UNIVERSITY OF CAPE COAST

SPOLIGOTYPE DIVERSITY AND DRUG RESISTANCE OF ARCHIVED *MYCOBACTERIUM TUBERCULOSIS* COMPLEX ISOLATED FROM PATIENTS PRESENTING AT THE GREATER ACCRA REGIONAL HOSPITAL

BY

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

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DECLARATION

Candidate's Declaration

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

Candidate's Signature: ……..... Date: ……

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Supervisors' Declaration

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

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ABSTRACT

Tuberculosis remains an important cause of infection in Ghana and Africa despite efforts put in place to eradicate it. Investigations to understand the population dynamics and circulating strains of the *Mycobacterium tuberculosis* complex will provide effective strategies in the control of the disease. This study was aimed at investigating and establishing the spoligotype diversity and drug-resistance profile of archived *Mycobacterium tuberculosis* complex isolates. A total of ninety (90) archived MTB isolates were analysed using the Capilia TB-Neo Immunochromatographic test and spoligotype to determine strain lineages. The samples were also subjected to the HAIN Genotype MTBDR*plus* assay to ascertain their drug resistance profiles. Spoligotyping results were used to query the International Spoligotyping database SITVIT2. The isolates generated 40 distinct spoligotype patterns with 5 major clusters. Identified families were Cameroon, T1, T3, AFRI_2, H3, LAM 9, AFRI_1, CAS, T4, AFRI, EAI 5, and Family 33. The dominant lineages were the Cameroon lineage with SIT 61 (31.11%), T1 family SIT 53 (11.11%), T3 family SIT 504 (6.67%), and *Mycobacterium africanum* (West African type 2, SIT 331) (3.33%). MIRU-VNTR*plus* database returned a cluster rate of 56.66% with Cameroon SIT 61 having the largest cluster. The HAIN Genotype MTBDR*plus* assay detected 20.45% (18/88) resistant isolates with 5.68% (5/88) being MDR. Significant mutations were seen in codon 526, 516, and 531 of the *rpoB* gene and on codon 315 of the *katG* gene for isoniazid and in *inhA* -15C/T of the promoter region.

KEYWORDS

Capilia TB-Neo Test

Drug-Resistant

Genetic diversity

HAIN Genotype MTBDR*plus* assay

Mycobacterium tuberculosis Lineage

Spoligotyping

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DEDICATION

To my children Jordan Eyram Kofi Bedzra, Faith Elorm Abena Bedzra, and in memory of my late wife Irene Narkie Bedzra

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LIST OF ABBREVIATIONS

AFB	Acid-fast Bacilli
BCG	Bacille-Calmette Guérin
bp	Base pair
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide triphosphate
DOTS	Directly Observed Treatment Short Course
DST	Drug Susceptibility Testing
EDTA	Ethylenediamine tetraacetic acid
GAEC	Ghana Atomic Energy Commission
INH	Isoniazid
LJ	Löwenstein-Jensen
MDR-TB	Multi-drug-resistant tuberculosis
mL	Millilitre
MTB	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis*-complex
NALC	N-acetyl cysteine
NTM	Non-tuberculous Mycobacteria
PCR	Polymerase Chain Reaction
RAMSRI	Radiological and Medical Sciences Research Institute
RIF	Rifampicin
TB	Tuberculosis
WHO	World Health Organization

ZN	Ziehl-Neelsen
SDG	Sustainable Development Goals
HIV	Human Immuno-deficiency Virus
RR-TB	Rifampicin-resistant Tuberculosis
NTP/NTCP	National Tuberculosis Control Programme
DR-TB	Drug-resistant tuberculosis
DR	Direct Repeat Region of the MTBC genome
LPA	Line Probe Assay
RRDR	Rifampicin-resistance Determining Region
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
IS	Insertion Sequence
XDR-TB	Extensively drug-resistant
NTHSSP	National Tuberculosis Health Sector Strategic Plan
MOH	Ministry of Health, Ghana
GHS	Ghana Health Service
CIDA	Canadian Government International Development Agency
OPD	Out Patient Department
CAS	Central Asian spoligotype family
LAM	Latin American-Mediterranean spoligotype family
CSF	Cerebrospinal Fluid
EMB	Ethambutol
PZA	Pyrazinamide

SIT	Spoligotype International Type
MAF	*Mycobacterium africanum*
SSPE	Sodium chloride – Sodium phosphate – EDTA solution
SDS	Sodium dodecyl sulphate solution
EAI	East African Indian spoligotype clade
KBTH	Korle Bu Teaching Hospital
M.	*Mycobacterium*
STR	Streptomycin
UCC	University of Cape Coast
UPGMA	Unweighted Pair Group with Arithmetic Mean tree
VNTR	Variable Number Tandem Repeat
MIRU	Multi-locus Interspersed Repetitive Unit
CRISPRs	Clustered Regularly Interspaced Short Palindromic Repeats
Min	Minute
s	Second
hr	Hour

CHAPTER ONE

INTRODUCTION

The disease, tuberculosis (TB), is classified as part of the oldest communicable diseases, a leading cause of death and infection on the human population. Notwithstanding the progress made in medical sciences and research, tuberculosis disease continues to ravage mankind (Myung-Sang *et al*., 2017). Tuberculosis (TB), an infectious bacterial disease also affects livestock and it exerts considerable morbidity. The disease is prevalent worldwide, although much more predominant in developing than in developed countries. The aetiological cause of tuberculosis is the MTBC. As a complex, it comprises the following: *M. tuberculosis*, *M. bovis*, *M. bovis* Bacillus Calmette-Guérin (BCG) (Calmette *et al*., 1927), *M. caprae (*Aranaz *et al*., 2003), *M. microti* (Reed, 1957; Wells & Oxon, 1937), *M. canetti* (van Soolingin *et al*., 1997), *M. pinnipedii* (Cousins *et al*., 2003), *M. suricattae* (Parsons *et al*., 2013), *M. orygis* (van Ingen *et al*., 2012) and *M. mungi* (Alexander *et al*., 2010).

Mycobacterium tuberculosis (MTB) has afflicted mankind for a very long time. The relevance of tuberculosis as a public health disease and the importance of the disease to human resource development and the economy of most countries in the world are well acknowledged (Konhya *et al*., 1980; Brosch *et al*., 2002). The mode by which the disease is transmitted is airborne and mostly human to human. Tracing infected persons and individuals who have come into contact with such persons helps in the prevention and spread of the disease. This is achieved by microscopically examining smears for the acid-fast bacilli by Ziehl-Neelsen stain (ZN stain).

The staining of the mycobacteria is one of the rapid procedures in the detection of the bacilli and a common first-line screening test, particularly in resource-limited settings. Although other identification tests are available, mycobacterial culture has remained as the "Gold Standard" in the confirmation of tuberculosis disease (Taylor *et al*., 2001). Despite this fact, the procedure is cumbersome and time-consuming.

In the year 2015, the United Nations under the Sustainable Development Goals (SDGs) targeted to end the tuberculosis epidemic by 2030. Before the setting of this target, the World Health Organization's "End TB Strategy" group which was formed in 2014, had called for a reduction in all forms of deaths to 90% and 80% for incidences by 2030 (WHO, 2016). Globally, the incidence of tuberculosis was largely under-reported. The burden of the disease globally in the year 2012 was estimated to be around 8.7 million (i.e., 125 cases per 100,000 populations) (WHO, 2012). In 2014, 9.6 million people were diagnosed as having TB by the WHO. The organization also projected that 3 million individuals with the disease are "missed" each year by health systems. This leads to the continuous infection of the disease by airborne transmission in our communities (WHO, 2014; Yuen *et al*., 2015). The estimated new TB case worldwide was 10.4 million out of which males, females, and children under 15 years were 56.0%, 34.0%, and 10.0% respectively (WHO, 2016). A population of 1.2 million had co-infection with HIV which was estimated to be around 11.0% (Naidoo *et al*., 2017). Case notification and detection is a big challenge. The WHO Africa Region contributed 1,333,504 (21.0%) to the global figure of 6,364,194.

The percentage of new and relapse cases identified from the notified cases was around 97.2%. The remaining 2.8% could be attributed to resistant strains. These six countries: Nigeria, South Africa, Indonesia, China, Pakistan, and India in total have contributed 60.0% of the new cases. In 2017, 10.0 million people were diagnosed as having TB and the death toll was 1.6 million (0.3 million were co-infected with HIV). The African continent contributed 25.0% of all new cases. In the year 2019, a WHO report states that an estimated population of infected persons with TB was in the range of 9.0 – 11.1 million in 2018; this figure has remained stable in recent years. The disease burden differs amongst nations with males aged 15 years and above accounting for 57.0% of all forms of the disease (WHO, 2019). Globally, 186,772 multi-drug resistant and rifampicin resistant tuberculosis cases were seen and reported in the year 2018, an increase from 160,684 in the year 2017. The report indicates 156,071 cases were registered for treatment, an increase in the 2017 figure of 139,114. There is an under-estimation of the disease with over 4,500 victims dying every single day and close to 30,000 people falling ill (World TB Day Bulletin, 2019). Untreated persons can infect as many as 15 individuals per year (WHO, 2015).

In line with these facts, the devastating consequence of the disease is enormous. Data from neighbouring countries within the sub-region also indicates that TB is a major public health challenge (WHO, 2008b; Yeboah-Manu *et al.*, 2011). The population of Ghanaians that are infected by the aetiological agent every year is estimated at 250,000 and approximately half of the population is likely to show signs of infection.

Case finding and treatment of active TB disease will be the key approach to interrupting transmission of TB and reducing incidence (WHO, 2010). The estimated National Prevalence of tuberculosis in Ghana during the period 2014 was 282 (111-530) per 100 000 population of TB cases with a projected 14668 new cases and a detection rate of 33.0% which fell short of the African regional average of 47% and the World Health Organizations target of 70.0% (WHO, 2015). In the year 2016, Ghana became the first African country represented by the National Tuberculosis Control Programme (NTP) to have collaborated with the London School of Hygiene and Tropical Medicine to survey to unearth the true impact of tuberculosis on patients in terms of cost to their treatment. The study focused on the public health facilities that were administering drugs to both groups of patients infected with susceptible strains and resistant strains (WHO, 2018).

In Ghana, surveillance systems put in place by the National Tuberculosis Control Programme (NTP) is projecting an estimated 54.2 per 100,000 populations. The male to female ratio of diagnosed cases is 2:1. The TB/HIV Co-infection Rate is 22.0% (GHS, 2017). For global control and fight against TB, the DOTS strategy was adopted as a WHO global policy. The TB BCG is currently the main available vaccine that is used to prevent severe childhood disease but it has not protected adults and is not expected to reduce the transmission due to TB (Narain & Lo, 2004). All members of the MTBC are related closely and belong to the family Mycobacteriaceae. This family consists of more than 197 species and 14 subspecies (including substitutes) within the genus Mycobacterium (Parrish, 2019).

These numbers increase as research utilizes molecular methods that ensure discriminatory power over orthodox phenotypic techniques to differentiate such organisms (Parrish, 2019). Species that belong to the MTBC are closely linked at the genetic level**.** The most common species that have predominately affected humans are *M. tuberculosis* (MTB) and *M. africanum* (MAF), while cattle are recognized as the host of *M. bovis* (*M. bovis*), a domesticated animal (Smith *et al*., 2006). Although differences exist in the type of host of MTB, the organisms have similarities at almost 99.9% at the genetic level and their 16S rRNA sequences are identical (Brosch *et al*., 2002). The introduction and improvement of molecular typing procedures in the last decade have contributed to the improvement in studies of communicable diseases especially tuberculosis. Typing MTBC strains have great potential for epidemiological studies. These techniques are used to investigate outbreaks, to study transmission and outbreaks within defined geographical settings, to detect MTB strains that have evolved into MDR strains (Small *et al*., 1993). Development and introduction of molecular typing methods have been one of the most essential epidemiological tools used to study the *Mycobacterium tuberculosis* complex.

van Embden *et al*. (1993) proposed a standardized procedure of DNA fingerprinting that employs restriction enzymes to generate strain-specific patterns based on the variations in the number of repeats and the position of the insertion sequence (IS*6110*) to investigate the transmission and strains causing disease. A total of 36 potential subfamilies or sub-clades of MTB complex were tentatively identified and divided into 8 main families worldwide (Brudey *et al.*, 2006; Filliol *et al*., 2002).

The Beijing strain is a successful strain of MTB. It is very common in geographical areas like South East Asia, South Africa and prevalent in the Russian Federation as well as in recently diagnosed tuberculosis patients and is strongly linked to MDR cases (Bjune Gunnar, 2005).

Spoligotyping

Spoligotyping, which is a PCR-centered molecular typing technique, identifies and categorizes members of the MTBC by multiplication of the vastly polymorphic direct repeat locus of the MTB genome. Information is generated and centered on the polymorphisms seen in the direct repeat region (Kremer *et al*., 1999; Kamerbeek *et al*., 1997). This technique identifies different strains of MTB causing infections in epidemiological studies. It is very efficient and reliable when undertaking surveillance work as well as the geographical distribution of strains and infections (Gori *et al*., 2005). It is extensively used to understand circulating strains in a specific area to identify the source of an outbreak. Spoligotyping is comparatively fast, generates easy to read DNA patterns that can be digitized, easy comparison between laboratories in terms of results communication. It can be performed directly on cell lysates and non-viable bacteria. Information gathered will assist in the development of control strategies against future outbreaks.

Drug-Resistance

The probability of a bacteria becoming resistant to a drug is mostly influenced by the genes present and the geographical location as well as the strain background (Brudey *et al*., 2006; Filliol *et al*., 2002; van Embden *et al*., 1993; Yeboah-Manu *et al*., 2011). Drug resistance is defined as the emergence of spontaneous gene mutations in bacteria rendering the bacteria to be

unaffected by most of the universally used anti-tuberculosis drugs (Palomino & Martin, 2014). The World Health Organization defines MDR-TB as the concomitant resistance to isoniazid and rifampicin in a strain. This calls for concentered efforts to find out the strains that are circulating within certain geographical areas. In response to this, the WHO introduced the GeneXpert or Xpert MTB/RIF assay methodology (Cepheid, Sunnyvale, Ca., USA). The GeneXpert or Xpert MTB/RIF assay platform is quite versatile as it offers prompt information on most of the mutations on the MTBC genome implicated in isoniazid and rifampicin resistance with very minimal sputum sample preparation. This assay detects both organism and drug resistance to rifampicin concurrently.

The advancement in the resistance to drugs is a result of the development of a buildup of alterations in specific genes related to drug resistance in MTBC isolates. These mutations arise due to incorrect treatment of infected patients and non-adherence to treatment regimens (Hillemann *et al*., 2007). The precise and rapid detection of the tuberculosis bacilli including multi drug-resistant and drug-resistant strains remains crucial for the effective treatment and management of patients as well as reducing the transmission rates (Steingart *et al*., 2014). Drug susceptibility testing allows the selection of the appropriate drugs or modification of the drugs used in treating infected persons thereby enabling healthier supervision of individuals for control and spread as well as surveillance of the disease within the community (Woldemeskel *et al*., 2005).

The World Health Organization recommends that its member states and National Tuberculosis Control Programmes should develop, extend, and

have a very formidable resource for the performance of culture-based drug susceptibility testing in their health facilities. They should also introduce new molecular-based tests and methods for diagnosing drug-resistant tuberculosis.

In furtherance to this, the Molecular Line Probe Assays were endorsed and introduced in the year 2008 for the speedy diagnosis and identification concerning multidrug-resistant tuberculosis in member states (WHO, 2008a). One of such available line probe assays is the Genotype MTBDR*plus* **(**Hain Life science GmbH, Germany) developed for the finding of alterations in resistance-associated genes to both rifampicin and isoniazid in cultured isolates or isolates worked-up directly from sputum. This assay uses extracted DNA amplified in a multiplex polymerase chain reactions and then hybridized to specific probes as oligonucleotides complementary to known resistance-associated mutations of interest, pre-attached onto a nylon strip for the identification of resistance-related alterations (i.e. *rpoB, katG* and *inhA*) genes (Woldemeskel *et al*., 2005).

The DNA sequence alterations or mutations that prevail in the rifampicin-resistant isolates are contained in the "core region" of the *rpoB* gene that generates an 81 bp band. There are 38 different point alterations-short insertions or deletions-within the core 81-bp region or the so-called Rifampicin Resistance Determining Region (RRDR) of the *rpoB* codons 507-533 encoding 27 amino acids (Ramaswamy & Musser, 1998) and most frequently seen in about 95.0% of all strains resistant to rifampicin (Riccardi *et al*., 2009).

The resistance that occurs in any bacteria to isoniazid is as a result of alterations in the catalase-peroxidase (*katG*) gene and the regulatory region

(*inhA*) (Alcaide & Coll, 2011; Zhang & Yew, 2009). The changes that occur in these genes indicate the level of resistance which could be at a high-level or low-level respectively (Vilcheze & Jacobs, 2007).

Limited studies employing Spoligotyping as a means to investigate the different strains and lineages of *M. tuberculosis* causing infection within the population in Ghana (Asante-Poku *et al.,* 2015b; Goyal *et al*., 1999; & Yeboah-Manu *et al*., 2011;). Addo *et al*. (2007) applying phenotypic DST methods reported in their study a low occurrence of drug resistance regarding studies targeted at communities than in health facilities. Though this study will produce valuable information on MTB strains causing tuberculosis and their drug resistance profiles in patients who had presented at the Greater Accra Regional hospital, the generated results cannot be used as a true reflection of the situation as of the year 2019. The basis of this work involved employing Spoligotyping and HAIN Genotype MTBDR*plus* assay to establish the circulation of MTB lineages and their drug resistance profiles in newly diagnosed cases of TB patients presenting at the Greater Accra Regional hospital in the Greater Accra Region using archived *Mycobacterium tuberculosis* isolates collected during the period January 2008 through July 2012.

Statement of the Problem

There is very little data on the diversity of strains causing human TB particularly in first-time infections in Ghana. Information about the genetic diversity in first-time cases would be useful in the formulation of control and management measures. Procedures to control tuberculosis require methods for

rapid detection and tracing sources of the infection to prevent further transmission.

The type of strains causing TB infections has not been clearly defined with populations. The association of these strains to recent transmission or uniqueness as well as recurrence of infection have not been fully established. This makes the fight against the disease a difficult one, therefore identification of the strains that are causing the infection is very crucial. The development of mutations in strains that lead to drug-resistance such as MDR and XDR tuberculosis is posing a huge task to the global fight against the prevention and spread of the disease thereby threatening global health.

Purpose of the Study

The aim of this research was to apply Spoligotyping and HAIN Genotype MTBDR*plus* assay to analyze a panel of archived TB samples to generate data on strain lineages and the mutations defining drug-resistance patterns thereof. Ghana has made a lot of efforts in the diagnosis of MTB concerning all the age ranges (i.e. from children to adults). Knowledge of the nature of mutations in strains causing an infection is very critical in the treatment of the disease. With the evolution of MTBC lineages/families, understanding the geographical distribution of these strains is crucial in policy formulation. Spoligotyping, which is a genotyping technique used in epidemiological studies of the MTBC is very reproducible, dependable, and easy to perform. The results to be generated can be used to beef up existing data in future research as the scientific community works to eradicate tuberculosis by the year 2030.

Research Questions

1. What were the species of *Mycobacterium* causing TB (for the first time) in patients who presented at this health facility?
2. Did these strains have different Spoligotype patterns?
3. Were these Spoligotyped strains epidemiologically related?
4. Did these strains belong to different genetic lineages?

Main Objective

This study aims at establishing the diversity of strains using Spoligotyping and the HAIN Genotype MTBDR*plus* assay to analyze a panel of archived TB samples in a population of presenting patients at the Greater Accra Regional Hospital.

Specific objectives

The specific objectives of this study were to:

1. Confirm if the isolates are truly members of the MTBC using the Capilia TB-Neo Test
2. Identify strain families/lineages of MTBC species using Spoligotyping
3. Determine the drug-resistant profiles of these isolated strains to isoniazid and rifampicin using HAIN Genotype MTBDR*plus* assay
4. Identify which spoligotypes depict resistance, and on which genes and codons?
5. Determine Spoligotype diversity, Spoligotype cluster rate, and Distribution of identified lineages.

Significance of the Study

Molecular typing techniques aimed at the study of the MTBC remain very useful in the control, monitoring, and detection as well as identifying outbreaks and to trace the source of these outbreaks (clonal spread of successful clones). Information gathered from molecular typing techniques such as spoligotyping is critically important to understanding the transmission and prevention of the extent of diseases. Typing methods must have high discriminatory power, easy to perform, must be reproducible, less costly, can be applied directly on a clinical specimen, and can be used to trace the association that exists between strains/families and their ecological locations.

Spoligotyping is a very useful technique for tracking epidemics and identifying new occurrences of MTB and helps to describe populations that are of high risk to focus prevention strategies on them and groups that need it most. As stated earlier, only three known articles had been published within the last two decades and their focus was on community bases. The distribution of strains causing infection among patients presenting in this health facility (Greater Accra Regional Hospital) is unknown. Archived samples were used to generate retrospective data from this facility. There was no genetic information on these MTB isolates in terms of the species and drug-resistant profiles. It is hoped that the information and data generated would enhance understanding of the *Mycobacterium tuberculosis* complex.

Limitations

This study had some limitations. Efforts were made to include very recent samples (isolates) to increase the sample size but logistical and financial limitations in the budget for this research work could not sustain it as

well as time constraints. The study focused on only one health facility within the Greater Accra Region for which samples had been collected and isolates obtained and preserved within the period 2008-2012. Despite these, the findings provided valuable information on the types of spoligotypes circulating within a particular period and since it was not population-based the results cannot be used generalize the actual situation in Ghana.

Organization of the study

This research work is organized into five chapters. Chapter 1 is the general introduction of the study. Chapter 2 gives an insight into relevant literature concerning the subject matter under study. Chapter 3 involves the design and experiments performed in the successful completion of the research. Chapter 4 is dedicated to the presentation of the findings of the work and its discussion. The last chapter which is chapter 5 is a summary, conclusion, and recommendations of the entire research work.

CHAPTER TWO

LITERATURE REVIEW

Tuberculosis in Ghana

Attempts to deal with the TB menace in Ghana dates back to the early 1900s and they were irregular and uncoordinated. The first funded survey of the burden of TB in Ghana was conducted with supervision of the World Health Organization (WHO) in 1957. The estimated annual risk due to infection was between 3 and 4 per 100 000 population in gold mining towns (National Tuberculosis Health Sector Strategic Plan for Ghana 2009-2013). In 1959, a more formalized institution was put in place for TB control named Ghana TB Services (Koch, 1960). Attempts in controlling the disease declined till the late 1990s but it was rekindled with the occurrence of the deadly HIV/AIDS disease (Amo-Adjei, 2013). With the pronouncement of TB as a disease of global importance in 1993 by the WHO, Ghana set up the National TB Control Programme (NTP) in 1994.

In the year 2007, a full review and report by Ghana's NTP indicated minimal case detection and this posed a serious threat to control and management measures in the country (Whalen *et al*., 2007). A study conducted by the National Tuberculosis Health Sector Strategic Plan (NTHSSP) for the Ministry of Health in Ghana between 2009-2013 observed exposure to the disease to be 27.0% in general and among sputum smear-positive patients to be 37.0% in the preceding year 2008. It stated that much focus should be placed on active case finding and proper interventions put in place (Ministry of Health, 2009). After the implementation of interventions, the case detection rate in Greater Accra saw a marginal increase initially from

2,517 detected TB cases in 2008 to 2,651 detected cases in 2009. The annual report of the Ghana Health Service in 2010 estimated that less than half of the TB cases were notified and 15,145 out of 47,632 the projected cases were diagnosed (GHS Annual Report, 2010). The prevalence of all forms of tuberculosis as projected by the World Health Organization in 2011 was at 92 per 100,000 populations (WHO, 2011). With support from WHO and the Canadian Government through its international development agency, the Canadian International Development Agency (CIDA), a strategy was initiated to search for cases within the capital city Accra. It was implemented for patients attending health facilities, Out-Patients Departments (OPD), HIV Clinics, Diabetes Clinics, and relations of persons recognized as having been infected with the disease to facilitate case detections (WHO, 2011b). Tuberculosis (TB) cases detected in 2014 were 2,071.

The reasons for this decline in the case of detection rates in the health facilities were unknown. The projected national occurrence of the disease in 2014 was 282 per 100,000 population and the incidence rate of 165 (Addo *et al*., 2017; WHO, 2015). New cases were estimated to be at 14,668 in 2014 and Ghana's case detection was 33.0% which was lower than the average rate of 47.0% set for the African region and 70.0% target by the WHO (WHO, 2015). Incidence of tuberculosis per 100,000 population reported in 2016 was 156 according to the World Bank on information gathered from recognized official sources about country development indicators.

The incidence is based on the projected number of new cases originating from smear microscopy positives from pulmonary sources and extra-pulmonary cases. Delays in the diagnosis of tuberculosis and late

reporting by patients pose a challenge to preventing the spread of the disease. This calls for other interventions that can quickly detect and help with better interventions. Communication from the outfit of the End TB Strategy group indicated that to achieve an increase in case detection universally, there should be intense education so that people with symptoms of the disease will seek early diagnosis and treatment and people belonging to high-risk groups should be screened periodically (WHO, 2014).

One such measure is the implementation of search and finds case methods using more sensitive diagnostic procedures that analyze samples beyond just the traditional sputum smear microscopy. International bodies involved in the eradication of TB are working at reducing the death toll of TB by 90.0% and increasing the detection of new cases by 80.0% by the end of 2030 (Uplekar *et al*., 2015). The rate of deaths attributed to tuberculosis in Ghana by the WHO is high and in 2015 this figure was estimated at 7.5 per 100 000 (WHO, 2015).

Mycobacterium tuberculosis strains are grouped into seven lineages that are classified based on their global geographical sites. These lineages are Lineage 1- Indo Oceanic; Lineage 2- East Asian, including "Beijing"; Lineage 3- Central Asian (CAS)/Delhi; Lineage 4- Euro-American, including the Latin American-Mediterranean (LAM), Haarlem, X type and T families; Lineage 5- West African; Lineage 6- West African 2 and Lineage 7- Ethiopia (Comas *et al*, 2013). Asante-Poku *et al.*, (2016 & 2015b) stated that in Ghana, Six (6) out of the Seven (7) *Mycobacterium* strains or lineages were found to be the main cause of TB infections within their study population. *Mycobacterium*

africanum (West Africa type I and type II) belonging to lineage 5 and 6 respectively were 20.0% of the strain population.

Further studies involving clinical isolates from the Southern part of Ghana also identified lineage 4 with the Cameroonian sub-lineage been prevalent in all human diagnosed cases (Asante-Poku *et al*., 2015a). It was also established that *M. africanum* still contributed to about 17.1% of all cases similar to findings of Yeboah-Manu *et al*. dated several years ago. Surveys have shown high adaptable occurrences of *M. africanum* in different regions of West Africa. Countries like Ivory Coast and Cameroon were recording approximately 5.0% and 10.0% of *M. africanum* infections respectively (Niobe-Eyangoh *et al*., 2003). Guinea-Bissau recorded at least 60.0% of its TB patients to be infected with *M. africanum* (Bonard *et al*., 2000; Källenius *et al*., 1999). Adesokan *et al*. (2019) reported a 19.1% prevalence of *M. africanum* in their study by spoligotyping.

This is similar to a 13.0% prevalence reported by Cadmus *et al*. (2006) in Ibadan, South-Western Nigeria but lower than the 60.0% reported by Bonard *et al.* (2000) from Guinea-Bissau. This nonetheless, *M. africanum* identified in West Africa, mostly in Guinea-Bissau is waning with current reports showing 47.1% of identified strains (Groenheit *et al*., 2011). Reports indicating a diminishing rate of occurrence of *M. africanum* could be attributed to misidentification and/or misclassification of this specie of the MTBC due to its significant phenotypic resemblance to *M. bovis* and *M. tuberculosis* (Adesokan *et al*., 2019). One reason attributed to the stability of *M. africanum* in Ghana might be due to the adaptation of the bacteria to this geographical location. One of the keys determining factors to the management

and prevention of diseases is the determination of a drug-resistant profile. Addo *et al*., (2007) reported in their study a lower occurrence of drug resistance in communities than in patients presenting at health facilities.

In this study by Addo *et al*., isoniazid (INH) mono-resistance was recorded at 10.9% relatively higher than rifampicin (RIF) mono-resistance at 1.2% (Addo *et al*., 2017). In their study, over 80.0% of the MTBC isolates tested for drug sensitivity were susceptible to isoniazid and rifampicin. *Mycobacterium tuberculosis* complex isolates that have been classified as MDR strains and patients infected are a possible risk to others, family, and the public in general. Ghana has previously recorded MDR-TB rates ranging from 2.2% - 2.5% (Asante-Poku *et al*., 2015a; Owusu-Dabo *et al*., 2006; Yeboah-Manu *et al*., 2012).

Diagnosis of Tuberculosis

Prompt and accurate diagnosis of Tuberculosis (TB) will go a long way in benefiting patients in the receiving of treatment and consequently reduce or stop disease transmission. The genus *Mycobacterium*, which includes the MTBC, was first introduced by Lehmann and Neumann (Buchanan & Gibbons, 1975). These mycobacteria are slightly curved, slender rod, acid-fast, immotile, and aerobic measuring 1-4 microns in length and 0.3-0.6 microns in breadth. They have a tough membrane of lipo-proteins that is responsible for its virulence aiding it to overcome adverse conditions, non-availability of air and nutrition can make it survive as dormant for a very long time in the host (Betegeri, 2001). They are usually slow-growing mycobacteria with a growth rate that requires over 7 days of incubation before visible

growth is seen and they form the majority of the potentially pathogenic atypical species (Grothuis & Yates, 1991).

Typically, mycobacteria are classified into 4 groups based on pigment production and based on the time required to detect visible growth (Runyon, 1958). The disease-causing mycobacterium can survive outside its host for a short period. When the bacteria are exposed to prolonged conditions such as exposure to heat (from the sun and other sources) and dry conditions it will die but will survive longer under conditions such as cold, dark, and moist areas. It does not grow outside its host except in cultured medium with an approximately mean multiplication time of 20 hrs.

Clinical Examination of Tuberculosis

The clinical manifestations largely depend on the size and type of infection (Primary or Post-primary). Primary tuberculosis is usually asymptomatic (Committee on Infectious Disease and American Academy of Pediatrics, 1991). In post-primary tuberculosis, the lungs are the main site of infection (Hopewell, 1994). The WHO (1982) defines the symptoms of pulmonary tuberculosis as limited to breathing difficulty, chest pain, a persistent cough (with mucus) for at least 2 weeks, coughing (blood-stained), night sweats, weight loss, etc. Non-respiratory symptoms such as (fatigue, fever, excessive night sweat, weakness, anorexia, loss of weight), and contact with persons with TB were added to this list (Teklu, 1993).

The disease is diagnosed by taking sputum from adults as well as aspiration of the gastric contents from children because they mostly swallow their sputum (Labato *et al*., 1998). Fine needle aspirates from infected sites or organs of patients with extra-pulmonary TB are also taken. Specimens are

taken normally depending on the affected sites and this may consist of cerebrospinal fluid (CSF), blood, peritoneal and pericardial fluid, early morning urine, lymph node aspirates, or tissue samples (Aggarwal, 2006). Routine identification and isolation of the MTBC are categorized into Conventional methods (Sputum Smear Microscopy (i.e. detection of AFB's), Culture of decontaminated samples, and Chest X-rays) and Molecular based (non-conventional) methods. However, the success or otherwise of a particular test result is dependent on the extent of disease prevalence as well as the sensitivity and specificity of the test procedure (Mausner & Kramer, 1994).

Conventional Methods for Detecting *Mycobacterium Tuberculosis*

Sputum smear microscopy

Microscopy using sputum smear is the most predominant technique worldwide for the diagnosis of patients assumed to have acquired TB and for detecting the existence of acid-fast bacilli (AFB's), particularly in most resource-stretched settings. To confirm that a patient has the disease, the bacteria must be isolated and identified from the specimen produced and it is a very important preliminary step in the diagnosis of active TB on either clinical samples of prepared tissues, especially in resource-constrained countries. Staining methods such as the Ziehl-Neelsen or Kinyoun (carbol-fuchsin based dyes) and the Auramine-Rhodamine are currently employed for the detection and diagnosis of TB. These methods are simple, low-cost, fast, and specific (Kubica, 1984). The concentration of a sample (specimen) after decontamination with appropriate methods is known to be more efficient than the direct method where smears are prepared directly from the sample (Apers *et al*., 2003; Lumb *et al*., 2013). This procedure eliminates other organisms

and makes culturing of the bacilli effective. One major disadvantage is its ability to detect bacteria in the range of 10^4-10^5 bacilli per mL^{-1} (Someshwaran *et al*., 2016; Toman, 1979). Direct smear microscopy detects approximately 60.0% of acid-fast bacilli with 1 x 10^3 AFB's per mL of sample (Pfyffer, 2003). The concentration of specimen by either centrifugation or filtration does greatly improve the sensitivity of microscopy (van Deun *et al*. 2000) but the un-concentrated procedure is extensively used due to limitations in resources. A handicap of this process is the inability to discriminate between MTBC and NTM species.

Light microscopy

The identification by staining of MTB was started with the microbiologist Robert Koch. Paul Ehrlich was the first person to describe the bacterium based on its acid-fast properties. The procedure for the acid-fast stain was modified by Frederich Neelsen, a German pathologist and Franz Ziehl a German bacteriologist by the introduction of phenol (carbolic acid) and basic fuchsin (Ziehl, 1882; Neelsen, 1883). This modification led to the introduction of the ZN staining procedure. In brief, the procedure involves the use of the mycobacteria's acid-fast properties by applying heat slowly to the cell walls of the bacteria to open it up.

This allows the penetration of dyes after which the stained bacteria is decolorized using an acid solution and counterstained with (methylene blue dye) to give it a red or pink color in a blue background that helps and improves its visualization under a microscope (WHO, 1998). An alternative to the ZN stain is the Kinyoun stain. Whiles in the ZN procedure heat is applied

as the staining technique, this procedure applies cold-staining with an increase in the concentration of the counterstain dye (methylene blue).

Fluorescence microscopy

Wide-ranging stains such as Acridine orange (AO) (Smithwick. 1995, Sethi, 2010) and Auramine-rhodamine (AR) (Sethi, 2010, Vildan, 2011) are currently in use. The auramine-rhodamine staining was described by Hagemann (Hagemann, 1937). The techniques allow for the rapid screening of sputum smear samples. The procedure is deemed to have superior diagnostic performance over other staining and microscopy methods (Hänscheid *et al*., 2007; Prasanthi & Kumari, 2005). Fluorescent microscopes decrease the evaluation time for slides. In the year 2010, the WHO recommended that, the conventional method of analysis using the light microscope be replaced by fluorescent microscopes (WHO, 2010).

This microscopy procedure is not widely used as compared to others because of the limited lifespan and cost of its short-arc mercury vapor lamp (MVP), which can last for typically 200-300 hrs. as well as its high cost of maintenance (Hung *et al*., 2007). A standard procedure for the fluorochrome staining with auramine and acridine (Siddiqi, 2005) is briefly discussed.

With auramine O staining, the bacterium appears as a bright yellow fluorescent rod when observed underneath an excitatory light source. Acridine orange (AO) stains specimen as orange/red fluorescing bacilli in a dull black background.

Culture of *Mycobacterium tuberculosis*-complex

Culture, a method employed to cultivate and isolate mycobacterial cells of isolates as well as recovery of the bacilli from both solid and liquid

media under specific conditions that favour the growth of the bacteria is generally considered as the "Gold Standard" when it comes to the confirmation of the disease (Taylor *et al*., 2001). *Mycobacterium tuberculosis* complex owing to its sluggish progress rate on solid media has a mean replication time between 18–30 hrs. (Salyers & Whitt, 1994).

The culture of the mycobacteria is very important to confirm its susceptibility to the available anti-TB drugs. It is crucially important because it provides starting material for phenotypic DST and genetic analysis. An advantage of culture over smear microscopy (detection of AFB's) is its sensitivity as it can identify as low as 10 bacilli/mL of processed concentrated sample (American Thoracic Society, 1990). Nonetheless, it is not 100% sensitive as a false-negative result may be realized especially in instances where the tubercle bacilli infection is at its early stages (Duffield *et al*., 1989).

Decontamination and cultivation methods

Before the cultivation of the mycobacteria, the sputa or tissue specimen must be decontaminated to prevent other contaminating microorganisms from outgrowing members of the MTBC. The most commonly used decontamination reagents or procedures as described by Della Latta, (2004) are:

1. The N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) method: A very common method used in laboratories worldwide. It uses N-acetyl-L-cysteine to digest the mucus and sodium hydroxide with sodium chloride as the decontaminant (Kubica *et al*., 1963)
2. Petroff or NaOH (Sodium hydroxide only) method: This is used at a concentration ranging between (2-4)% to digest and decontaminate the

specimen. Each laboratory determines the lowest concentration for optimal work (Petroff, 1915; Sharma *et al*., 2012)

3. Oxalic acid method: Mostly used on tissue samples and is especially recommended to decontaminate experimental specimens that might be contaminated with *Pseudomonas aeruginosa* (e.g. specimen originating from patients with cystic fibrosis as well as from urine) (Kent & Kubica, 1985)
4. Sodium Lauryl Sulphate method (Narasiman *et al*., 1972)
5. Chlorhexidine and Squalamine method (Asmar & Drancourt, 2015)
6. Universal Sample Processing solution (USP): Very useful in processing extra-pulmonary TB cases (Chakravorty *et al*., 2005)
7. Cetyl Pyridinium Chloride (CPC) method: This contains 1.0% Sodium Chloride (known as 1%CPC) (Pardini *et al*., 2005)
8. Tri-sodium Phosphate (TSP) method (Jena & Panda, 2004).

Some decontaminates also serve as a transport medium for the preservation of the organism when samples have to be transported over a long distance before reaching the laboratory for analysis.

Detection of MTB visible growth on media

It takes 2-3 weeks to detect visible growth (colonies) on solid egg-based media like Loewenstein-Jensen (LJ) and 4-16 weeks to confirm as negative (Grooms & Molesworth, 2000; Grange *et al*., 1996). Other solid mediums include the Ogawa medium and Coletsos® medium (Coletsos, 1971; Idigoras *et al*., 1995) with agar-based media such as Dubos oleic acid albumin, Middlebrook 7H10, and Middlebrook 7H11 (Kent & Kubica, 1985). Loewenstein-Jensen (LJ) egg-based media is a modification of both Ogawa

medium and Coletsos® medium with the incorporation of L-asparagine and pyruvic acid (sodium pyruvate) that enhances the growth of *M. bovis.*

The liquid culture medium used to cultivate bacteria that are classified as belonging to the MTBC is more sensitive and faster as compared to the use of solid culture media but it is predisposed to contaminations. BACTEC 12B is a 7H9 broth supplemented with casein hydrolysate, bovine serum albumin, catalase, and radio-labeled (^{14}C). This system is a radiometric respirometry system where growth is detected based on a growth index value amount of $^{14}CO_2$ produced, a reflection of the metabolism of the radiolabelled substrate. The BACTEC 12B media is supplemented with PANTA supplement containing polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin to subdue other interfering microorganisms from growing (Heifets, 1991).

This radiometric assay has recently been substituted with fluorescence reducing systems that can improve safety drastically. The semi-automated (BACTEC 460TB) or the automated systems (BACTEC MGIT 960, MB/BacT/Alert 3D) (Becton Dickinson, Sparks, Massachusetts, USA), alternatively, can be used for TB drug analysis by the application of kits that are commercially available and widely used. This system has a detection mean time of 10-12 days (Hanna *et al*., 1999; Kubica & Wayne, 1994; Piersimoni *et al*., 2001; Woods *et al*., 1997). It is a combined modification of Middlebrook 7H11 broth with oleic acid albumin dextrose catalase (OADC) that has been enhanced through the addition of PANTA antibiotic mixture (Kontosa *et al*., 2004; Siddiqi & Rusch-Gerdes, 2006; Tortoli *et al*., 1999; Tortoli *et al*., 2002).

Other automated systems include the Versa TREK system (Thermo Scientific, USA) and Bract/ALERT 3D system (bio Merieux Diagnostic, France).

Treatment of MTB Infection

Anti-tuberculosis drugs

The WHO (2010c) has classified anti-tuberculosis drugs into five classes namely:

1. First-class: ethambutol, isoniazid, pyrazinamide, and rifampicin.
2. Second class: amikacin, capreomycin, kanamycin, and streptomycin.
3. Third class: old- and new-generation fluoroquinolones.
4. Fourth class: cycloserine, ethionamide, para-aminosalicylic acid, prothionamide, terizidone, and thioacetazone.
5. Fifth class: amoxicillin/clavulanate, clarithromycin, clofazimine, imipenem, and linezolid.

Table 1: ***Drugs, target sites, and their outcomes***

Drug Name	Mode of Drug Activity and its Effect on Bacteria	Site of Target	Remarks
Rifampicin	It is bactericidal. (It inhibits the synthesis of RNA)	The polymerase β subunit of RNA that is encoded by the *rpoB* gene	Its active is against vigorously growing and sluggishly metabolizing non-growing bacteria
Isoniazid	It is bactericidal. (It inhibits the synthesis of mycolic acid in the cell wall)	Acyl carrier protein reductase encoded by *inhA*; other multiple targets	Pro-drug, activation through *katG* required
Streptomycin	It is bactericidal. (It inhibits protein synthesis)	The S12 protein in the small 30S ribosomal subunit that is coded by the *rpsL* gene and The 16S rRNA coded by the *rrs* gene	Contraindicative during pregnancy
Ethambutol	It is bactericidal. (It inhibits the synthesis of arabinogalactan in the cell wall)	Arabinosyl transferase encoded by *embCAB*	
Pyrazinamide	It is both bactericidal and bacteriostatic. It depletes energy and interferes with membrane transport systems	Metabolism of energy in the membrane	Active only in acid pH and more active at low oxygen or anaerobic conditions; pro-drug activated by PZase/ nicotinamidase encoded by *pncA*
Ethionamide	It is bacteriostatic. (It inhibits the synthesis of mycolic acid in the cell wall)	Acyl carrier protein reductase encoded by *inhA*	Pro-drug, activated by the enzyme EthA
Quinolones	It is bactericidal. (It inhibits the synthesis of DNA)	DNA gyrase encoded by *gyrA*	Examples: moxifloxacin, gatifloxacin
Kanamycin, Capreomycin Amikacin	It is bactericidal. (It inhibits the synthesis of proteins)	16S rRNA (encoded by *rrs*)	Injectables, used in second-line treatment

Adapted from Zhang (Zhang 2005)

Standard Drugs for TB Management

New cases of individuals that have been diagnosed with tuberculosis and have not started taking anti-tuberculosis have to be treated for 6 months. This consist of two months intensive phase and four months continuation phase as described in (Table 2).

Table 2: ***Summary of the standard TB drug regimen***

Treatment	Drugs	Treatment duration
Drug-susceptible TB	Isoniazid(INH) Rifampicin(RIF) Pyrazinamide(PZA) Ethambutol(EMB)	6-months regimen: The intensive phase of 2 months (HRZE) The next 4 months (HR)
MDR-Patients	Fluoroquinolone (FLQ) (belonging to group A): Levofloxacin,moxi,gati Injectable drug (belonging to group B): amikacin (AMK), capreomycin (CAP), kanamycin (KAN), streptomycin (STR) At least two drugs (group C): ethionamide/prothionamide, cycloserine, linezolid, clofazimine PZA (group D1)	20-months regimen: 8 months intensive phase 12-18 months continuation phase
MDR-TB short regimen	AFLQ (groupA): MFX or (GFX) An injectable drug (group B): KAN Two drugs (belonging to group C): PTO and clofazimine High-dose INH, PZA, and EMB (group D1)	9-12-months regimen: 4 months intensive phase 5 months continuation phase
Multidrug resistance (MDR): $INH^R + RIF^R$ - Extensively drug resistance (XDR): $INH^R + RIF^R + FLQ^R +$ $(AMK/CAP/KAN)^R$		

(WHO, 2016)

Based on their efficacy and relative toxicity, these anti-TB drugs are classified as first-line and second-line drugs. The first-line drugs have been established to have low toxicity to the immune system and have high efficacy.

Molecular Basis of Drug-Resistance

Drug-resistant tuberculosis treatments involve the use of effective drugs that are divided into first-line drugs (rifampicin, isoniazid, ethambutol, and pyrazinamide) and second-line drugs. The most crucial challenge for clinicians worldwide is the forced start of therapy without waiting for results of drug susceptibility test (DST) information. Phenotypic DST procedures take a long time to complete because of the slow growth of MTBC strains. The World Health Organization now requires prompt diagnosis and treatment of patients and also the identification of patients who should be placed on different treatment choices (Falzon *et al*., 2017; Pym *et al*., 2016). Genotypic drug susceptibility tests deliver results in a short time frame and are very accurate. There should be a clear-cut understanding of resistance to the available anti-tuberculosis drugs. Mutations that confer resistance in the MTB strains, as well as the specific genes and the mode of action of these drugs, must be understood (Dheda *et al*., 2017).

Mutations do not describe absolutely the resistance in all cases, signifying that other mechanisms could be involved which are unknown as to how the MTB strains can resist these drugs (Ramaswamy *et al*., 1998). The MTBC exhibits two types of resistance. The first is genetic resistance that involves mutations in the genomic regions with a focus on targeted genes that indicates the ability to escape the drug influence and the second is the phenotypic resistance that involves variations and alterations of the protein

structures that generates resistance to drugs without mutation on the DNA (Chaoui *et al*., 2009; Juarez-Eusebio *et al*., 2017). The most frequent mutations seen in MTB isolate that indicate resistance to the first-line drugs are discussed below.

Isoniazid

It is a bactericidal drug that is widely used as one of the first-line anti-tuberculosis drugs against MTB. It is known to act against metabolically active reproducing bacilli. It is a pro-drug and resistance to the drug is associated with the most frequent mutations on the *katG* and *inhA* genes (Ramaswamy *et al*., 1998; Zhang *et al*., 1992). The mutation found in the *katG* gene is S315T that occurs in approximately 50-95% of all resistant strains (Zhang & Yew, 2009). This mutation is linked with high-level resistance with MIC greater than 1 µg/mL and is very frequent in MDR strains (Fenner *et al*., 2012; Hazbón *et al*., 2006).

In the promoter region of the *inhA* gene, mutations also do occur. The most dominant mutation is found at position −15C/T and is commonly linked to low-level resistance with MIC less than 1 µg/mL. Identification of mutations in the *inhA* promoter region also gives an indication of cross-resistance to ethionamide (Banerjee *et al*., 1994; Larsen *et al*., 2002). Other genes that have been associated with isoniazid resistance are *ahpC* (which encodes for alkyl hydroperoxidase reductase); *kasA* (which encodes for β-ketoacyl-ACP synthase) and *ndh* (encoding for NADH dehydrogenase) (Peñuelas-Urquides *et al.* 2018).

Rifampicin

The drug rifampicin is bactericidal for *Mycobacterium tuberculosis.* It facilitates the formation of hydroxyl radical in susceptible strains of MTB by binding to the β-subunit of RNA polymerase thereby impeding the extension of the mRNA (Blanchard, 1996). Resistance to this drug is developed as a result of a mutation in the known 'hot-spot region' of 81bp covering codons 507-533 of the *rpoB* gene and has been identified as the resistance region (Ramaswamy & Musser, 1998). This gene encodes the β subunit of RNA polymerase and mutations have been established in approximately 96.0% of MTB strains.

The most common mutations are sited in codons 516,526 and 531 (Caws *et al*., 2006; Somoskovi *et al*., 2001). One crucial point worth noting is that, not all mutations in the *rpoB* gene are associated resistance (Zhang & Yew, 2015). Mono-resistance that occurs in strains to rifampicin is fairly rare and in most cases, strains shown as resistant also became resistant to other drugs specifically isoniazid and it is the cause considering these two as an indicator for MDR-TB (Traore *et al*., 2000).

Ethambutol

This is a bacteriostatic drug administered against reproducing bacilli which interfers with the biosynthesis of arabinogalactan located in the cell wall (Takayama & Kilburn, 1989). The target for this drug is to inhibit the enzyme arabinosyl transferase. This enzyme is associated with the biosynthesis of the cell wall arabinogalactan and is encoded by the *embB* gene which is concealed in the *embCAB* operon. Mutation in this gene confers ethambutol resistance. Most mutations on the gene are located in codon 306

with more than 68.0% of MTB strains having the mutation (Zhang & Yew, 2015). About 30.0% of resistant strains to ethambutol do not show mutations in the *embB* gene indicating the importance to identify the other means of drug resistance to ethambutol.

Studies have shown that *M. tuberculosis* isolates that were found to contain mutations in the *embB* gene at codon 306 were not automatically resistant to ethambutol but are predisposed to developing resistance to several drugs (Hazbón *et al*., 2005). Mutations that have been identified in the *embB* and *embC* genes as a result of its accumulation give rise to a series of MICs of ethambutol subject to the mutation type and number (Safi *et al*., 2013). Most mutations that occur in the *embB* gene at codon 306 are responsible for various degrees of ethambutol resistance but not sufficient enough to cause high-level resistance to the drug.

Pyrazinamide

The drug pyrazinamide is a pro-drug that is changed to pyrazinoic acid which is its active form by the enzyme pyrazinamidase/nicotinamidase which is coded by the *pncA* gene (Konno *et al*., 1967; Scorpio & Zhang, 1996). It destroys non-growing or semi-dormant persistent bacteria located in the lesions of TB patients (Mitchison, 1985). Resistance is associated with mutations in the *pncA* gene. Strains that becomes resistant to the drug ranges between (72-99) percent. These mutations are distributed all over the gene with most occur in a 561-bp region in the open reading frame or an 82-bp region of its putative promoter (Juréen *et al*., 2008; Scorpio *et al*., 1997).

Notwithstanding this, there are silent mutations that do not confer

resistance and this is likely to be a result of mutations in another not yet identified regulatory gene (Zhang & Yew, 2015).

Drug resistance occurs as a result of rapid changes in the genes of *M. tuberculosis.* Testing and surveillance are very critical in the development, protection, and usage of new drugs globally. These mutations have led to the use of faster and efficient molecular techniques to identify and distinguish drug resistance in MTB isolates within a short time. The development of mutations in certain specific genes of the mycobacterial DNA came up as a consequence of patients not taking their drugs on time (treatment failure) and leading to drug resistance (Palomino, 2005; van Rie *et al*., 1999). The recently identified Extensively Drug-Resistant strains (XDR) have complicated the fight against the disease. The World Health Organization and its partners in 2006 defined Extensively drug-resistant TB (XDR-TB) as strains that have developed resistance to at least isoniazid and rifampicin (first-line anti-TB drugs), any one of the fluoroquinolones, and at least one of (amikacin, capreomycin, or kanamycin) which are second-line injectables (Centre for Disease Control, 2006).

Phenotypic Methods of Testing MTB

Phenotypic methods used for drug susceptibility testing (DST) employ both solid and liquid mediums with incorporated drugs at various concentrations (i.e. breakpoint concentrations). Some of these methods are the Absolute Concentration Method, Resistance Ratio Method, MGIT 960 test**,** the Proportion Method performed on Loewenstein-Jensen Media and others like Middlebrook 7H10, 7H11, 7H12 (Hazbón *et al*., 2000; Tortoli *et al*., 2002).

These methods were previously considered as the "Gold Standard" for DST studies but due to medium variability, low-level resistance, susceptibility challenges, and difficulties in obtaining an optimum inoculum size it is now classified as the imperfect "Gold Standard" (van Deun *et al*., 2013). It is carried out on isolates that have been sub-cultured after initial diagnosis which is about 3-8 weeks and an additional 4 weeks susceptibility testing. These methods will continue to be in use because molecular methods are very expensive, requiring high levels of skills and expertise.

Genotypic Methods for Testing MTB

Genotyping methods identify genetic variations based on the amplification of the start material DNA by PCR and then analyzing the product for exact mutations related to resistance (García de Viedma, 2003; Palomino, 2005). The techniques employed are fast and offer a rapid screening process of the isolates. Some of these techniques are DNA sequencing, Probe-based hybridization methods (Dot-blot, Line blot assays, INNO-LIPA RIF-TB), Restriction Fragment Length Polymorphism PCR (RFLP-PCR) (which employs restriction enzymes to digest amplified PCR products to generate specific polymorphic DNA sequences followed with gel electrophoresis), Single Strand Conformation Polymorphism (SSCP) (used to determine short stretches of DNA).

Others include Heteroduplex analysis (HA), Molecular beacons (used as single-stranded oligonucleotide hybridization probes in diagnostic assays), Amplification refractory mutation system (ARMS)-PCR: This is known as allelic specific PCR (ASPCR) or PCR amplification of specific alleles

(PASA). They are used to identify point mutations or small deletions as well as alterations associated with rifampicin resistance.

Reversed Line Blot HAIN Genotype MTBDR*plus* assay

The HAIN Genotype MTBDR*plus* assay was officially certified by the WHO as an as test procedure to be employed for the screening of patients that are deemed to be highly pre-disposed to acquiring drug-resistant strains of *Mycobacterium tuberculosis* in 2008 (WHO, 2008c). This molecular method is very useful in the identification of bacteria strains that have mutations occurring in the genes. A mutation conferring resistance to the *rpoB* gene is as a result of changes that have occurred in the RNA polymerase b-subunit. In the *katG* gene, a mutation that encodes the catalase-peroxidase and *inhA* (i.e., enoyl-acyl carrier protein reductase) indicates a high and low-level resistance to INH, respectively (Mäkinen *et al*., 2006). The assay is a very accurate and fast procedure that is applied to samples that have been cultured for bacteria growth and direct specimen from patients (new, treatment failure and relapse) cases for the detection and identification (Federal Ministry of Health, 2013). This assay can be used for rapid DST without going through the traditional phenotypic DST option.

The test is a three-step procedure that involves the extraction of DNA from the bacteria cells; multiplex PCR amplification of targeted genes and the reverse hybridization of all amplified genes. The profiling and identification of resistance to the drug rifampicin (RIF) are mainly targeted at the *rpoB* gene which encodes the β-subunit of the RNA polymerase. In isoniazid resistance, multiple genes are analyzed for mutations with the most frequent once being *katG and inhA* (Laurenzo & Mousa, 2011). The first is targeted at the high

level resistant *katG* gene that encodes the catalase-peroxidase and secondly, a low-level resistance found in the promoter region of the *inhA* gene which encodes the NADH enoyl ACP reductase.

The HAIN Genotype MTBDR*plus* assay test is made up of a nylon membrane strip on which species-specific oligonucleotide probes containing the most frequently occurring mutations associated with resistance to INH and RIF have been immobilized on as parallel lines. The test kit has been designed to detect mutations in the *rpoB, katG, and inhA* genes of clinical isolates of MTB. The test kit is made up of 27 probes as follows. The control zones consist of (conjugate control (CC), amplification control (AC), *Mycobacterium tuberculosis* complex control (TUB), rifampicin gene control (*rpoB*), high resistance isoniazid gene control (*katG*) and low resistant isoniazid gene (*inhA*). The remaining 21 reaction test zones consist of 12 zones that contain probes which confer resistant or susceptibility to the *rpoB* gene ((8 wild types (WT1-WT8) and 4 mutations (Asp516Val (MUT1); His526Tyr, (MUT2A); His526Asp (MUT2B) and Ser531Leu (MUT3)). Resistant or susceptibility to isoniazid is characterized by 9 zones that contained probes (3 for *katG* gene and 6 for *inhA* gene) ((*katG* WT (315), Ser315Thr1 (*katG* MUT1) and Ser315Thr2 (*katG* MUT2); (*inhA* WT 1-(-15/-16) and *inhA* WT2-(-8); Cys15Thr (MUT1); Ala16Gly (MUT2); Thr8Cys (MUT3A) and Thr8Ala (MUT3B)) (www.stoptb.org/wg/gli). For easy identification of band colours, a colour intensity bar, M, is added. The examination and interpretation of results are based on the existence or absence of an identifiable band pattern that has been generated in the various zones indicating either a wild type (WT) band or a mutant (MUT) band on the LPA

strip. The absence of a wild type band in each of its zones indicates resistance. The presence of a band in the mutant zones indicates resistance to the associated drugs.

Strain Identification of *Mycobacterium tuberculosis*-complex

Immunochromatographic Assay (Capilia TB-Neo Test)

The application of liquid culture in detecting and identifying MTB in clinical samples has been internationally endorsed (WHO, 2007) The BACTECTM MGITTM 960 (MGIT) system (BD, Franklin Lakes, NJ, USA) is rapid, reliable and easy to operate and has being widely accepted to replace the current conventional solid culture procedures (Stop TB Department, 2007). This method is proficient in isolating both MTBC other pathogenic mycobacteria but lacks a definitive identification in positive cultures (Srisuwanvilai, *et al.,* 2008)

To overcome the challenge of distinguishing mycobacteria, the Capilia TB-Neo assay (TAUNS, Numazu, Japan; Nippon Becton Dickinson Co, Ltd, Tokyo, Japan) was developed. It was aimed at differentiating members of the MTBC from NTM's and is a simple, inexpensive, and rapid immunochromatographic assay for the identification of bacteria isolates (Abe *et al*., 1999; Nagai *et al*., 1991). The sensitivity as well as the specificity associated with the original assay ranging from 92.0% to 100% respectively (Abe *et al*., 1999; Hillemann *et al*., 2005; Shen *et al*., 2009). Further studies reported sensitivity and specificity to be at 99.2% and 100% respectively (Hirano, 2004). With this rate of detection, in 2007, the Capilia TB-Neo test became the official detection kit used for detecting *Mycobacterium tuberculosis* complex in Japan (http://www.tauns.co.jp/english/contact.php).

The underlining principle of the assay is based on a reaction of the MPB64 protein derived from one mouse (Mollenkopf *et al*., 1999; Yamaguchi *et al*., 1989). The kit is made up of a sample placement area and a reagent area. The reagent area is made up of a nitrocellulose membrane placed on a carrier strip with a colloidal gold labelled immobilized anti-MPB64 mouse monoclonal antibodies for the detection of MPB64 proteins in the sample development area (Hillemann *et al*., 2005; Muyoyeta *et al*., 2010). Bacteria suspensions of about 100 µL are dropped into the sample placement area; the MPB64 antibody then forms an antigen-antibody with the monoclonal antibodies then migrates to the test area by capillary action. It generates a visible red-purple colour band on both the Control (C) and Test (T) reading area of the kit indicating a member of the MTBC and a band at the Control (C) only indicating an NTM. The detection time is within 15-20 min.

The differentiation of MTBC from other mycobacterial employs this simple and rapid assay that is based on the presence of the protein MPB64 antigen that was discovered in 1984 (Nagai *et al*., 1991). The MPB64 antigen is part of the numerous proteins that are predominately excreted by members of the MTBC when they are cultured on solid media and liquid mediums (Li *et al*., 1993).

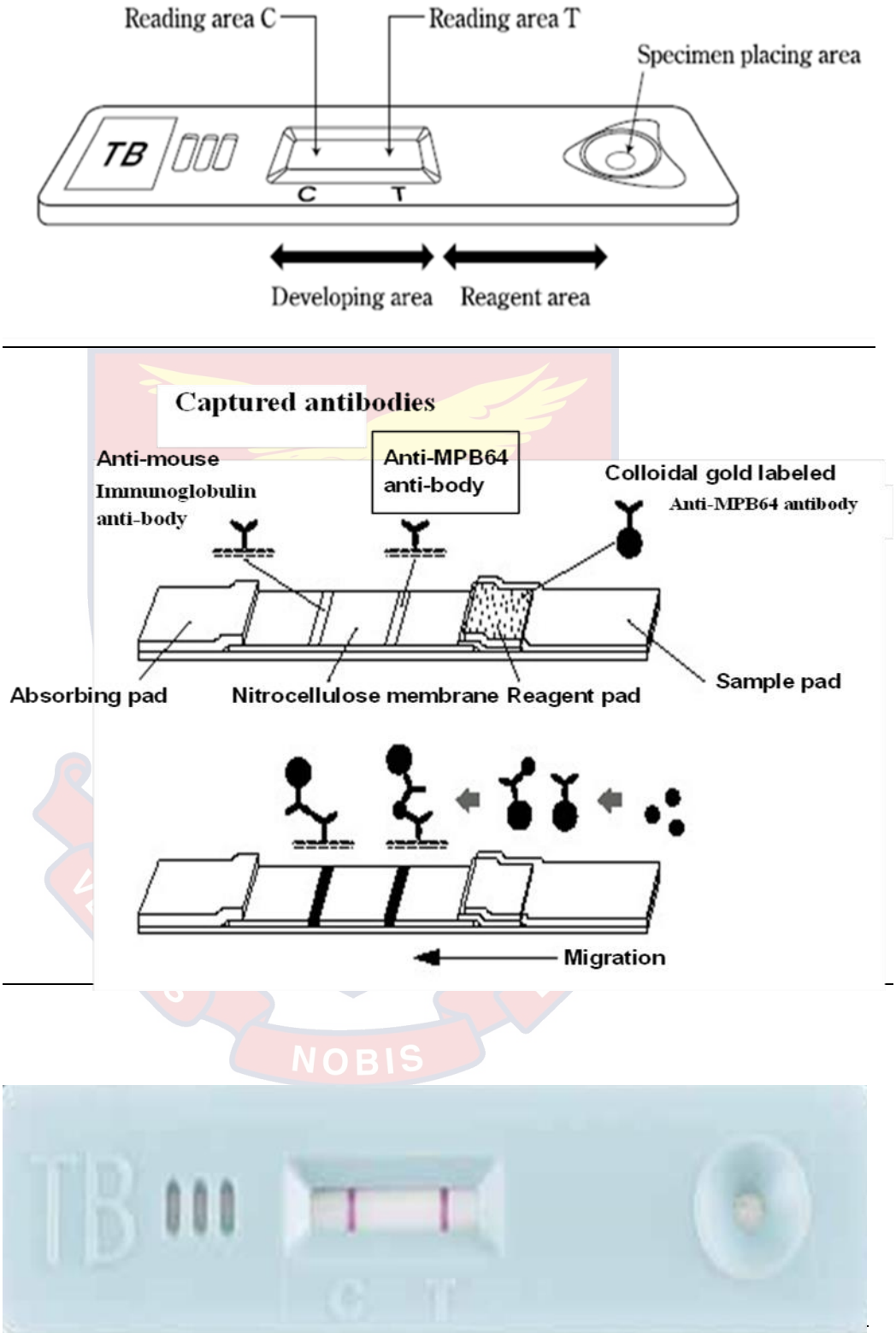


Figure 1: The principle underlying the detection of MTBC with the Capilia TB-Neo cartridge (TAUNS, 2010)

Spoligotyping

Spacer Oligonucleotide typing also known as spoligotyping is one of the methods in genotyping members of the MTBC. It is centered on a polymerase chain reaction (PCR) amplification that detects and type members of the MTBC. The method determines the strain causing the infection and gives the epidemiologic information of the strain. It is based on polymorphism in the Direct Repeat locus. This has become a very important locus used to study the evolution of clinical isolates of MTBC. The Direct Repeat locus is part of a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) family of recurring DNA. In spoligotyping, the whole Direct Repeat locus is amplified by PCR (Skuce & Neill, 2001). The procedure was first described by Hermans *et al*., based on *M. bovis* (BCG) Direct Repeat (DR) locus which contains direct repeat sequences of 36 bp, interspersed by the non-repetitive DNA spacers of 35-41 bp in length (Hermans *et al*., 1991). Groenen *et al*., proposed the use of this locus for epidemiological studies of the MTBC (Groenen *et al*., 1993). It is suitable in the surveillance of disease transmission and to avoid further spread of the disease (Gori *et al*., 2005). This procedure is relatively fast in the generation of DNA band patterns, the data easily digitalized and can be performed directly on lysed cells as well as non-viable bacteria. This hybridization assay detects an inconsistency in the direct repeat (DR) region in the DNA of MTB.

This is made up of several copies of a conserved 36-base-pair sequence divided by many distinctive spacer sequences (the standard spoligotyping assay uses 43). Different MTB strains have different patterns of the 43 spacers and this is the principle upon which the assay was developed (Kamerbeek *et*

al., 1997). The 43 spacers produce either a dark band (indicating the presence of a spacer) or no band (indicating the absence of a spacer).

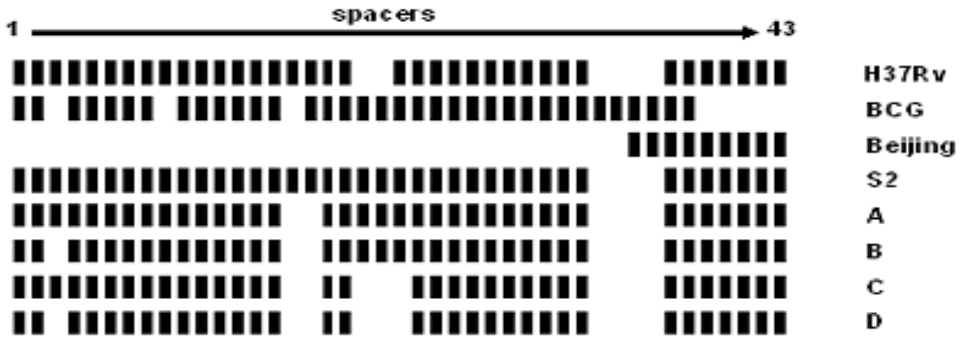


Plate 1: A representation of spoligotype patterns with certain strains of MTBC. Strains H37Rv and BCG are control strains and showing all 43 spacers. (Centre for Disease Control, 2012)

These patterns are then converted into binary code of either 1s or 0s (1 indicate band present and 0 indicate band absent). This is further simplified into a 15-digit octal code (base 8 with digits 0-7). This gives the sample its unique band pattern. The banding pattern generated is entered into a Microsoft Excel spreadsheet.

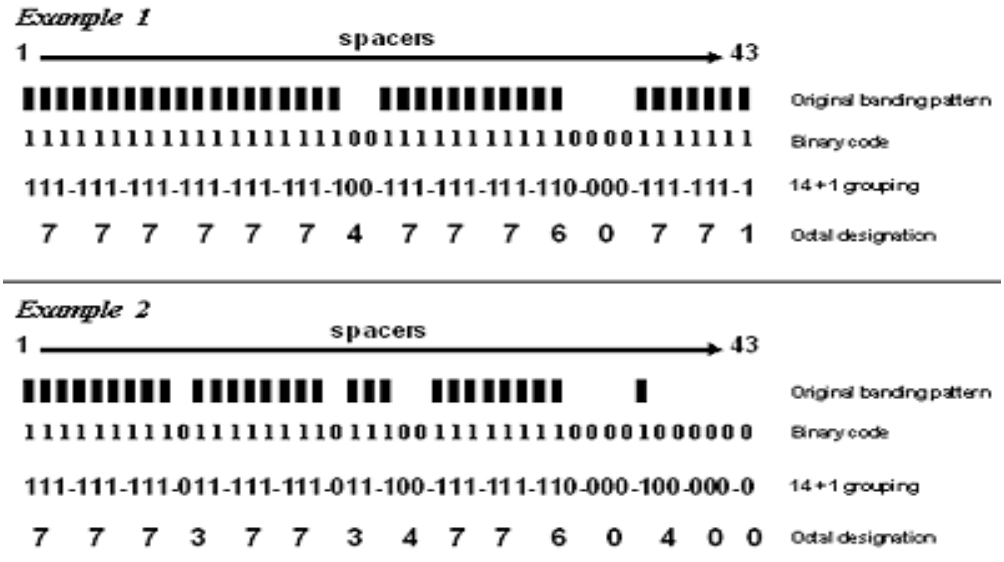


Plate 2: Spoligotype results with original band patterns with their pattern conversion into octal code designation. (Centre for Disease Control, 2012)

Differentiation of the spoligotype isolates is subject to the existence or non-existence of any of the 43 specific spacer sequences that are found in the direct repeat (DR) locus on the TB Chromosome which serves as the target for the assay (Filliol *et al*., 2002). It is used to identify strain types, clades, lineages/ families that belong to the MTBC such as Cameroun, Ghana, Beijing, Haarlem, East-African Indian (EAI), etc.

Global Diversity of Spoligotypes

Eight regions of the world are described below (Hans, 1999). The Central-Asia (CAS) family was essentially localized, emerging from India and (Middle-East and Central Asia) with 75.0% and 21.2% respectively. The distribution of the Haarlem (H) family was estimated to be at 25.0% globally but within the South American region, it constituted around 50.0% of all isolates identified. Latin-American-Mediterranean (LAM) constituted 50.0% of the other half of the strains from South America. The T family is less well defined and includes more than 600 unclassified Shared Types (STs). This lineage is currently stratified into 5 sub-clades (T1-T5). East-African-Indian (EAI) family was more abundant in South-East Asia, particularly in Vietnam and Thailand (32.0%). The (X) family is extremely dominant around the Americas at 21.5% and 11.9% respectively. Beijing and its related strains represented approximately 50.0% of strains that were emerging from Asia and the Far East with a global contribution of 13.0% (Pham, 2007). *Mycobacterium tuberculosis*-complex has been divided into seven core lineages established extensively on the sequence polymorphisms and characterized as belonging or associated with specific geographical regions as

well as particular human populations with different occurrence and risk to the disease (Coscolla & Gagneux 2010; Yen *et al*. 2013).

1. Lineage 1 (also known as Indo-Oceanic), which occurs around the Indian Ocean and the Philippines
2. Lineage 2 (East-Asian lineage, includes the Beijing family of strains) that is widely distributed in East Asian countries
3. Lineage 3 that has a relatively narrow distribution occurring in East Africa, Central, and South Asia
4. Lineage 4 (also known as Euro-American) that has a broad distribution in Europe and America but also in Africa and the Middle-East
5. Lineage 5 (also known as *M. africanum* West African 1)
6. Lineage 6 (*M. africanum* West African 2), which are geographically restricted to Western African countries
7. Lineage 7 recently described as the Ethiopian lineage is limited to Ethiopia only (de Jong *et al*., 2010; Firdessa *et al*., 2013; Gagneux *et al*. 2006; Hershberg *et al*., 2008).

Subsequently, all these spoligotypes have been classified into lineages using the spoligotyping technique (Kamerbeek *et al*. 1997).

Spoligotype Diversity in West Africa

Studies have revealed that there is usually a high genetic diversity amongst MTB strains exhibiting a low prevalence. This reactivation will be widespread and not necessarily from the dominance of a single strain (Lopez-Avalos *et al*., 2017; Michel *et al*., 2008). Abebe *et al*. (2018) did report that most of their isolates analyzed belonged to the SIT 37 and SIT 53 families (Bedewi *et al*., 2017; Belay *et al*., 2014; Getahun *et al*., 2015; Maru *et al*.,

2015). The specie *M. africanum* is divided into two types *M. africanum* type I and *M. africanum* type II. The *M. africanum* type I (WAF I) which lacks spacers 37-39 is very common in the West African sub-region particularly in Cameroon. However, studies have indicated a decline in the strain (Mbelle *et al*., 1999; Niobe-Eyango *et al*., 2003) and confirmed by (Asante-Poku *et al*., 2015b). The Spoligotype pattern of *Mycobacterium africanum* type II (WAF II) which lacks hybridization to spacer 40 has mostly been found in isolates from Uganda (van Loo & Mooi, 2002). Cadmus *et al*. (2011) confirmed that strains of LAM10_CAM family were responsible for most TB transmissions in Ibadan under their study period and diverse strains of *M. tuberculosis* (i.e., SIT 838, 580, 403, 373, 71, 53, and 61) were found as stated early by (Lawson *et al*., 2012).

Significantly, diverse strains of *M. tuberculosis* were found and the SIT strains have also been reported in earlier studies where most of them clustered into the LAM10_CAM and T1 families (Cadmus *et al*., 2011; Lawson *et al*., 2012). The findings of Cadmus *et al*. (2018) confirm the underestimation of DR-TB in Nigeria and calls for concerted regional and technical efforts at regular surveillance and use of modern diagnostic tools to define the problem of resistance in Nigeria and the rest of Africa where the disease has remained unabated. A much more recent study conducted by Adesokan *et al*., in Nigeria on 64 MTBC isolates which were genotyped using spoligotyping revealed 22 different spoligotype patterns with the occurrence of 17 patterns (Adesokan *et al*., 2019). Five different clusters were revealed ranging from 2–29 isolates per cluster. What was worrying in their study was the emergence of Uganda I family with SIT 46 (45.3%) which is an indication

of a zoonotic link (Adesokan *et al*. 2019). This isolated spoligotype identified in their study of livestock workers and cattle suggests an infection originating from the cattle.

The following patterns were also identified LAM_CAM 10 family of SIT 61 (14.1%) and T family of SIT 53 (1.6%). There identified 14 new MTBC spoligotype patterns (3 *M. tuberculosis* strains, 6 *M. africanum strains,* and 5 *M. bovis* strains). Their study is the first to identify Uganda I family in Nigeria which is an indication of a rare lineage emerging in West Africa. This strain has previously been identified in Cameroon and Ghana (Asante Poku *et al.*, 2015a; Assam *et al*., 2013; Koro *et al*., 2015). Nonetheless, the LAM_CAM 10 clade is the most prevalent strain circulating in Nigeria (Lawson *et al*., 2012; Uzoewulu *et al*., 2016). An article review by Gehre *et al*. in 2016 of more than 16 original research articles from 12 out of the 15 West African countries indicated that the main lineages causing tuberculosis in these countries were the Euro-American lineage (L4), *M. africanum* lineages (L5 and L6). They also found out that 32 different families were responsible for infections with 8 contributing to 84.0% of all the infections (*M. africanum* West Africa 1 (MAF1), *M. africanum* West Africa 2 (MAF2), LAM 9, LAM 10, Haarlem 1, Haarlem 3, T 1 and Beijing families). The most common family was the T1 family. Nigeria and Benin were dominated by the MAF1 (L5) whiles MAF2 (L6) were common to Gambia and Guinea-Bissau (Gehre *et al*., 2016).

Affolabi *et al*. (2017) reported 40 different spoligotypes in Benin, West Africa with genotypic diversity of 40.0%. A diversity of 49.0% had been previously reported in Cotonou among new cases only in 2005 (Affolabi *et al*.,

2009). This indicates a similarity in both new and previously treated patients. A worrying trend is the emergence of the Beijing family that caused a major outbreak in Cotonou (Affolabi *et al*., 2009). The most frequent spoligotype isolated by Affolabi *et al*. (2017) was ST61 (LAM) that accounted for 33.0% of the studied isolates with lineage 4-Euro-American, including the Latin American-Mediterranean (LAM), Haarlem, X type and T families been the most prevalent at 74.0%.

CHAPTER THREE

MATERIALS AND METHODS

Chemicals, Reagents, and Equipment

Basic Fuchsin and Methylene Blue were obtained from Eurostar Limited, (UK). Absolute ethanol from Sigma-Aldrich, (USA). Sulphuric acid was obtained from BDH, (UK). Agarose gel, Ethylenediamine tetraacetic acid (EDTA), Tris-base, and ethidium bromide were also obtained from Sigma Chemical Corporation, USA. The Carbol-fuchsin stained slides were examined using a Leica CME microscope (Buffalo NY, USA). Centrifugation, vortexing, incubation, and hybridization were carried out in an Eppendorf microcentrifuge 5415C, Heidolph Vibramax Vortex Mixer, and a GFL Water Bath and GFL 7601 hybridization chamber respectively. Hain reagents and test strips were from Hain Lifescience GmbH, Nehren, Germany. Polymerase chain reactions (PCR) were carried out in a Dyad Peltier thermal cycler (BIO-RAD Richmond, California.). Agarose gel electrophoreses (AGE) were run on EC320 Mini Gel Electrophoresis System, USA, and first verified by viewing on a Vilber- Lourmat UV Trans-illuminator. Gel pictures were taken with an Olympus digital camera (Olympus imaging Corporation, Jakarta, Indonesia) connected to an Alpha Digidoc Gel analysis system (Alpha-Innotech/Cell Biosciences, USA).

Study Design and Set-Up

The main focus of this study was to take a retrospect look at the strains that were causing tuberculosis infections among patients based on research conducted at the Greater Accra Regional Hospital formerly known as the Ridge Hospital located within the central business part of Accra. The defined

population was taken from out-patients presenting at the hospital to undergo TB testing from January 2008 through July 2012. Sputum samples were decontaminated and then subjected to culture on Loewenstein Jensen media for isolation of bacteria. All positive cultures that indicated the growth of acid-fast bacteria were harvested and stored at -20^{o}C until analyzed. Efforts were made to include very recent samples (isolates) but logistical and financial limitations in the budget for this research work could not sustain it as well as time constraints.

Study Population

As stated earlier, positive TB isolates/samples had been harvested and stored after the culture of sputum samples. These sputum samples had been elicited from a population of patients presenting at the hospital for testing with a report from a certified Medical Doctor within the period January 2008 through July 2012. The phenotypic, genetic, and drug resistance characteristics of these isolates were not known at the time of its preservation. The retrieved isolates were sub-cultured on Loewenstein Jensen (LJ).

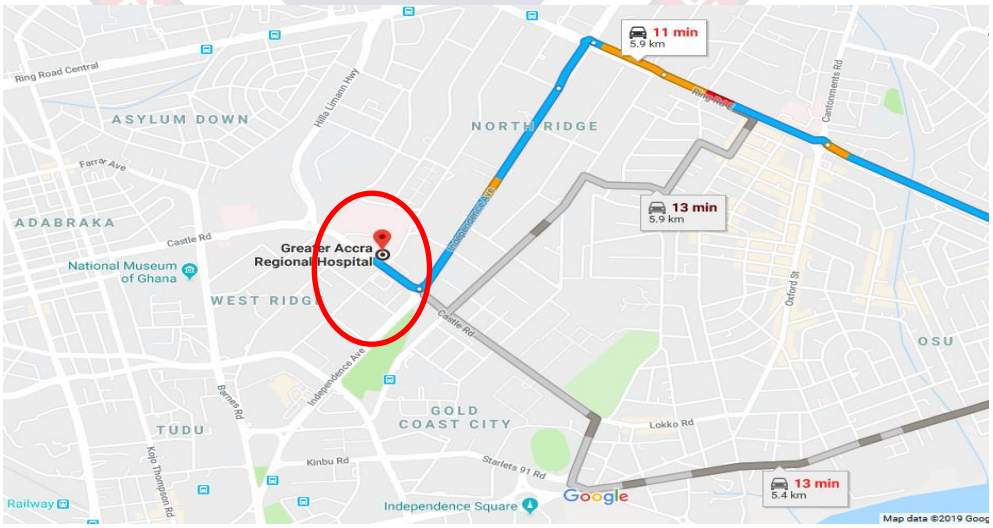


Figure 2: Google map indicating the location of Greater Accra Regional Hospital. (Source: Google maps (Accessed on 16/01/2019))

Ethical Clearance

Ethical clearance for these archived samples had been obtained from the Ghana Health Service Ethics Review Committee (ID No.:GHS-ERC:05/5/06) (Appendix I). Permission was also obtained from the Greater Accra Regional Hospital where samples were collected from the laboratory. All information obtained within the period of sample collection and analysis was kept confidential.

Sample Selection

Sample size and technique

Regarding analytical kits and consumables available and also to make the sample as large as possible, the convenient sampling technique was used and ninety (90) archived MTBC samples were selected. The convenience sampling method is relatively easy and inexpensive to conduct. The identification of strains and their geographical distribution as well as drug-resistant profiles had not been done.

Inclusion criteria

All archived MTBC isolates used in this study were obtained from the sputa of first-time infected patients who were not undergoing chemotherapy.

Data Collection

Bio-data information and mycobacterial culture data on all the ninety (90) isolates were retrieved from files from the laboratory of the Cellular and Clinical Research Centre (CCRC) Ghana Atomic Energy Commission (GAEC). This information was captured from the laboratory register which had been compiled and completed by the laboratory technician or technologist in-charge of the health facility. Sputum samples had been elicited from

presenting patients into 50 mL polypropylene tubes with screw caps, and an equal volume of 1.0% Cetyl pyridinium chloride (CPC) added in each instance. The CPC served both as a transport medium and a decontaminant. These were finally transported in a double sealed container to research laboratory of the Cellular and Clinical Research Centre of the Ghana Atomic Energy Commission for further analysis and examination.

Laboratory Methods

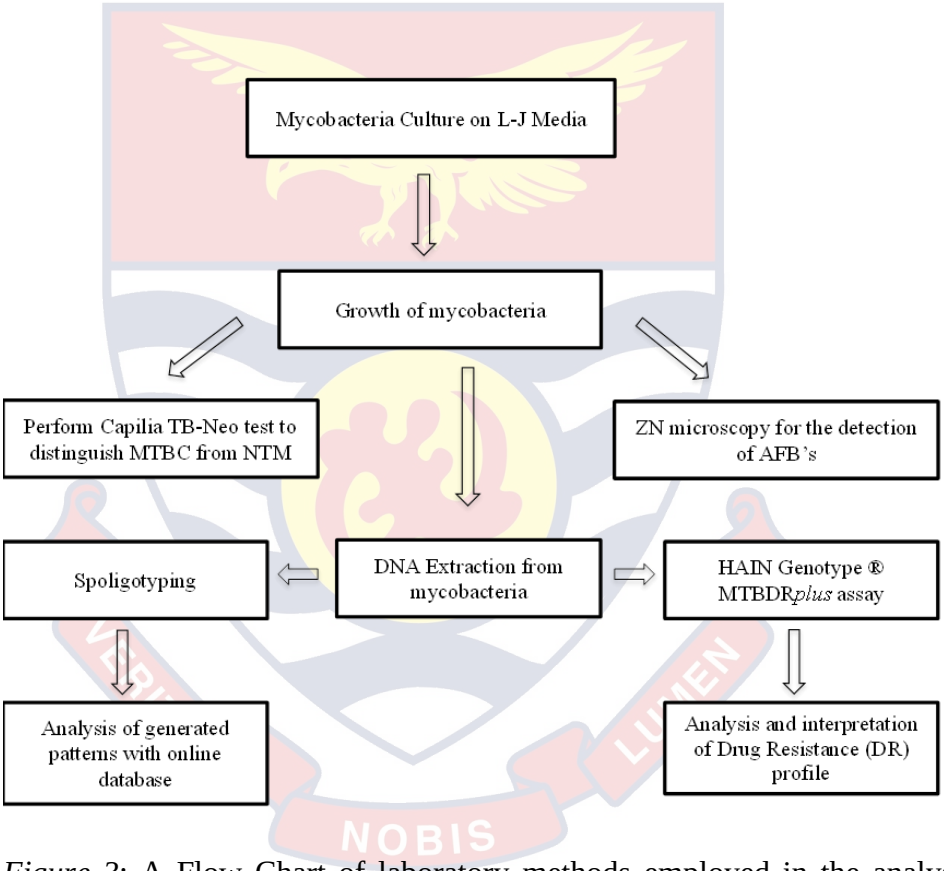


Figure 3: A Flow Chart of laboratory methods employed in the analysis of selected isolates

Sub-culture of Archived *Mycobacterium tuberculosis* Isolates

The archived samples that had been stored in Middlebrook 7H9 liquid media (a nutrient and preservative medium) at -20^{o}C were retrieved and the following experimental procedures were performed. All experiments were performed in a P2 laminar flow hood. The samples were allowed to thaw and

100 µL was inoculated on two (2) slants of LJ egg-based media that were prepared in-house at the laboratory of the Cellular and Clinical Research Centre (CCRC, GAEC). The slants were then placed in a Mermert incubator at 37^{o}C for 4-12 weeks. The incubated samples were checked weekly for growth and morphology. Tubes with sufficient bacterial colonies were selected for further testing.

Confirmation of acid-fastness of sub-cultured isolates

To confirm that the sub-cultured archived samples were indeed acid-fast, ZN staining and microscopy were carried out. Smears were prepared on frosted slides and fixed by passing them through a flame. The ZN staining and microscopy were performed according to standard protocol. This procedure is briefly explained. All heat-fixed slides were carefully placed on a stainless steel rack over a sink and flooded with Carbol-fuchsin. The bottom of the slides was heated slowly until the first appearance of vapour and left on the rack for 5 min and later rinsed under running tap water. The slides were flooded with 20.0% Sulphuric acid for 5 min and then rinsed again and stained with 0.3% methylene blue as a counterstain for 1 min to give it a blue background. All the slides were examined with a light microscope (Leica CME). The positive control used was a well-characterized MTB control strain (MTB Control strain) with sterile double distilled water as the negative control. A straight or slightly curved pink or pale red rod on a blue background indicated the presence of acid-fast bacilli whereas a negative result was characterized by the only blue background.

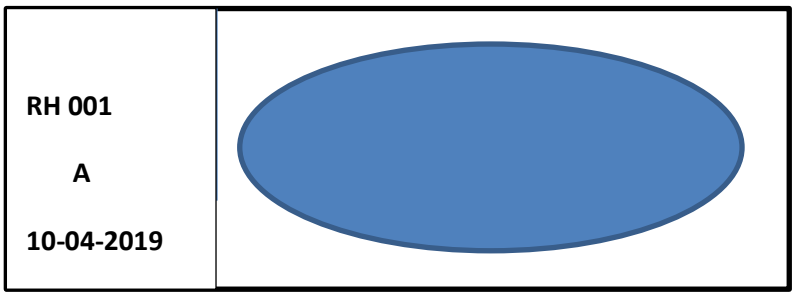
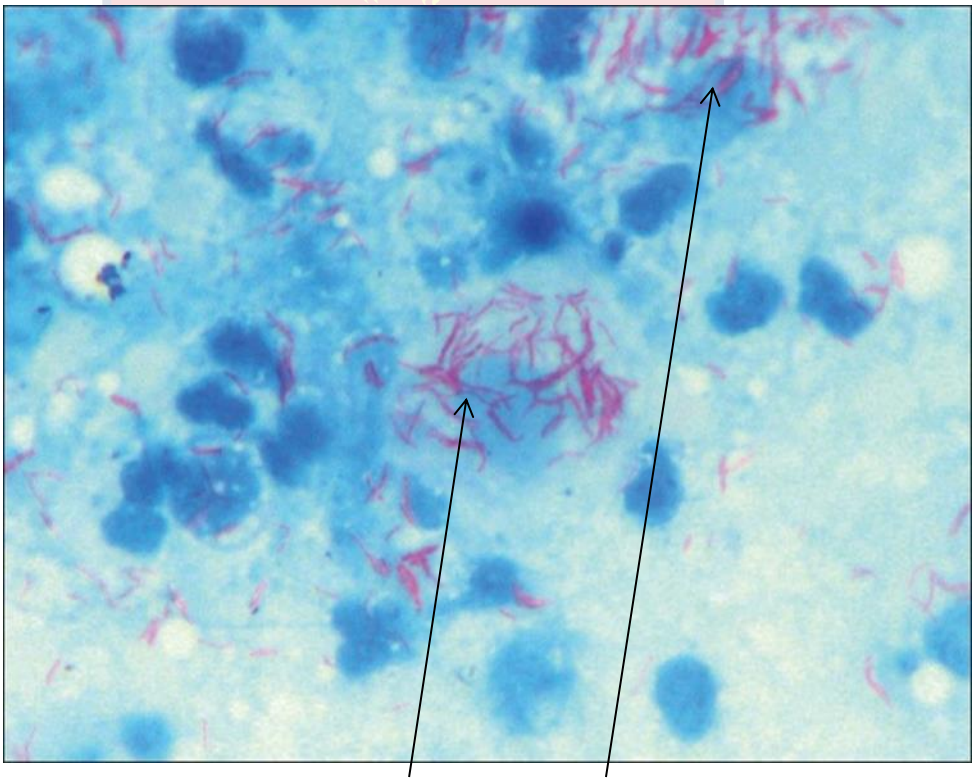


Plate 3: Diagram indicating how the glass microscopic slides were labelled. The labels 'RH 001' and 'A' represents sample identification number and sequence of smear preparation respectively. The date 10-04-2019 represents the date of smear preparation.



Pink rods (Acid-fast bacilli)

Plate 4: A positive microscopic field view of a slide depicting acid-fast MTB bacilli as pink rods in a blue background.

(https://www.google.com/dipaperjrta.ioptout.org _acid-fast-bacilli)

Screening of MTB Isolates

Capilia TB-Neo test (Immunochromatographic Assay)

The test was performed by dispensing 0.2 mL (200 µL) of the extraction buffer into appropriately labelled sterile 2.0 mL micro-centrifuge tubes. Sterile loops were used to transfer a bacterial colony into the tubes. This was vortexed vigorously for 5 min until a uniformly dispersed suspension was obtained. Approximately 100.0 µL of the specimen was aliquoted into the sample placement area of the test cartridge. This was covered and the result was observed in the reading area after 15 min. The results were recorded and interpreted. The development of purple colour in both the control and test band sections (C and T) indicates that the sample is positive for *M. tuberculosis*. When the purple colour is seen in only the control section that sample is classified as been negative for *M. tuberculosis*.

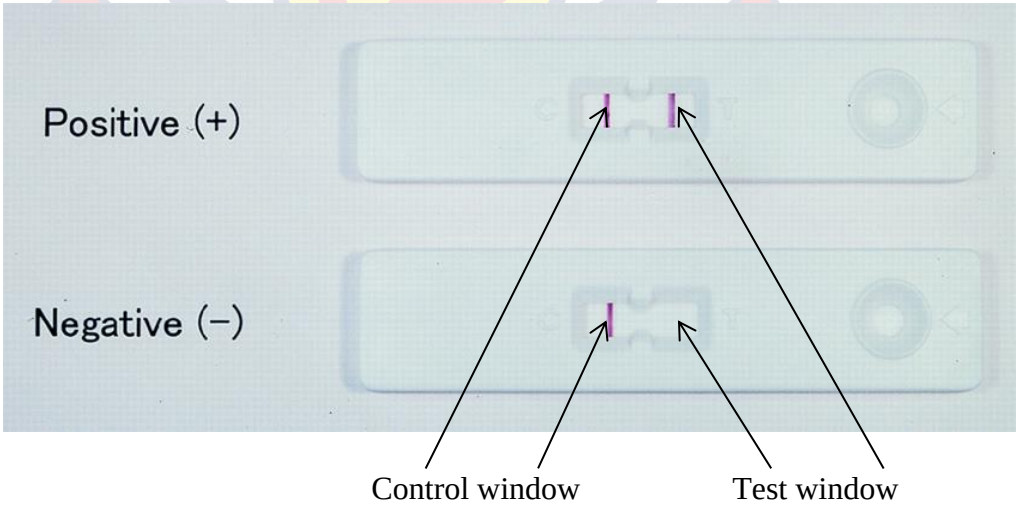


Plate 5: Image of Capilia TB- Neo cartridge depicting positive and negative test result respectively (http://www.tauns.co.jp/english/contact.php)

Isolation of Mycobacterial DNA

For each sample isolate, 150 µL of lysis buffer containing 10 mM Tris-HCl, 1 mM EDTA, and 1.0% Triton X-100 was aliquoted into the tube containing the suspended cell and vortexed vigorously for 10 s. Specimen tubes were then placed on a covered heating block and allowed to heat to 95ºC for 30 min. The cell lysates were vortexed briefly for 5 s and then centrifuged for 10 min at 14,000 rpm. One hundred and thirty-five microlitres (135 µL) of the supernatant were carefully transferred into a correspondingly labelled sterile 1.5 mL Eppendorf tube (ensuring that the cellular pellets formed were not disturbed). This supernatant obtained, therefore, contained DNA of acceptable purity to be used for further genetic analysis. The samples were placed in labelled cryogenic boxes and stored at -20ºC before molecular work.

Spoligotyping of Isolates

To amplify the Direct Repeat (DR) locus, the following primers were used for the PCR: DRa-bio: 5'– GGT TTT GGG TCT GAC GAC – 3', DRb: 5'– CCG AGA GGG GAC GGA AAC – 3'. DRa-bio is biotinylated to reveal hybridized patterns after autoradiography. The reaction for the PCR master mix was prepared as follows: 5.0 µL of PCR 10x Buffer, 5.0 µL of template DNA, 4.0 µL of primer DRa (at 20 pmol), 4.0 µL of primer DRb (at 20 pmol), 2.0 µL of dNTP mix (at 10mM), 0.2 µL of Taq Polymerase (at 5 units/µL) and 29.8 µL of HPLC grade water (which brought the total volume to 50.0 µL). All reagents were purchased from (Ocimum Biosolutions Hyderabad, India). The tubes were then placed in a Bio-Rad Dyad DNA Peltier Thermal Cycler (Bio-Rad Laboratories Inc., Ca., USA) for amplification. The cycling

parameters were: 95°C for 15 min, 1 Cycle; 94°C for 1 min, 55°C for 1 min, 72°C for 30 s, 30 Cycles; 72°C for 5 min, 1 Cycle; and finally held at 4°C.

Detection of hybridized PCR products

After the successful completion of the PCR, the products (amplicons) obtained were hybridized perpendicular to a Biodyne C membrane that contains 43 immobilized synthetic oligomeric spacers. The following buffers were pre-warmed at these various temperatures before use 2XSSPE/0.1% SDS at 42°C; 2XSSPE/0.5% SDS at 60°C; 2XSSPE/0.5% SDS at 42°C and 2XSSPE at room temperature. The PCR products of approximately 25.0 µL were mixed with 150 µL 2XSSPE/0.1% SDS and allowed to stand at 55°C for 60 min. This resulting mixture was heated at 100°C for 10 min and immediately placed on ice. During this period, the membranes to be used were then washed in a bowl containing 250 mL of 2XSSPE/0.1%SDS by gentle shaking at 42°C for 5 min. The washed membrane was then put on a supporting pad on a 45 lane blotter (Miniblotter 45, Immunetis, Cambridge, Ma., USA). The top right corner of the membrane was cut off as a mark of orientation on the cushion. The line patterns that had been generated by the oligonucleotide on the membrane were positioned at right angles to the slots on the mini-blotter. Excess fluid from the washing process that was left in the slots of the blotter was removed and the screws under the blotter tightened to prevent sample spill-over of reaction that could contaminate other slots.

The diluted PCR products were then transferred using a pipette into each well as labelled and then allowed to hybridize at 60°C for 60 min by placing it horizontally in a hybridization oven (GFL 7601, Gesellschaft für, Labortechnik mbH, D-30938 Burgwedel, Germany) without shaking. The

fluid in the slots of the 45-lane blotter was removed by aspiration. The membrane was then removed into a clean bowl using a pair of sterile forceps. It was washed (2X) with 250 mL 2XSSPE/0.5 %SDS at 60°C for 10 min and immediately put in a rolling bottle (Schott, Duran, Sigma Aldrich Co., USA) and left to cool. After the cooling, streptavidin-peroxidase conjugate 5.0 µL of 500 U/mL, (Boehringer Mannheim, Germany) was added to 14 mL of 2XSSPE/0.5% SDS and the membrane placed in this and incubated at 4°C for 60 min. After this incubation, the membrane was washed twice in 250 mL of 2XSSPE/0.5% SDS at 42°C for 10 min. It was immediately rinsed twice in 250 mL of 2XSSPE at room temperature for 5 min. The membrane was then incubated in a 20 mL volume of ECL detection solution which consisted of 10 mL each of Amersham ECL detection reagents 1 and 2 respectively (GE Healthcare Sciences, UK) for 1min. The membrane was removed and placed in a transparent plastic sheet and secured in a film cassette (Eastman Kodak, USA).

The membrane was then developed in a dark room after exposure for 5 min to a light-sensitive X-ray film (Eastman Kodak, USA) and then washing the X-ray film for 2 min in a Kodak GBX 'fixer and replenisher' (Kodak USA) and rinsing for a further 90s in a Kodak GBX 'developer and replenisher' (Kodak, USA). The resulting images generated were input into a Spoligo Excel spreadsheet. Image data was captured as a binary code of 0 and 1. White square dots (absence of spacers and consequently lack of hybridization) were indicated by '0' whilst black square dots (presence of spacers and consequently occurrence of hybridization) were indicated by '1'.

The obtained codes were used to enquire for signatures from the SITVITWEB database (Demay, *et al.*, 2012).

Drug-Resistance Testing using the Genotype MTBDR*plus* Assay

The HAIN Genotype MTBDR*plus* test was performed on the extracted DNA samples. The drug-resistant profiles of all the isolated strains were accomplished using the Genotype MTBDR*plus* assay version 2.0 from (Hain Lifescience GmbH, Germany) by following the manufacturer's instructions on a GT-Blot 48 Automated hybridization (Hain Lifescience, Nehren, Germany).

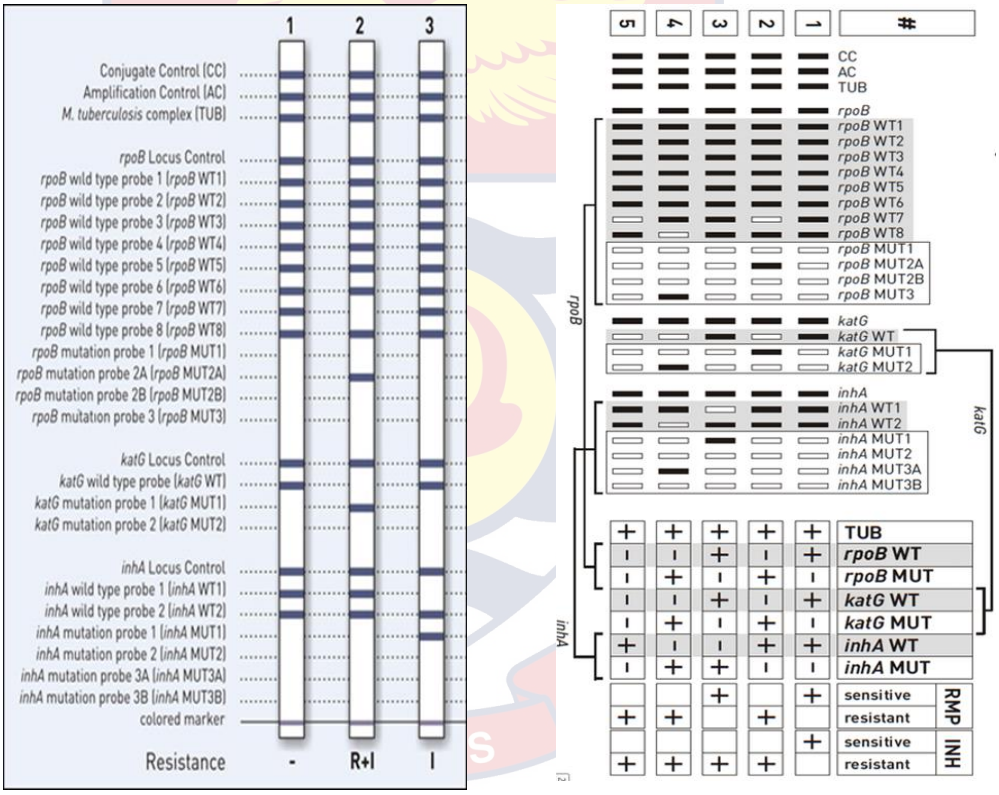


Figure 4: Image of HAIN Genotype MTBDR*plus* test strips format (www.hain-lifesciences.de)

Multiplex PCR amplification for the HAIN Genotype MTBCDR*plus* assay

The PCR amplification master mix was prepared according to the manufacturer's instructions as follows: An aliquot 10.0 µL of the reagent AM-A and 35.0 µL of the second reagent AM-B in a total volume of 45.0 µL and 5.0 µL of the DNA serving as the target template was then added. The amplification cycling parameters as provided by the manufacturer was as follows: initial pre-denaturing at 95°C for 15 min, 1 cycle; a second step of (95°C for 30 s and 65°C for 2 min), 10 cycles; a third step of (95°C for 25 s, 50°C for 40 s, 70°C for 40 s), 20 cycles; and finally 70°C for 8 min, 1 cycle. The amplicons after the amplification were verified by agarose gel electrophoresis and then stored at 4ºC until retrieved for further analysis.

Gel electrophoresis

PCR amplifications were verified by running a 2.0% agarose gel electrophoresis i.e. 1.2 g agarose (Sigma Aldrich, USA) in 60 mL 1X TBE incorporating 2.0 µL of 10 mg/mL Ethidium bromide. A volume of 10.0 uL each of the PCR product amplicon was thoroughly mixed with 5 µL of Orange G loading buffer (obtained by mixing 50 mL glycerol with 10 mL 10X TE buffer, pH 8, and 0.125 g Orange G). Molecular weight marker, 5 µL (100bp DNA ladder, Metabion, Germany) was also loaded for each row of PCR product amplicons, and the gels run at 100V for periods between 45-60 min. After electrophoresis, the gels were observed on a UV trans-illuminator and pictures of DNA bands taken with an Olympus digital camera coupled to an AlphaDigidoc gel analysis system.

Two close bands indicated the presence of members of the MTBC except for *M. bovis* BCG, a single band indicated non-tuberculous

mycobacteria (NTM) or atypical mycobacteria and three bands indicated specifically *M. bovis* BCG.

Reversed Line Blot HAIN Genotype MTBDR*plus* assay

After the successful amplification of these genes (i.e. *rpoB, katG and inhA*), the PCR products or amplicons were subjected to the Reversed Line Blot Genotype MTBDR*plus* Assay using GT-Blot 48-Automated hybridization machine *(*Hain Lifescience, Nehren, Germany) following the manufacturer's operating instructions with slight modifications as follows: Before loading the equipment, all reagents were preheated for 15 min. 20 µL of denaturing solution (DEN, blue) was mixed with 20 µL of corresponding amplified DNA sample in each good tray which was mixed and incubated at room temperature for 5 min. Each labelled LPA strip was placed in a well and transferred into the GT-Blot 48 hybridization machine. The hybridization was completed after 100 min. The tray was removed and the LPA strips dried on a clean tissue paper. The strips were then transferred to the Genotype MTBDR*plus* results sheet provided with the kit for interpretation (Barnard *et al*., 2012; Hain Lifescience GmbH, 2012).

CHAPTER FOUR

RESULTS

A total of 90 preserved isolates (i.e., new cases only) from a pool of isolates that had been collected from the Greater Accra Regional Hospital were analyzed in this study. Descriptive data on the population from which the archived isolates were obtained are shown in (Table 4). The proportion of male to female was males 70.0%, female 30.0%, with a male to female ratio of 2.3:1. The clinical information on all the isolates used indicated that they were new cases and had not been treated with any anti-TB drug before.

Table 3: ***Age characteristics of sampled patients yielding positive isolates***

Category	Number of Patients	Age range (years)	Average age (years) (s.d.)
Male	63	15 - 76	38.3 (7.3)
Female	27	15 - 88	35.9 (15.9)
Total	90	15 - 88	37.6 (14.0)

Legend: s.d. – standard deviation

The number of presenting patients and their age range categorization is shown in (Table 4).

Table 4: ***Number of patients presenting per isolates***

Age Range	Males	Female	Total
15 - 25	13	8	21
25 - 35	20	10	30
35 - 45	14	4	18
45 - 55	8	2	10
55 - 65	4	1	5
65 - 75	2	1	3
75 - 85	2	0	2
85 - 95	0	1	1
Total	**63**	**27**	**90**

Legend: The ages of the presenting patients and their frequencies

Confirmation of Acid-Fastness of Sub-Cultured Isolates

All the bacterial colonies harvested after the sub-culture on the LJ medium were observed to be generally rough eugenic buff as depicted in the picture below. The results from the ZN microscopic analysis showed that all the isolates were acid-fast.

Plate 6: A representation of colony formation on LJ Media (https://en.wikipedia.org/wiki/*Mycobacterium tuberculosis*)

Capilia TB-Neo test (Immunochromatographic Assay)

The Capilia TB-Neo assay revealed that 96.7% of the isolates were members of the MTBC and 3.3% were NTMs as shown in (Table 5)

Table 5: ***Categorization of isolates as either MTBC or NTM using Capilia TB Neo Assay***

Category	Number of isolates	Presence of MPB64 Protein (MTBC)	Absence of MPB64 Protein (NTM)
Male	63	61	2
Female	27	26	1
Total	90	87	3

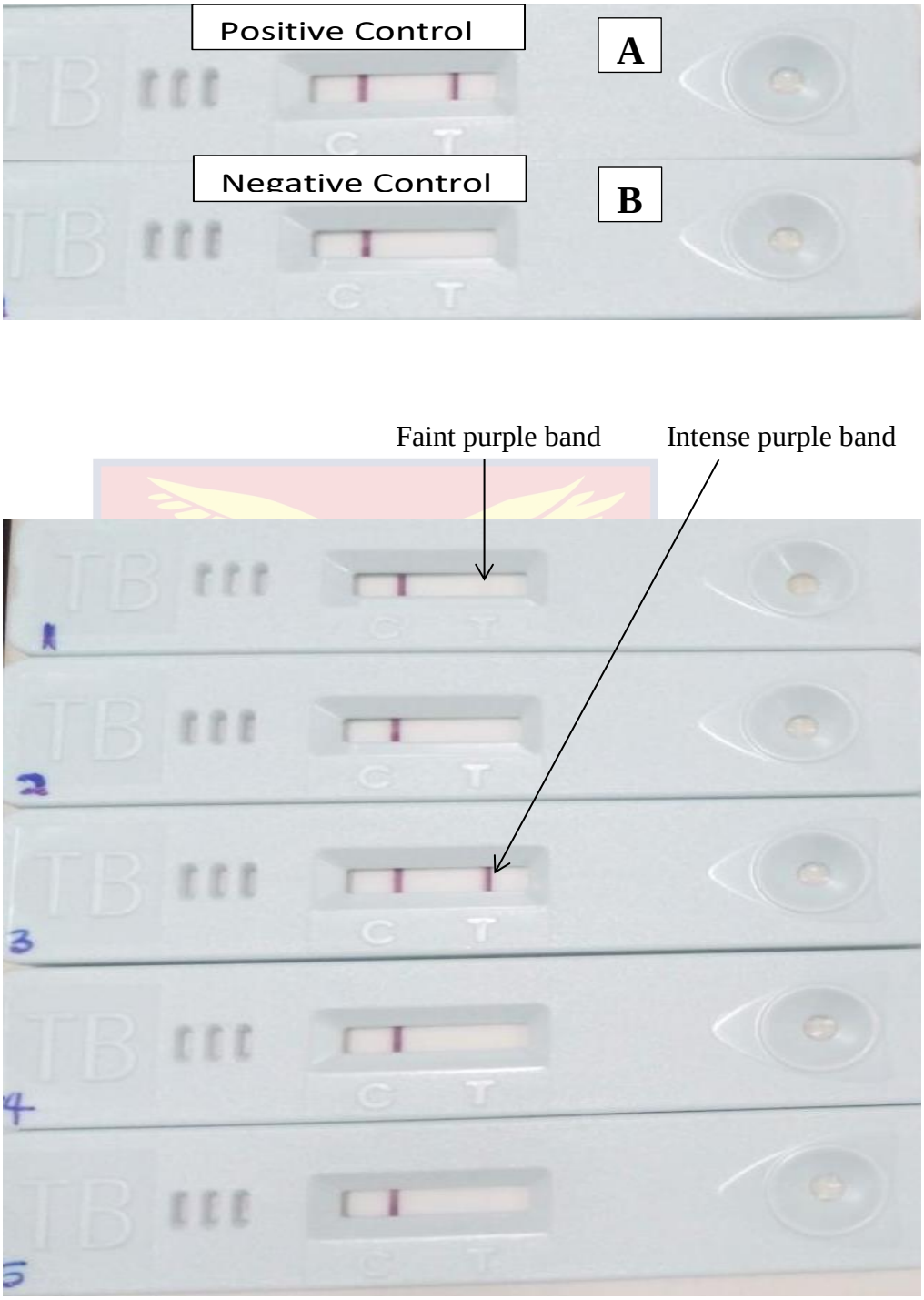


Plate 7: Cartridge of Capilia® TB-Neo test kit (Legend: **A** is a positive control test; a clear distinguishable reddish-purple band appears in the test (T) window. **B** is a negative control test; only one reddish-purple band appears in the control (C) window. Samples 1 and 3 are positive samples indicating they belong to the MTBC; Samples 2, 4, 5 are negative samples indicating they are NTM)

Spoligotyped Patterns

Result of isolates that were successfully spoligotyped.

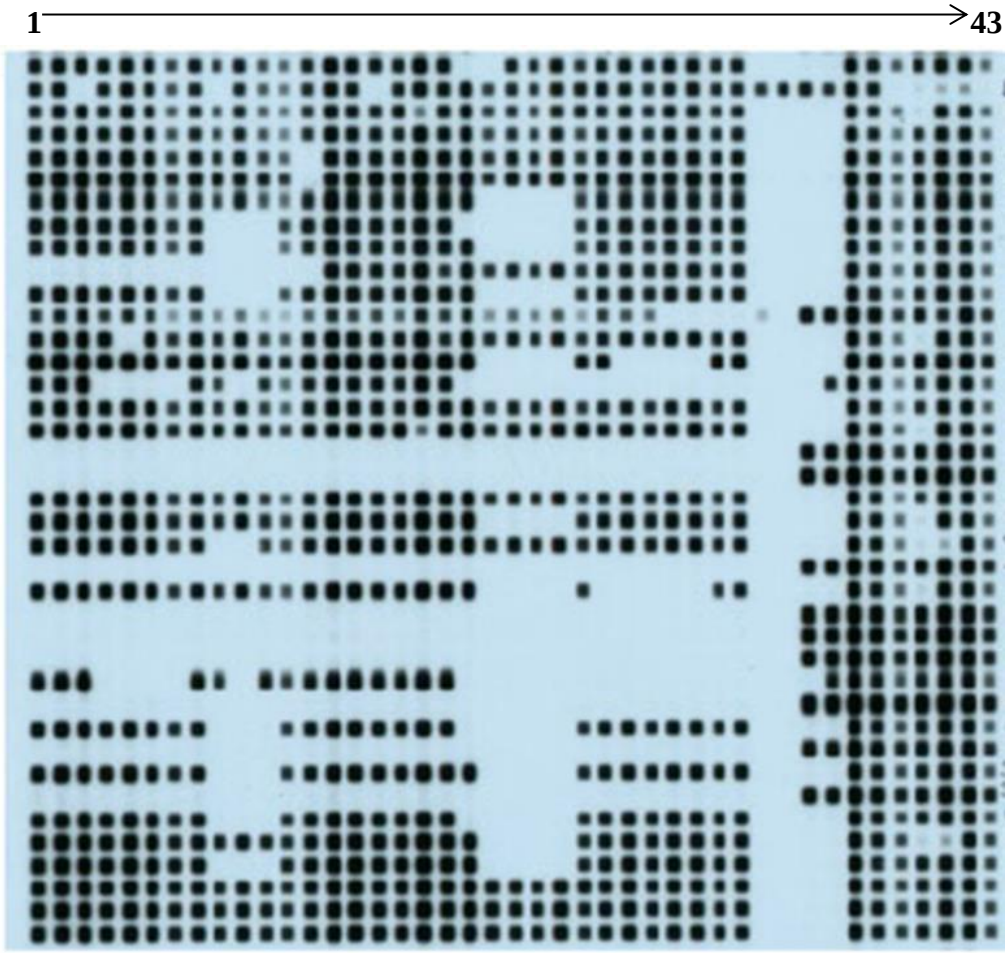


Plate 8: Spoligotype patterns of some selected isolates. (Legend: Dark squares indicate hybridization (presence of spacers) and blank or no squares indicate no hybridization (absence of spacer) in the direct repeat (DR) region of the MTB genome).

The SITVIT2 database which is an updated version of the SITVIT WEB database (Demay *et al*. 2012) an open access *M.tb* molecular marker database was accessed between October-November 2019. The Spoligotyped isolates were converted to lineages as described by Shabbeer *et al*. 2012. Tables 6, 7, and 8 describe the spoligotype lineages among the isolates as well as their frequencies and their SIT designations generated from the SITVIT2 database (http://www.pasteur-guadeloupe.fr:8081).

The spoligotype lineages and their frequencies as classified by the SITVIT2 database in with respect to family type are shown in (Table 6).

Table 6: ***Distribution of different lineages of isolates***

Lineage (Sub-Lineage)	SIT	No. of Isolates	Prevalence in %, n = 69
Cameroon	61	28	40.58
	57	1	1.45
	852	1	1.45
T1	53	10	14.45
	2900	1	1.45
T3	504	6	8.70
	331	3	4.35
AFRI_2	2984	1	1.45
	319	2	2.90
	50	2	2.90
H3	49	1	1.45
LAM 9	42	2	2.90
AFRI_1	181	2	2.90
CAS	1789	1	1.45
T4	40	1	1.45
AFRI	Orphan	1	1.45
EAI 5	1407	1	1.45
			Prevalence in %, n = 90
Not defined	Orphan	20	22.22
	1498	2	2.22
Unknown	2748	1	1.11
	2781	2	2.22
	1338	1	1.11

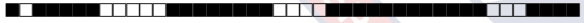
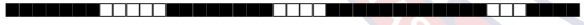
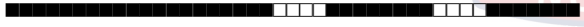
Legend: Signature International Type (SIT), T (T family), AFRI (*Mycobacterium africanum*), AFRI_1 (*Mycobacterium africanum* type 1), AFRI_2 (*Mycobacterium africanum* type 2), H (Haarlem), LAM (Latin American-Mediterranean), CAS (Central Asian), EAI (East African Indian)

The number of strains as analysed in the SITVIT2 database generated 40 distinct strain types. Sixty-nine (69) out of the 90 isolates generated 20 unique SIT numbers and the remaining 21 isolates were all orphan or probably new strains that have not been assigned an SIT number yet in the SITVIT2 database. Eleven major families were identified in the analysis of the 69 isolates. Sixty-eight of these isolates were assigned with SIT numbers and 1 was orphan or probably a new strain. The Cameroon family with SIT (61 (28 isolates); 57 (1 isolate); 852 (1 isolate)) were the dominant family 43.5% (n = 30) out of the total defined isolates. The second most dominant was the T1 family (15.9%) with SIT (53 (10 isolates); 2900 (1 isolate)). The other families were as follows: T3 with SIT 504 (8.7%) (6 isolates); AFRI_2 (8.7 %) with SIT (331 (3 isolates), 319 (2 isolates) and 2984(1 isolate); H3 (4.4%) with SIT (50 (2 isolates), 49 (1 isolate); LAM 9 with SIT (42) and AFRI_1 with SIT (181) contributed 2 isolates each (2.9%) each, CAS SIT (1789); T4 SIT (40) and EAI5 SIT (1407) contributed 1 isolate (1.5%) each with AFRI an orphan strain also at (1.5%).

Among the remaining 21 isolates, 20 were not properly defined and therefore were not assigned with SIT numbers indicating that they could be new or emerging strains. 6 of the isolates were classified as an unknown family but were assigned with SIT numbers (1498 (2 isolates); 2748 (1 isolate); 2781 (2 isolates) and 1338 (1 isolate).

The assigned spoligotype international type (SIT) of the MTB isolates indicating the frequencies of lineages in the population studied and defined in the SITVIT2 database (accessed between October-November, 2019), is shown in (Table 7).

Table 7: ***Distribution of Spoligotypes and their Lineages***

SIT	Spoligotype Pattern	Octal Code	Frequency	% in study	Lineage
1338		777777777777671	1	1.1	Unknown
1407		775377777413771	1	1.1	EAI 5
1498		777777776000371	2	2.2	Unknown
1789		703777740000171	1	1.1	CAS
181		770777777777671	2	2.2	AFRI_1
2748		774077600000031	1	1.1	Unknown
2781		777777777767771	2	2.2	Unknown
2900		777777763760771	1	1.1	T1
2984		770003607777071	1	1.1	AFRI_2
319		574077607777071	2	2.2	AFRI_2
331		774077607777071	3	3.3	AFRI_2
40		777777377760771	1	1.1	T4
42		777777607760771	2	2.2	LAM9
49		777777777720731	1	1.1	H3

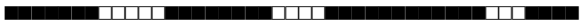
Legend: T (T family), AFRI_1 (*Mycobacterium africanum* type 1), AFRI_2 (*Mycobacterium africanum* type 2), H (Haarlem), LAM (Latin American-Mediterranean), CAS (Central Asian), EAI (East African Indian)

Table 7, Continued

SIT	Spoligotype Pattern	Octal Code	Frequency	% in study	Lineage
50		777777777720771	2	2.2	H3
504		777737737760771	6	6.7	T3
53		777777777760771	10	11.1	T1
57		777777143760771	1	1.1	Cameroon
61		777777743760771	28	31.1	Cameroon
852		400003743760771	1	1.1	Cameroon
New		774067400707031	1	1.1	ND
New		770367707777671	1	1.1	ND
New		374177607777031	1	1.1	ND
New		737777677760731	1	1.1	ND
New		770777703737231	1	1.1	ND
New		374177607777031	1	1.1	ND
New		777771000000771	1	1.1	ND
New		674077717777071	1	1.1	ND

Legend: SIT (Spoligotype International Type), ND (Not Defined), H (Haarlem), T (T family),

Table 7, Continued

SIT	Spoligotype Pattern	Octal Code	Frequency	% in study	Lineage
New		774017607777071	1	1.1	AFRI
New		777777543760771	1	1.1	ND
New		774077600000000	1	1.1	ND
New		774067600000031	1	1.1	ND
New		777367647777671	1	1.1	ND
New		002203034000000	1	1.1	ND
New		777757743760771	1	1.1	ND
New		777761077760771	1	1.1	ND
New		477777077720771	1	1.1	ND
New		777377637760671	1	1.1	ND
New		400003743760671	1	1.1	ND
New		477777777721771	1	1.1	ND

Legend: SIT (Spoligotype International Type), AFRI (*Mycobacterium africanum*), ND (Not Defined)

Spoligotype lineages among the isolates as well as their frequencies and their SIT designations generated from the SITVIT2 database (http://www.pasteur-guadeloupe.fr:8081) are shown in (Table 8).

Table 8: ***Isolates from Presenting Patients: SITVIT2 SITs and Lineages of spoligotypes of Archived MTB isolates***

Lineage	Sub-Lineage/ Family	SIT	No. of Isolates	Prevalence in %, n = 90
M. africanum	AFRI (1)	Orphan	1	1.11
Lineage 5 ***West Africa type I***	AFRI_1 (1)	181	1	1.11
Lineage 6 ***West Africa type II***	AFRI_2 (6)	331	3	6.67
		2984	1	
		319	2	
Lineage 4 ***Euro-American***	Cameroon (30)	61	28	33.33
		57	1	
		852	1	
	T1 (11)	53	10	12.22
		2900	1	
	T3 (6)	504	6	6.67
	T4 (1)	40	1	1.11
	Haarlem H3 (3)	50	2	3.33
		49	1	
	LAM 9 (2)	42	2	2.22
Lineage 3 ***East African-Indian***	CAS	1789	1	1.11
Lineage 2 ***East Asian***	EAI 5	1407	1	1.11
	Not defined (20)	Orphan	20	22.22
	Unknown with SIT (6)	1498	2	6.67
		2748	1	
		2781	2	
		1338	1	

Spoligotypes that were assigned with SITs, but could not be placed in any family or defined lineage in the SITVIT2 database were further analysed. The SPOTCLUST online resource (http://tbinsight.cs.rpi.edu/run_spotclust.html) was used to search for *M. tuberculosis* family similarity. It is also an online resource or tool used in advancing the study of MTB genotype data based on different models to identify families within the MTBC based on their spoligotype patterns. In the analysis, the family designation was retained when the probability was greater than or equal to (≥) 90% (Table 9).

Table 9: ***Isolate s of Unknown Lineage: Spoligotypes analysed with TB-Insight web tool***

SIT	Number of isolates	TB-Insight (SPOTCLUST) Most probable family	Probability
1498	2	M. tuberculosis Haarlem1	0.978860719
2748	1	M. tuberculosis EAI1	0.998297772
2781	2	Family33	1
1338	1	Family33	1

Clustering analysis was carried out using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) by assessing the MIRU-VNTR online tool (http://www.miru-vntrplus.org/MIRU/index.faces) as depicted in Figure 5 and Figure 6. The strains were defined as forming a cluster when two or more share the same and indistinguishable spoligotype pattern. They were grouped and categorized as a small cluster (2 isolates), medium cluster (3-5 isolates), large cluster (6-20 isolates), and very large cluster (> 20 isolates).

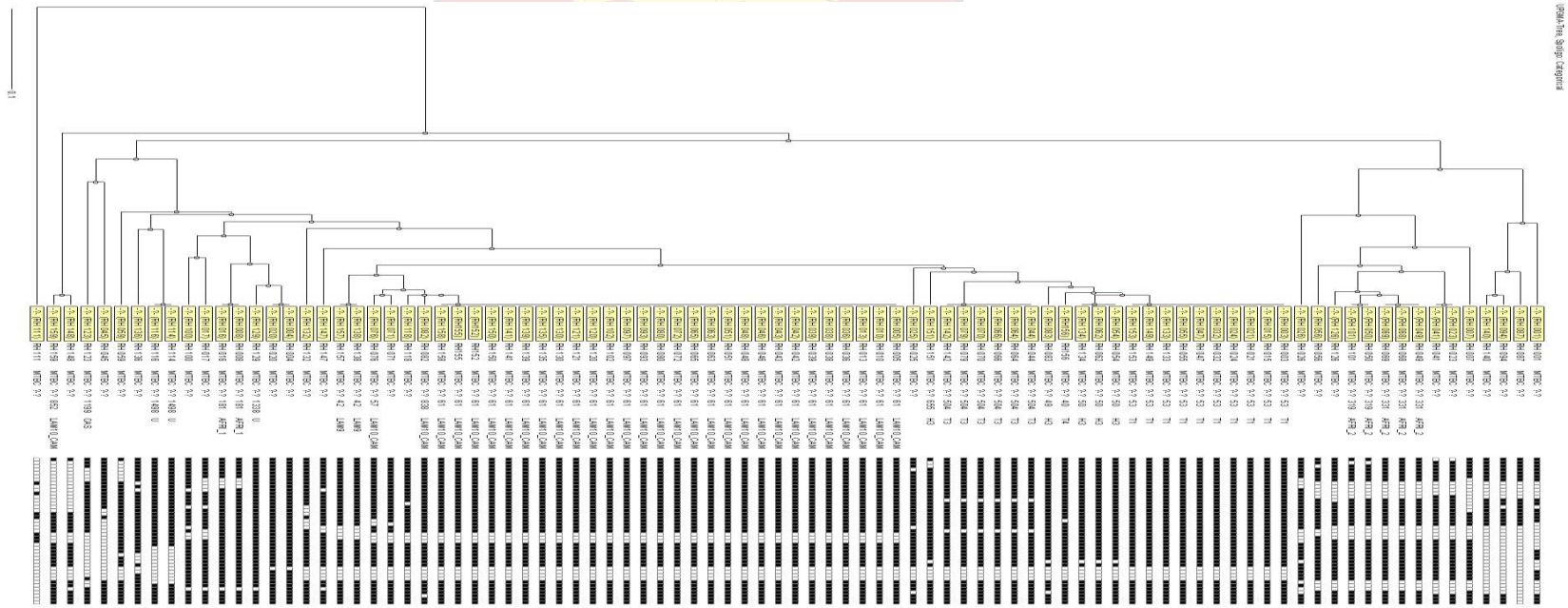


Figure 5: A UPGMA tree showing the Spoligotype lineage diversity of the 90 isolates from patients presenting at the Greater Accra Regional Hospital. The weighting distance measure is set as Categorical =1. (Legend: UPGMA=Unweighted Pair Group Method with Arithmetic Mean)

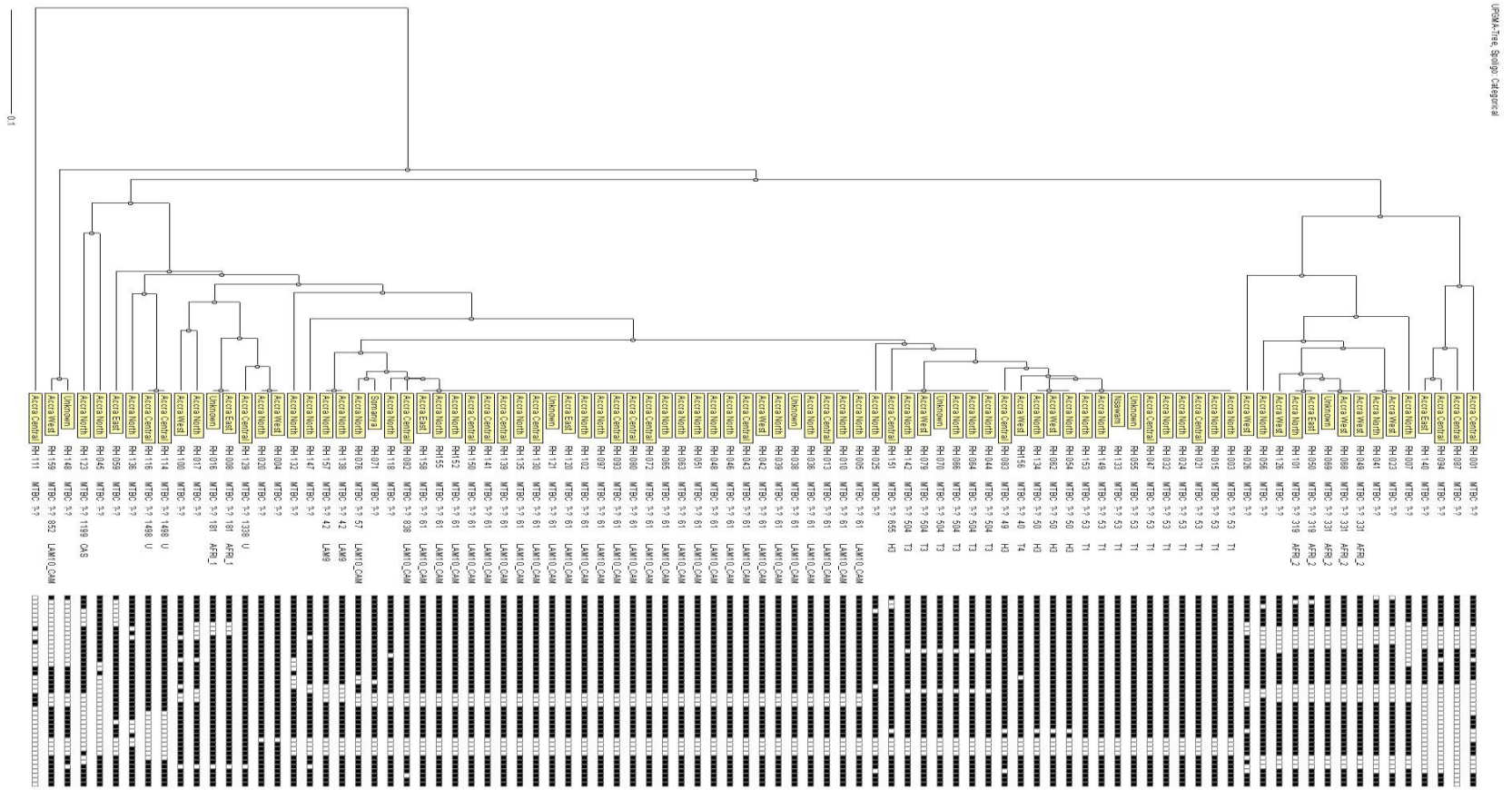


Figure 6: A UPGMA tree showing the Spoligotype Suburb diversity of the 90 isolates from patients presenting at the Greater Accra Regional Hospital. The weighting distance measure is set as Categorical =1. (Legend: UPGMA=Unweighted Pair Group Method with Arithmetic Mean)

PCR products of controls that were used in this study are shown in Plate 10 as well as studied isolates in Plate 11.

Gel Electrophoregram of HAIN Genotype MTBDRplus Assay

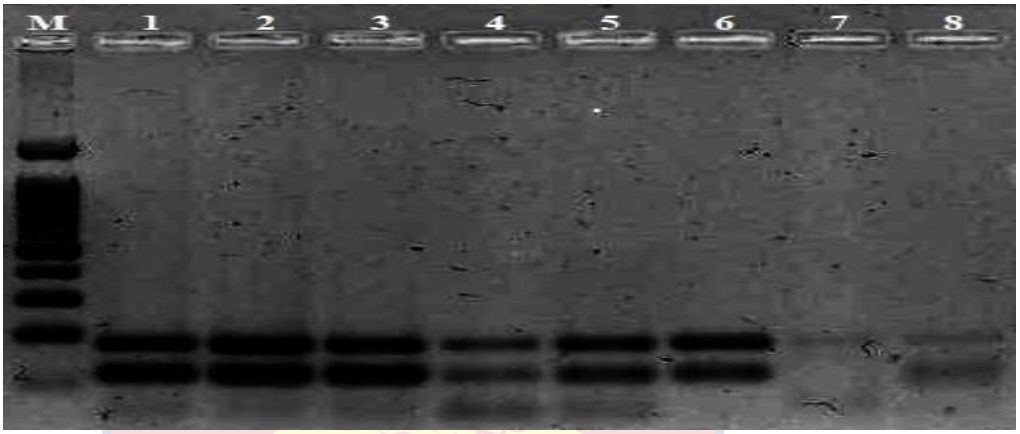


Plate 9: Agarose gel electrophoregram of PCR products of control (reference) strains using the HAIN Genotype MTBDR*plus* assay (Legend: M = 100 base pair (bp) marker; 1 = $H_{37}Rv$; 2 = MDR strain; 3 – 6 and 8 = MTBC; 7 = NTM).

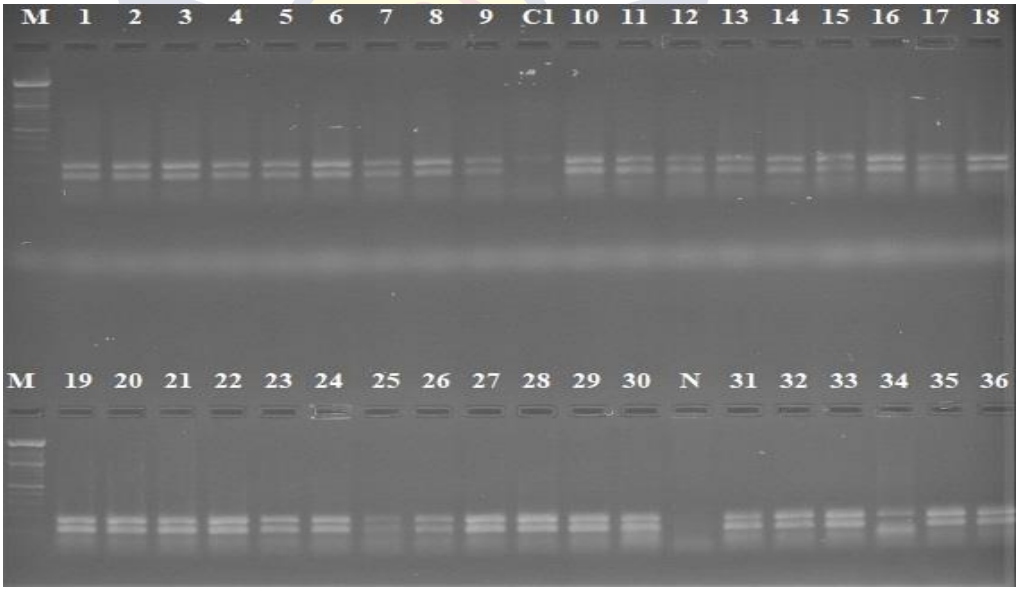


Plate 10: A representative agarose gel electropherogram of HAIN Genotype MTBDR*plus* assay of isolates before the determination of their drug resistance profiles (Legend: Single bands indicate NTM; double bands indicate MTBC, C1 = NTM negative control, N = negative control)

The results of the line probe assay analysis performed on the reference strains and the isolates studied are shown in (Plate 12) and (Plate 13).

Genetic resistance based on HAIN Genotype MTBDRplus Assay

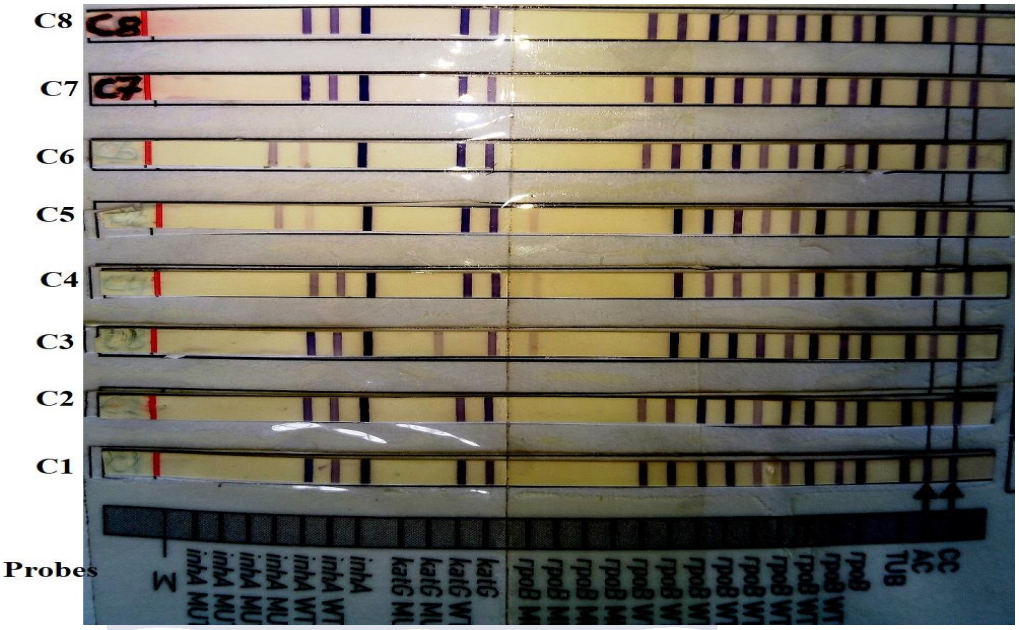


Plate 11: Drug resistance profile of reference strains used in the determination of isolate profiles (Legend: C1 = H37Rv – susceptible strain, C2 = Bovis BCG – susceptible strain, C3 = MDR strain, C4 = Rifampicin resistant strain, C5 = MDR strain, C6 = Isoniazid resistant strain, C7 = MTB susceptible strain, C8 = MTB susceptible strain)

Plate 12: A representative layout of the drug-resistant profile of isolate analyzed

A total of 88 isolates were analysed to confirm their resistance to isoniazid and rifampicin drugs. A predictive indicator of multi-drug resistance using the commercially available rapid DNA-based line probe assay (Genotype MTBDR*plus* kits) from Hain life science, Germany. Eighteen of the isolates were resistant to either isoniazid or rifampicin as depicted in (Table 10).

Table 10: ***Drug Resistance Profile of isolates from the study population***

(N=88)

Resistance Profile	Gene	Number of isolates			Prevalence (%)
		Male	Female	Total	
RIF only	*rpoB*	4	1	5	5.68
INH only	*katG* (High level resistance)	2	2	8	9.09
	inhA (Low level Resistance)	2	2		
RIF and INH (MDR)	*rpoB/katG* or *inhA*	4	1	5	5.68
Total (%)		**13.63**	**6.81**	**20.45**	**(20.45)**

Legend: RIF – rifampicin, INH – Isoniazid, MDR – Multi Drug Resistance

This study revealed mutations in both INH and RIF targets. The most prevalent mutations in the rifampicin mono-resistant isolates were found in codon 526 and codon 531 of the *rpoB* gene whiles with the isoniazid resistance isolates, mutations were seen in the *katG* gene at codon 315 and *inhA* -15C/T promoter region as shown in (Table 11).

Table 11: ***Frequency of Genetic Resistance based on HAIN Genotype MTBDRplus Assay*** **(N=88)**

Mono Resistant Isolates				
	Gene	**Mutations**	**Point of Mutation**	**Number of isolates**
Rifampicin Resistance	*rpoB*	MUT 2A	His526Tyr	3
		MUT 2B	His526Asp	2
		MUT 3	Ser531Leu	3
		MUT 2A/2B/3	His526Asp His526Tyr Ser531Leu	2
		WT 8	-	1
Isoniazid Resistance	*katG*	MUT 1	Ser315Thr1	1
	inhA	MUT 1 & WT 1	Cys15Thr	3
		MUT 1 & WT 1	Cys15Thr	1
		MUT 1 & WT (1&2)	Cys15Thr	2
		WT (1&2)	WT 1-(-15/-16) WT2-(-8)	2

Legend: MUT–Mutation, WT–Wild Type, His-Histidine, Tyr-Tyrosin, Asp-Aspartic acid, Ser-Serine, Leu-Leucine, Try-Tyrosine, Thr-Threonine, Cys-Cysteine

There were 5 MDR isolates among the samples analysed. These MDR strains depicted patterns with resistance to at least the *rpoB* gene and *inhA* gene or the *rpoB* gene and the *katG* gene. All these 5 isolates harboured mutation in the *rpoB* MUT 2A (His526Tyr). Four isolates had mutations in the *inhA* gene in MUT 3A (Thr8Cys) whiles the other isolate had a mutation in *katG* MUT 1 (Ser315Thr1) as depicted in (Table 12)

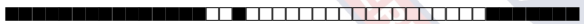
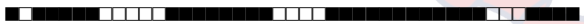
Table 12: ***Multi-Drug Resistant isolates and their associate mutations***

Drug	Gene	MDR Isolates Mutation	Point of Mutation	Frequency
Rifampicin Resistance	*rpoB*	MUT 1	Asp516Val	3
		MUT 2A	His526Tyr	5
		MUT 2B	His526Asp	2
Isonizid Resistance	*katG*	MUT 1	Ser315Thr1	1
	inhA	MUT 3A	Thr8Cys	4

Legend: MUT–Mutation, Asp-Aspartic acid, Val-Valine, His-Histidine, Tyr-Tyrosin, , Ser-Serine, Thr-Threonine, Cys-Cysteine

The resistance profile of the 5 MDR isolates and the spoligotype lineage they belong to are shown in (Table 13). The most dominant lineages, that is Cameroon (SIT 61), T1 (SIT 53), AFRI_2 (SIT 319), and H3 (SIT 50) all had an MDR strain among the isolates.

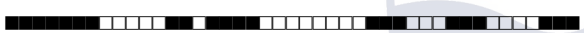
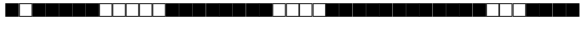
Table 13: ***Multi-Drug Resistant (MDR) isolates and their Spoligotype lineage distribution***

SIT	Spoligotype pattern	Octal Code	Lineage	MTBDR*plus* assay		Mutation Spot
				RIF	INH	
61		777777743760771	Cameroon	R	R	*rpoB*MUT1 (+), *rpoB*MUT2A (+), *rpoB*MUT2B (+), *inhA*MUT3A(+)
53		777777777760771	T1	R	R	*rpoB* WT7(-), *rpoB* MUT2A(+), *katG* WT(-), *katG*MUT1(+)
New		777771000000771	Unknown	R	R	*rpoB*MUT1 (+), *rpoB*MUT2A (+), *inhA*MUT3A(+)
319		574077607777071	AFRI_2	R	R	*rpoB*MUT2A (+), *inhA*MUT3A(+)
50		777777777720771	H3	R	R	*rpoB*MUT1 (+), *rpoB*MUT2A (+), *rpoB*MUT2B (+), *inhA*MUT3A(+)

Legend: R-Resistant, MUT-Mutatoin, *rpoB*-Rifampicin, *katG*-Isoniazid (High level resitance), *inhA*-Isoniazed (Low level resistance)

Table 14 shows spoligotype lineages and detailed mutations detected and their core region of resistance-conferring mutations. The identified mutations for *inhA* and *katG* gene both low and high resistance to isoniazid and *rpoB* gene, resistance to rifampicin are described.

Table 14: ***Selected Spoligotype isolates and their drug-resistant profiles***

SIT	Spoligotype Pattern	Octal Code	Lineage	MTBDR*plus* assay		Mutation Spot
				RIF	INH	
New		774067400707031	ND	S	R	*katG* WT(-), *katG* MUT1(+)
61		777777743760771	Cameroon	R	R	*rpoB*MUT1 (+), *rpoB*MUT2A (+), *rpoB*MUT2B (+), *inhA*MUT3A(+)
New		770367707777671	ND	S	R	*inhA* WT 1& 2(-), *inhA* MUT 1(+)
53		777777777760771	T1	R	R	*rpoB* WT7(-), *rpoB* MUT2A(+), *katG* WT(-), *katG*MUT1(+)
New		770777703737231	ND	S	R	*inhA* MUT 1(+), *inhA* WT 1(-)
New		777771000000771	Unknown	R	R	*rpoB*MUT1 (+), *rpoB*MUT2A (+), *inhA*MUT3A(+)
61		777777743760771	Cameroon	R	S	*rpoB* MUT 3 (+)
53		777777777760771	T1	S	R	*katG* WT (-); *katG* MUT 1(+)
319		574077607777071	AFRI_2	R	R	*rpoB*MUT2A (+), *inhA*MUT3A(+)

Legend: ND-Not determined, R-Resistant, S-Susceptible, WT-Wild type, MUT-Mutation, *rpoB*-Rifampicin, *katG*-Isoniazid (High level resistance), *inhA*-Isoniazid (Low level resistance)

Table 14, Continued

SIT	Spoligotype Pattern	Octal Code	Lineage	MTBDR*plus* assay		Mutation Spot
				RIF	INH	
New		403777777560771	ND	R	S	*rpoB* WT8 (-)
50		777777777720771	H3	R	R	*rpoB*MUT1 (+), *rpoB*MUT2A (+), *rpoB*MUT2B (+), *inhA*MUT3A(+)
504		777737737760771	T3	S	R	*inhA* MUT 1(+), *inhA* WT 1&2 (-)
1498		777777776000371	Unknown	R	S	*rpoB* MUT {2A, 2b, 3} (+)
New		777761077760771	ND	R	S	*katG* MUT 1(+)
61		777777743760771	Cameroon	R	S	*rpoB* MUT {2A, 2b, 3} (+)
1407		775377777413771	EAI5	S	R	*katG* WT(-); *katG* MUT 1(+)
61		777777743760771	Cameroon	R	S	*rpoB* MUT 2A (+)

Legend: ND-Not determined, R-Resistant, S-Susceptible, WT-Wild type, MUT-Mutation, , *rpoB*-Rifampicin, *katG*-Isoniazid (High level resistance), *inhA*-Isoniazid (Low level resistance).

CHAPTER FIVE

DISCUSSION

A lot of efforts and policies have been put in place for the diagnosis of tuberculosis concerning all the age ranges (i.e. from children to adults). The introduction of new methods for the detection and diagnosis of cases has improved although light microscopy is still widely used for sputum examinations. Spoligotyping is extensively used as a method of analysis to understanding the circulating strains within a specific geographical area or region. It generates knowledge of the lineages of the MTBC. This study was based on archived MTBC clinical isolates aimed at establishing spoligotype diversity in a population of presenting patients at a health facility in the Greater Accra Region of Ghana and also to determine the drug resistance profiles of these isolated strains to isoniazid and rifampicin using commercially available Hain Genotype MTBDR*plus* assay (Hain Life Sciences, Nehren, Germany, 2009).

Population characteristics; Microscopy, Sub-culture; Capilia TB-Neo test

The bio-data gathered from 90 MTBC isolates indicated that there were 70% males and 30% females which are consistent with other studies (Addo *et al*., 2017; Asante-Poku *et al.,* 2015a). As stated in the results section in (Table 3), males in the age range of 15-76 had a (mean age = 38.3 years, SD = 7.3 years) and females in the range 15-88 had a (mean age = 35.9 years, SD = 15.9 years) with a general population group (mean age 37.6 years, SD = 14.0 years). The male-to-female ratio observed was at 2.3:1 and it is comparable to the national average of approximately 2:1 as stated by (Asare *et*

al., 2018). Individuals below the age of 55 years were more likely to be infected with the disease contrary the findings of (Asare *et al*., 2018; Vluggen *et al*., 2017; Hamblion *et al*., 2016). Almost all isolates were identified as *M. tuberculosis* 96.7% with 3.3% as NTM by the Capilia TB-Neo test.

Spoligotyping (Lineages/families)

These findings are similar to studies that had been conducted within the West African sub-region. In the study by Adesokan *et al*. (2019), 22 different spoligotype patterns were identified with diversity at 34.4%. Fourteen new MTBC spoligotype patterns were recognized. They identified the following families as prevalent, Cameroon family (SIT 61), T1 family (SIT 53), and *M. africanum* (MAF 1- West Africa type 1). Studies conducted in Nigeria by Pokam *et al*. (2019) saw 58 different spoligotype patterns with 22 identified SITs with 36 new or orphan strains. The dominant families were Cameroon (SIT 61), Uganda I, Haarlem (H3), and *M. africanum* (West African 1). Affolabi *et al.* (2017) reported 40 different spoligotype patterns in their study in Benin corresponding to the genetic diversity of 40%. Nineteen isolates were found to be new spoligotypes. The predominant spoligotypes were found to be Cameroon family (SIT 61), T1 family (SIT 53), and *M. africanum*.

Niobe-Eyangoh *et al.*, (2003), first reported the Cameroon family (SIT 61) in Cameroon with a prevalence of 34.0%. It is a very dominant strain in Chad (Diguimbaye-Djaibe *et al.*, 2006), Benin (Affolabi *et al.*, 2009), Burkina Faso (Godreuil *et al.*, 2007), Guinea Bissau (Groenheit *et al.*, 2011), Mali (Traore *et al*., 2012) Nigeria (Ani *et al.*, 2010) and Ghana (Yeboah-Manu *et*

al., 2011). These findings are in agreement with the results generated from this study.

The indication of the Cameroon family being the predominant cause of TB in this research at 33.3% conforms to a previous study that had been done in Ghana (Yeboah–Manu *et al*., 2011). Asante-Poku *et al.* (2015a) which indicated that 65.0% of isolates they studied were from the Cameroon family with SIT 61. Other studies that have been done in neighbouring West African countries have reported similar findings; Nigeria, 52.1% (Molina-Moya *et al*., 2018), Burkina Faso 25.0% (Intemann *et al*., 2009).

Mostowy *et al*. (2004), in their study of *M. africanum,* established that two lineages out of the seven MTBC lineages, (*M. africanum* subtype I and *M. africanum* subtype II) predominantly originate from West Africa. *M. africanum* subtype I which is the (West African clade) has been sub-divided into (MAF 1) located around the Gulf of Guinea and (MAF 2) is prevalent in western West-Africa and exhibits *M. bovis*-like properties (de Jong *et al.,* 2010). The *M. africanum* type II (East African clade) has been reclassified into MTBss and is indicated as "Uganda" genotype (de Jong *et al.,* 2010; Mostowy *et al.,* 2004) and exhibits *M. tuberculosis*-like properties. de Jong *et al.,* (2010) indicated in their review paper that, 8.4% and 21.0% of isolates belonged to MAF 1 and MAF 2 which is similar to reports by (Asante-Poku *et al*., 2015b; Yeboah-Manu *et al*., 2011) which is in agreement with findings of this study MAF 1 (1.1%) and MAF 2 (6.7%).

Gomgnimbou *et al.* (2012) also detected *M. africanum* spoligotypes (West African 1 and West African 2) in 20.0 % of their studied isolates. In

Nigeria, the situation was not different as reported by other researchers, and the L5 (*M. africanum* - West African 1) genetic diversity was 15.8% and L6 (*M. africanum* - West African 2 was 2.7% (Molina-Moya *et al*., 2018). The prevalence of *M. africanum* reported by Adesokan *et al.* (2019) was 19.1% and 13.0% by Cadmus *et al.,* (2006) in South Western Nigeria which is comparable to findings in this study at 8.9% but lower than the 60.0% reported in Guinea-Bissau by Bonard *et al.* (2000). Notwithstanding this, Groenheit *et al.*, 2011 reported a 47.1% rate in Guinea-Bissau. This could be due to methodology, as molecular genotyping results are more definite and accurate compared to biochemical speciation (de Jong *et al.*, 2010; Niobe-Eyangoh *et al.*, 2003).

Studies conducted by Uzoewulu *et al.* in (2016) recorded 11.0% of isolates belonging to the T1 family (SIT 53) which is similar to the findings of this study at 12.2%. The most prevalent lineage in this study was the Euro-American lineage (L4) as seen in other studies in Ethiopia (Garedew *et al.,* 2013) and Guinea (Ejo *et al.,* 2015). In their study in Ghana, (Asare *et al.,* 2018) found the most prevalent lineage to be lineage 4, lineage 5, and lineage 6 whiles this study observed lineage 4, lineage 6, and lineage 5 contrary to their findings. Studies in Mali have indicated that the T1 family and Cameroon family from lineage 4 and *M. africanum* (West African 2) from lineage 6 are the leading causes of TB in Bamako as well as the spoligotypes from T3 family, Haarlem 2, EAI3 and family 33 are the commonly seen isolates from 2010 to 2016 (Togo *et al*., 2017) which is similar to findings in this study and others conducted in Ghana.

UPGMA analysis (clusters)

This study recorded 11 different clusters with 2-28 isolates per cluster at a clustering rate of 56.7% similar to studies done by Affolabi *et al.*, 2017. They identified 9 clusters with 2-3strains per cluster giving a clustering rate of 69.0% in their study. Adesokan *et al*. (2019) reported 5 different clusters with 2-29 isolates per cluster suggesting a clustering rate at 73.4%. Five major clusters 3-28 of 52 isolates were seen in this study. The cluster rate per spoligotype family was Cameroon (SIT 61) 31.1% (28 isolates), T1 (SIT 53)-Ghana clade 11.1% (10 isolates), T3 (504) 6.7% (6 isolates), H3 (50) and ARFI_2 contributed 3.3% (3 isolates) each.

HAIN Genotype MTBDR*plus* test (Drug-Resistant profiles)

The Beijing lineage has often been associated with drug resistance for reasons that remain unclear (Shanmugam *et al.*, 2011) but this lineage was not detected in the study. Studies carried out by Addo *et al.* (2017) revealed significant variations in isoniazid (INH) resistance-conferring mutations in different MTBC lineages using the HAIN Genotype MTBDR*plus* assay which conform to findings of this study as depicted in (Table 11). Homolka *et al.* (2010) saw a significantly higher proportion of *katG* 315 mutations in MTBss whiles *M. africanum* type I (MAF I) strains remained more possible to harbour a mutation in the promoter region of the *inhA* gene which was similar to findings of other studies (Otchere *et al*., 2016). Notwithstanding this, it was observed in the MDR resistant isolates mutations in codon 526 and 516 for the *rpoB* gene and codon 315 and -8T/C for the *inhA* promoter region that compares to studies done in Ghana by Addo *et al*. (2017), in Barcelona Spain

by Bernard *et al*. (2008) and in South Africa by Lacoma *et al*. (2008). Addo *et al*. (2017) stated that resistance against INH and RIF are commonly associated with mutations in *katG* (Ser315Thr) and *rpoB (*Asp516Val) that was also seen in this study but a significant number of isolates were harbouring a mutation in *katG* (His526Tyr) and (His526Asp).

Drug-resistance and its associated mutations complicate tuberculosis control and management in general. This leads to the use of second line drugs which are more expensive (D'Ambrosio, *et al.*, 2015). The resistance of isolates to specific drugs are related to mutations in specific genes as seen in this study and confirmed by (Somoskovi, *et al.*, 2001). Although mutations as stated by Addo *et al.*, (2017) are commonly associated with drug resistance, these mutations do not explain entirely the resistance that are observed (Chaoui *et al.*, 2009; Juarez-Eusebio *et al.*, 2017), indicating that other mechanisms may possibly be complicating the cases. The uncontrolled and unsupervised use of various antibiotics could justify the detection of drug-resistance among the studied isolates which is comparable to findings of (Yeboah-Manu *et al.*, 2012).

CHAPTER SIX

SUMMARY, CONCLUSION, AND RECOMMENDATIONS

Summary

Tuberculosis remains as one of the commonest communicable diseases in Ghana although the country is not listed as part of the high-burden TB nations in the world by the WHO. As stated by Valcheva *et al.* (2015), the characterization of the MTBC and its sub-populations in different geographical locations will facilitate the control and management of the disease. The addition of molecular analysis of isolates and epidemiological data from clinical samples will help in understanding the association that exists between strain type epidemiological characteristics of the disease. To control and manage tuberculosis, identification, and characterization of MTBC strains circulating within a population is very crucial as well as its susceptibility or resistance to drugs that are administered to patients must be a priority. This characterization will help in identifying strain families/lineages and also determine their diversity, distribution, and cluster rate. Notwithstanding this, the drug-resistant profile and genes harbouring mutations will help facilitate the treatment of patients.

The diversity of strains within geographical areas should be a major concern in finding a lasting solution to the TB menace. Approximately 96.7% were identified as members belonging to the MTBC and 3.3% as NTMs by the Capilia TB-Neo test. The typing method employed in this study (i.e., Spoligotyping) successfully revealed different MTB families and genetic diversity among the isolates studied. The high observance of isolates from

lineage 4 (Cameroon, SIT 61) and (T1, SIT 53) (Ghana clade) indicates the importance of these pathogens in the treatment of the disease. In terms of strain diversity, 5 out of the 7 TB lineages had isolates associated with them but the distribution of the strains was concentrated around Accra North 48.1%. As indicated in this study, the high prevalence of isolates from lineage 4 as to lineage 5 and lineage 6 does not mean outcompeting but further studies by Asare *et al.*, (2018) and Yebaoh-Manu *et al.*, (2016) have reported similar findings.

The Ghana sub-lineage T1 (SIT 53) has been reported to be associated with drug resistance (Otchere *et al.*, 2016) and becoming an important Public Health issue for the National Tuberculosis Control Programme requiring facilities to be accurately resourced to detect the spread of the Ghana sub-lineage. Fortunately, this study recorded a low prevalence of drug-resistant among the T family (T1 (SIT 53 – 2 isolates)) of which 1 was INH resistant and 1 MDR with (T3 (SIT 504 – 1 isolate)) that was INH resistant. Among the drug-resistant isolates, the dominant Cameroon family was 22.2% and what was worrying was the number of unknown or probably new isolates 38.9%. In the study population, total drug-resistant was seen to be 20.5%. Unknown strains that were causing resistance were 7.8% and therefore pose a risk to disease control. The most prevalent mutations in rifampicin were found in codon 526, codon 531, and codon 516 of the *rpoB* gene whiles with isoniazid mutations were seen in the *katG* gene at codon 315 and *inhA* -15C/T and -8T/C promoter region.

Analysis of spoligotype isolates by the MIRU-VNTR online tool MIRU-VNTR*plus* database (http://www.miru-vntrplus.org/MIRU/index.faces) revealed a clustering rate of 56.6% with the Cameroon family been prevalent 34.4% followed by the T family 18.9%. This observed trend from other studies done in Ghana (Asante–Poku *et al*, 2015a; Asare *et al*., 2018 & Otchere *et al*., 2016) could probably be indicating lineage stability.

However, because the archived samples collected were not representative of the region, the current study does not allow us to estimate the proportion of TB caused by the various MTBC clades in this region of the country. Such a systematic survey of the MTBC population structure in Ghana is currently in progress from various studies being carried out.

Conclusion

This study aimed to determine the spoligotype diversity and drug resistance of archived MTBC isolates originally obtained from the sputa of presenting patients at a health facility within the periods January 2008 through July 2012. The findings of this study will contribute to the understanding of the population dynamics of strains isolated from patients presenting at health facilities that they visit. A total of 90 archived MTB isolates were studied. This consists of 63 males and 27 females. Eighty-seven of the isolates were confirmed as members of the MTBC and three as NTMs. The predominant strains that were identified belonged to the Cameroon family (SIT 61), T family (SIT 53), and *Mycobacterium africanum* (West African type 2). Lineage 4 (Euro American) contributed the highest number of isolates 58.9% followed by Lineage 6 (West African type 2) 6.7% of the isolates spoligotyped

and 20 isolates were orphans or probably new strains. The clustering rate of the isolates after analysis with MIRU-VNTR online tool MIRU-VNTR*plus* database was 56.7%. The Hain Genotype MTBDR*plus* assay detected drug resistance to either rifampicin or isoniazid among 18 of the isolates and 5 were multi-drug resistant strains (MDR) (i.e. resistant to both rifampicin and isoniazid). Substantial mutations were seen in codons 526, 516, and 531 of the *rpoB* gene and on codon 315 of the *katG* gene for isoniazid and in *inhA* -15C/T of the promoter region of the *inhA* gene. To avert the transmission of MDR-TB and XDR-TB, there must be renewed efforts in drug resistance testing, infection control as well as capacity to detect outbreaks.

The National Tuberculosis Control program must enforce the strict use of drugs meant for TB treatment only so that it is not used for treating other infectious diseases. Disease control officers should be encouraged to monitor patients so as to improve compliance to treatment and early reporting.

Recommendation

1. With the more recent programme by Ghana Health Service for the introduction of GeneXpert machines into the TB diagnosis system for the detection of rifampicin-resistant strains by health facilities, efforts must be made to complement this with genotyping techniques and assays such as the Capilia TB-Neo assay for the detection of NTMs.
2. A worrying trend that was observed during this study is the seemingly increasing trend of the dominance of the Cameroon family (SIT 61) and T family (SIT 53) that must be further investigated.

3. With the higher percentage of isolates clustering, it will be prudent if mass screening programmes are done to find active cases.
4. Since the first-line anti-TB drugs include Pyrazinamide (PZA) and Ethambutol efforts should be made to include it in drug-susceptibility testing.

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GHANA HEALTH SERVICE ETHICAL REVIEW COMMITTEE

In case of reply the number and date of this Letter should be quoted

Health Research Unit

Ghana Health Service
P. O. Box G P- 184
Accra

17th August 2006

My Ref. :GHS-ERC: 3
Your Ref. No.

Tel. +233-21-681109
Fax + 233-21-226739
Email: Hannah.Frimpong@hru-ghs.org

ETHICALCLEARANCE
ID NO: GHS-ERC:
05/5/06

The Ghana Health Service Ethics Review Committee has given approval for the implementation of your protocol title:

"Application of Molecular Techniques in the Control and Management of Drug-Resistant Tuberculosis (DR-TB) in Selected Ghanaian Prisons"

PRINCIPAL INVESTIGATOR: Oti Kwasi Gyamfi

This approval requires that you submit periodic review of the protocol to the Committee and a final full review to the Ethical Review Committee (ERC) at the completion of the study. The ERC may observe or cause to be observed procedures and records of the study during and after implementation.

Please note that any modification of the project must be submitted to the ERC for review and approval before its implementation.

You are also required to report all serious adverse events related to this study to the ERC within seven days verbally and fourteen days in writing.

You are requested to inform the ERC and your mother organization before any publication of the research findings.

Please always quote the protocol identification number in all future correspondence in relation to this protocol.

SIGNED..... ...ALBERT GEORGE BAIDOE

ART PROF. ALBERT GEORGE BAIDOE AMOAH
(GHS-ERC CHAIRMAN)

Cc: The Administrative
GHS Ethics Review
Committee Health
Research Unit Accra