAKPALI&lt;br&gt;TONG&lt;br&gt;TAMALE MOSHIE ZONGO&lt;br&gt;LUNGBUNGBA&lt;br&gt;KUSA WGU&lt;brKPANDAI</td>
</tr>
<tr>
<td><strong>UPPER EAST</strong></td>
<td>BALANSA AKUYERI&lt;br&gt;KPUGRI KPASALKO&lt;br&gt;ZUARONGO DABORO&lt;br&gt;BAWKU DADURE&lt;br&gt;DUAA- APWONGO</td>
</tr>
<tr>
<td><strong>UPPER WEST</strong></td>
<td>KPALIWOGO&lt;br&gt;JIRAPA&lt;br&gt;KONYANGANGU</td>
</tr>
</tbody>
</table>
APPENDIX B: MUTATIONS ASSOCIATED WITH RESISTANCE TO ISONIAZID AND RIFAMPICIN

[I] GenoType MTBDRplus version 2.0 mutations in the katG gene and the corresponding wild type and mutation bands

<table>
<thead>
<tr>
<th>Failing wild type band</th>
<th>Codon analyzed</th>
<th>Developing mutation band</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>katG WT</td>
<td>315</td>
<td>katG MUT1</td>
<td>S315T1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>katG MUT2</td>
<td>S315T2</td>
</tr>
</tbody>
</table>

[II] GenoType MTBDRplus version 2.0 mutations in the inhA promoter region and the corresponding wild type and mutation bands

<table>
<thead>
<tr>
<th>Failing wild type band</th>
<th>Analyzed nucleic acid position</th>
<th>Developing mutation band</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>inhA WT1</td>
<td>-15</td>
<td>inhA MUT1</td>
<td>C-15T</td>
</tr>
<tr>
<td></td>
<td>-16</td>
<td>inhA MUT2</td>
<td>A-16G</td>
</tr>
<tr>
<td>inhA WT2</td>
<td>-8</td>
<td>inhA MUT3A</td>
<td>T-8C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>inhA MUT3B</td>
<td>T-8A</td>
</tr>
</tbody>
</table>
GenoType MTBDRplus version 2.0 mutations in the *rpoB* gene and the corresponding wild type and mutation bands

<table>
<thead>
<tr>
<th>RpoB WT</th>
<th>Codons analyzed</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>505-509</td>
<td></td>
<td>F164S</td>
</tr>
<tr>
<td>510-513</td>
<td></td>
<td>L311P*</td>
</tr>
<tr>
<td>510-517</td>
<td></td>
<td>Q313L*</td>
</tr>
<tr>
<td>513-516</td>
<td></td>
<td>S516V</td>
</tr>
<tr>
<td>516-520</td>
<td></td>
<td>deT518*</td>
</tr>
<tr>
<td>518-525</td>
<td></td>
<td>S522L</td>
</tr>
<tr>
<td>526-529</td>
<td></td>
<td>H526D</td>
</tr>
<tr>
<td>530-533</td>
<td></td>
<td>L535P</td>
</tr>
</tbody>
</table>

* This rare mutation has only been detected theoretically in silico yet. It is therefore possible that it cannot be detected in vitro.
### APPENDIX C: GenoType Mycobacterium ASSAY KIT CONTENTS

<table>
<thead>
<tr>
<th>Item</th>
<th>Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane strips coated with specific probes (STRIPS)</td>
<td></td>
<td>12/96</td>
</tr>
<tr>
<td>Primers Nucleotide Mix (PNM), contains primers, nucleotides, &lt; 1%</td>
<td></td>
<td>0.5 ml/4 ml</td>
</tr>
<tr>
<td>Dimethyl Sulfoxide, dye; Master Mix A; Master Mix B</td>
<td></td>
<td>0.3 ml/2.4 ml</td>
</tr>
<tr>
<td>Denaturation Solution (DEN) contains &lt; 2% NaOH, dye (ready to use)</td>
<td></td>
<td>30 ml/120 ml</td>
</tr>
<tr>
<td>Hybridization Buffer (HYB) contains 8-10% anionic tenside, dye (ready to use)</td>
<td></td>
<td>20 ml/120 ml</td>
</tr>
<tr>
<td>Stringent Wash Solution (STR) (ready to use) contains &gt; 25% of a quaternary ammonium compound, &lt; 1% anionic tenside, dye</td>
<td></td>
<td>50 ml/360 ml</td>
</tr>
<tr>
<td>Rinse Solution (RIN) (ready to use) contains buffer, &lt; 1% NaCl, &lt; 1% anionic tenside</td>
<td></td>
<td>0.2 ml/1.2 ml</td>
</tr>
<tr>
<td>Conjugate Concentrate (CON-C) contains streptavidin-conjugated alkaline phosphatase, dye</td>
<td></td>
<td>20 ml/120 ml</td>
</tr>
<tr>
<td>Conjugate Buffer (CON-D) contains buffer, 1% blocking reagent, &lt; 1% NaCl</td>
<td></td>
<td>0.2 ml/1.2 ml</td>
</tr>
<tr>
<td>Substrate Concentrate (SUB-C) contains Dimethyl Sulfoxide, Substrate solution</td>
<td></td>
<td>20 ml/120 ml</td>
</tr>
<tr>
<td>Substrate Buffer (SUB-D) contains buffer, &lt; 1% MgCl, &lt; 1% NaCl</td>
<td></td>
<td>20 ml/120 ml</td>
</tr>
<tr>
<td>Tray, evaluation sheet</td>
<td></td>
<td>2/4</td>
</tr>
<tr>
<td>Template</td>
<td></td>
<td>1/4</td>
</tr>
</tbody>
</table>

*Adapted from OSR Line Probe Assay (LiPA) IOS EBP-TM 002, Section 5.1 -5.2
APPENDIX D: WHO/IUATLD GRADING SCALE FOR SPUTUM SMEAR MICROSCOPY

[I] Grading Chart for ZN Microscopy (100x oil immersion objective and 10x eye piece)

<table>
<thead>
<tr>
<th>ZN staining grading</th>
<th>Reporting /Grading</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;10 AFB/field after examination of 20 fields</td>
<td>Positive, 3+</td>
</tr>
<tr>
<td>1-10 AFB/field after examination of 50 fields</td>
<td>Positive, 2+</td>
</tr>
<tr>
<td>10-99 AFB/100 field</td>
<td>Positive, 1+</td>
</tr>
<tr>
<td>1-9 AFB/100 field</td>
<td>Positive, scanty</td>
</tr>
<tr>
<td>No AFB per 100 fields</td>
<td>Negative (No AFBs Seen)</td>
</tr>
</tbody>
</table>

[II] Recommended number of AFBs and fields for grading fluorescent stained smears

<table>
<thead>
<tr>
<th>200-250x; 1 length=30 fields=300 HPF</th>
<th>400x; 1 length=40 fields=200 HPF</th>
<th>FM Reporting/ Grading</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;100 AFB/1 field</td>
<td>&gt;50 AFB/1 field</td>
<td>Positive, 3+</td>
</tr>
<tr>
<td>10-100 AFB/1 field</td>
<td>5-50 AFB/1 field</td>
<td>Positive, 2+</td>
</tr>
<tr>
<td>30-299 AFB/1 Length</td>
<td>20-199 AFB/1 Length</td>
<td>Positive, 1+</td>
</tr>
<tr>
<td>1-29 AFB/1 Length</td>
<td>1-19 AFB/1 Length</td>
<td>Positive, Scanty</td>
</tr>
<tr>
<td>Zero AFB/1 Length</td>
<td>Zero AFB/1 Length</td>
<td>Negative (No AFBs Seen)</td>
</tr>
</tbody>
</table>

http://ugspace.ug.edu.gh/
APPENDIX E: BD MGIT TBC ID TEST KIT

TBcID cassettes for immunochromatographic identification of *M. tuberculosis*  
(Adapted from [www.bd.com](http://www.bd.com))
APPENDIX F: OVERVIEW OF LINE PROBE ASSAY PROCEDURE

Illustration of the DNA-strip technology by Hain Life Science. The GenoType MTBC, Mycobacterium CM, Mycobacterium AS, MTBDR\textit{plus} and NTM-DR molecular LPA are divided into three procedures: (1) DNA extraction from NaOH-NALC decontaminated smear positive specimens or from cultured isolates (solid or liquid media), (2) a multiplex Polymerase Chain Reaction amplification (PCR) and (3) the reverse hybridization (including detection and evaluation).
APPENDIX G: EVALUATION AND INTERPRETATION OF HYBRIDIZATION RESULTS

[1] Interpretation chart for GenoType MTBC

Approximately 5% of the subspecies *M. bovis caprae* exhibit a banding pattern according to the right column.
1) Does not include other species of the *M. avium* complex.

2) *M. immunogenum* (belongs to the *M. abscessus*/*M. chelonae* group) shows the same banding pattern as *M. chelonae* or *M. abscessus*. In case the quality and/or quantity of the extracted DNA does not allow an efficient amplification, the amplicon hybridizing both to the Genus Control and to band 6 may have been supplanted due to competition of the single reactions during amplification. In this case, *M. abscessus* shows the banding pattern identifying *M. chelonae*. However, as long as the specifications given in these instructions for use are observed and the DNA polymerase used for performance evaluation is applied, this does not occur.

3) Due to sequence variations within the species two different *M. fortuitum* banding patterns do occur.

*M. mageritense* shows the *M. fortuitum* banding pattern as depicted in the right column.

4) *M. chimaera* shows the same banding pattern as *M. intracellulare*.

5) *M. paraffinicum* and *M. parascrofulaceum* show the same banding pattern as *M. scrofulaceum*.

6) *M. haemophilum*, *M. palustre*, and *M. nebraskense* show the same banding pattern as *M. malmoense*. *M. haemophilum*/*M. nebraskense* can be identified with the GenoType Mycobacterium AS kit.

7) *M. ulcerans* can be identified with the GenoType Mycobacterium AS kit.

8) If band 15 has also stained positive, additional detection methods must be applied.

9) *M. alvei* and *M. septicum* show the same banding pattern as *M. peregrinum*. 
### Interpretation chart for GenoType Mycobacterium AS

<table>
<thead>
<tr>
<th>High GC gram-positive bacteria</th>
<th>Mycobacterium species</th>
<th>M. smegmatis</th>
<th>M. celatum</th>
<th>M. gordonae</th>
<th>M. szulgai</th>
<th>M. intermedium</th>
<th>M. kansasii</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1) When using liquid medium, contaminating bacteria may generate the same banding pattern. The banding pattern indicating the presence of *M. celatum* or *M. smegmatis* is therefore only valid when the DNA was extracted from bacteria grown on solid medium (single colony/morphologically identical colonies).

2) *M. triplex* shows the same banding pattern as *M. genavense*.

3) *M. szulgai* and *M. intermedium* can be identified using the GenoType Mycobacterium CM kit. *M. szulgai* will display the banding pattern 1, 2, 3, 10, and 11, *M. intermedium* will display the banding pattern 1, 2, 3, and 10.

4) *M. nebraskense* shows the same banding pattern as *M. haemophilum*.

5) Due to sequence variations 4 different *M. kansasii* banding patterns are possible.
If all wild type bands display a signal, this is classified as positive and marked in the WT column of the respective gene as “+”.

If at least one of the wild type bands is absent, this is classified as negative and marked in the WT column as “−”. Negative entries are only made to the mutation columns when none of the mutation bands display a coloration.

If at least one of the mutation bands display a coloration, this is classified as positive and the MUT column of the respective gene is marked with a “+”.

Below, two of the examples shown above are explicated:

Example 1 shows the wild type banding pattern. All wild type probes, but none of the mutation probes display a signal; hence, the evaluation chart shows “+” in the three wild type columns and “−” in the three mutation columns. Accordingly, the boxes “RMP sensitive” and “INH sensitive” are marked with a “+”.

One of the rpoB and the katG wild type probes are missing in example 5; hence, the boxes for “rpoB WT” and “katG WT” are marked with a “−”.

As none of the mutation probes are developed, these boxes are also marked with a “−”. The inhA promoter region does not deviate from the wild type pattern. The strain is evaluated as resistant to RMP and INH.
[V] Interpretation chart for GenoType NTM-DR

Conjugate Control (CC)
A line must develop in this zone, documenting the efficiency of conjugate binding and substrate reaction.

Universal Control (UC)
This zone detects, as far as is known, all mycobacteria and members of the group of gram-positive bacteria with a high G+C content. If this zone and the Conjugate Control zone stain positive but the remaining banding pattern cannot be assigned to a specific mycobacterium, additional methods have to be applied to identify the respective bacterial species.

Species-specific probes (SP1-SP10)
Specific probes, for evaluation see interpretation chart (above)
Locus Controls (\textit{erm}(41), \textit{rrl}, and \textit{rrs})

The Locus Control zones detect a gene region specific for the respective locus. Since the \textit{erm}(41) locus is only present in members of the \textit{M. abscessus} complex, the \textit{erm}(41) probe will only stain positive for members of this complex. In case of a positive test result (evaluable wild type or mutation banding pattern), the signals of the Locus Control bands may be weak.

\textit{erm}(41)

The \textit{erm}(41) gene is examined for detection of resistance to macrolides (clarithromycin or azithromycin) and is only present in members of the \textit{M. abscessus} complex.

The \textit{erm}(41) C28 probe detects a genotype that carries a C at position 28 of the \textit{erm}(41) gene. When the \textit{erm}(41) C28 probe stains positive, this indicates that the tested strain is sensitive to macrolides (except for strains with an additional \textit{rrl} mutation).

The \textit{erm}(41) T28 probe detects a genotype that carries a T instead of a C at position 28 of the \textit{erm}(41) gene. When the \textit{erm}(41) T28 probe stains positive, this indicates that the tested strain is resistant to macrolides.

The probes \textit{erm}(41) C28 and \textit{erm}(41) T28 are only relevant for \textit{M. abscessus} subsp. \textit{abscessus} and \textit{M. abscessus} subsp. \textit{bolletii}, but not for \textit{M. abscessus} subsp. \textit{massiliense}. Due to deletions in the \textit{erm}(41) gene of \textit{M. abscessus} subsp. \textit{massiliense} the gene is nonfunctional, leading to macrolide sensitivity in spite of a developed \textit{erm}(41) T28 band (except for strains with an additional \textit{rrl} mutation).

\textit{rrl}

The \textit{rrl} gene is examined for detection of resistance to macrolides (clarithromycin or azithromycin). The wild type probe comprises the most important resistance region of the \textit{rrl} gene. When the wild type probe stains positive, there is no detectable mutation within the examined region. In case of a mutation, the respective amplicon cannot bind to the wild type probe resulting in the absence of the wild type probe signal. The mutation probes detect the most common resistance-mediating mutations. Each pattern deviating from the wild type pattern indicates, as far as is known, a macrolide resistance of the tested strain.

\textit{rrs}

The \textit{rrs} gene is examined for detection of resistance to aminoglycosides (kanamycin, amikacin, gentamicin). The wild type probe comprises the most important resistance region of the \textit{rrs} gene. When the wild type probe stains positive, there is no detectable mutation within the examined region. In case of a mutation, the respective amplicon cannot bind to the wild type probe resulting in the absence of the wild type probe signal. The mutation probe detects the most common resistance-mediating mutation. Each pattern deviating from the wild type pattern indicates, as far as is known, an aminoglycoside resistance of the tested strain.
# APPENDIX H: STATISTICAL ANALYSIS

<table>
<thead>
<tr>
<th>Gender</th>
<th>OR MTBC</th>
<th>95% CI</th>
<th>Fisher's exact test</th>
<th>OR NTM</th>
<th>95% CI</th>
<th>Fisher's exact test</th>
<th>OR MDR</th>
<th>95% CI</th>
<th>Fisher's exact test</th>
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<tbody>
<tr>
<td>Male</td>
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<td>1.5133, 3.5407</td>
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</table>

<table>
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<th>Age</th>
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<th>Fisher's exact test</th>
<th>OR NTM</th>
<th>95% CI</th>
<th>Fisher's exact test</th>
<th>OR MDR</th>
<th>95% CI</th>
<th>Fisher's exact test</th>
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<td>15-24</td>
<td>0.8820</td>
<td>0.5005, 1.5544</td>
<td>0.7738</td>
<td>1.1337</td>
<td>0.6433, 1.9980</td>
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<td>25-34</td>
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<th>Occupation</th>
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<th>Fisher's exact test</th>
<th>OR NTM</th>
<th>95% CI</th>
<th>Fisher's exact test</th>
<th>OR MDR</th>
<th>95% CI</th>
<th>Fisher's exact test</th>
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86
<table>
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<tr>
<th>Region</th>
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<th>OR NTM</th>
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<th>OR MDR</th>
<th>95% CI</th>
<th>Fisher's exact test</th>
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</table>
APPENDIX J: ETHICAL CLEARANCE CERTIFICATE

[Image of the document]

7th March, 2012

ETHICAL CLEARANCE

FEDERALWIDE ASSURANCE FWA 00001824
NMIMR-IRB CPN 031/10-11 amend. 2012
IRB 00001276
IORG 0000908

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

TITLE OF PROTOCOL: Assessing tuberculosis disease prevalence in Ghana through a population based survey

PRINCIPAL INVESTIGATOR: Dr. Frank Bonu

CO-INVESTIGATORS: Dr. Kwasi Addo, Prof. John Gyapong & Dr. Margaret Gyapong & Prof. Kwadwo Koram

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

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

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

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

Signature of Chairman: ………………………………………………………………………………………………………

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

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

http://ugspace.ug.edu.gh/