

**UNIVERSITY OF GHANA**

**COLLEGE OF BASIC AND APPLIED SCIENCES**

**EVALUATION OF OMNIGENE SPUTUM, A NOVEL SPUTUM TRANSPORT  
AND DECONTAMINATION REAGENT FOR MICROSCOPY, CULTURE AND**

**DNA-BASED ASSAYS**

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**(10305818)**

**THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON IN  
PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF  
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DEGREE.**

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## DECLARATION

This is to certify that this thesis is the result of research undertaken by Diana Asema Asandem towards the award of Master of Philosophy in Molecular Cell Biology of Infectious Diseases in the Department of Biochemistry, Cell and Molecular Biology, University of Ghana.

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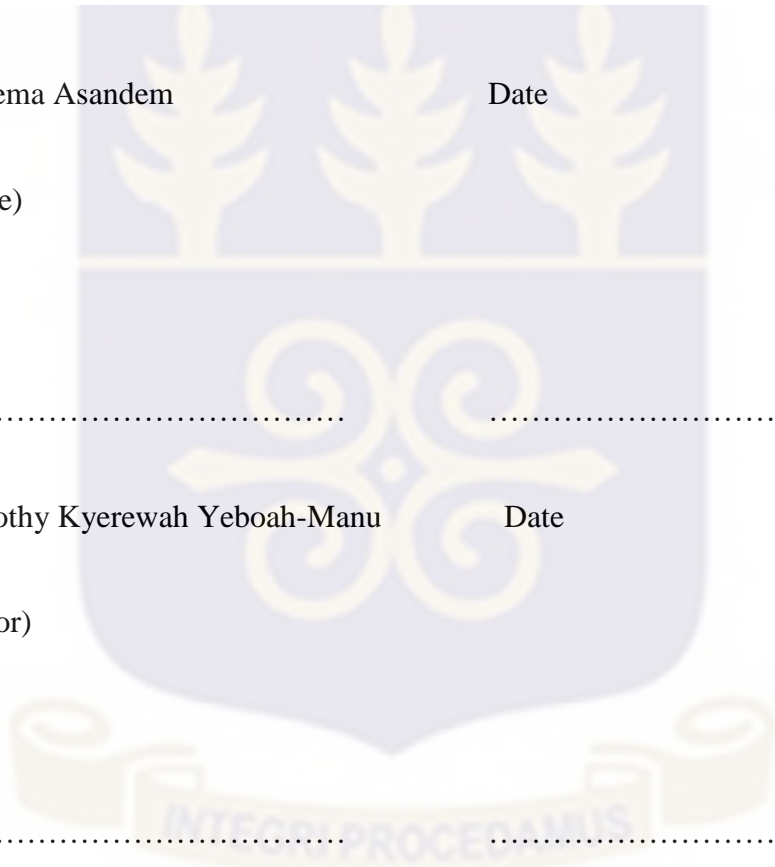
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## ABSTRACT

Tuberculosis (TB), continues to kill millions of people each year. Sputum culture for laboratory confirmation of TB diagnosis and treatment monitoring is limited to centralized facilities. Thus, samples require transportation from peripheral laboratories to these facilities, which compromises specimen quality since it is difficult to maintain cold chain. This project evaluated Omnigene Sputum reagent (OMS) for preserving sputa in transport.

One hundred and four sputa were collected from clinically diagnosed TB patients. Sputum contaminants were first characterized using blood agar cultures and MALDI-ToF MS then sputa were decontaminated with NALC-NaOH and OMS. The treated samples were first inoculated on blood agar to isolate persisting contaminants, then on Lowenstein Jensen (LJ) media for mycobacteria recovery before smears were prepared for microscopy. The compatibility of OMS for molecular analysis was determined using Xpert MTB/RIF analysis.

Before decontamination, 89.4% sputa had bacterial growth on blood agar and 4.8% and 5.8% still had contaminants after OMS and NALC-NaOH treatment respectively. On LJ media, higher contamination rate was recorded for NALC-NaOH (12.8%) samples than for OMS (4.3%) samples. However, mycobacteria positivity was comparable between both methods: OMS; 78.7% and NALC-NaOH 74.4%. Acid fast bacilli positivity after OMS and NALC-NaOH treatment was 89.4% and 75.9% respectively and 97.9% of the samples analyzed by Xpert MTB/RIF assay after each of the treatments were positive for MTBC with no detection of rifampicin resistance. Findings from this project clearly show that OMS can effectively be used as a sputum transport and decontamination reagent in Ghana, however I will recommend for further work involving field-testing.

## **DEDICATION**

This thesis is dedicated to Jehovah almighty, in whom I find my strength and zeal.



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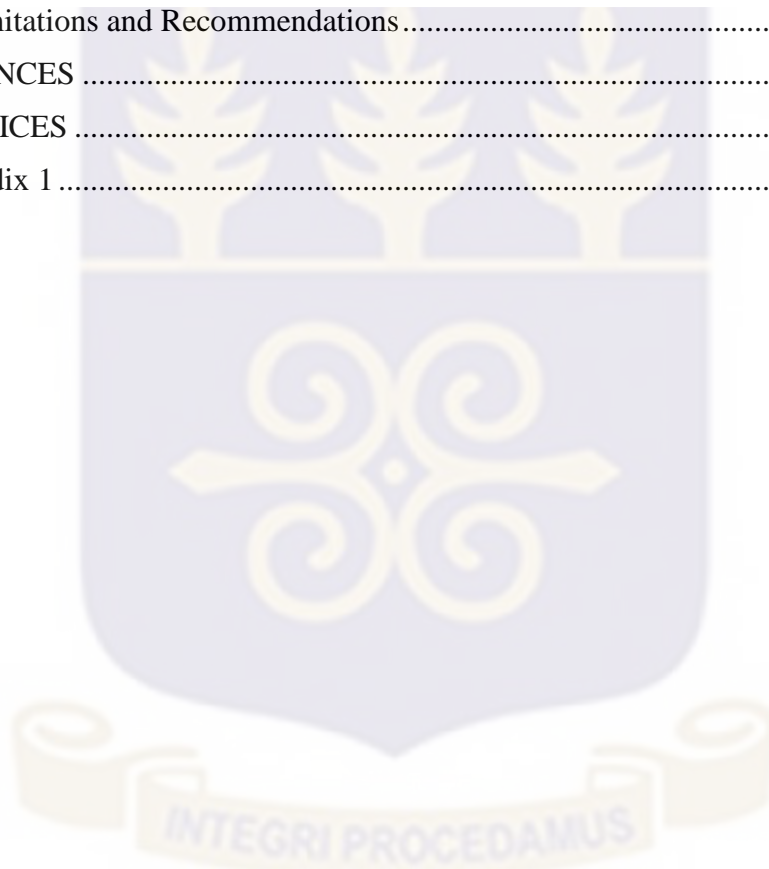
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## LIST OF ABBREVIATIONS

AFB	Acid Fast Bacilli
BCG	Bacillus Calmette Guérin
DOTS	Directly Observed Short Course Therapy
DST	Drug Susceptibility Test
INH	Isoniazid
MDR	Multi- Drug Resistance
MTBC	<i>Mycobacterium tuberculosis</i> complex
MTBss	<i>Mycobacterium tuberculosis</i> sensu stricto
MAF	<i>Mycobacterium africanum</i>
NALC-NaOH	N-Acetyl-L-Cysteine Sodium Hydroxide
NTMs	Non-tuberculous Mycobacteria
NTP	National Tuberculosis Control Programme
OMS	Omnigene Sputum reagent
PCR	Polymerase Chain Reaction
RIF	Rifampicin
TB	Tuberculosis
WHO	World Health Organization
ZN	Zeihl-Neelsen staining

## CHAPTER ONE

### 1.0 INTRODUCTION

#### 1.1 Background

Tuberculosis (TB), an ancient disease that has killed more humans than any infectious disease, is still of public health concern as it kills millions of people each year. TB is ranked one of the top 10 causes of death worldwide causing more deaths than Human Immunodeficiency Virus (HIV) (WHO, 2015b). A third of the world's population is latently infected with the causative agent, and this population serves as a pool for future cases (Dye & Williams, 2010). In 2015 alone, the estimated number of new TB cases was 10.4 million worldwide; 56%, 34% and 10% occurred in men, women and children respectively (WHO, 2016). The TB epidemic is fueled by the synergy with the HIV pandemic, urbanization leading to overcrowding, lack of effective vaccine and the emergence of strains resistant to anti-TB drugs (Friedland *et al.*, 2007; Keshavjee & Farmer 2012). If drug resistance is not controlled; a treatable disease can be rendered untreatable. In 2015, TB/HIV co-infected cases alone were 1.2 million, out of which 0.4 million died while the number of multi-drug resistant cases were 490 000 out of which 190 000 died (WHO, 2016). The main regions affected by the TB epidemic are sub-Saharan Africa and South-East Asia.

Tuberculosis is caused by a group of mycobacteria which together are termed the *Mycobacterium tuberculosis complex* (MTBC). This group of pathogens share up to 99% genetic similarity but show marked phenotypic differences ranging from their varying host adaptations to their preferred carbon source on solid agar (Cole *et al.*, 1998; Keating *et al.*,

2005). Organisms in this group include *Mycobacterium tuberculosis* sensu stricto (MTBss) and *Mycobacterium africanum* (MAF) which are human-adapted pathogens, *Mycobacterium microti*, *Mycobacterium pinnipedii*, *Mycobacterium caprae*, *Mycobacterium bovis*, *Mycobacterium mungi* and *Mycobacterium suricatta* which preferably infect varying animal species (Gagneux, 2012). MTBC have unique properties, which are crucial for their survival in the host. These include their distinct multi-layered cell wall comprising mainly of fatty acids and glycoproteins cross-linked to form a complex impervious structure as well as their slow growing nature (Hett & Rubin, 2008; Trifiro *et al.*, 1990).

Ghana remains burdened with the TB epidemic and currently has an estimated incidence of 281 per 100,000 people (WHO, 2015b). A survey carried out showed that the actual burden was more than three times the World Health Organization (WHO) estimated burden attributable to stigmatization of the disease leading to low case detection in many of the districts. At the community level, suspected TB patients are banned from trading at public markets and from participating in public events making them refrain from health centers, hide in the community and avoid being noticed (Dodor & Afenyandu, 2005; Dodor *et al.*, 2008). These people do not get the urgent medical attention they need serving as transmission sources of the disease. Post-mortem studies at the Korle-Bu teaching hospital, a tertiary health facility in Ghana shows tuberculosis as the leading cause of death amongst HIV patients (National TB programme, 2011). Furthermore, 19% of TB deaths in Ghana, occur in HIV patients (WHO, 2016). Ghana is not immune to the growing TB drug resistance burden, with an estimated 1500 drug resistant cases in 2015. Alarmingly, only a

quarter of these cases are detected, out of which only half get cured (WHO, 2015a). This poses a threat to the control program and requires urgent attention.

Due to lack of an effective vaccine, the main strategy of TB control programmes worldwide is prompt diagnosis and treatment to reduce transmission (Addo *et al.*, 2010). Central to TB case management is the laboratory, as results from microbiological confirmation determine the drug regimen given and for categorizing treatment outcomes. However, many laboratories especially those in developing countries are challenged with resources and lack the expertise to perform most detection methods (L. M. Parsons *et al.*, 2011).

Tuberculosis can be diagnosed using sputum smear microscopy, culture as well as DNA based techniques such as the Xpert MTB/RIF and the Genotype MTBDRplus (Ryu, 2015). Smear microscopy is the most widely used method of diagnosis in developing countries because it is simple, rapid and inexpensive. On the other hand, it relies on the collection of a quality sample with a high bacilli load (> 10,000) for acid fast bacilli (AFB) to be detected (Desikan, 2013). It is not a reliable tool for diagnosis in children and HIV patients who usually have a low bacilli load (Swai *et al.*, 2011). Moreover, it cannot be used to determine drug susceptibility. The current war waged against drug resistant TB necessitates early detection of drug resistance so that clinicians can take informed treatment decisions for appropriate patient management (Desikan, 2013; Kurz *et al.*, 2016).

Culture remains the gold standard for TB diagnosis because it is the main method for detecting viable organisms. (Centers for Disease Control and Prevention, 2009). Furthermore, sputum culture is used to monitor TB treatment especially in drug resistant or treatment failing cases, where sustained culture conversion from positive to negative is

used as a marker of clinical improvement and cure. Additionally, isolates retrieved from culture positive samples can also be used for drug susceptibility testing (Hopewell *et al.*, 2006). In recent times, many DNA based diagnostics which are fast, sensitive and specific have been developed and approved for TB diagnosis. The problem with these diagnostics is that they are expensive and require trained personnel to perform (L. M. Parsons *et al.*, 2011).

## **1.2 Rationale**

The global burden of TB and the rise in drug resistance calls for good laboratory support that could impact disease surveillance and research especially in developing countries to improve treatment outcomes. Due to biosafety, expertise and infrastructural requirements, TB diagnosis is limited to only sputum smear microscopy (Perkins & Cunningham, 2007; Speers, 2006). In Ghana for instance, there are only 4 laboratories that have the capacity to perform mycobacteria culture and not all can perform downstream DNA based analysis. Therefore, samples need to be transported to a reference laboratory through courier services for analysis, which sometimes can take several days before arriving at the reference laboratories.

In addition to surveillance, culture and DNA-based assays are needed for case monitoring. One key strategy in TB control is monitoring of cases under treatment. The intensive phase (first 2 months) of treatment involves the use of 4 drugs (isoniazid, rifampicin, pyrazinamide and ethambutol/streptomycin) after which smear results are expected to be negative (WHO, 2010). Subsequently, microscopy analysis at the 3<sup>rd</sup> and 5<sup>th</sup> months are also expected to be negative for treatment to be declared successful. In cases where smear

results turn out positive at any of the stages of monitoring, it is recommended that drug susceptibility testing be performed following culture, mainly to confirm mycobacteria viability and elucidate drug susceptibility pattern (WHO, 2010).

During sputum expectoration, several resident microbes are collected along with the TB bacilli. Due to the relatively faster growth rate of these microbes, there is a tendency for them to over grow in the sputum samples especially while they are transported to the laboratory for analysis (Allen *et al.*, 2016). Therefore, the samples need to be decontaminated with acids or alkali at specific concentrations and for specific durations to remove unwanted bacteria and fungi before culturing on selective media for mycobacteria growth (Kubica *et al.*, 1963). This is a key step in mycobacteria cultures because it prevents culture overgrowth and improves mycobacteria isolation. Mycobacteria can withstand these harsh treatments due to their hydrophobic and impervious cell wall structure (Brennan, 2003; Hett & Rubin, 2008). However, prolonged treatment may lead to the inactivation of the TB bacilli.

Furthermore, to reduce culture contamination, it is recommended that samples arrive at the reference laboratory for analysis the same day or be maintained at 2-8 °C for a maximum of 48 hours (Global Laboratory Initiative, 2014). However due to lack of logistics at the peripheral facilities and cost of courier services, samples are usually batched before transport, which in some cases takes more than the recommended 48 hours (Bolduc, 2016; Maharjan, Shrestha, *et al.*, 2016). Cetylpyridinium chloride (CPC), which was formerly used to preserve mycobacterium viability during transport, has been discontinued due to several adverse effects on bacilli and incompatibility with DNA-based tests (Sankar *et al.*, 2009; Selvakumar *et al.*, 2004; Smithwick *et al.*, 1975). The current war against TB

requires a decontaminant that is compatible for further analysis by both culture and DNA-based rapid diagnostics.

OMNigene sputum reagent is a newly formulated decontaminant produced by DNA Genotek (Ottawa, Canada) and according to the manufacturers, it is a highly stable non-toxic reagent designed to liquefy sputum, decontaminate and preserve TB bacilli at ambient temperature (4 °C – 40 °C) for up to 8 days; removing the requirement of daily transport of sputum, use of fridge and freezers for storage as well as maintenance of cold chain during transport (Kelly-Cirino *et al.*, 2016); hence very useful for low-resourced countries. This assertion needs to be evaluated in the Ghanaian setting to determine the feasibility in applying it to support the laboratory algorithm of the National Tuberculosis Program (NTP) for sample collection, processing and analysis.

### **1.3 Research questions**

**The questions that this thesis answered are:**

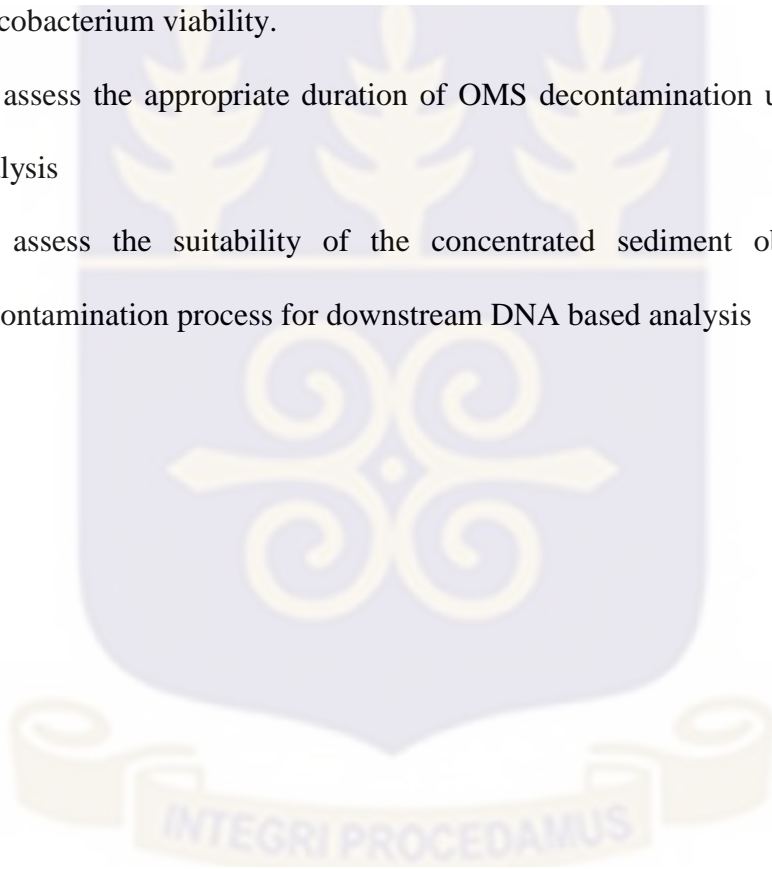
- Will OMS be as effective as NALC-NaOH for sputum decontamination?
- How long will mycobacterial viability be maintained in Omnigene sputum reagent (OMS)?
- Can OMS treated samples be used directly for DNA-based tests?

#### **1.4 Aim**

To evaluate the efficiency and suitability of OMS for sputum transport and decontamination, as well as its compatibility with DNA-based tests for use in Ghana.

##### **1.4.1 Objectives**

1. To assess the ability of OMS to decontaminate samples in transit while maintaining mycobacterium viability.
2. To assess the appropriate duration of OMS decontamination using a time course analysis
3. To assess the suitability of the concentrated sediment obtained after each decontamination process for downstream DNA based analysis



## CHAPTER TWO

### 2.0 LITERATURE REVIEW

#### 2.1 Historical facts of Tuberculosis

Tuberculosis (TB) is an ancient disease that has plagued mankind throughout history. The disease has killed more people than any other infectious disease known to man and genetic studies suggest that the disease has been present for at least 40,000 years (Galdston, 1954; Kapur *et al.*, 1994; Wirth *et al.*, 2008). The disease has been given different names: 'phthisis' by the Greeks (G. Cook, 1999), 'the white death' (Dormandy, 1999) and 'the great white plaque' (Galdston, 1954) by 17<sup>th</sup> century Europeans, and 'raja yaksma' by Indians, (Daniel, 2006; Prasad, 2002). Other names such as 'robber of youth', 'graveyard cough' and 'captain of all these men of death' were also used to describe tuberculosis, reflecting the horror and despair the disease aroused (Galdston, 1954).

In the 17<sup>th</sup> century, the disease was associated with cold weather, poverty, overcrowding and filth (Spence *et al.*, 1993; Trask, 1917). In the search for a cure, many theories sprung up claiming that fresh air and altitude could cure the disease. Isolated places of rest (Sanatoria) were created where mainly rich people who could afford it went to be cured (Hurt, 2004). There was no reliable treatment for TB at that time and treatment in these facilities were mainly bed rest and breathing in fresh air. Some physicians prescribed bleedings and purging and later surgical resection of the affected lungs was used to treat infected patients (Arthur, 1940). One in four patients died and the few people who recovered were plagued by harsh recurrences that ruined any hope of a normal life (Daniel, 2006).

Many studies were initiated that sought to understand the pathogen to control and eradicate the disease. Jean-Antoine Villeman and Robert Koch were the first to prove that TB is an infectious disease in 1868 and 1882 respectively. Robert Koch identified the bacilli, which is now known to be *Mycobacterium tuberculosis* (Cambau & Drancourt, 2014). Their findings changed the scientific community's view on the disease but led to stigmatization against infected individuals because they were considered contagious. Individuals suspected of the disease lost their jobs and were kicked out of their homes by landlords (Pôrto, 2007).

The hope for an intervention emerged in 1920 when Albert Calmette and Camille Guerin through a series of animal experiments produced an attenuated version of *M. bovis* which was approved for human trials as a vaccine called Bacille Calmette-Guerin (BCG) after the discoverers (Calmette Albert, 1927). Even though the BCG vaccine has been successful in preventing severe forms of TB in children, it is not effective in adults (Barreto *et al.*, 2006). The first chemotherapeutic agent against TB, streptomycin was discovered by Schatz, Bugie and Waksman in 1944 and not long after, other anti-tuberculous drugs, isoniazid and rifampicin were also discovered ("Streptomycin Treatment Of Pulmonary Tuberculosis: A Medical Research Council Investigation," 1948). These drugs have remained the backbone for the treatment of TB till today. Due to the upsurge of TB cases globally, WHO in 1993 declared TB as a public health emergency.

## 2.2 Global Tuberculosis burden

Even though TB is largely treatable, it is still a major cause of morbidity and mortality worldwide. According to the WHO, about 10 million people globally get sick with TB each year of which 10% are children and 62% are males. In the year 2015, 1.8 million people died from TB making it one of the top ten causes of death worldwide and claiming more lives than HIV in a single year (WHO, 2016). A third of the world's population is latently infected with the disease, serving as a pool of individuals for future occurrence of active disease (Ahmad, 2010).

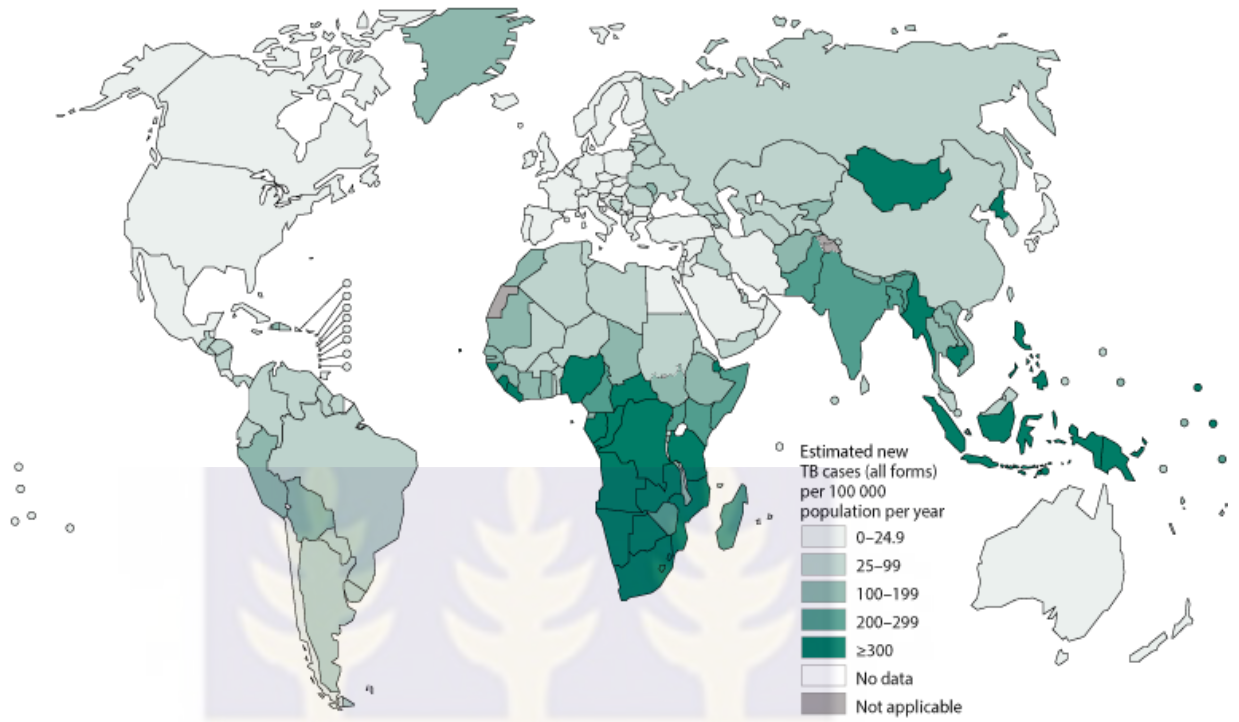
Majority of TB incidents were reported in developing countries in Asia (61%) and Africa (26%). Countries including China, Indonesia, India, Pakistan, Nigeria and South Africa reported 60% of all TB cases worldwide. India has the highest burden of TB with 40% of its population latently infected (WHO, 2016). In Africa, close to a million people died of TB in 2015 alone, 450,000 in non-HIV patients and 300,000 in HIV patients many of which are male and children below 18 years Old (Kanabus, 2016; WHO, 2016). Nigeria has the highest TB incidence in Africa with an estimated 586,000 people having the disease. Africa, which houses only 11% of the world population is estimated to contribute to over 26% of the global burden making Africa, the continent with the highest disease burden when population size is considered (WHO, 2016). Figure 2.2 shows the global TB burden for 2015.

Several factors including the HIV epidemic, late reporting of cases which increase community transmission, the increasing number of drug resistant TB which is fueled by non-compliance to treatment regimen and the ineffective BCG vaccine in adults is driving the TB epidemic. TB is the most important opportunistic infection in HIV and apart from

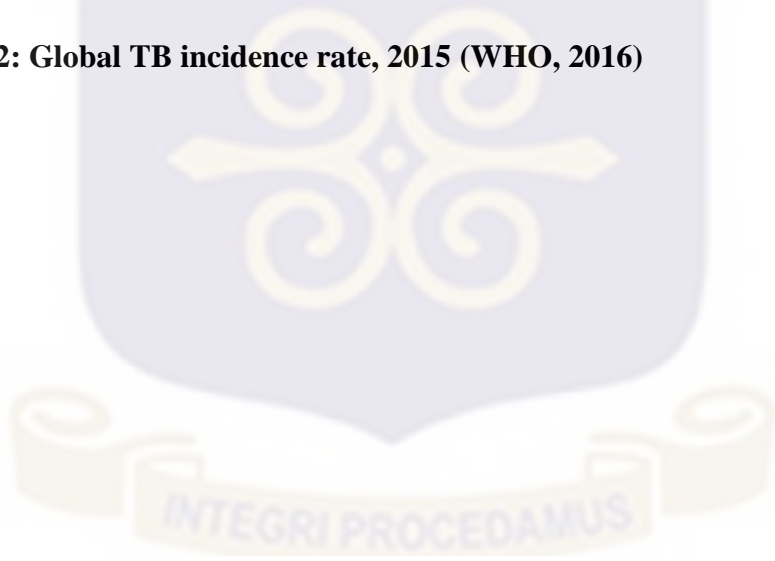
increasing the risk of reactivation of latent TB, it also increases the risk of progressing faster to active TB disease after infection (Daley *et al.*, 1992). Thus TB is a common cause of morbidity in HIV- positive adults and in 2015, 11% of TB cases worldwide were in HIV infected people out of which 0.4 million died (WHO, 2016).

A major complication to TB control is the emergence of mycobacteria strains that are resistant to the current anti-TB drugs. Drug resistant TB is a key threat to TB control programs because they can render a treatable disease untreatable (Liang *et al.*, 2017). Globally, 3.9% of new TB cases and 21% of previously treated cases are drug resistant cases. Additionally, close to 300, 000 people died from drug resistant TB in 2015 (WHO, 2016). Apart from the lengthy treatment regimen these patients are put on, they are also required to take more drugs (Hopewell *et al.*, 2006). The side effects of the prolonged usage of these drugs render patient's lives uncomfortable and stressful.

Additional factors such as overcrowding, poor sanitation and housing as well as poverty have also been implicated in the persistence of TB (Santos *et al.*, 2007). The socio-economic impact of TB cannot be overemphasized seeing that majority of affected people are either children or adults in their productive years (Millet *et al.*, 2013). In addition to affecting productivity especially in high burdened countries and hence directly fueling poverty, TB also has non-treatment costs socially and psychologically, which leads to lower standards of living of TB patients and their families (Morris *et al.*, 2013).



**Figure 2.2: Global TB incidence rate, 2015 (WHO, 2016)**



### **2.3 Tuberculosis burden in Ghana**

In Ghana, TB associated mortality was reported even before independence. The disease is believed to have come into the country through European conquerors and nomads migrating for trade purposes (Koch, 1960). The first tuberculosis clinic was set up in 1953 by Dr. Pointon-Dick in Accra. However, due to limited resources, progress in controlling the disease was slow (Koch, 1960). A formalized control unit was established in 1959 to lead the fight against TB but activities towards the control of the disease declined between 1960 and 1990 (Amo-Adjei & Awusabo-Asare, 2013). The emergence of HIV in 1988 fueled re-emergence of TB and caused the disease to re-gain interest in the country (Amo-Adjei & Awusabo-Asare, 2013). The National Tuberculosis control Programme (NTP) was established in 1994 with the mandate to champion the fight against TB (Whalen, 2007).

Currently, Ghana has a TB prevalence of 281 per 100 000 population making it one of the high burdened TB countries (WHO, 2016). Being equally burdened with HIV, it is not surprising that the TB burden continues to increase, accounting for up to 20% of deaths in HIV patients. At the Korle-Bu Teaching Hospital (KBTH), TB is responsible for 40-50% of all HIV deaths (Ghana Health Service, 2007). Additionally, 1500 cases were estimated to be resistant to at least one of the most potent first line anti-TB drugs, that is rifampicin only or both rifampicin and isoniazid (MDR/RR-TB) (WHO, 2016). However, only a quarter of these cases were detected and only half of the detected cases were cured after treatment ("The End TB Strategy: a global rally," 2014).

Per the 2014 NTP report, the regions in Ghana reporting the highest numbers of cases are the Ashanti and Greater Accra regions, reporting 3,041 and 2,901 TB cases respectively. The Northern, Upper East and Upper West regions reported the least number of cases (642,

708 and 330 cases respectively). The formal reported cases in Ghana is generally low compared to WHO estimates and the prevalence rates obtained after the current National Prevalence Survey conducted in 2014. The lower number of cases reported may be due to stigmatization of affected persons leading to low hospital attendance, as well as limited number of diagnostic facilities present in these regions for cases to be reported to (Abdul-Karim, 2013; Amenuvegbe *et al.*, 2016).

#### **2.4 Causative organism**

Tuberculosis is caused by a group of acid-fast bacilli termed the *Mycobacterium tuberculosis complex* (MTBC). These organisms exhibit low genetic variations yet have some phenotypic differences which are obvious in their host adaptations, though there are incidents of cross infections (Frothingham *et al.*, 1994; Gordon *et al.*, 2015). The MTBC comprises nine pathogens and has been divided into human and animal-adapted pathogens. *Mycobacterium tuberculosis sensu stricto* and *Mycobacterium africanum*, are known to infect mainly humans, hence referred as the human adapted MTBC. The remaining 7 are the animal adapted-species; *Mycobacterium microti* infects rodents, *Mycobacterium pinnipedii* infects seals and sea lions, *Mycobacterium caprae* infects sheep and goat, *Mycobacterium bovis* infect cattle while *Mycobacterium mungi* infect banded mongooses and very recently, *Mycobacterium suricatta* has been added which infects meercats (Gordon & Behr, 2015; S. D. C. Parsons *et al.*, 2013)

*Mycobacterium tuberculosis complex* belong to the family Mycobacteriaceae and the genus Mycobacterium which contain over 170 species (Ng *et al.*, 2014). Majority of the species of this genus are widely distributed in the natural environment and are known to be

saprophytes and only cause diseases in low-immune individuals making them opportunistic pathogens especially in clinical settings, yet other members such as *M. leprae* and *M. ulcerans* are known to cause leprosy and skin infections respectively in immunocompetent individuals (Lory, 2014).

Mycobacteria are non-motile, rod-shaped, non-spore forming aerobes and even though they stain poorly by Gram's stain, they are considered gram positive because their cell wall lacks the external lipid membrane found in gram-negative bacteria (Wayne & Kubica, 1986). Another unique feature of mycobacteria is the differences in their growth rates on solid media. Those that grow and form macroscopic colonies on selective media within a week are termed as fast growers examples of which are *M. smegmatis* and *M. chelonae* whilst those that grow beyond a week are termed as slow growers and examples are *M. tuberculosis* and *M. ulcerans* (G. M. Cook *et al.*, 2009).

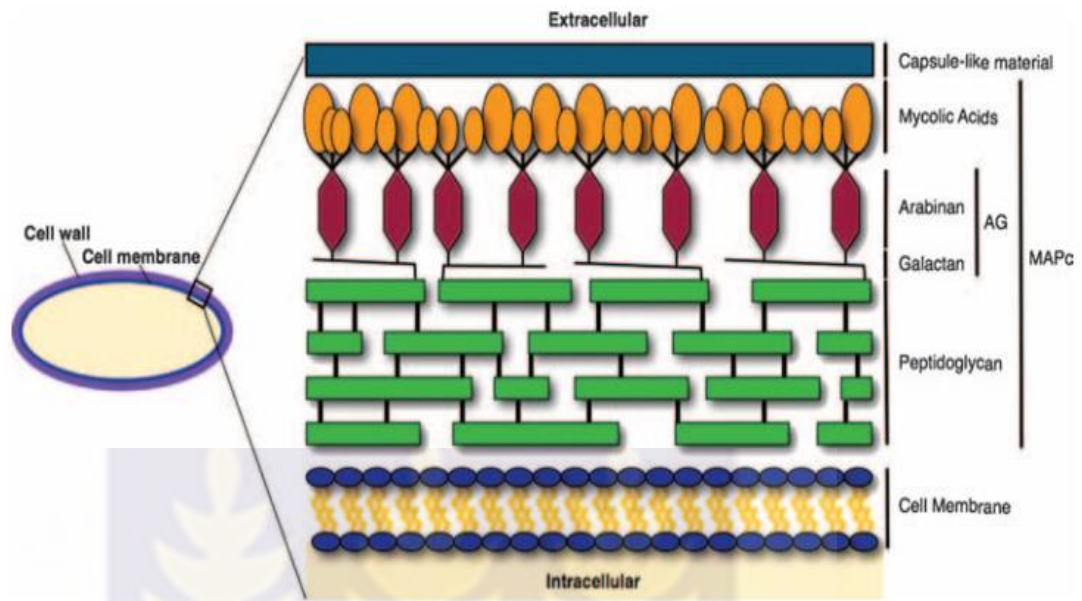
The human adapted MTBC is divided into genetic groups known as lineages. Seven lineages have been identified and they show a phylogeographic structure meaning distinct lineages are associated with specific geographical settings and distinct human geographical populations. MTBss has a global distribution and consists of lineages 1 to 4 plus 7 while lineages 5 and 6, also known as West Africa-1 and 2 respectively fall under MAF and are restricted to West Africa (Gagneux *et al.*, 2006).

### 2.4.1 The mycobacterial cell wall

One of the unique characteristic features of mycobacteria is their cell wall that is impermeable to many chemical agents and resistant to many antimicrobials and nutrients. The cell wall is a multi-layered hydrophobic entity which surrounds the plasma membrane (D. Chatterjee, 1997). The plasma membrane is first surrounded by a peptidoglycan layer covalently attached to arabinogalactan, which is in turn non-covalently linked to mycolic acids. The mycolic acids are also linked non-covalently to an outer capsule comprising mainly of proteins and lipid-linked polysaccharides (Hett & Rubin, 2008) (Figure 2.4.1).

The peptidoglycan layer is made up of repeated polymers of N-acetyl glucosamine and N-acetyl-muramic acid (NAG-NAM), playing more of a structural role (Hett & Rubin, 2008). The lipid-linked polysaccharides are composed of lipoarabinomannan (LAM), lipomannan and phthiocerol-containing lipids and together with proteins, they are involved in adhesion and penetration of the organism into the host cell while serving as the first barrier preventing macromolecules from entering the cell (D. Chatterjee, 1997; Daffe & Etienne, 1999).

In slow growing pathogenic mycobacteria, such as *M. tuberculosis* and *M. bovis*, the LAM's are mannosylated whereas fast growing non-pathogenic mycobacteria such as *M. smegmatis*, have phosphoinositol-capped LAM's (Hett & Rubin, 2008). This difference has been shown to play an immunomodulatory role allowing the pathogenic mycobacteria to persist in human macrophages without host immune detection and clearance (D. Chatterjee *et al.*, 1992).

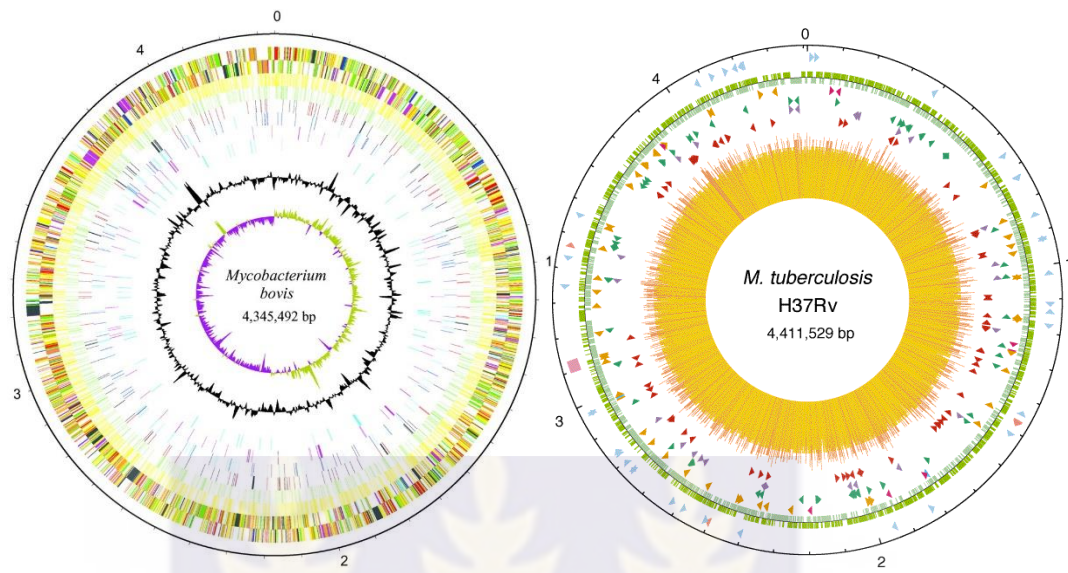


**Figure 2.4.1: The mycobacterial cell wall showing the multi-layered cell wall composition (Hett and Rubin, 2008)**

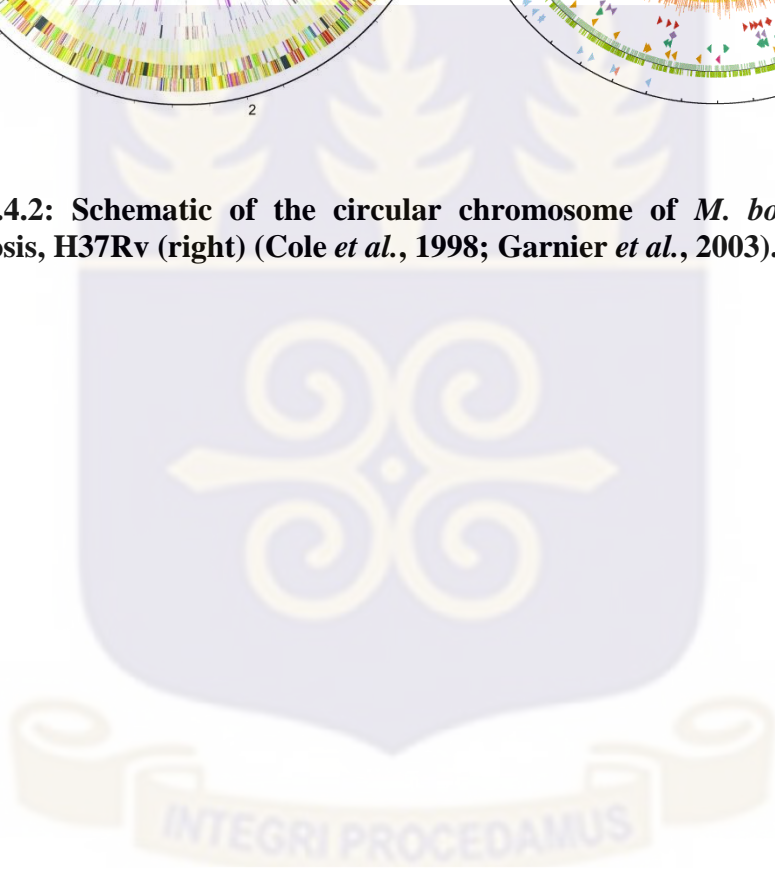
#### **2.4.2 The *Mycobacterium tuberculosis* genome**

The first genome of *M. tuberculosis*, H37Rv was sequenced in 1998, providing valuable data for understanding this unique pathogen. With a genome size of over 4MB, the H37Rv has the second largest genome available after *Escherichia coli*, 60% of which is mainly guanine and cytosine pairs (G-C) (Cole *et al.*, 1998). Importantly, over 4000 putative genes have been identified, 6% of which are dedicated to fatty acid synthesis, explaining the lipid rich cell wall the organism possess. Several genes were found to have either an in-frame stop codon or frame shift mutations making them pseudogenes (Cole *et al.*, 1998) (Figure 2.4.2). Before the genome sequences were made available, scientist had used hybridization methods to compare the similarity between members of the MTBC and found them to be highly identical (Behr *et al.*, 1999) sharing up to 99% nucleotide similarity even though they display marked phenotypic differences (Garnier *et al.*, 2003).

Whole genome comparisons have revealed the presence of non-random deletions in MTBC genome creating regions of difference (RD's) of varying sizes in different MTBC species, sub-species and lineages, which is suggested to contribute to the phenotypic diversity and host specificity that they exhibit (Gordon *et al.*, 1999). Furthermore, repetitive DNA elements such as transposable elements and short repeats have been recognized, including the numerous copies of the promiscuous insertion sequence 6110 (IS6110) which is exploited for MTBC identification and the relatively stable IS1081 (Poulet & Cole, 1995). Another interesting feature of mycobacteria that has been exploited for molecular typing is the presence of 36-bp direct repeats (DR) interspersed with non-repetitive spacers of varying lengths (van Embden *et al.*, 2000).



**Figure 2.4.2: Schematic of the circular chromosome of *M. bovis* (left) and *M. tuberculosis*, H37Rv (right) (Cole *et al.*, 1998; Garnier *et al.*, 2003).**



## 2.5 Mode of transmission and immunopathology

Tuberculosis is an aerosol transmitted disease although other routes of transmission including oral route by ingestion of unpasteurized milk containing the TB bacilli have been reported (Davies, 2006; Riley *et al.*, 1962). Through everyday activities such as talking, laughing, singing, sneezing and coughing infected people can generate aerosols, which may contain TB bacilli. These bacilli can persist as aerosols in the atmosphere for up to 6 hours depending on factors such as ventilation and humidity (Dooley *et al.*, 1990).

Inhalation of these droplet nuclei by an uninfected person may lead to several interactions with the host immune system and produce one of these three outcomes, namely, 1) Clearance of the bacilli by the immune system leaving no memory 2) Active/Primary infection and 3) Latent infection. Active infection occurs when the host immune system is overcome by the bacilli and infection is established within 6 months to 1 year of contact with the bacilli (Loudon & Spohn, 1969). Unlike active infection, latent TB occurs when the host immune system mounts a potent response leading to containment of the infection. Nonetheless, when the immune system becomes compromised, latent infection may be reactivated to produce an active infection (Smith, 2003). Interestingly, only 5% of infected immunocompetent people develop active disease within the first five years after infection, an additional 5% of immunocompetent individuals will develop TB in their lifetime while majority (90%) develop latent infection. (Getahun *et al.*, 2015; Mack *et al.*, 2009).

Both innate and humoral immune systems play important roles to determine infection outcomes. *Mycobacterium tuberculosis*, which is an obligate aerobic pathogen mainly affects the lungs (Pulmonary TB) an oxygen rich organ. However, infection may spread and affect other organs such as the lymph nodes, bones and the meninges (Extrapulmonary

TB) (Harisinghani *et al.*, 2000). After inhalation of the tubercle bacilli, the pathogen first encounters the mucosal lining of the respiratory tract, which consists of the epithelial cells, the connective tissue layer (Lamina propria) and the basement membrane. The epithelium is covered by fluid known as the airway surface liquid (ASL) which consists of mucus and Immunoglobulin A (IgA). Together, they form a primary barrier and prevent the bacilli from attaching and invading while the connective tissue harbours lymphocytes called the mucosal-associated invariant T-cells (MAIT) and macrophages (Lerner *et al.*, 2015).

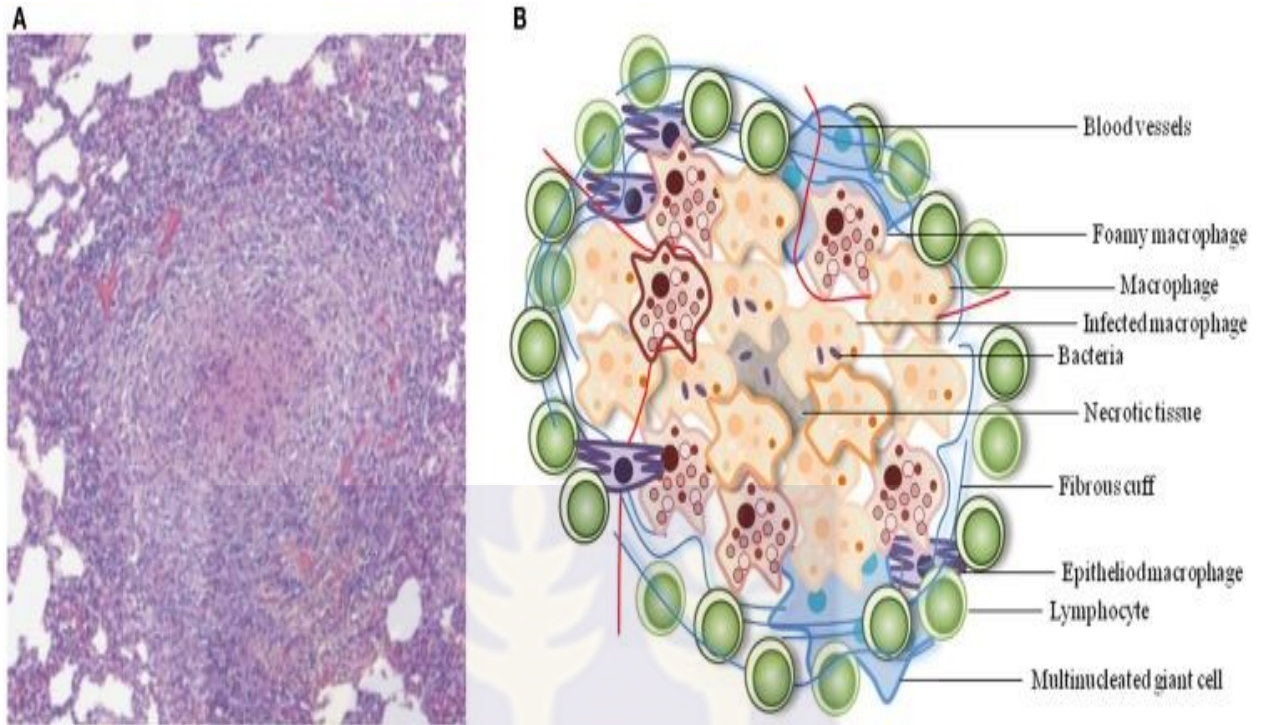
The mucosal epithelial cells are known to constitutively express many pathogen recognition receptors including Toll-like receptors (TLR's), C-type lectin receptors (CLR's) and nucleotide oligomerization domain-containing protein 2 (NOD 2) (Li *et al.*, 2012). These receptors recognize bacteria unique molecules called pathogen associated molecular patterns (PAMP's) on the surface of tubercle bacilli leading to antigen presentation and the production of chemokines (Interferon (IFN)- $\gamma$ , Tissue Necrosis Factor (TNF)- $\alpha$  and granzyme) and pro-inflammatory cytokines (IL-4 and IL-12) by MAIT. This results in the recruitment of phagocytes to the site of infection which assist in mopping up the bacilli (Lerner *et al.*, 2015).

Upon reaching the alveoli, the bacilli trigger a cascade of similar events mainly mediated by macrophages. Bacilli are engulfed by the macrophages, which secrete pro-inflammatory cytokines such as TNF- $\alpha$ , IL-12 and IL-1 (Raja, 2004) . These mediate the recruitment of other phagocytes including neutrophils, monocytes and more macrophages. Neutrophils and monocytes are the first to arrive at the site of infection followed by more macrophages and together, they encapsulate infected macrophages at the site of infection to avoid its dissemination (Raja, 2004). Some of the infected macrophages fuse with other phagocytes

to form multinucleated giant cells which mainly present antigens to T-cells (Sakamoto, 2012). Furthermore, T-helper 1 (TH-1) cells and dendritic cells (DC's) are also recruited to the site of infection where, dendritic cells take up tubercle bacilli and present antigens to TH-1 cells who in turn produce IFN- $\gamma$  to enhance phagosome and lysosome fusion to form phago-lysosome in macrophages (Cooper & Khader, 2008; Raja, 2004).

In some cases, the formation of the phago-lysosome containing nitric oxides and reactive oxygen intermediates results in the killing of the bacilli and total clearance of the infection (Pieters, 2008). However, in others, the MTBC have evolved to survive in the macrophages thereby inhibiting the formation of the phago-lysosome. Encapsulated infected macrophages form a granuloma (Figure 2.5) where the bacilli can survive in a low metabolic state for a long time (Getahun *et al.*, 2015)





**Figure 2.5: Tuberculosis histopathology** (Gil *et al.*, 2010; Guirado & Schlesinger, 2013) A) *Histological section of a granuloma from the lung tissue of a minipig*  
B) *Schematic showing the components of a granuloma*

## **2.6 Clinical presentation**

Tuberculosis presents in various individuals differently. However, some of the classical symptoms of active pulmonary disease include persistent cough lasting more than 2 weeks, hemoptysis, fever, night sweats and weight loss (Heemskerk D, 2015.). In severe cases, patients may have acute respiratory distress which is shown as difficulty in breathing and chest pains (Raina *et al.*, 2013). In latent infection, the patient is asymptomatic because the bacilli are contained in a somewhat controlled environment (Chapman & Lauzardo, 2014). In extrapulmonary TB, clinical signs depend on the site of infection. For instance, in TB meningitis, patients experience severe headaches, confusion and weakness whilst lymphatic TB patients may have swollen lymph nodes (Golden & Vikram, 2005). Tuberculomas may also occur in the brain which can lead to impaired vision and subsequent death (Monteiro *et al.*, 2013).

## **2.7 Tuberculosis control**

Before the 21<sup>st</sup> century, TB control was mainly by the implementation of social interventions and life style changes such as improved sanitation and housing, nutritional changes, exercising and better living standards (Arthur, 1940; Daniel, 2006). However, there was still an urgent need for other treatment options since these social interventions produced only a small decline in mortality (Lienhardt, 2001). So in the early 1900's, the first vaccine was developed by Albert Calmette and Camille Guerin using an attenuated version of the *M. bovis* which is named Bacillus Calmette-Guerin (BCG) after the discoverers (Luca & Mihaescu, 2013). This vaccine is currently the only approved TB vaccine available and offers many children protection against severe forms of TB but is

not effective in adults (Barreto *et al.*, 2006) . In Ghana, it is the first vaccine given to newly born infants and forms part of the Expanded Program of Immunization (EPI).

Currently, TB control programmes rely mainly on detecting active TB cases and treating them, to cut the source of infection and prevent mortality of the affected Treatment is carried out using a combination of drugs depending on whether the bacilli causing infection is drug resistant or drug susceptible (WHO, 2002). To ensure that patients take the right drugs at the right time for the appropriate duration, the WHO introduced the Directly Observed Treatment Short course (DOTs) treatment strategy in the early 1990's. Through the DOTs' treatment strategy default is reduced and many people under treatment get cured (Out, 2013). The Ghana NTP adopted this strategy in 1994 (National Tuberculosis Control Programme, 2001).

### **2.7.1 Diagnosis of tuberculosis**

Diagnosing TB has been by using both clinical observations and laboratory analysis. Clinicians look out for symptoms such as unexplained persistent cough lasting 2 weeks or more, fever, night sweats and fatigue. Individuals presumed to have TB may be required to take a chest radiograph which is observed for infiltrates with cavitation in the lungs (Campbell & Bah-Sow, 2006; Woodring *et al.*, 1986). Even though, these clinical features may be suggestive of TB, they are not specific and conclusive enough. The definite diagnosis is bacteriological detection and identification of the causative mycobacteria.

Several laboratory diagnostic methods have been approved for TB diagnosis including sputum smear microscopy, culture, immune based tests and DNA based detection methods (Sulis *et al.*, 2016). Depending on availability, cost and technical expertise needed to

perform the tests, countries have adopted different combinations of tests to aid in successful diagnosis. The diagnostic methods rely on the uniqueness of the mycobacterium from other organisms as well as the distinct immune response it generates in the host for differentiating it from other organisms in clinical samples.

### **2.7.1.1 Sputum smear microscopy**

Sputum smear microscopy is the most widely used method for diagnosis of TB especially in resource-constrained settings. It is fast and inexpensive and can be performed with minimal resources. In Ghana, almost all point of care health facilities are equipped to carry out this test (Addo *et al.*, 2006). It involves preparation of smears from sputum samples, staining these smears by the Zeihl-Neelsen (ZN) or auramine stains followed by observation under a microscope. Briefly, the heat fixed smears are flooded with a primary stain and heat is applied to aid the penetration of the stain into the bacilli. An acid decolourisation step is performed followed by counter staining (Global Laboratory Initiative, 2014). The stained slides are then observed under immersion oil if ZN stained or a fluorescence microscope for auramine stain.

Although it is the easiest method of diagnosis, it has a low sensitivity and lacks specificity as clinical samples require a bacilli load of 10,000 or more to be detected (Desikan, 2013). So, in children and HIV-infected persons who have low bacilli load, sputum smear microscopy is not reliable for diagnosis of TB. Also, this method, cannot distinguish among the different MTBC species and Non-tuberculous mycobacteria (NTM's) (Lipsky *et al.*, 1984; Swai *et al.*, 2011). Several modifications have been developed to improve sensitivity of ZN methods including concentration methods which utilize mechanical or

chemical preparations to liquefy viscid sputum samples and centrifugation to concentrate the bacilli before smear preparation (Gebre *et al.*, 1995; R. Singhal & Myneedu, 2015), thereby enhancing the sensitivity by up to 18% (Karen R. Steingart *et al.*, 2006). These modifications can be cost intensive and require specialized equipment, which may not be available in point of care laboratories.

### **2.7.1.2 Culture**

Isolating viable bacilli by culture remains the gold standard method for TB diagnosis and is recommended at various stages of TB treatment for monitoring purposes (Hopewell *et al.*, 2006). It involves isolation of mycobacteria from clinical samples using selective media. Some of the clinical samples utilized are sputum, gastric lavage and bronchoalveolar lavage. Sputum is the most frequently used sample since the most affected site is the lungs, however in extra-pulmonary cases and in vulnerable groups like children and the aged who cannot produce sputum, the other specimen types are more appropriate (Baghaei *et al.*, 2011; Somu *et al.*, 1995).

Culture media for mycobacterial growth include solid media such as Lowenstein-Jensen (LJ) solid egg-based selective media and Middlebrook 7H11 media as well as liquid media including BACTEC 460TB media (Saito, 1998). *In-vitro*, mycobacteria use either glycerol or pyruvate as a carbon source for growth (G. M. Cook *et al.*, 2009). Due to the slow growth nature of the MTBC, mycobacterial cultures are time consuming. It may take 4-12 weeks to observe macroscopic growth on solid media and 2 weeks for growth in liquid media (Shah *et al.*, 2010). Furthermore, because sputum samples are not sterile, they require special sample processing before inoculation on media. Without optimum sample

preparation (decontamination), cultures may contaminate. Thus, culture cannot be used for routine diagnosis, nevertheless due to its, specificity and sensitivity it is useful for treatment monitoring in difficult to treat cases and for obtaining viable organisms for drug susceptibility testing.

Drug susceptibility testing (DST) is becoming a routine requirement for monitoring TB treatment because of the increasing drug resistant TB cases and consequent expansion of anti-tuberculosis drug resistance campaigns in many parts of the world. DST can be done by observing for growth or metabolic inhibition of mycobacteria in a growth media containing the drug being tested or by detecting specific mutations in drug resistance DNA loci (Kim, 2005). The conventional agar-based or broth methods involve growing organisms in drug containing media while monitoring for growth or inhibition (Canetti *et al.*, 1969). These methods have a long turnaround time so they are not convenient for routine monitoring of treatment. Several other modifications have been developed which aim at shortening the turnaround time by detecting early growth inhibition. Examples of these methods include the BACTEC and MB/BACT systems which measure CO<sub>2</sub> production and oxygen consumption (Diaz-Infantes *et al.*, 2000).

#### **2.7.1.3 DNA based detection methods**

DNA based detection methods utilize the uniqueness of an organism's genetic material to differentially distinguish it from other organisms. Unique DNA sequences that are used include the MTBC unique insertion sequence 6110 (*IS6110*) and RNA polymerase (*rpoβ*) gene sequence (Raviglione & Sulis, 2016). Currently, the commonest DNA-based methods

are the Line Probe Assay (LPA) by Hain Lifesciences, Xpert MTB/RIF by Cepheid, Gene-Probe AccuProbe system and the Amplicor *Mycobacterium tuberculosis* test.

The LPA identifies the presence of MTBC by detecting their unique *rpoβ* sequence as well as resistance to isoniazid and rifampicin, the 2 most important first line anti-TB drugs. Drug resistance testing is by detecting mutations associated with drug resistance in the *rpoβ*, *katG* and *inhA* genes, using multiplex PCR and reverse hybridization steps (Barnard *et al.*, 2012). In contrast, the Xpert MTB/RIF detects MTBC specific *rpoβ* gene sequences along with resistance to rifampicin only using real time PCR (Dorman *et al.*, 2012). The core region of the *rpoβ* gene is amplified and probes are used to distinguish between wild type and mutants with rifampicin resistance (Rahman *et al.*, 2016).

Compared to culture, DNA based methods can detect MTBC in just a few hours in majority of samples (Moore *et al.*, 2005). Using AFB smear positive samples, positive predictive value of DNA based methods increases significantly and it has been shown to be effective in identifying MTBC in patients suspected to have TB but whose sputum smear microscopy results are negative (Taegtmeier *et al.*, 2008). With these methods, drug susceptibility profile of MTBC can be determined simultaneously making them important for TB control (Dinnes *et al.*, 2007).

#### **2.7.1.4 Immunological based detection methods**

*Mycobacterium tuberculosis complex* generates unique immunological response in the human body which has been exploited to determine its presence or absence in the host. Several diagnostics have been developed which utilize such responses including the tuberculin skin test (TST) and the interferon Gamma release assay (IGRA).

The tuberculin skin test works by detecting exposure to mycobacterial antigens using delayed-type hypersensitivity reaction (Teixeira *et al.*, 2007a, 2007b). The patient is exposed intradermally to purified protein derivative (PPD) which contains mycobacterium specific antigens, monitored for up to 72 hours for reaction which is observed as an induration of the exposed skin (H. Yang *et al.*, 2012). Additionally, IGRA measures a patients T-cell based immune response to mycobacteria when exposed to TB-specific antigens (ESAT-6, CFP-10) using whole blood (Lalvani & Pareek, 2010).

Even though immune based tests are more rapid compared to mycobacterial cultures, they are largely inaccurate and inconsistent and it is not advisable to solely rely on them for diagnosis (Hopewell *et al.*, 2006; K. R. Steingart *et al.*, 2007) as it is difficult distinguish between latent infection and active disease in most cases.

### **2.7.2 Treatment of tuberculosis**

Effective treatment of TB reduces transmission and relapse, as well as restores quality of life and prevents development of resistance against the current anti-tuberculosis drugs and death. Tuberculosis is treated by administering a combination of drugs for 6 – 24 months depending on the patient's clinical situation (primary infection, relapse, drug resistant TB and re-infection) as well as the drug susceptibility pattern of the infecting pathogen (American Thoracic Society, 2003).

New TB cases are treated for a total of 6 months in two phases, an intensive two-month phase and a continuous 4-month phase. During the intensive phase, the patient is put on a 4-drug combination therapy made up of Isoniazid (INH), Rifampin (RIF), Pyrazinamide (PZA) and Ethambutol (EMB) (WHO, 2010). For drug-susceptible TB, the patient

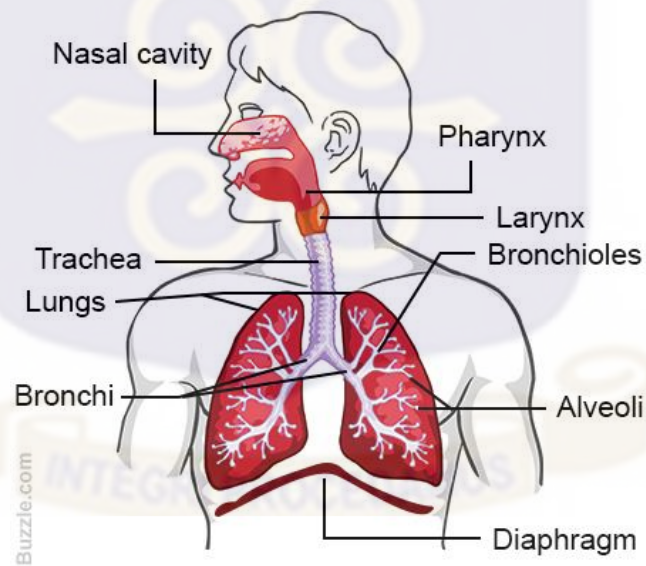
continues with only INH and RIF for the following 4-month continuous phase period. Treatment is monitored by smear microscopy at two and five months. However, for cases that are failing treatment and suspected drug resistant cases, isolation of viable mycobacteria from the patients sample for further analysis is required (Hopewell *et al.*, 2006; WHO, 2010). This is done mainly to detect drug resistance early for appropriate intervention (Hopewell *et al.*, 2006). Currently this can be supported by the Xpert MTB/RIF assay. The patient is said to be cured if sputum smear microscopy after the 5<sup>th</sup> month of treatment is negative otherwise it is termed as treatment failure (WHO, 2010). Failed treatment may be due to development of drug resistance or non-compliance or other inherent host factors.

The long duration of TB treatment and the side effects of the drugs on certain patients lead to a tendency for patients to default in treatment. Defaulting treatment leads to relapse and development of drug resistance. Thus, control programmes worldwide have implemented the DOTs strategy to improve compliance and this strategy has yielded positive results (Brust *et al.*, 2010; Demissie & Kabebe, 1994).

Patients who are infected with drug resistant forms of TB go through a much longer treatment duration depending on the extent of drug resistance. For multi-drug resistant (MDR) TB which can be defined as resistance to the 2 most important first line drugs (isoniazid and rifampicin), a minimum of 20 months treatment with a combination of second line anti-TB drugs is recommended but a new regimen has been approved by the WHO called the Bangladesh regimen which is for treating uncomplicated MDR-TB patients for 9 months using novel combinations of already existing drugs (Aung *et al.*, 2014; WHO, 2010).

## 2.8 Respiratory tract and sputum production

The primary function of the respiratory tract is gaseous exchange where air is inhaled and exhaled through the nostrils to and from the lungs for distribution via the blood stream (Perry & Burggren, 2007). The respiratory tract is divided into two; the upper and lower respiratory tracts. The upper respiratory tract consists of the nostrils, pharynx and larynx while the lower respiratory tract is made up of the trachea, bronchial tree, pulmonary alveoli and the lungs (Figure 2.8.1) (De Graaf, 2001). The respiratory tract is constantly exposed to foreign particles which are inhaled with the air. To overcome these foreign materials, the tract is equipped with various defense mechanisms.

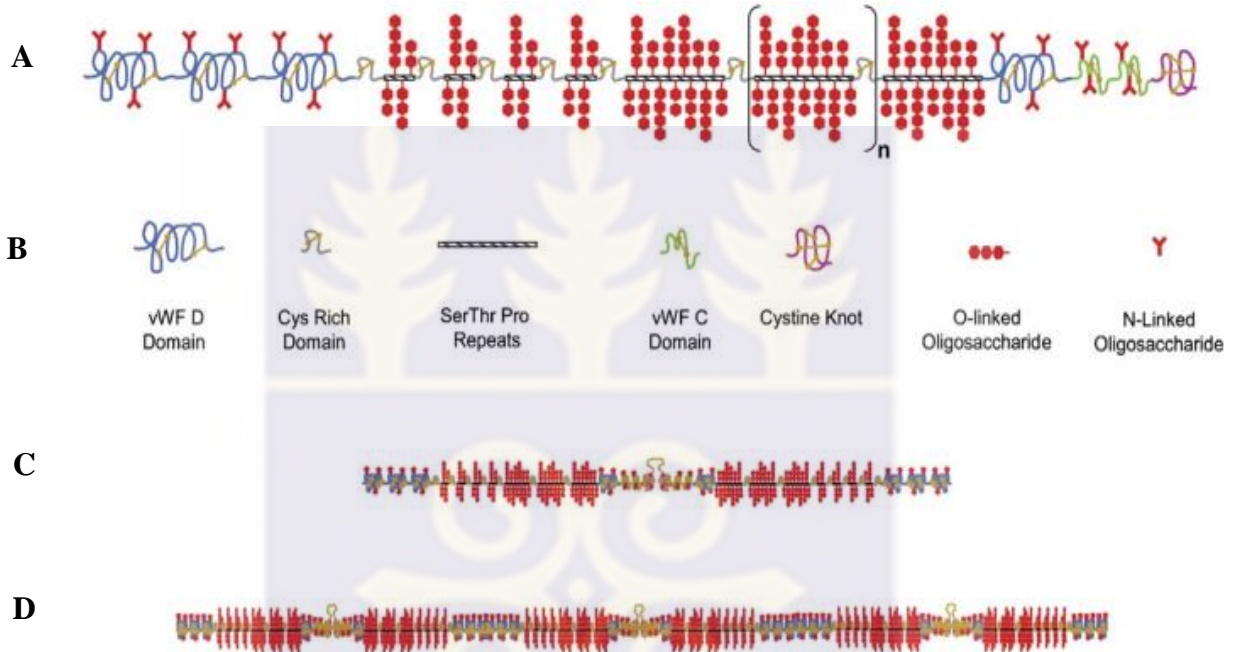


**Figure 2.8.1: The respiratory tract of human (www.mvstudyguide.com)**

The epithelial cells of the respiratory tract which comprise ciliated cells, mucous secreting cells and Clara cells form part of the defense mechanism. Furthermore, resident macrophages within the respiratory tract known as Kupffer cells are responsible for detection and engulfing hazardous particles and organisms (Richardson, 2003). Although the respiratory tract is equipped with these defense mechanisms it is still not sterile but rather rich in bacterial flora in controllable quantities (Aho *et al.*, 2015). This resident flora poses no harm to the body unless they proliferate in excess and succeed in colonizing the epithelia cells. Normal flora in the respiratory tract include staphylococci, diphtheroid, pneumococci, Neisseria and mycoplasmas (Charlson *et al.*, 2011; Laurenzi *et al.*, 1961). To inhibit attachment of bacteria and other particles, mucous is secreted by mucous secreting cells to cover the epithelial surface. It is this mucous that is expectorated as sputum for TB diagnosis.

Sputum is a mixture of secreted mucous by secretory cells (goblet cells) lining the respiratory tract, exogenous and endogenous fluid, microorganisms, cells from the surrounding tissues, proteins, lipids and foreign particles (Farzan, 1990). Saliva on the other hand is watery fluid secreted by salivary glands located in the mouth and which also harbours microbes from the mouth (Tiwari, 2011). Mucins, the major component of mucous forms a greater portion of expectorated sputum. They are high molecular weight, heavily glycosylated glycoprotein polymers linked by disulfide bonds and are responsible for the viscid nature of sputum (Voynow & Rubin, 2009). The chemical composition of mucins play a role in its observed properties; 80% carbohydrate comprising mainly of N-acetylgalactosamine, N-acetylglucosamine, fucose, sialic acid plus 20% protein (repeating units of serine, threonine and proline interspersed with cysteine rich domains) forms the

core of the glycoprotein (Bansil & Turner, 2006) (Figure 2.8.2) . It is within this complex structure that the other sputum components are trapped.



**Figure 2.8.2: Structure of mucins (Bansil & Turner, 2006)**

- A) Monomeric structure of mucins
- B) Molecular domains present in mucins
- C) Mucin dimer
- D) Mucin polymer

## 2.9 Sputum storage and processing for mycobacterial analysis

Sample quality is important in clinical diagnosis. Quality and accurate laboratory results depend on the collection of a quality sample and maintaining the integrity of the sample until it is ready to be analyzed. In many resource-constrained countries, point of care laboratories are under resourced and so rely on central laboratories or reference laboratories to perform certain important tests such as mycobacterial cultures, drug susceptibility testing and molecular analysis. The need to ensure that samples are transported to these centralized reference laboratories in the right conditions cannot be overemphasized.

The WHO recommends that sputum samples be analyzed immediately they are collected otherwise, they should be maintained at 2-8 °C for a maximum of 48 hours (Global Laboratory Initiative, 2014). Maintaining a cold chain during transport is expensive and due to transport constraints, these time limits are exceeded frequently. This affects mycobacterial viability leading to false negative results (Šula *et al.*, 1960). Moreover, due to the septic nature of sputum, bacterial flora may over grow while in transit, complicating the sample processing procedures. In geographical settings where samples cannot be analyzed immediately nor maintained in cold chain, chemical preservatives have been used to help maintain mycobacterium viability, while inhibiting other bacterial species during transport. Preservatives that have been used include cetylpyridinium chloride (CPC) and sodium carbonate (Bobadilla-del-Valle *et al.*, 2003).

Sputum decontamination is an important sample-processing step that is performed during mycobacterial cultures (Kubica *et al.*, 1963). It involves the use of chemical agents to inactivate fungi and bacteria other than mycobacteria, neutralization of the chemical agent and concentration of the mycobacteria bacilli before inoculation on selective media (P. C.

A. M. Buijtels & P. L. C. Petit, 2005). The constituents of expectorated sputum provide a conducive environment for microorganisms to thrive. Many of these microorganisms are fast growers and within a short time (18-24 hours), macroscopic growth can be seen on solid media unlike mycobacteria, which are slow growers. If not eliminated, the fast growers may over grow the media surface and digest it before mycobacteria get a chance to grow (Allen *et al.*, 2016).

In addition to removal of unwanted microorganisms, decontamination also helps to digest the viscid sputum and homogenize it to expose tubercle bacilli for detection (WHO, 2009). Acids, bases and detergents have been the main decontamination reagents utilized including hydrochloric acid (HCL), sodium hydroxide (NaOH), sulfuric acid (H<sub>2</sub>SO<sub>4</sub>) and phenol. These take advantage of the hydrophobic nature of the mycobacteria cell wall that does not readily allow hydrophilic substances to enter, hence giving it the ability to selectively resist the decontamination treatments. However, at higher concentrations and for prolonged periods, mycobacterium viability may be compromised by these chemicals.

### **2.10 Cetylpyridinium chloride (CPC)**

Cetylpyridinium chloride is a quaternary ammonium compound that has antiseptic properties and has been used in mouthwashes to prevent plaque formation (Pitten & Kramer, 2001). It has been applied in mycobacteriology at specific concentrations for transporting sputum samples for mycobacteria isolation. Cetylpyridinium chloride acts by keeping the tubercle bacilli at a low metabolic state and by decreasing mycolic acid synthesis (Sekar *et al.*, 2014).

At a 1% concentration, CPC can liquefy sputum samples and maintain mycobacterial viability for up to 8 days without the need for a cold chain (Pal *et al.*, 2009). However, time is taken to remove CPC completely from the sample before inoculating on selective media otherwise it reduces mycobacterial viability significantly (Sankar *et al.*, 2009). Also, samples preserved in CPC cannot be used directly for DNA based analysis since the remnants of the quaternary ammonium compound interfere with PCR reagents. Yet, the current fight against TB makes it imperative that chemicals used for processing samples for bacteriological analysis be compatible for DNA-based rapid diagnostics. This makes its use very limited because direct clinical samples analysis by PCR based assays are now very integrated in TB control activities. This has slowly made the use of CPC for sample transport obsolete.



**Figure 2.10: Chemical structure of Cetylpyridinium Chloride (Reza Zarei *et al.*, 2013)**

### **2.11 Petroff decontamination**

The Petroff decontamination method has been in use for a very long time (Yajko *et al.*, 1993). This method employs the bactericidal effects of the alkali sodium hydroxide (NaOH) for bacteria inactivation through the alteration of pH and production of OH<sup>-</sup> species which bind to the bacteria proteins and interfere with their function, while inhibiting macromolecular synthesis of the organism (Martínez, 2005). After its discovery, modifications have been made to improve its effectiveness. The original method uses 4% NaOH and a twenty to thirty minute incubation followed by a neutralization step with either an acid or sterile phosphate buffered saline (Kent *et al.*, 1985). This proved to be harsh even for mycobacteria so Kent and Kubica in 1985 made a modification to the original method by the addition of phenol red and the use of a shorter incubation time. This resulted in reduced destruction to tubercle bacilli compared to the original method (de Kantor & Laszlo, 1998; Tomita *et al.*, 2008).

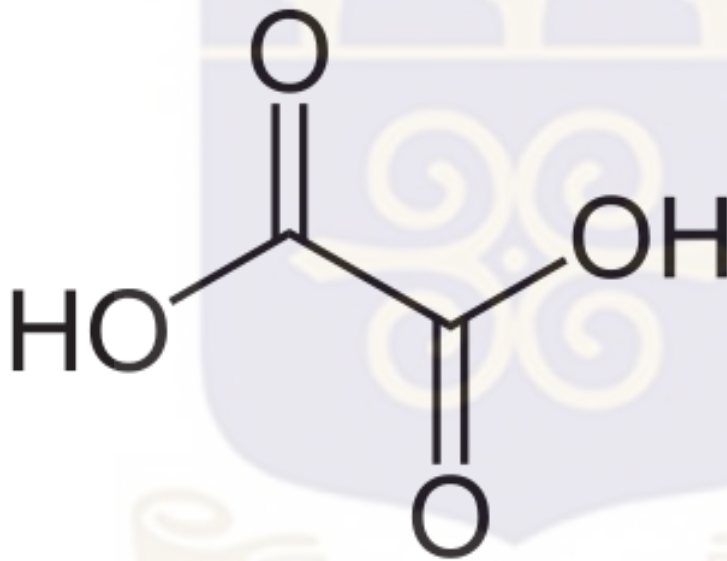
The drawback remains that about 60% of tubercle bacilli are still killed by this method and the modified method offers little improvement to maintaining mycobacteria viability (Burdz *et al.*, 2003). Thus, the use of the petroff decontamination method may result in false culture negatives.

### **2.12 Oxalic acid decontamination**

Oxalic acid is a dicarboxylic organic acid which also acts as a reducing agent. Its use as a decontaminating agent for respiratory specimen was first described by Corper and Uyei in 1930 where samples were incubated for 30 minutes in 5% oxalic acid solution, followed by neutralization and centrifugation (Yajko *et al.*, 1993). Ever since, studies have evaluated

oxalic acid for decontamination and found that it is more efficient, yielding higher mycobacterium recovery rates (Yajko *et al.*, 1993; Yeboah-Manu *et al.*, 2004).

Because of its reducing ability, oxalic acid is converted to oxalate in solution which in turn binds to metals in the cell walls of bacteria leading to their instability and subsequent lysis (Cho *et al.*, 2004). The main disadvantage of this method is that, if not properly neutralized, oxalic acid destroys mycobacteria even after inoculation on selective media and interferes with PCR processes (Palomino & Portaels, 1998).



**Figure 2.12: Chemical structure of Oxalic acid (www.study.com)**

### **2.13 Sulfuric acid decontamination**

Various concentrations (2-6%) of sulfuric acid have been used to decontaminate samples for mycobacteria isolation (Ambrosio *et al.*, 2008; P. C. Buijtelts & P. L. Petit, 2005). Sulfuric acid alters the pH of the sample and dissociates to generate free radicals which bind to macromolecules and interfere with their function in microorganisms leading to inactivation of the organism (Seymour S. Block, 1983). Mycobacteria due to their hydrophobic cell wall structure can withstand this treatment for a specific interval (Hett & Rubin, 2008). Aside from the relatively lengthy decontamination time, sulfuric acid decontamination has been shown to destroy up to 60% tubercle bacilli making it largely unsuitable for decontamination (Ambrosio *et al.*, 2008).

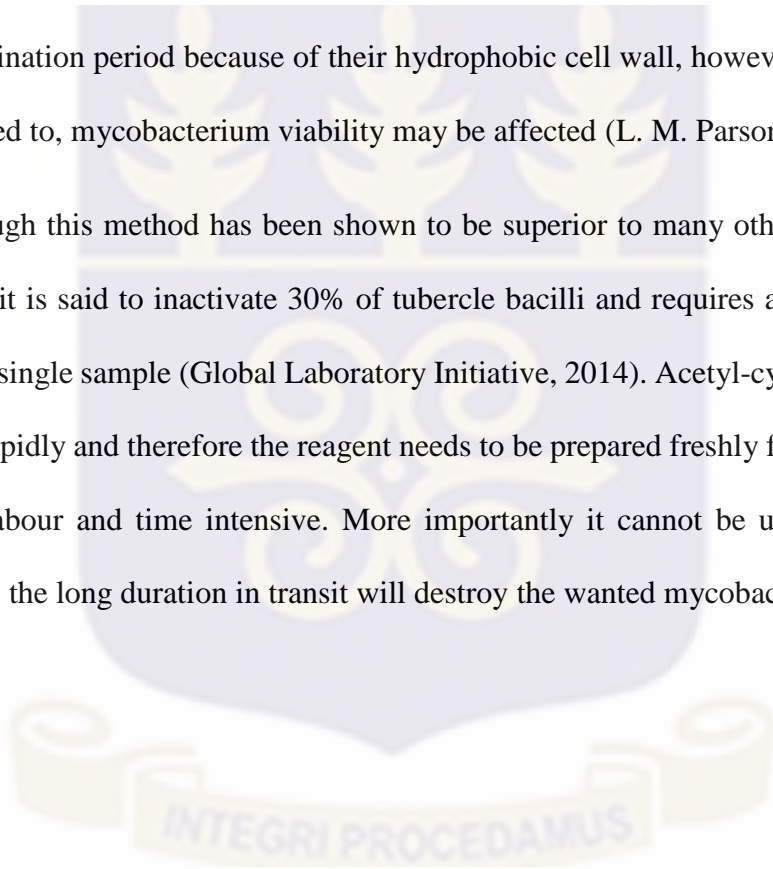
### **2.14 N-acetyl-L-cysteine sodium hydroxide method (NALC-NaOH)**

The NALC-NaOH decontamination method is currently the most widely used method and it is also the WHO approved method of decontamination, since processing clinical samples with NALC-NaOH, allows the sample to be analyzed by both conventional and DNA-based assays. The reagent is made up of a combination of 0.5 % N-acetyl-L-cysteine (NALC), 2.9% sodium citrate and 4% sodium hydroxide (Global Laboratory Initiative, 2014). This method was first described in 1963 by Kubica *et al* who employed the mucolytic activity of NALC to aid the release of tubercle bacilli from mucoid sputum for detection (Kubica *et al.*, 1963). Each of the components plays a role that helps digestion of sputum and inhibition of unwanted bacteria.

N-acetyl-L-cysteine is a Sulphur containing protein which disrupts the disulfide bonds that link the mucin monomers in a reduction reaction, thereby breaking down the mucin

polymer and causing the sputum to lose its viscosity and be liquefied (Kubica *et al.*, 1964). In this way, the bacilli, which are trapped in the mucous are, now exposed for detection. Non-mycobacteria are also exposed to the action of NaOH. Sodium citrate also binds heavy metal ions that may be present in the sputum. These heavy metals if unbound would bind to and inhibit the activity of NALC. NaOH inactivates the non – mycobacteria present by generating OH<sup>-</sup> radicals which interfere with DNA, lipid and protein synthesis (Martínez, 2005). Mycobacteria can withstand the devastating effects of NaOH for the decontamination period because of their hydrophobic cell wall, however if strict timing is not adhered to, mycobacterium viability may be affected (L. M. Parsons *et al.*, 2011).

Even though this method has been shown to be superior to many other decontamination methods, it is said to inactivate 30% of tubercle bacilli and requires about 40 minutes to process a single sample (Global Laboratory Initiative, 2014). Acetyl-cysteine also loses its activity rapidly and therefore the reagent needs to be prepared freshly for each use making it more labour and time intensive. More importantly it cannot be used in transporting sputum as the long duration in transit will destroy the wanted mycobacterial species.



## CHAPTER THREE

### 3.0 MATERIALS AND METHODS

#### 3.1 Materials

##### 3.1.1 Consumables and disposables

Powdered latex examination gloves (Diamed, South Africa), Powder-free nitrile gloves (Polyco-Bodyguard, UK), sterile disposable inoculating loops (Thermo Scientific, USA), sterile single wrapped disposable Pasteur pipette (Alpha laboratories, UK), disposable laboratory coats (Medline, France), 1.5ml sterile microfuge tubes (Sarstedt, Germany), autoclave Bags (Greiner Bio-One, Germany), Microscope slides with frosted edge (Thermo Scientific, USA), Sterile disposable petri dishes (Fischer Scientific, USA).

##### 3.1.2 Reagents

Blood agar base (Oxoid, England), Nutrient agar (Oxoid, England), Sabouraud dextrose agar (Oxoid, England), Tri-sodium citrate (Sigma Aldrich, USA), N-acetyl-L-cysteine (Sigma Aldich, USA), sodium hydroxide crystals (Sigma Aldrich, USA), Omnigene sputum (DNA Genotek, Canada), Xpert MTB/RIF cartridges (Cepheid, USA), Glycerol (Timstar laboratories, UK), Middlebrook ADC (Becton, Dickson and Company, USA), Crystal violet (Sigma Aldrich, USA), Methylene blue (Sigma Aldrich), Sulfuric acid (Fast cycling PCR Master Mix (Qiagen), Primers for PCR (Macrogen), Hot-start Taq polymerase (Qiagen), Agarose (AGTC Bioproducts, UK), Ethidium bromide (Sigma Aldrich, USA).

### **3.1.3 Equipment**

Heratherm Incubator (Thermo Scientific, USA), Thermal cycler (Applied Biosystems 2720, USA), Vortex mixer (Scientific Industries, USA), Heating block (Heat Labor Consult, Germany), Digital coagulator (Hirasawa works, Japan), Weighing balance (A&D Instruments, Japan), Water bath (Grant Technical, England), Autoclave (TOMY SEIKO CO. LTD., Japan), Class 2 Safety cabinet (Air Tech, UK), GeneXpert Module GX-IV (Cepheid, USA), MALDI-TOF Biotyper (Bruker Daltonics, Germany), Electrophoresis tank (Bio-Rad, USA).

## **3.2 Methods**

### **3.2.1 Study site and specimen collection**

The study was conducted at the instance of the National Tuberculosis Control Program (NTP); the procedure for sampling followed national guidelines. The sites for specimen collection were the Korle-Bu Teaching Hospital (KBTH) and the La General hospital. Korle-Bu Teaching Hospital is a tertiary health facility in the Greater Accra region of Ghana where most pathological cases are referred. Moreover, it has a specialized facility where TB cases are managed. The La General hospital also in the Greater Accra region, serves the communities in and around the La suburban area and receives most of the TB cases from these communities. These hospitals receive the first and third highest number of cases in the Greater Accra Region respectively and importantly have a comparatively effective system that facilitates sample collection hence were chosen for the study. The laboratory staff of the respective health facilities were trained on the objectives of the study

and procedure for sample collection and storage before the commencement of sample collection.

One hundred and four sputum samples confirmed microscopically as acid fast bacilli (AFB) positive according to the International Union Against Tuberculosis and Lung diseases (IUTLD) guidelines at the chosen sites were collected and transported to the bacteriology laboratory of the Noguchi Memorial Institute for Medical Research (NMIMR) the same day (Rieder *et al.*, 2007). Sputum characteristics are shown in table 3.1 Before transportation, the sputum samples were packaged using a triple packaging system (WHO, 1997). At NMIMR, the samples were sent immediately to the biosafety Level-3 laboratory where they were processed. Samples were collected from May to September 2016.

This study forms part of a tuberculosis molecular epidemiology study in Ghana and ethical clearance was sought from the NMIMR-Institutional Review Board (IRB). The ethical clearance is shown in Appendix 1.

**Table 3.1: Macroscopic Features and Acid Fast Bacilli Load**

<b>Characteristics</b>				
<b>Macroscopic features</b>	<b>Mucopurulent</b>	<b>Bloody</b>		
	<b>n (%)</b>	<b>n (%)</b>		
	104 (100)	5 (4.8)		
<b>Acid fast bacilli load n=93</b>	<b>Scanty</b>	<b>1+</b>	<b>2+</b>	<b>3+</b>
	<b>n (%)</b>	<b>n (%)</b>	<b>n (%)</b>	<b>n (%)</b>
	11 (11.8)	16 (17.2)	25 (26.9)	41 (44.1)

### **3.2.2 Media preparation**

#### **3.2.2.1 Blood agar (BA) preparation**

Blood agar was used to isolate bacteria and fungi species before and after each decontamination treatment. The media was prepared by following the manufacturer's instructions. Briefly, 40 g of the blood agar base powder was mixed with 1 L distilled water, then boiled briefly to dissolve. The suspension was sterilized by autoclaving at 121 °C for 15 minutes, cooled to 50 °C in a water bath, then sterile sheep blood was added aseptically to a concentration of 5%. After inverting several times to mix, 20-25 mL of the solution was dispensed into sterile petri dishes to obtain a 4 mm depth. This was then allowed to solidify and incubated overnight at 37 °C to check for sterility. Subsequently, the plates were examined for contamination and those that were sterile stored in sealed plastic bags at 4 °C if not used immediately.

#### **3.2.2.2 Nutrient agar (NA) preparation**

Bacteria colonies obtained from blood agar cultures were sub-cultured on to nutrient agar to obtain pure colonies for identification. Nutrient agar was prepared according to the manufacturer's instructions by accurately weighing 28 g of the dehydrated media into 1 L distilled water. The solution was dissolved by briefly boiling, before autoclaving at 121 °C for 15 minutes to sterilize. Subsequently, the mixture was left to cool to 50 °C in a water bath, and then dispensed into sterile petri dishes in 20-25 mL volumes, to obtain a depth of 4 mm. The plates were left to solidify at room temperature, before incubating at 37 °C to check sterility. After overnight incubation, plates were examined for contamination after which sterile plates were stored at 4 °C in sealed bags if not used immediately.

### 3.2.2.3 Sabouraud dextrose agar (SDA) preparation

Fungal isolates were sub-cultured on sabouraud dextrose agar after blood agar cultures. The media was prepared following the manufacturer's instructions by weighing 65 g of the dehydrated media into 1 L distilled water. The mixture was briefly boiled to dissolve, then sterilized by autoclaving at 121 °C for 15 minutes. Afterwards, the media solution was cooled to 50 °C in a water bath, dispensed into petri dishes in 20-25 mL volumes to obtain a 4 mm depth before leaving at room temperature to solidify. Next, the plates were incubated at 37 °C overnight to check sterility. Finally, they were examined for contamination then sterile plates were packed into sealed bags for storage at 4 °C if not immediately used.

### 3.2.2.4 Lowenstein-Jensen (LJ) media

Mycobacteria was isolated by culturing sputum samples on pyruvate and glycerol supplemented LJ media; pyruvate for *M. africanum* and *M. bovis* growth, and glycerol for growth of other mycobacteria. First, glassware used were cleaned and sterilized for 1 hour at 160 °C. Afterwards, the mineral salts (Potassium phosphate, magnesium sulphate, magnesium citrate, L-asparagine, glycerol/sodium pyruvate) were dissolved in 1 L distilled water, before autoclaving at 121 °C for 15 minutes to sterilize. For glycerol supplemented media, glycerol alone was added whilst for pyruvate supplemented media, only pyruvate was added. Next, 167 mL egg homogenate and 2% filtered malachite green solution were added in succession. The media was aliquoted into sterile media tubes in 5-6 mL volumes then slanted and coagulated in an inspissator at 85 °C for 55 minutes. To check for sterility,

the media was incubated overnight, then observed for contamination before use. Unused media was stored at 4 °C in sealed bags.

### **3.2.3 Preparation of decontamination solution**

#### **3.2.3.1 N-acetyl-L-cysteine Sodium Hydroxide (NALC-NaOH)**

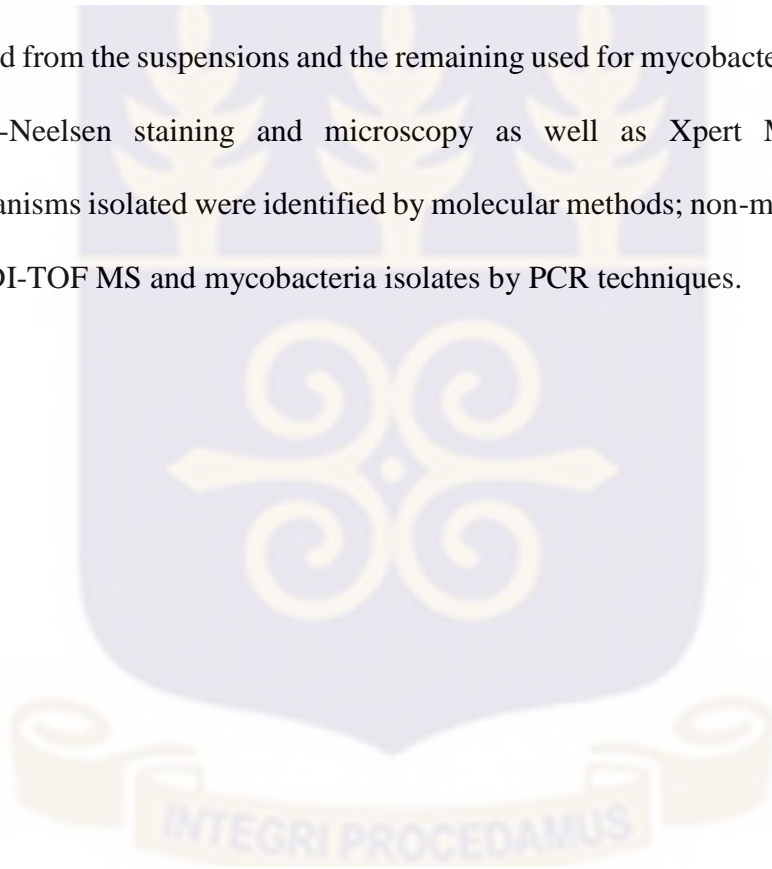
N-acetyl-L-cysteine sodium hydroxide solution was prepared by combining sodium hydroxide solution (NaOH), sodium citrate solution and N-acetyl-L-cysteine (NALC). First, 1 M NaOH was prepared by dissolving 40 g of NaOH crystals in 1 L distilled water. Then, 0.1 M sodium citrate was prepared by dissolving 29 g of tri sodium citrate crystals in 1 L distilled water after which both solutions were sterilized by autoclaving at 121 °C for 15 minutes. The reagents were stored at 4 °C from which the working NALC-NaOH solution was prepared on the day of analysis. NALC-NaOH solution was prepared by adding 0.5% NALC powder to a mixture containing 50 mL each of the sodium citrate and sodium hydroxide solutions then allowed to dissolve completely before use (G. P. Kubica; Global Laboratory Initiative, 2014).

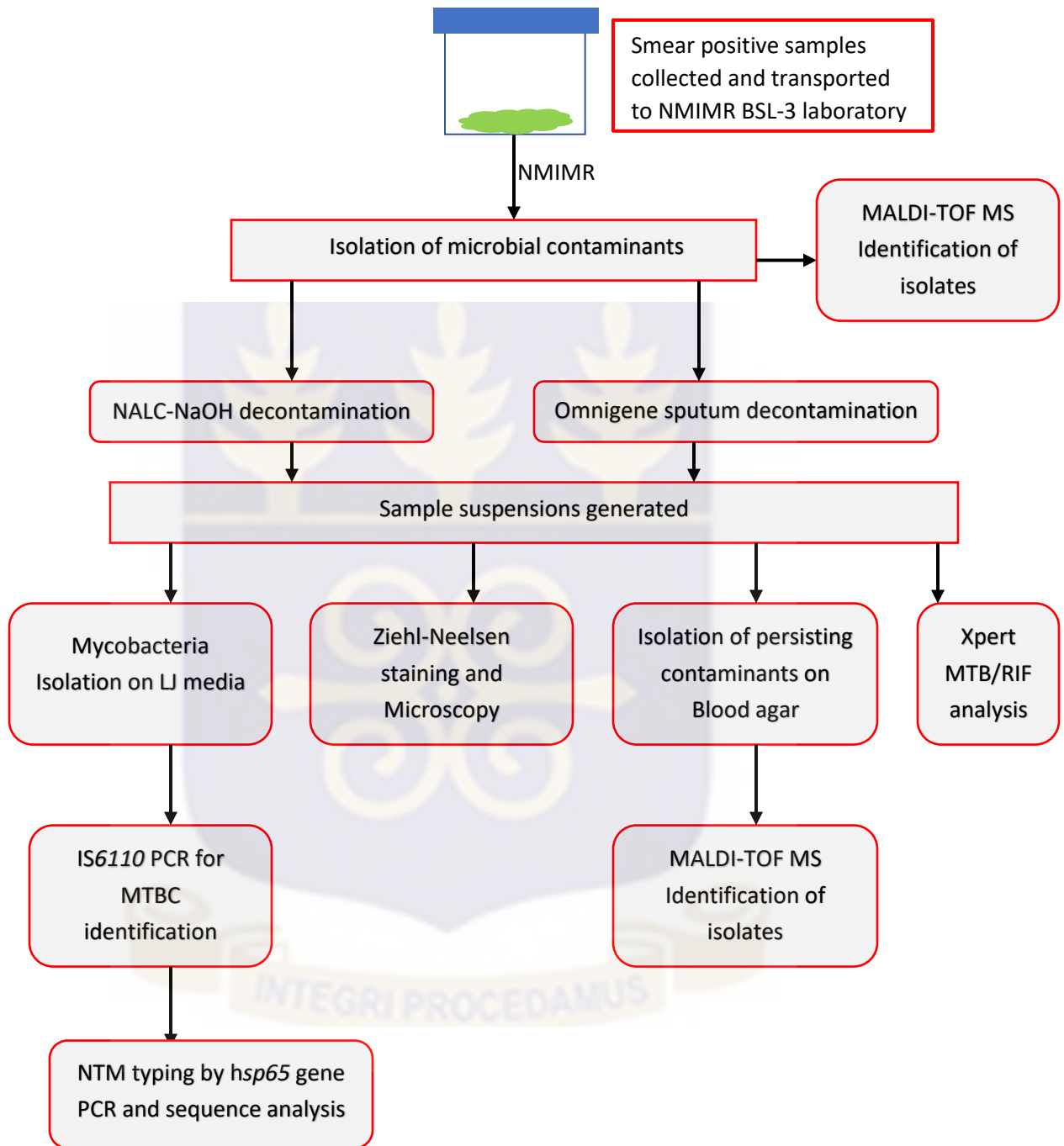
#### **3.2.3.2 Omnigene Sputum reagent (OMS)**

Omnigene sputum reagent was received from DNA Genotek (Ottawa, Canada). Being a ready to use solution, OMS required no prior preparation or reconstitution. The reagent was stored in its original container according to the manufacturer's instructions in a cabinet away from sunlight.

### **3.2.3.3 Study design for the evaluation**

This was an analytical study that evaluated the efficacy of a laboratory reagent for transporting and decontaminating sputum samples for TB diagnosis. The work flow is as indicated in figure 3.1. The microbial contaminants of the sputum samples were first determined, followed by chemical treatment (decontamination) using Omnigene sputum reagent and NALC-NaOH. Concentrated suspensions were generated from the samples by centrifugation after each treatment process. Persisting microbial contaminants were determined from the suspensions and the remaining used for mycobacteria cultures, smears for Ziehl-Neelsen staining and microscopy as well as Xpert MTB/RIF analysis. Microorganisms isolated were identified by molecular methods; non-mycobacteria isolates by MALDI-TOF MS and mycobacteria isolates by PCR techniques.





**Figure 3.1: Diagram indicating the work flow for Omnigene Sputum assessment using NALC-NaOH as control**

### **3.2.4 Determination of other microbial contaminants before decontamination**

Sputum samples were cultured directly on blood agar before taken through chemical decontamination. First, a loopful ( $\approx 10 \mu\text{L}$ ) of each sample was inoculated on the blood agar media and incubated aerobically at  $37^{\circ}\text{C}$  overnight. After overnight incubation, the plates were examined for macroscopic growth. Distinct bacteria and fungi colonies were purified and amplified by sub-culturing onto NA and SDA respectively to obtain pure growth for identification using MALDI-TOF mass spectrometry.

### **3.2.5 Evaluation of the decontamination solution-OMS**

Sputum samples were incubated in equal volume of OMS for 2, 4 and 8 days at ambient temperature to assess the ability of OMS to decontaminate, appropriate duration of decontamination and its ability to maintain mycobacteria viability while using NALC-NaOH as control. An additional 14-day incubation was performed to test the usability of samples if the time to their arrival at the laboratory exceeds the required 8 days.

### **3.2.6 N-acetyl-L-cysteine – Sodium Hydroxide (NALC-NaOH) decontamination**

Equal volume of freshly prepared NALC-NaOH solution was added to each sputum sample and vortexed briefly to obtain a uniform mixture. Sample mixture was allowed to incubate at room temperature for 20 minutes with intermittent vortexing. The reaction was stopped by topping up the mixture with sterile phosphate buffered saline (PBS) to the 45 mL mark. The mixture was centrifuged at  $3800 \times g$  for 30 minutes at  $4^{\circ}\text{C}$  and the supernatant was decanted leaving a sediment which was re-suspended in 1-2 mL sterile PBS.

### **3.2.7 Cultivation of samples after decontamination**

#### **3.2.7.1 Non-mycobacteria isolation**

To assess the effectiveness of decontamination reagents to inhibit growth of other microorganisms, the decontaminated samples were inoculated on blood agar and incubated aerobically at 37 °C overnight. After overnight incubation, the plates were examined for macroscopic bacteria or fungal growth. Distinct bacteria and fungi were sub-cultured on to NA and SDA respectively for identification using MALDI-TOF mass spectrometry.

#### **3.2.7.2 Mycobacteria isolation**

Two hundred microliter (200 µL) each of the suspension was inoculated in quadruplets on LJ media supplemented with either pyruvate to support the growth of *M. bovis* and *M. africanum*, or with glycerol to support the growth of other mycobacteria species. Cultures were incubated aerobically at 37 °C and observed for macroscopic growth daily for the first 7 days then at weekly intervals for 12 weeks. In addition to colonial morphology, growth was recorded according to the classifications in table 3.1 while contamination was recorded, if at least a quarter of the tube was covered with non-AFB (Global Laboratory Initiative, 2014; Sathianathan & Khalil, 1981) and no growth when no microbial colony was observed after the 12 weeks of incubation according to the protocol used at the bacteriology department, NMIMR.

**Table 3.2: Culture reading classification**

Grading	Interpretation
Negative	No growth
Scanty	1-9 colonies
1+	10-100 colonies
2+	100-200 colonies
3+	200-500 colonies
4+	>500 colonies

### 3.2.8 Smear preparation and microscopy

For each of the decontaminated samples, smears were prepared for detection of acid-fast bacilli (AFB) by ZN staining. Drops (3) of each sample suspension was placed on a labelled microscope slide and allowed to air dry. The slides were first heat fixed and then flooded with carbol fuchsin. Heat was applied gently beneath the flooded slides until fumes appeared to make pores in the hydrophobic mycobacterial cell wall for uptake of the stains. Slides were allowed to stand for 5 minutes after which the stain was washed off under running tap water and then flooded with 20% sulfuric acid for another 5 minutes to decolourize. Following decolourization, the counter stain (methylene blue) was flooded on the slide and left for 1 minute before washing off under running tap water (Cheesbrough, 1984). The slides were air-dried, observed under the 100X magnification using immersion oil and graded according to the International Union against Tuberculosis and Lung Diseases grading system.

### **3.2.9 Xpert MTB/RIF analyses**

To assess the compatibility of the decontamination treatment with rapid DNA-based tests, the decontaminated samples were analyzed by the Xpert MTB/RIF assay according to the manufacturer's instructions. Aseptically, 1.5 mL of the sample reagent (mixture of sodium hydroxide and isopropanol) provided in a ready-to-use form was added to 0.5 mL of sample suspension in a falcon tube. The mixture was incubated for 15 minutes at room temperature with intermittent vortexing after which it was aspirated into the cartridge provided. The cartridge contains three beads of freeze dried PCR reagents and two reagent solutions as follows: Bead 1 (polymerase, deoxynucleoside triphosphates, bovine serum albumin), Bead 2 (primers, probes, bovine serum albumin), Bead 3 (sample processing control-~6000 *Bacillus globigii* spores), and reagent 1 and 2 (tris buffer, surfactant, EDTA). The GeneXpert software was launched and the manufacturer's instructions followed to run the test (Cepheid, 2015). One Xpert MTB/RIF cartridge was used for each sample.

### **3.2.10 Isolate identification**

#### **3.2.10.1 Gram staining of isolates**

Smears prepared from bacteria colonies obtained after subcultures were stained by Gram's staining technique to ascertain their purity before molecular identification. First, the prepared smears were heat fixed and flooded with the primary stain crystal violet for 1 minute. The stain was washed off under running tap water, and then smears flooded with Lugol's iodine, which fixes the primary stain into the bacteria cell wall. After washing under running tap water, a decolourization step was performed by flooding the smear with acetone-alcohol for few seconds followed by washing under running tap water. The smear

was then covered with the counter stain neutral red for 1 minute, washed off then allowed to air dry before observing under 100X magnification using immersion oil (Cheesbrough, 1984). Colonies that stained purple were considered Gram positive whereas those that stained red were classified as Gram negative.

### **3.2.10.2 Identification of non-mycobacteria species by Matrix Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS)**

MALDI-TOF MS, a novel molecular identification tool that works by targeting organisms' unique proteomic fingerprint was used to identify bacterial and fungal contaminants (microorganisms other than mycobacteria in sputum). Organisms were first cultivated to the log phase of growth then infused into a small crystalline organic compound (matrix) as follows; a single colony was spread evenly onto a spot on the MALDI target plate to form a thin film. The spot was overlaid with 1  $\mu$ L 70% formic acid and air dried at room temperature. Immediately after drying, 1  $\mu$ L of the matrix (HCCA,  $\alpha$ -cyano-4-hydroxycinnamic acid) solution was added and allowed to dry at room temperature.

The target plate was loaded into the mass spectrometer, where a laser beam was directed on to the matrix embedded organisms to achieve protein ionization. Following manufacturer's instructions, a unique protein fingerprint was generated for each isolate (N. Singhal *et al.*, 2015). Proteomic fingerprint of each isolate was compared to the MALDI-Biotyper database which currently houses proteomic fingerprints of over 3000 organisms (Panda *et al.*, 2014).

### 3.2.10.3 Identification of mycobacteria species

A colony on LJ media was first stained by ZN technique and those confirmed to be AFB positive identified by DNA-based methods. PCR amplification of the MTBC-specific insertion sequence *6110* (*IS6110*) was used to identify MTBC while amplification of the mycobacteria species-specific 65-kDa antigen gene (*hsp65*) followed by sequence analysis was used for identification of non-tuberculous mycobacteria (NTM).

#### 3.2.10.3.1 Polymerase Chain Reaction (PCR) detection of MTBC by *IS6110* PCR

Mycobacteria isolates were suspended in sterile distilled water, then heated for 60 minutes at 95 °C to inactivate the tubercle bacilli and release the nucleic acid content in solution. The crude DNA in solution was used for the PCR.

Amplification of *IS6110* was performed as described by Yeboah-Manu *et al.*, (2001). Briefly, each PCR reaction consisted of 5 µL 10X PCR buffer, 10 µL Q solution, 2.5 µL MgCl<sub>2</sub>, 2.5 µL forward primer (TB284-5'GGACAACGCCGAATTGCG3'), 2.5 µL reverse primer (TB850-5'TAGGCGTCGGTGACAAAGGCCAC3'), 1 µL deoxynucleoside triphosphates (dNTP's), 5 µL 10X coral dye, 16.2 µL nuclease free water, 0.3 µL hot start taq polymerase and 7 µL template DNA to a volume of 45 µL.

Instead of the template, H37Rv was added to the positive control while nuclease free water was added to the negative control. The reaction was run in a conventional PCR thermal cycler (Applied Biosystems 2720), using the conditions shown in table 3.2. PCR products were resolved on a 2% agarose gel (Yeboah-Manu *et al.*, 2001). MTBC isolates yielded a band size of 550 bp.

**Table 3.3: Cycling conditions for IS6110 PCR**

Temperature (°C)	Time (minutes)	Number of cycles
96	5	1
95	1	35
65	1	
72	1	
72	10	1

### 3.2.10.3.2 Mycobacterium-specific 65-kDa antigen gene (*hsp65*) sequence analysis for NTM identification

First, amplification of the *hsp65* gene was carried out as previously described (Yeboah-Manu *et al.*, 2001): each reaction mix was made up of 3 µL 10X PCR buffer, 3 µL Q solution, 1.8 µL MgCl<sub>2</sub>, 1.8 µL forward primer (TB11-5'ACCAACGATGGTGTGTCCAT3'), 1.8 µL reverse primer (TB12-5'CTTGTCGAACCGCATACCCT3'), 0.6 µL deoxynucleoside triphosphates, 3 µL 10X coral dye, 14.8 µL nuclease free water, 0.2 µL hot start taq polymerase and 5 µL template DNA to a volume of 35 µL. The reaction was run in a conventional PCR thermal cycler (Applied Biosystems 2720), using the conditions shown in table 3.3. After resolving on a 2% agarose gel, the DNA sequence of the amplified product was determined by outsourcing (Macrogen, Europe). A band size of 439 bp corresponding to the mycobacterium-specific 65-kDa antigen gene was used to confirm the presence of mycobacteria species.

**Table 3.4: Cycling conditions for *hsp65* PCR**

Temperature (°C)	Time (minutes)	Number of cycles
95	10	1
96	1	35
60	1	
68	1	
72	10	1

### 3.3 Data Analysis

The positivity, negativity and contamination rates were computed by dividing the number of tubes that showed mycobacterial growth, no growth and other bacterial growth over total number of inoculated tubes respectively. The culture positivity, negativity and contamination rates of the OMS decontamination method was compared to the NALC-NaOH method using the Chi-square test, where a p-value < 0.05 was interpreted as significant.

Agreement of the two methods with respect to ZN microscopy and the Xpert MTB/RIF assay were compared using the Cohen's kappa test performed using the Stata version 14.2. Sequences of *hsp65* gene were cleaned as follows: vector sequences were first removed and the sequences processed into gap experimental files before editing them using the pregap4 and gap4 of the Staden package respectively (Staden, 1996). A blast search of the clean sequences on the NCBI nucleotide blast page was used to identify NTM's.

## CHAPTER FOUR

### 4.0 RESULTS

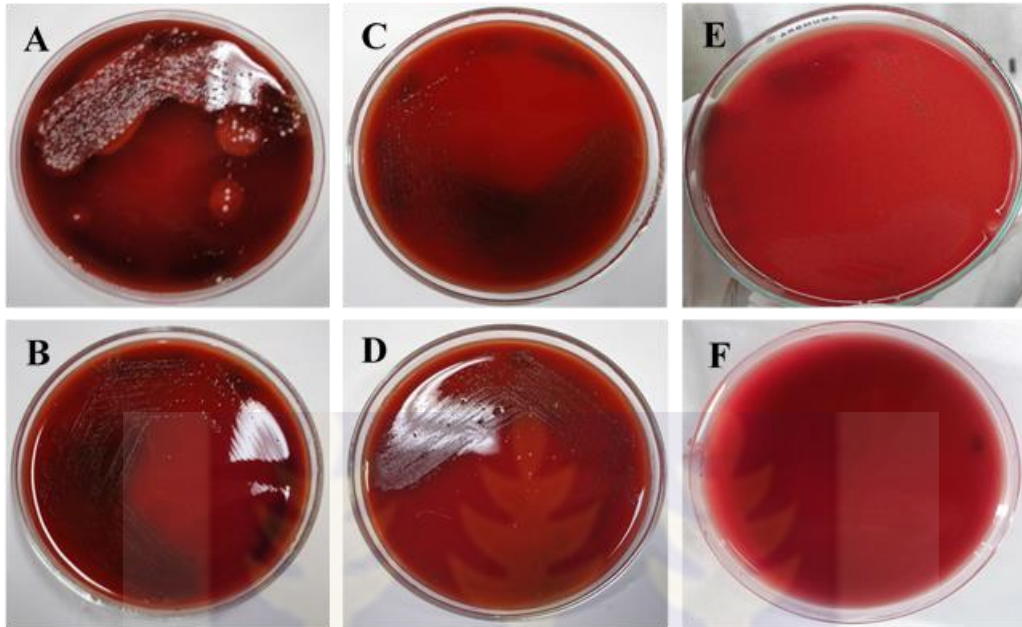
#### 4.1 Macroscopic features of sputum samples and direct sputum smear positivity

A total of one hundred and four sputum samples collected from pulmonary TB patients were received from two peripheral health facilities and analyzed. All the 104 (100%) samples were mucopurulent and 5 (4.8%) were also bloody. Of the 104 sputa, direct sputum smear result was indicated for 93 (89.4%) out of which 11 (11.8%) had a scanty bacilli load, 16 (17.2%) had 1+, 25 (26.9%) had 2+ and the remaining 41 (44.1%) had 3+.

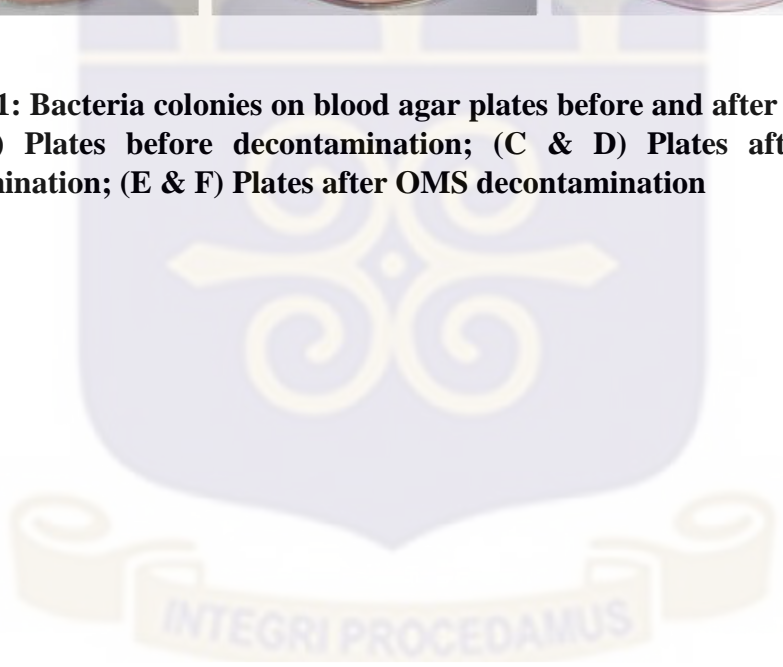
#### 4.2 Decontamination efficiency

##### 4.2.1 Microbial contaminants on blood agar before chemical treatment

Overall, one-hundred and four sputum samples were cultured on blood agar before chemical treatment out of which 93 (89.4%) yielded microbial growth, with 27 of the 93 samples having more than one organism isolated; 2 organisms were isolated from 22 samples and 3 organisms from 5 samples giving a total of 125 different isolates. In addition, 101/125 isolates comprising 91 (90.0%) bacteria and 10 (10.0%) fungi were identified. In all, 44 distinct microbial species were identified. Figure 4.1 shows some blood agar cultures before and after decontamination. Among the organisms isolated, the most predominant bacterial species were *Streptococcus oralis* (20, 18.0%), *Staphylococcus aureus* (10, 9.0%) and *Pseudomonas species* (10, 9.0%) whereas *Candida species* (8, 7.2%) was the most dominant fungi (Table 4.1).



**Figure 4.1: Bacteria colonies on blood agar plates before and after decontamination. (A & B) Plates before decontamination; (C & D) Plates after NALC-NaOH decontamination; (E & F) Plates after OMS decontamination**



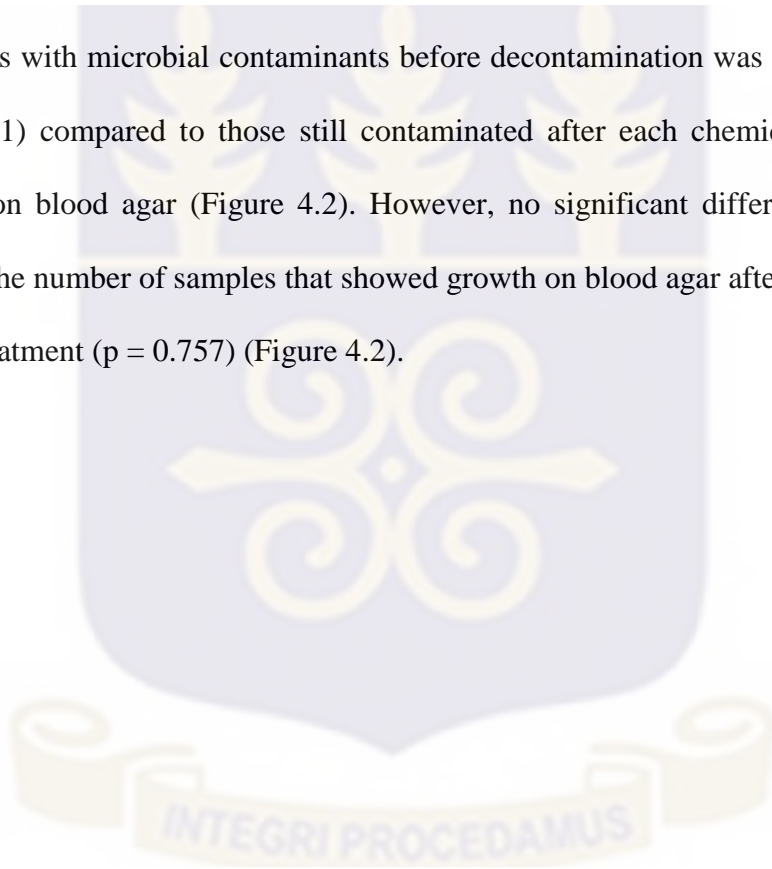
**Table 4.1: The spectrum of microbial organisms isolated from sputum samples before and after decontamination on blood agar**

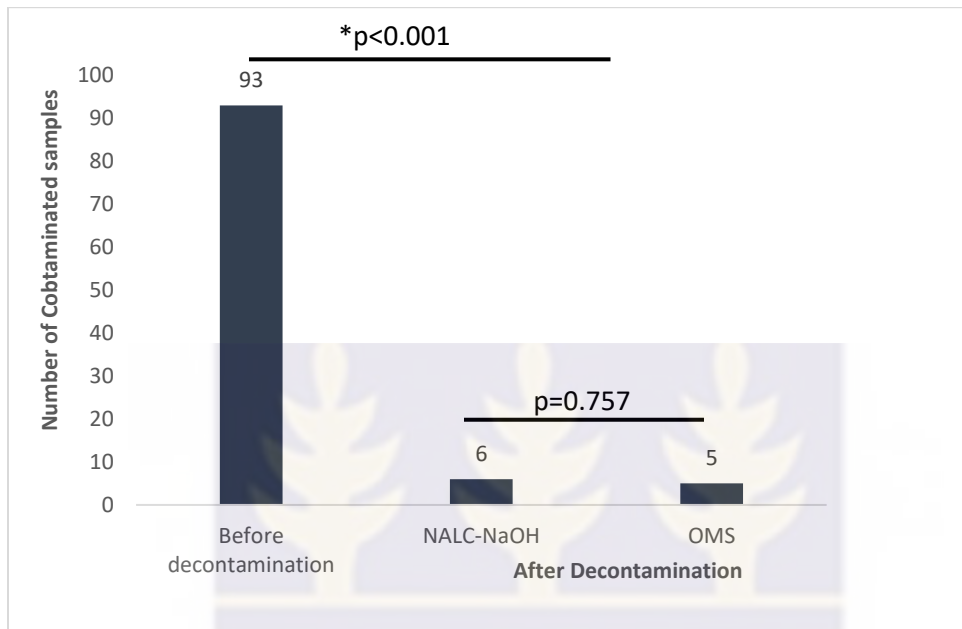
Microorganism	After Treatment	
	OMS (n=5)	NALC-NaOH (n=6)
<b>Before Treatment (n=125)</b>		
<b>Bacteria</b>		
<b>20 (16.0%) <i>Streptococcus oralis</i></b>	1 (20.0%) <i>Bacillus pumilus</i>	1 (16.7%) <i>Bacillus pumilus</i>
<b>10 (8.0%) <i>Staphylococcus aureus</i></b>		1 (16.7%) <i>Pseudomonas species</i>
<b>10 (8.0%) <i>Pseudomonas species</i>*</b>		1 (16.7%) <i>Micrococcus luteus</i>
<b>5 (4.0%) <i>Bacillus pumilus</i></b>		
<b>5 (4.0%) <i>Klebsiellae pneumoniae</i></b>		
<b>4 (3.2%) <i>Stenotrophomonas maltophilia</i></b>		
<b>3 (2.4%) <i>Staphylococcus epidermidis</i></b>		
<b>3 (2.4%) <i>Acinetobacter baumannii</i></b>		
<b>2 (1.6%) <i>Escherichia coli</i></b>		
<b>2 (1.6%) <i>Staphylococcus haemolyticus</i></b>		
<b>2 (1.6%) <i>Streptococcus parasanguinis</i></b>		
<b>2 (1.6%) <i>Streptococcus salivarius</i></b>		
<b>2 (1.6%) <i>Corynebacterium argenterantense</i></b>		
<b>2 (1.6%) <i>Proteus mirabilis</i></b>		
<b>1 (0.8%) <i>Bacillus firmus</i></b>		
<b>1 (0.8%) <i>Neisseria subflava</i></b>		
<b>1 (0.8%) <i>Streptococcus pneumoniae</i></b>		
<b>1 (0.8%) <i>Streptococcus infantis</i></b>		
<b>1 (0.8%) <i>Providentia stuarti</i></b>		
<b>1 (0.8%) <i>Ochrabactrum intermedium</i></b>		
<b>1 (0.8%) <i>Achromobacter xylosoxidans</i></b>		
<b>1 (0.8%) <i>Bacillus megaterium</i></b>		
<b>1 (0.8%) <i>Acinetobacter haemolyticus</i></b>		
<b>1 (0.8%) <i>Granulicatella adiacens</i></b>		
<b>1 (0.8%) <i>Neisseria flavescens</i></b>		
<b>1 (0.8%) <i>Issatchenkia orientalis</i></b>		
<b>1 (0.8%) <i>Enterobacter cloacae</i></b>		
<b>1 (0.8%) <i>Micrococcus luteus</i></b>		
<b>1 (0.8%) <i>Staphylococcus warneri</i></b>		
<b>1 (0.8%) <i>Corynebacterium diphtheriae</i></b>		
<b>1 (0.8%) <i>Rothia mucilaginosa</i></b>		
<b>1 (0.8%) <i>Kocuria aegyptia</i></b>		
<b>1 (0.8%) <i>Streptococcus agalactiae</i></b>		
<b>Fungi</b>		
<b>4 (3.2%) <i>Candida albicans</i></b>		
<b>3 (2.4%) <i>Candida tropicalis</i></b>		
<b>1 (0.8%) <i>Candida rugosa</i></b>		
<b>1 (0.8%) <i>Magnusiomyces capitatus</i></b>		
<b>1 (0.8%) <i>Cryptococcus neoformans</i></b>		
<b>Unidentified organism</b>		
<b>24 (19.2%)</b>	<b>4 (80.0%)</b>	<b>3 (50.0%)</b>

\**Pseudomonas species* comprises *P. paucinobilis* (1), *P. composti* (1), *P. mendocina* (2), *P. stutzeri* (3), *P. plecoglossida* (1), *P. otitidis* (1) and 1 unidentified specie.

#### 4.2.2 Microbial contaminants on blood agar after chemical treatment

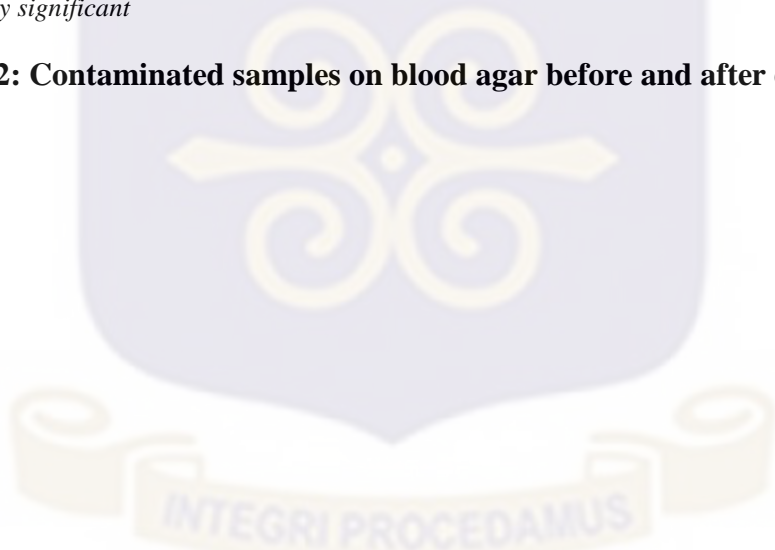
Following cultivation of sputum samples on blood agar after chemical treatments, 6 (5.8%) of the samples treated with NALC-NaOH had microbial growth whereas 5 (4.8%) samples treated with OMS also had microbial growth (Figure 4.2). Three of the 6 isolates obtained from the NALC-NaOH treated samples were identified as *Micrococcus luteus*, *Bacillus pumilus* and *Pseudomonas species* while the remaining 3 could not be identified. Only one of the OMS isolates could be identified as *B. pumilus* (Table 4.1). Overall, the number of samples with microbial contaminants before decontamination was significantly higher ( $p < 0.001$ ) compared to those still contaminated after each chemical treatment when checked on blood agar (Figure 4.2). However, no significant difference was observed between the number of samples that showed growth on blood agar after OMS and NALC-NaOH treatment ( $p = 0.757$ ) (Figure 4.2).





*\*statistically significant*

**Figure 4.2: Contaminated samples on blood agar before and after decontamination**



#### 4.2.3 Contamination rate on Lowenstein-Jensen media

Overall, 94 samples each were treated with NALC-NaOH and OMS and each inoculated on LJ media in quadruplets giving a total of 376 inoculated LJ slants per treatment. The number of contaminated LJ slants (that is quarter of the tube grew microorganism other than mycobacteria) for each treatment was recorded after a 12-week culture duration.

A sample is indicated contaminated when all 4 inoculated tubes got contaminated; 12 (12.8%) NALC-NaOH treated samples got contaminated. Among the 12 contaminated samples, 7 (7.4%) were recorded in the first week, 4 (4.3%) during the second week and 1 (1.0%) in the third week of culture (Table 4.2). A total of 70 (18.6%) tubes contaminated after NALC-NaOH treatment; 36 (9.6%) in the first week, 25 (6.6%) during the second week, 8 (2.1%) in the third week and 1 (0.3%) within the eighth week of culture as shown in table 4.3.

Four (4.3%) samples got contaminated after OMS treatment. Among these, 2 (2.1%) were recorded in the first week, 1 (1.1%) during the second week and 1 (1.1%) in third week of culture. Overall, 25 (6.6%) LJ slants contaminated after OMS treatment of which 14 (3.7%) were recorded in the first week, 6 (1.6%) during the second week, 4 (1.1%) in the third week and the remaining 1 (0.2%) in the eighth week of culture (Table 4.2).

The contamination rate was significantly higher after NALC-NaOH treatment than after OMS treatment ( $p = 0.037$  for number of contaminated samples and  $p < 0.001$  for number of contaminated LJ slants).

**Table 4.2: Contamination rate after NALC-NaOH and OMS treatments and the week of observed contamination**

Period of observed contamination (weeks)	Number of contaminated samples and LJ slants			
	NALC-NaOH		OMS	
	Samples (%) n=94	LJ slants (%) n=376	Samples (%) n=94	LJ slants (%) n=376
1	7 (7.4)	36 (9.6)	2 (2.1)	14 (3.7)
2	4 (4.3)	25 (6.6)	1 (1.1)	6 (1.6)
3	1 (1.1)	8 (2.1)	1 (1.1)	4 (1.1)
8	0 (0)	1 (0.3)	0 (0)	1 (0.2)
Total	*12 (12.8)	#70 (18.6)	*4 (4.3)	#25 (6.6)

\* $p=0.037$  # $p<0.001$

#### 4.3 Mycobacterium viability after chemical treatments

Of the 94 NALC-NaOH treated samples, 70 (74.5%) had mycobacteria growth, indicating viability after chemical treatment: 45 (47.9%) recorded within the first three weeks of culture, 21 (22.3%) between the fourth and sixth weeks and the remaining 4 (4.3%) from the seventh to tenth weeks of culture (Table 4.3). Furthermore, 235 (62.5%) of the 376 inoculated LJ slants were mycobacteria positive after NALC-NaOH treatment out of which 123 (32.7%) were recorded in the first three weeks, 89 (23.8%) from the fourth to sixth weeks and 23 (6.1%) within the seventh and tenth weeks.

Seventy-four (78.7%) of the 94 OMS treated samples had mycobacteria growth after the 12 weeks of culture. Of the 74 positive samples, 26 (27.7%) were recorded in the first three weeks of culture, 42 (44.7%) within the fourth and sixth weeks and the remaining 6 (6.4%) in the following seventh to tenth weeks. Additionally, mycobacteria growth was observed

in 228 (60.6%) of the 376 inoculated LJ slants after OMS treatment: 70 (18.6%) observed in the first three weeks, 136 (36.2%) from the fourth to sixth weeks and the remaining 22 (5.9%) from the seventh to tenth weeks (Table 4.3).

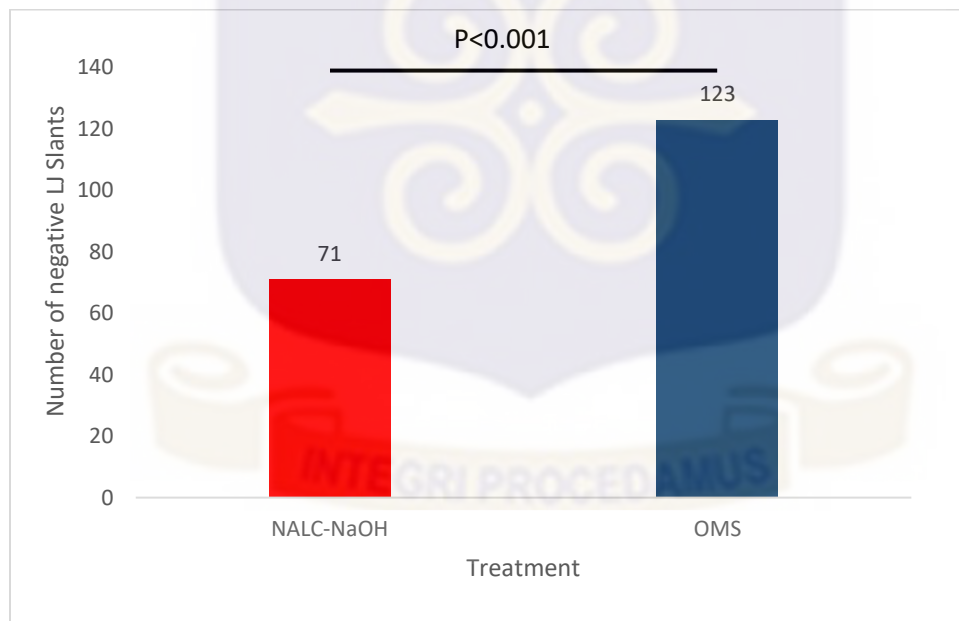
The mycobacteria positivity rate of the samples was higher after OMS treatment than after NALC-NaOH treatment. Furthermore, the number of mycobacteria positive LJ slants was higher after NALC-NaOH treatment than after OMS treatment. A significant difference ( $p = 0.002$ ) in the time to culture positivity between NALC-NaOH treated samples and OMS treated samples was observed. The NALC-NaOH treated samples showed a significantly higher rate of culture positivity (47.9% vs. 27.7%;  $p = 0.004$ ) within the first three weeks of culture whereas the OMS treated samples rather showed a significantly higher culture positivity during the 4-6 weeks period (22.3% vs. 44.7%;  $p = 0.001$ ).

Comparatively, 71 (18.9%) of the 376 inoculated LJ slants after NALC-NaOH treatment had no growth whereas 123 (32.7%) of 376 inoculated LJ slants after OMS treatment also had no growth (Figure 4.3).



**Table 4.3: Mycobacteria positivity rate computed after NALC-NaOH and OMS treatment**

Period of observed positivity (weeks)	Mycobacteria Positivity Rate			
	NALC-NaOH		OMS	
	Samples (%) n=94	LJ slants (%) n=376	Samples (%) n=94	LJ slants (%) n=376
1-3	45 (47.9)	123 (32.7)	26 (27.7)	70 (18.6)
4-6	21 (22.3)	89 (23.7)	42 (44.7)	136 (36.2)
7-10	4 (4.3)	23 (6.1)	6 (6.4)	22 (5.9)
Total	70 (74.5)	235 (62.5)	74 (78.7)	228 (60.6)



**Figure 4.3: Negative cultures after OMS and NALC-NaOH treatment**

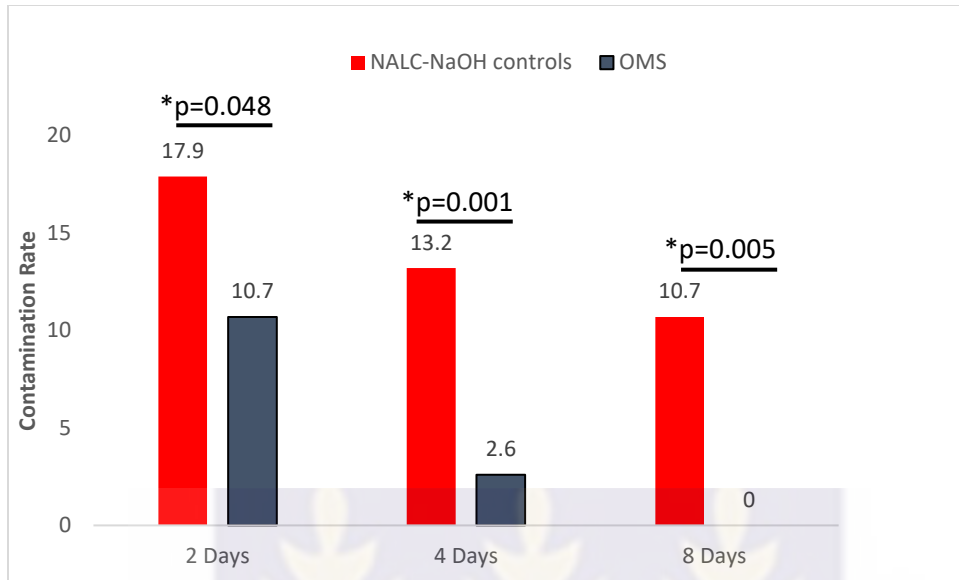
#### 4.4 Appropriate time for OMS decontamination

A time course analysis was used to evaluate the duration of OMS sample transport and decontamination. Twenty-eight of the samples were treated with OMS for 2 days, 38 samples for 4 days and the remaining 28 samples for 8 days with corresponding NALC-NaOH controls at each time point. Figure 4.4 summarizes the contamination rate for each OMS incubation period relative to the respective NALC-NaOH controls.

After NALC-NaOH treatment, 5 (17.9%) out of 28, 5 (13.2%) out of 38 and 2 (10.7%) out of 28 samples contaminated for the respective OMS incubation times (Figure 4.4). However, of the 28 samples treated with OMS for 2 days, 3 (10.7%) contaminated whereas only 1 (2.6%) of the 38 samples treated for 4 days contaminated. None of the samples treated with OMS for 8 days got contaminated.

At each time point, the contamination rate was significantly higher after NALC-NaOH treatment compared to after OMS treatment (2 days:  $p = 0.048$ , 4 days:  $p = 0.001$ , 8 days:  $p = 0.005$ ) as shown in figure 4.4. In addition, the contamination rate significantly reduced with increasing OMS treatment duration ( $p < 0.001$ ).





**Figure 4.4: Culture contamination rate after OMS and NALC-NaOH treatment in a time series**

The mycobacteria positivity rate for the time series is summarized in table 4.4. At the 2-day time point, 20 (71.4%) of the 28 NALC-NaOH treated samples showed mycobacteria growth, 27 out of 38 (71.1%) were also mycobacteria positive at the 4-day time point and at the 8-day time point, 23 (82.1%) of 28 NALC-NaOH treated samples were mycobacteria positive. After OMS treatment, 21/28 (75%) samples had mycobacteria growth at the 2-day time point, 30/38 (78.9%) at the 4-day time point and 23/28 (82.1%) at the 8-day time point.

Compared to the NALC-NaOH treated samples, OMS samples had higher mycobacteria positivity at both the 2-day and 4-day time points even though the differences were not significant (2-day:  $p = 0.761$ , 4-day:  $p = 0.432$ ) (Table 4.4). Even though the observed number of mycobacteria positive samples increased with increasing OMS treatment duration, it was not significant ( $p = 0.227$ ).

**Table 4.4: Mycobacteria positivity rate after OMS and NALC-NaOH treatment in a time series**

OMS Incubation Time (Days)	Positivity rate		
	NALC-NaOH (%)	OMS (%)	p-value
2 [n=28]	20 (71.4)	21 (75.0)	0.761
4 [n=38]	27 (71.1)	30 (78.9)	0.432
8 [n=28]	23 (82.1)	23 (82.1)	1.000

#### 4.4.1 Extended incubation time of sputum samples in OMS (14-day incubation)

A total of ten smear positive samples were treated with OMS for 14 days to assess the usability of samples which may exceed the recommended 8-days of OMS treatment while in transport. The ten samples were inoculated on LJ media in quadruplets to a total of 40 slants.

Seven (70%), 2 (20%) and 1 (10%) of the samples were mycobacteria positive, contaminated and negative respectively, using NALC-NaOH as controls. Furthermore, 28 (70%) of the 40 LJ slants inoculated after NALC-NaOH treatment were positive for mycobacteria growth, 7 (17.5%) contaminated while 5 (12.5%) had no growth (Table 4.5). Eight (80%) out of 10 OMS treated samples had mycobacteria growth while 2 (20%) had no growth. Additionally, 20 (50%) of the 40 inoculated LJ slants were mycobacteria positive after OMS treatment whereas the remaining 20 (50%) were negative. No contamination was recorded.

**Table 4.5: Assessment of OMS for 14-day incubation time relative to NALC-NaOH**

Parameter	NALC-NaOH		OMS		‡p-value
	Samples, n (%)	LJ slants, n (%)	Samples, n (%)	LJ slants, n (%)	
Positivity	7 (70)	28 (70)	8 (80)	20 (50)	0.068 <sup>+</sup>
Contamination	2 (20)	7 (17.5)	0 (0)	0 (0)	0.006*
Negativity	1 (10)	5 (12.5)	2 (20)	20 (50)	0.531 <sup>+</sup>
Total	10 (100)	40 (100)	10 (100)	40 (100)	

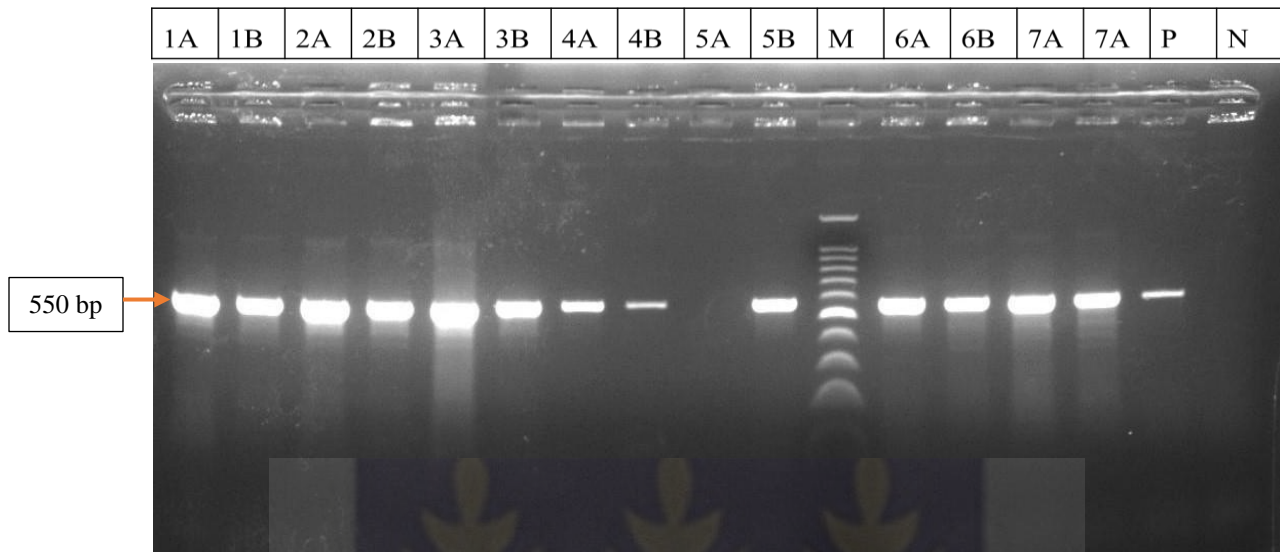
‡Tests of significance was computed for each parameter based on the sample numbers

\*Statistically significant

<sup>+</sup>not significant

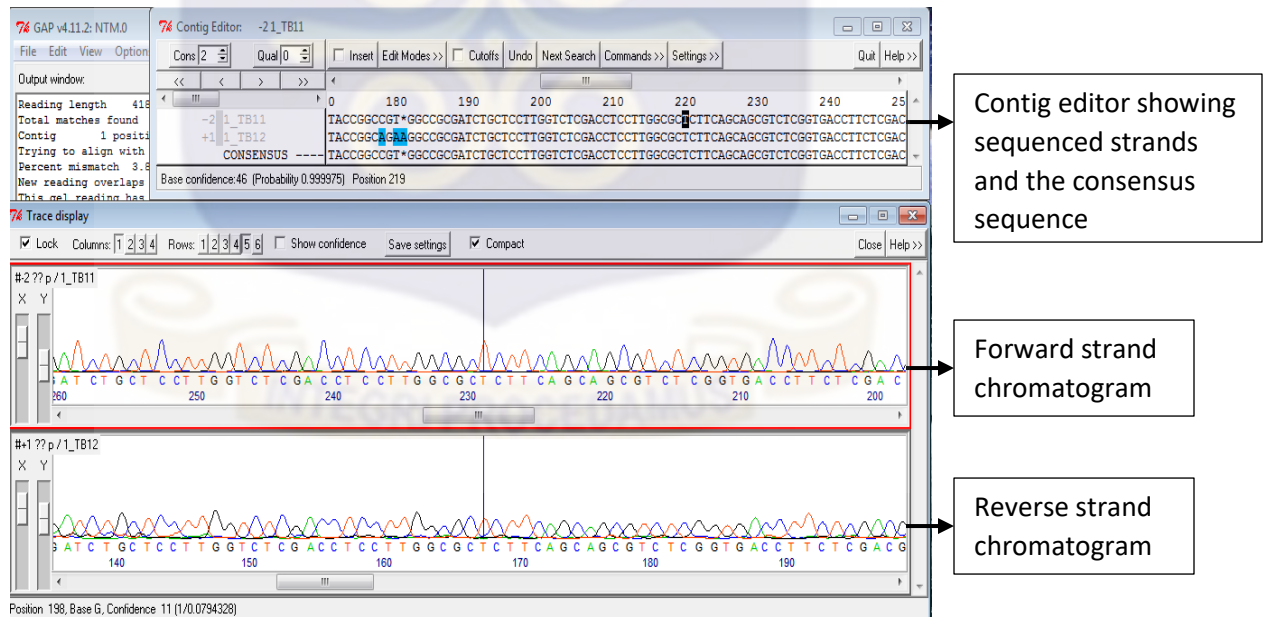
#### 4.4.2 Identification of Mycobacteria species

Colony morphology and AFB positivity was used to distinguish between macroscopic mycobacteria growth and other organisms. Overall, 159 mycobacteria isolates were obtained after both treatments: OMS 82 (78.8%) and NALC-NaOH 77 (74.0%). All the isolates obtained were positive for IS6110 PCR, having a 550 bp band size on 2% agarose gel indicating members of the *Mycobacterium tuberculosis complex* except for one isolate obtained after OMS treatment (Figure 4.5). This isolate was further typed by sequence analysis of the mycobacteria genus specific *hsp65* gene and NCBI Blast search, which identified it as *Mycobacterium abscessus* with a 99% identity. Figure 4.6 displays a chromatogram of the *M. abscessus* sequences.



*M*: 100 bp molecular weight marker, *P*: positive control, *N*: negative control

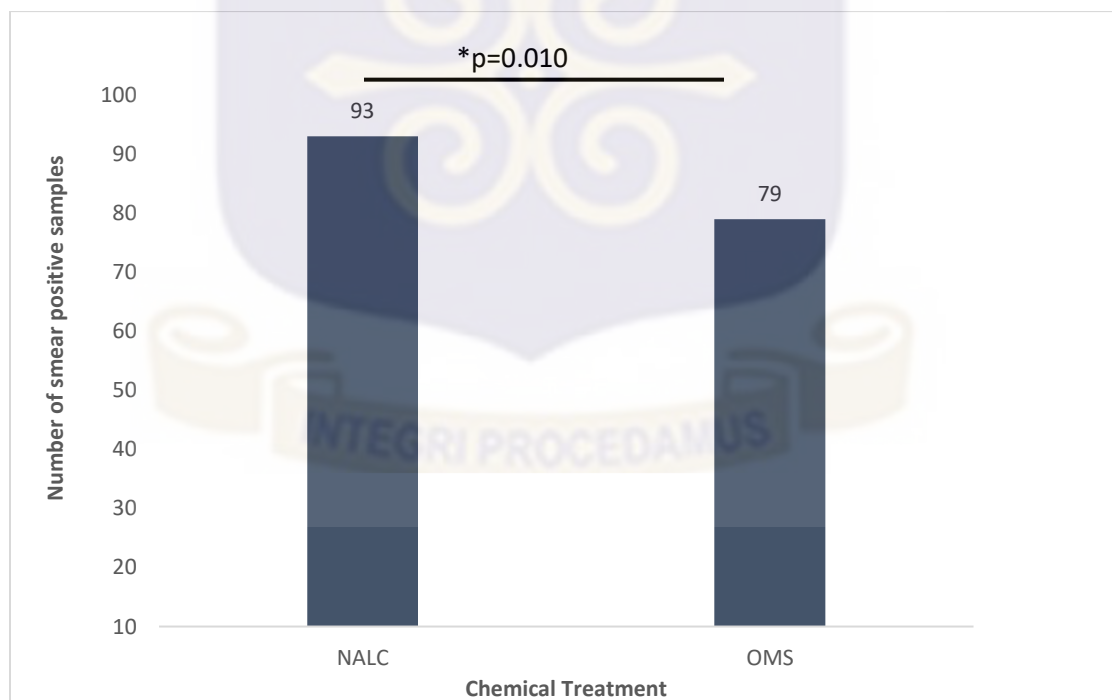
**Figure 4.5: Gel image showing IS6110 PCR positive isolates**



**Figure 4.6: Chromatogram showing a part of the *M. abscessus* sequence**

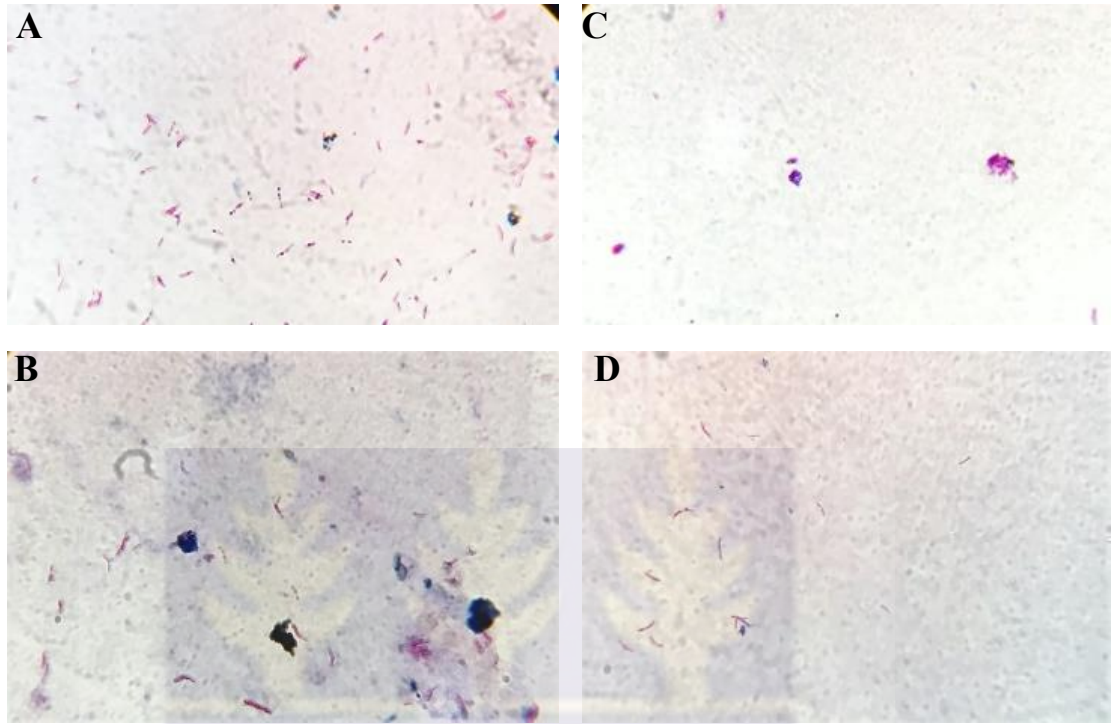
#### 4.5 Acid fast bacilli detection by Ziehl-Neelsen (ZN) staining and microscopy

To test the compatibility of the chemical treatments with ZN staining, smears were made directly from the resulting sample suspensions after each treatment and stained by ZN technique. Of the 104 smears examined per treatment, 93 (89.4%) were acid-fast bacilli (AFB) positive after NALC-NaOH treatment while 79 (75.9%) were AFB positive after OMS treatment (Figure 4.7). Smear positivity after NALC-NaOH treatment was significantly higher than after OMS treatment ( $p = 0.010$ ) (Fig. 4.7). However, using the Cohen's Kappa test which measures the level of agreement between different methods, both methods were found to agree 78% more often than expected by chance ( $k=0.392$ ,  $p < 0.001$ ). Figure 4.8 displays some images of ZN stained smears after each chemical treatment.

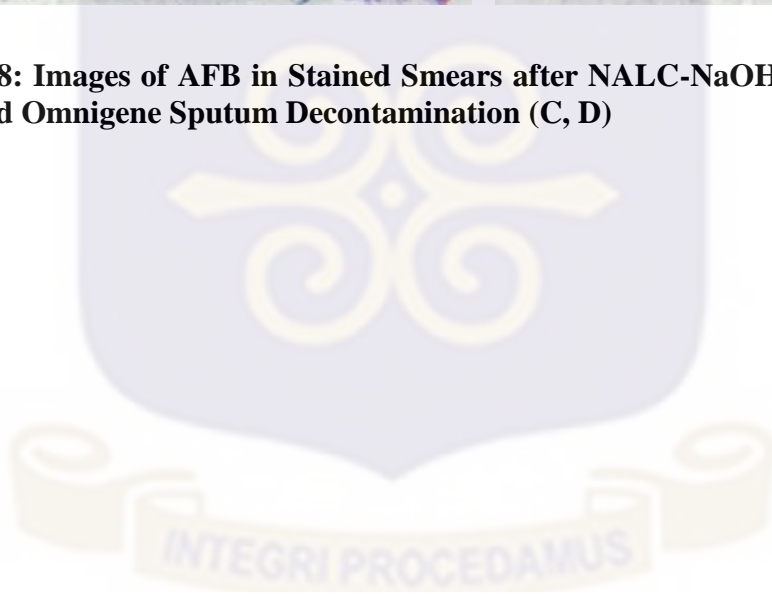


*\*Statistically significant*

**Figure 4.7: Acid fast bacilli Positivity Rate for Omnigene Sputum treated samples compared to NALC-NaOH treated Samples**



**Figure 4.8: Images of AFB in Stained Smears after NALC-NaOH Decontamination (A, B) and Omnigene Sputum Decontamination (C, D)**



#### 4.6 Compatibility of OMS with Xpert MTB/RIF assay

The Xpert MTB/RIF analysis was used to test the compatibility of OMS treatment with DNA-based assays using NALC-NaOH treatment as control. Forty-seven samples were randomly selected after each chemical treatment and analyzed by the Xpert MTB/RIF assay. All the samples tested were positive for MTBC with no rifampicin resistance detected except for one which was MTBC negative. The negative sample was also direct smear and mycobacteria culture negative.

Of the 47 samples analyzed per treatment, 32 (68.1%) of the NALC-NaOH samples recorded a high bacilli load, 12 (25.5%) had a medium bacilli load and 2 (4.3%) had a low bacilli load while 29 (61.7%) OMS samples recorded high bacilli load, 16 (34.0%) had a medium load and 1 (2.1%) had a low bacilli load (Table 4.6). Overall, both methods agreed 93.6% more often than expected by chance ( $k=0.689$   $p < 0.001$ ).

**Table 4.6: Bacilli load results from Xpert MTB/RIF assay**

<b>Xpert MTB/RIF assay (n=47)</b>		
<b>Bacilli load</b>	<b>OMS (%)</b>	<b>NALC-NaOH (%)</b>
High	29 (61.7)	32 (68.1)
Medium	16 (34.0)	12 (25.5)
Low	1 (2.1)	2 (4.3)
Negative	1 (2.1)	1 (2.1)

## CHAPTER FIVE

### 5.0 DISCUSSION

The current TB control strategy, Directly Observed Therapy Short course (DOTs) rely heavily on the laboratory for case confirmation, determination of treatment regimen based on microbiological result, monitoring of treatment progress, especially for difficult to treat cases as well as for drug susceptibility testing (DST). Tuberculosis microbiological determination depends very much on the quality of sputum (Sakundarno *et al.*, 2009; Dunn & Revell, 2016). Due to infrastructural, biosafety, and expertise requirement, TB culture, which is the gold standard method and DST are done in centralized facilities, requiring long distance travel, compromising specimen quality, as it is difficult to maintain cold chain. Sputum samples that are usually lost to contamination due to long period of time in transit from peripheral health facilities to the few designated reference laboratories in country continue to pose a greater challenge to the isolation of viable mycobacteria for the needed laboratory testing, which are crucial for TB control. This thesis therefore sought to evaluate the effectiveness of Omnigene sputum reagent for preserving sputum samples by assessing its ability to maintain mycobacterium viability while at the same time inhibiting the growth of contaminants as well as compatibility with downstream DNA based tests.

#### **Sputum Contaminants: the need to decontaminate before mycobacterial culture**

The respiratory tract which is the organ mainly affected in pulmonary TB disease harbours a diverse microbial population including but not limited to organisms of the genera *Streptococcus*, *Neisseria*, *Haemophilus* and *Candida* (Yi *et al.*, 2014). This diverse

microbial population is often collected together with mycobacteria during sputum expectoration for TB diagnosis (Cui *et al.*, 2012; Robinson, 2004). Moving away from the oral cavity down to the upper and lower respiratory tracts, the microbial flora changes with few organisms such as *Candida sp.* persisting in multiple sections. Majority (89.94%) of the sputa analyzed had microbial growth on blood agar prior to decontamination. This clearly confirms the unsterile nature of the respiratory tract as well as sputum samples expectorated from this site.

The main microorganisms isolated prior to chemical treatment were *Streptococcus oralis* (a commensal of the oral cavity), *staphylococcus aureus* (a commensal of both the skin and lungs) as well as *Pseudomonas species* (a commensal of the skin and throat). In addition, *Candida sp.* found mainly in both the oral cavity and lower respiratory tract of healthy individuals was also isolated. Though these organisms are normal microbial flora, they could also be sources of secondary respiratory infection in TB patients especially when the immune system is compromised (Azoulay *et al.*, 2006; Robinson, 2004; Shishido *et al.*, 1990). For instance, *Pseudomonas sp.* has been associated with recurrent and treatment failure in TB (Wu *et al.*, 2013) whereas *Klebsiella pneumoniae* causes infection even in healthy individuals (Patel *et al.*, 2014).

Whether in synergy or in isolation, these microbes may play an important role in pulmonary complications observed in TB patients especially those also living with HIV which is essential to public health since more than 20% of TB patients in Ghana are co-infected with HIV (National Tuberculosis Control Programme & National AIDS Control Programme, 2007). Furthermore, these bacteria contaminants are fast growers with average generation time ranging from 12 to 30 minutes resulting in macroscopic growth within 24 hours of

aerobic incubation (L. Yang *et al.*, 2008). On LJ media, metabolic activities of these microbes result in their rapid proliferation, media lyses and liquefaction to the detriment of mycobacteria which are rather slow growing (generation time of 20-24 hours and may take up to 12 weeks to observe macroscopic growth) (Smith, 2003). Aside their fast growing nature and shorter generation time, growth characteristics on media such as the swarming motility of *Proteus sp.* (Hernandez *et al.*, 1999) and lysis of media by others severely hinders mycobacteria isolation. It is therefore important to remove these microbes by decontamination to improve mycobacteria isolation.

The presence of microbial contaminants after chemical decontamination was assessed on both blood agar and LJ media. Microorganisms have evolved different adaptation strategies to survive in varying environmental conditions. Stress response in microbes include sporulation, biofilm formation and upregulation of stress modulators such as heat shock proteins and sigma factors (Poole, 2012; Russell, 1990). Within this project, *B. pumilus*, a spore forming bacterium survived the harsh chemical treatment of both NALC-NaOH and OMS. *Bacillus pumilus* has previously been inactivated using radiation and high pressures (Clouston & Wills, 1969; Ha *et al.*, 2017) but this may not be feasible to selectively eliminate *B. pumilus* from clinical specimen. Higher concentrations of decontaminants need to be assessed to remove this contaminant in future. Furthermore, *Pseudomonas sp.* isolated after NALC-NaOH treatment on both blood agar and LJ is a persistent microbial contaminant in LJ cultures which can withstand various decontaminants and disinfectants because of its ability to form biofilms (Kassaza *et al.*, 2014). However, OMS treatment was able to inhibit its growth in this study unlike other decontaminants. The only other effective decontaminant against *Pseudomonas sp.* is oxalic acid which acts by generating

H<sup>+</sup> ions and altering pH to disrupt normal bacteria metabolism leading to their death (S.S. Block, 2001; McClean *et al.*, 2011; Yeboah-Manu *et al.*, 2004). In other instances, some microbes could induce and live in a low metabolic state to evade the harsh treatments of chemical agents until favourable conditions are restored (Padan *et al.*, 2005). This likely explains why after decontamination, certain organisms such as *Magnusiomyces capitatus* were not isolated on blood agar following overnight aerobic incubation yet were recovered from the LJ cultures mostly within one week of culture.

### **Balancing removal of unwanted bacteria and maintaining mycobacteria viability**

One of the key features that allow mycobacteria to selectively survive decontamination is their unique multilayered and hydrophobic cell wall which allows them to withstand these mostly hydrophilic chemical decontaminants (Hett & Rubin, 2008; Maris, 1995). However, the mycobacteria cell wall is not completely inert to hydrophilic chemicals and contains porins and other channels which mediate hydrophilic exchange between the organism and its environment even though at a slower rate (Danilchanka *et al.*, 2015; Kartmann *et al.*, 1999). Thus while treating to remove unwanted microbes which improve mycobacteria isolation, it is important to ensure that mycobacteria viability is not compromised in the process. A good decontaminant should therefore inhibit the growth of unwanted microbes while enhancing mycobacteria recovery. The optimum duration of OMS decontamination was evaluated.

Generally, the findings from this thesis found OMS to have a lower contamination rate (4.3%) compared to the rate of 12.8% recorded for NALC-NaOH. The differences in contamination rate observed could be attributed to the unique effects that each chemical

agent may be exerting which makes one more effective than the other. Most decontaminants are either acids or bases. NALC-NaOH being mainly alkaline works by altering pH, generating free  $\text{OH}^-$  ions which bind macromolecules and interfere with bacteria metabolism leading to their inactivation (S.S. Block, 2001). Additionally, the final working concentration of NaOH that is available to work in each sputum is 0.25 M which upon dissociation yields 0.25 M  $\text{OH}^-$  to act on microbes. This concentration of  $\text{OH}^-$  may be too small to inhibit all the microbial contaminants present leading to the survival of some. Also, microbial stress responses such as sporulation, biofilm formation and upregulation of stress modulators such as heat shock proteins and sigma factors may play a role in the survival of some of the organisms against the chemical agents. Even though the manufacturer does not make the composition of OMS public, the pH of the reagent (9.4) implies that it is also alkaline. It's effectiveness against the microbial contaminants implies that a higher  $\text{OH}^-$  concentration is released into solution giving it a longer half-life than NALC-NaOH and making it better at removing unwanted microbes.

it may be acidic thereby releasing  $\text{H}^+$  ions which protonates macromolecules and interfere with their function leading to bacteria inactivation, especially considering its ability to inactivate *Pseudomonas sp.*

The effectiveness of OMS treatment against contaminating microbes may mean that even in the stress responsive low metabolic state of organisms, OMS is still able to act on the organisms which may not be so with NALC-NaOH. The OMS contamination rate also falls within the allowable range (2-5%) for mycobacteria cultures on LJ media ("Diagnostic standards and classification of tuberculosis in adults and children," 2000) and this compares well with other studies further emphasizing the effectiveness of OMS as a

decontaminant (Maharjan, Kelly-Cirino, *et al.*, 2016; Maharjan, Shrestha, *et al.*, 2016). Thus, one can clearly indicate that OMS has a greater efficiency to effectively remove unwanted microorganisms.

The overall recovery rate of mycobacteria on LJ after OMS treatment (78.7 %) was comparable to the mycobacteria recovery rate achieved after NALC-NaOH treatment (74.5%). Even though both decontaminants exhibit selective toxicity against non-mycobacteria, OMS seems to maximize mycobacteria recovery slightly better than NALC-NaOH. Furthermore, the relatively higher contamination rate of NALC-NaOH does not afford mycobacteria the opportunity to grow which may explain the slightly lower mycobacteria positivity observed.

Notwithstanding, delayed positivity was observed for OMS treated samples. It is possible that OMS treatment might be causing mycobacteria to go into a state of low metabolism as a response to the harsh environment making them require more time to recover on LJ media hence the observed delay. Even though mycobacteria are known to withstand harsh chemical treatments, prolonged exposure to these chemicals could also compromise their viability (M. Chatterjee *et al.*, 2013). These treatments also constitute stress environments to the mycobacteria, which may enter into a state of low metabolism characterized by stationary phase growth just like they do to evade host defenses (DeMaio *et al.*, 1996; Oлару *et al.*, 2014). The delayed positivity of OMS treated samples may also be attributed to the harshness of the chemical which may be reducing the number of viable mycobacteria in the sample. This could also explain why the number of negative samples were higher after OMS treatment than after NALC-NaOH treatment.

The contamination rate decreased significantly with increased duration of OMS treatment as observed in the time course analysis where samples were treated for 2, 4 and 8 days. After 2 days OMS treatment, the contamination rate was 10.7% while after 4 days it was 2.6%. The difference could be attributed to prolonged exposure of contaminants to chemical agent, increasing the contact time of contaminants with the chemical agent resulting in their efficient inhibition (McDonnell & Russell, 1999). There was no contamination after 8 days which may also be effects of the long exposure of microbes to the decontamination agent. As contaminants are inhibited, mycobacteria of interest can grow in culture hence the increased positivity especially at the end of day 8.

Extending OMS treatment beyond the recommended 8 days to 14 days, 80% mycobacteria positivity was recorded although the positivity was observed after the eighth week. Compared to the NALC-NaOH treated samples, the OMS samples yielded significantly lower contamination rate at each point of the time series including the 14-day time point with comparable mycobacteria positivity rate. From these findings, OMS may effectively be used as a transport medium for an optimum duration of 8 days but in the presence of undue delays, it is clear that viable mycobacteria are retrievable for up to 14 days at a time cost. However, since these experiments were undertaken in a controlled laboratory environment, field testing is recommended.

The ability of OMS to effectively decontaminate samples while maintaining mycobacteria viability for such long duration may be attributed to the concentrations of the effective chemicals that make up the reagent. The concentrations of chemicals used require modulation in order to achieve a balance between removal of unwanted microbes and preservation of mycobacteria viability. This explains why even in the use of other

decontaminants such as sulfuric acid, oxalic acid and sodium hydroxide, strict adherence to concentration of the chemical during reagent preparation as well as exposure time is advised.

### **Compatibility with DNA-based Tests and Microscopy**

In TB diagnosis, it is important that the methods used to process samples do not interfere with existing diagnostics as well as recent technologies to avoid costly retooling of existing laboratories when these new methods are applied. Sputum smear microscopy is one of the commonest and most widely used TB diagnostic test and can be carried out in almost all point of care laboratories.

This thesis therefore assessed the effects of OMS treatment on AFB detection and one major finding was that OMS had significantly lower AFB positivity compared to NALC-NaOH. This finding may be because NALC-NaOH is a well-known good digestant which efficiently breaks down mucin polymers to release tubercle bacilli to readily pick up the stains used. The inherent high lipid content of mycobacteria make them naturally buoyant, requiring high centrifugal force to sediment them during concentration steps. Certain chemical agents may further alter this buoyancy making them lighter and harder to sediment by centrifugation (Thornton *et al.*, 1998). In this regard, tubercle bacilli may remain in suspension leading to them being discarded with the supernatants during the concentration step. Additionally, the cell wall alterations may affect the bacilli's permeability to staining dyes which directly influences uptake of the dye and hence detection by microscopy (Fukunaga *et al.*, 2002). Cell wall alterations include loss of mycolic acids which can dissolve in organic solvents and since ZN staining relies on uptake

of the carbol fuchsin dye by mycolic acids in the mycobacteria cell wall, such alterations can influence staining reaction. Lastly, differences were observed in bacilli load categorization where NALC-NaOH treated samples had slightly higher load than the OMS counterparts. This further stresses the relatively poor digestibility of OMS. However, it could also be a reflection of the imprecision associated with manually splitting mucoid sputum samples prior to liquefaction.

It is important for OMS treated samples to be testable using ZN staining and microscopy because, it will eliminate the need to burden the patient with collection of two different samples which may be very uncomfortable and may not even contain bacilli in both collected sputa. Simplicity is afforded if with one sample, several tests can be carried out without hindrances.

In the current drug-resistance TB control era, molecular diagnosis has become important and indispensable for detecting and understanding pathogens. This is mainly because they are rapid and relatively more informative than conventional methods. The ability of OMS treated samples to be directly used for DNA-based tests using the Xpert MTB/RIF analysis was assessed with NALC-NaOH as control. From the results, all except one of the samples tested were MTBC positive after both treatments. The few differences observed were with respect to bacilli load categorization which may arise because of the imprecision of manually splitting mucoid sputum samples into two prior to their liquefaction. From these findings, it is clear that OMS treatment does not interfere with the Xpert MTB/RIF assay.

In TB diagnosis, the common molecular tests employed include the Genotype MTBDRplus and the Xpert MTB/RIF assay. These are both PCR based methods which detects drug resistance in addition to causative pathogen. Without adequate sample preparation, PCR

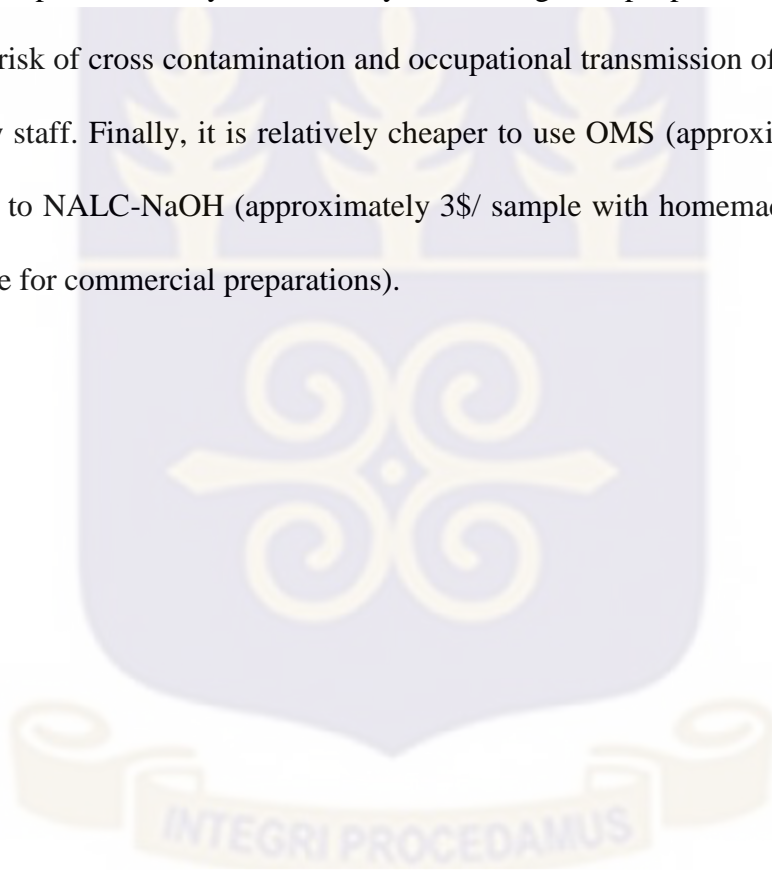
based tests can be prone to inhibition which influences the detection of pathogens by these methods (Wilson, 1997). This is especially important in the case of the Xpert MTB/RIF assay which relies heavily on probes for the detection of both *Mycobacterium tuberculosis complex* and rifampicin resistance (Trombley Hall *et al.*, 2013). Some compounds commonly associated with PCR inhibition include phenolic compounds, organic compounds and heavy metals (Wilson, 1997). Since the composition of OMS is not public, This assessment was performed to ascertain its compatibility with the Xpert MTB/RIF test. Moreover, the Ghana National Tuberculosis Control Programme is currently rolling out the Xpert MTB/RIF assay to regional hospitals to improve TB diagnosis. As such peripheral health facilities at the district and municipality levels still need to transport samples to these regional health centers for testing. Thus, it is necessary to test the usability of OMS treated samples for this test especially. Omnigene Sputum treated samples performed comparably to NALC-NaOH treated samples in the Xpert MTB/RIF assay.

All the mycobacteria isolated from this study were members of the *Mycobacterium tuberculosis complex* except one from the OMS arm which was identified to be *M. abscessus*. This is clinically significant since *M. abscessus* is a non-tuberculous mycobacteria (NTM) causing a TB-like disease which does not respond to treatment with TB drugs and hence is difficult to treat.

### **Economic and public health implications**

The findings of this study show that OMS is advantageous over NALC-NaOH for sputum transport and decontamination based on practical factors. The reduced culture contamination rate has several economic implications particularly for resource constrained

laboratories and poor high burdened countries. Aside from eliminating cost of retesting samples that are lost due to contamination, unnecessary delays and discomfort of patients having to produce another specimen for retesting is eliminated. Additionally, OMS comes as a ready to use reagent which can be stored in ambient temperature up to 40°C. There is therefore no need for daily preparation of reagents as required in the use of NALC-NaOH which requires daily preparation because of the short half-life of NALC in solution. This affords a simpler laboratory work flow by eliminating multiple processing steps which may surge the risk of cross contamination and occupational transmission of infectious agent to laboratory staff. Finally, it is relatively cheaper to use OMS (approximately 2\$/ sample) compared to NALC-NaOH (approximately 3\$/ sample with homemade preparations and 9\$/ sample for commercial preparations).



## CHAPTER SIX

### 6.0 CONCLUSION, LIMITATIONS AND RECOMMENDATIONS

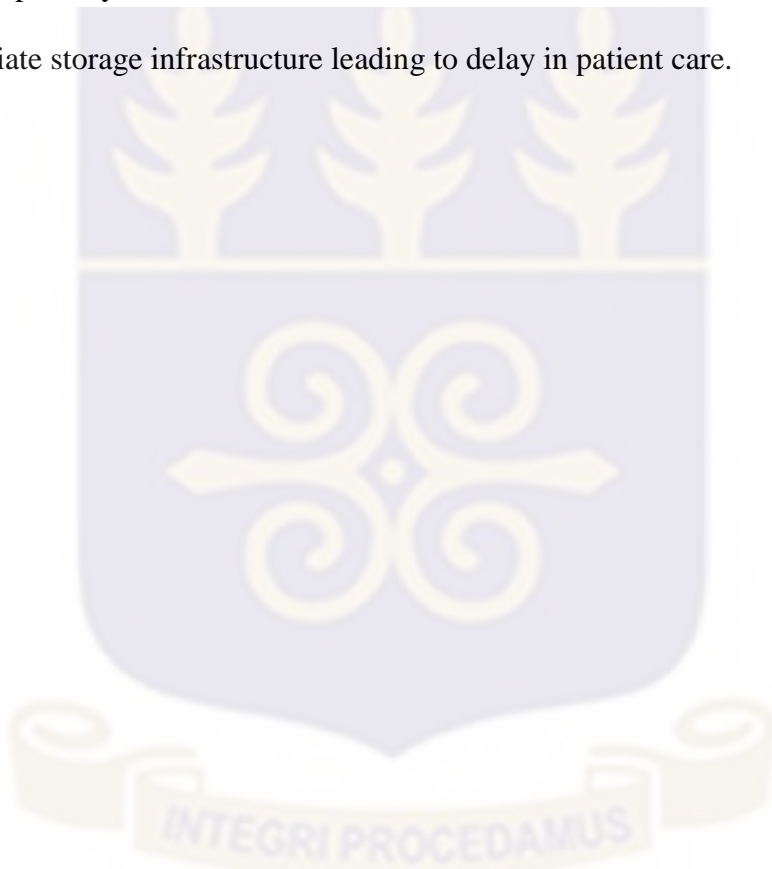
#### 6.1 Conclusion

This study, which to the best of my knowledge is the first study assessing Omnigene Sputum reagent for use in Ghana, confirmed the unsterile nature of sputum samples collected for TB diagnosis. The most predominant microbes isolated from the sputa used for this study prior to decontamination are *Streptococcus oralis*, *Staphylococcus aureus*, *Pseudomonas species* and *Candida species*, all commensals of the respiratory tract with pathogenic tendencies in the immunocompromised.

Omnigene sputum reagent was effective at inhibiting the growth of microbial contaminants in sputa and this is supported by other studies that evaluated OMS in other countries (Kelly-Cirino *et al.*, 2017; Maharjan, Shrestha, *et al.*, 2016). Additionally, mycobacteria viability was preserved in OMS beyond the recommended 8 days, even up to 14 days though there was delayed positivity after the eighth day. As the OMS treatment duration increased, the number of contaminated samples reduced with mycobacteria positivity also increasing. Omnigene Sputum was found to decrease microscopic detection of AFB detection after ZN staining. Samples treated with OMS were also directly compatible with Xpert MTB/RIF analysis. The findings of this thesis support the use of OMS as a transport and decontamination reagent in Ghana.

## 6.2 Limitations and Recommendations

The limitation of this study is that, the evaluation was carried out in a controlled laboratory environment. Thus, it is recommended that a larger field-based study be carried out to further ascertain the efficacy of OMS as a transport and decontamination reagent under real life conditions. Also, the manufacturer needs to improve the digestibility of OMS to improve AFB detection. Within the findings of the limited study, OMS is recommended for use especially in resource limited countries to reduce loss of samples due to inappropriate storage infrastructure leading to delay in patient care.



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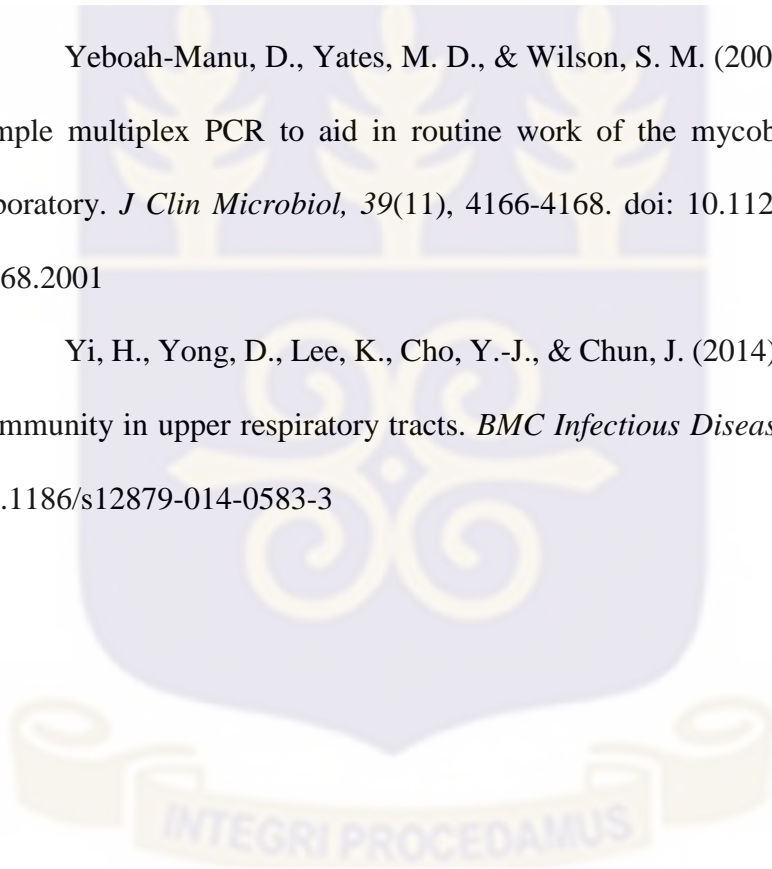
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## APPENDICES

### Appendix 1

#### Ethical Clearance

**NOGUCHI MEMORIAL INSTITUTE FOR MEDICAL RESEARCH**  
*Established 1979* *A Constituent of the College of Health Sciences*  
University of Ghana

**INSTITUTIONAL REVIEW BOARD**

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

7<sup>th</sup> March, 2012

**ETHICAL CLEARANCE**

**FEDERALWIDE ASSURANCE FWA 00001824** **IRB 00001276**

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

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

**TITLE OF PROTOCOL** : **Understanding the genetic diversity between *Mycobacterium africanum* and *Mycobacterium tuberculosis***

**PRINCIPAL INVESTIGATOR** : **Dr. Dorothy Yeboah-Manu, PhD**

**CO-INVESTIGATORS** : **Dr. Audrey Forson & Dr. Frank Bonsu**

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

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

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

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

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

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