COMPARISON OF DECONTAMINATION METHODS FOR ISOLATION OF *MYCOBACTERIUM* SPECIES FROM THE ENVIRONMENT

BY

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DECLARATION

I, Zuliehatu Nakobu do hereby declare that except for references to other people's work which I have acknowledged, this thesis is the product of my own research, and it has not been presented in its entirety or part elsewhere for another degree.

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DEDICATION

This thesis is dedicated to my late mother Mariama Iddirisu, my beloved husband Labram Massawudu Musah, my son Faisal Massawudu and my siblings: Kobshie Nakobu and Hawa Nakobu. Thank you for your support and love.

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ABSTRACT

The environment harbours many bacterial species, some of which include non-tuberculous mycobacteria which have recently become important in public health. Isolation of mycobacteria from the environment has not been easy because of the presence of other fast growing bacteria and fungi. For isolation of mycobacteria from the environment, decontamination methods that minimize contamination but maximize recovery of mycobacteria are needed. This study sought to compare three decontamination methods for isolation of mycobacteria from the environment. Sixty-five samples were collected from both Buruli ulcer disease endemic and non-endemic villages. Polymerase chain reaction (PCR) was done to detect the biomarker IS*2404* as a first screening procedure. Direct microscopy was performed on the IS*2404* positive samples and three decontamination methods were evaluated, namely; 4% NaOH/ 5% simplified OA method, 0.3% malachite green/ 0.75 g/50 ml cycloheximide/ 4% NaOH and 3% SDS/ 4% NaOH decontamination methods. Three different media with antibiotic supplementation and one without antibiotic supplementation were used for isolation of mycobacteria from the environment. Thirty-seven out of the 65 samples were positive for IS*2404* marker and 5/37(13.5%) were acid-fast positive. Decontamination by NaOH/OA method gave the highest number of total tubes that confirmed mycobacterial growth (42/91, 46.1 %) and the least contamination. The medium containing PANTA-mycobactin-J (PM) was best among the four media used. Isolates obtained from this study were identified by Hain GenoType CM® line probe assay. Forty-four *Mycobacterium* species were identified and *Mycobacterium chelonae* was the most frequently isolated species. Decontamination with 4% sodium hydroxide/5% oxalic acid and L-J medium containing PANTA and mycobactin J (PM) were the most efficient in supporting the growth of mycobacteria and may be used as standard for isolating mycobacteria from the environment.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AFB - Acid- fast bacilli

AIDS - Acquired immune deficiency syndrome or acquired immunodeficiency syndrome

ATM - Atypical mycobacteria

ATS - American Thoracic Society

BU - Buruli ulcer

CDC - Centers for Disease Control

CPC - Cetylpyridinium chloride

DNA - Deoxyribonucleic acid

DNTPs - Deoxynucleoside triphosphates

EB - Ethambutol

HIV - Human Immunodeficiency Virus

H_2SO_4 - Sulfuric acid

INH - Isoniazid

IUALTD- International Union against Tuberculosis and Lung Diseases

LAM - Lipoarabinomannan

L-J - Löwenstein-Jensen

MAC - *Mycobacterium avium* complex

MAI - *Mycobacterium avium -intracellulare*

MTC - *Mycobacterium tuberculosis* complex

MOTT - *Mycobacteria* other than tuberculolosis

MU - *Mycobacterium ulcerans*

NALC - N-acetyl cysteine

NaOH - Sodium hydroxide

NMIMR- Noguchi Memorial Institute for Medical Research

NTM - Non-tuberculous mycobacteria

OA - Oxalic acid

PANTA - Polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin

PBS - phosphate buffered saline

PCR - Polymerase chain reaction

PIMs - Phosphatidylinositol mannosides

PM	-	PANTA-Mycobactin J
SDS	-	Sodium dodecyl sulphate
SLS	-	Sodium lauryl sulphate
TB	-	Tuberculolosis
WHO	-	World health organization
ZN	-	Ziehl- Neelsen

CHAPTER ONE

INTRODUCTION

1.1 Background to Study

The genus *Mycobacterium* belongs to the phylum of Actinobacteria and the family Mycobacteriaceae. The genus consists of more than 140 species (American Thoracic Society, 2007; Slany *et al*., 2010). This genus is noted for the rigid and thick cell wall that allows them to resist many chemical solutions including acids and alcohol. Mycobacteria adapt easily on simple substrates and are ubiquitous (Falkinham, 2002). They can be found in many natural and artificial environments; these include soil, rivers, treated water in distribution systems, biofilms, aerosols, equipment; bronchoscopes, catheters and food (Rahbar *et al*., 2010; Falkinham, 2002). Their distribution is also influenced by biotic factors such as soil type and local vegetation (Chilima *et al*., 2006; Rahbar *et al*., 2010).

The genus includes pathogens known to cause serious diseases such as tuberculosis (*Mycobacterium tuberculosis*) and leprosy (*Mycobacterium leprae*). The other members of the genus other than *Mycobacterium tuberculosis* (Mtb) and *Mycobacterium leprae* are referred to as non tuberculous mycobacteria (NTM), *Mycobacterium* species other than *Mycobacterium tuberculosis* (MOTT) or atypical *Mycobacterium* (ATM). Infections due to NTMs are increasingly becoming more of a public health problem. These NTMs are not so pathogenic, and because they are found ubiquitously in the environment than causing disease, they are also referred to as environmental pathogens. Non-tuberculous mycobacteria that were previously known to be non pathogenic have now been shown to cause infections. Some however can cause

serious infections in both immunocompetent (*Mycobacterium ulcerans*) and immunocompromised (*Mycobacterium avium*) persons.

Disease such as Buruli ulcer which is an ulcerative skin disease mainly affects the skin and the subcutaneous tissue. Lymphadenitis which mostly affects lymph nodes in children is caused by *M. avium – intracellulare* complex (known as the MAI). From the stand point of human health, the most significant of the environmental Mycobacteria include MAI and *M. ulcerans*.

Non-tuberculous mycobacteria have become a significant cause of infection with the emergence of HIV/AIDS. *Mycobacterium avium – intracellulare* is the predominant cause of disseminated mycobacteremia in about 25% to 50% of patients with HIV/AIDS in the United States and in Europe (Falkinham, 1996). In addition, *Mycobacterium* species that are members of the MAI also cause majority of NTM infections in developing countries (von Reyn *et al*., 1993).

The environment may be the likely reservoir for these infections as there is no evidence of human to human transmission and the environment where NTMs occupy are shared by humans (Wolinsky *et al*., 1979; Griffith *et al*., 2007). Studies have implicated that *Mycobacterium* species such as *Mycobacterium ulcerans* could be transmitted from aquatic environments to humans. Insects, aquatic plants, amoebae, and aquatic vertebrates and invertebrates have been suggested by various studies to be reservoirs of NTMs in natural environments (Heckert *et al*., 2001; Marion *et al*., 2010). However, most of the studies on *M. ulcerans* ecology have been conducted through PCR based methods for detection. Different modes of transmission have been hypothesized for diseases caused by NTMs by different studies; none of them have been proven up to now (Griffith *et al*., 2007). For instance, several different mechanisms have been proposed

for the transmission of *M. ulcerans*. These include contact with contaminated environment, aerosol resulting from vapourisation of contaminated water and insect bite (Marion *et al*., 2010).

Therefore, there is the need to link data from isolates obtained from culture, genetic characterization and epidemiological studies to allow more inference on the actual sources and mode of transmission of these mycobacteria.

Bacteria culture is considered the gold standard for the detection of *Mycobacterium* species as it proves viability of the bacteria in the sample. However, obtaining pure culture of mycobacterial species is a very difficult procedure due to several reasons. The environment contains other microorganisms other than mycobacteria such as fast growing bacteria and fungi. While other bacteria grow very fast such that within 18 hours macroscopic growth can be achieved, the fast growing mycobacteria grows within seven days, it can take the slow growers even more than six months to grow. Thus the cultures set for isolating *Mycobacterium* species from the environment is usually contaminated by these fast growing bacterium and fungi. Moreover, *Mycobacterium* which is grouped into fast and slow growers and the slow growing *Mycobacterium* species are the most pathogenic.

The main factor that determines the growth rate is the number of rRNA operons; the slow growing mycobacteria possess one operon and rapid growers possess two while the other fast growing bacteria such as Escherichia coli have seven operons (Falkinham, 2008; Condon *et al*., 1995). To arrest contamination of cultures, basic and or acidic reagents are introduced to remove all non-mycobacterial species from the samples and to improve the recovery of mycobacteria from both clinical and environmental samples, in a decontamination step during samples processing for culture.

Several decontamination solutions such as, cetylpyridinium chloride (CPC), sodium hydroxide (NaOH) (also known as Petroff's method), N-acetyl cysteine (NALC), sulfuric acid (H2SO4) oxalic acid (OA), centrimide, sodium dodecyl sulphate (SDS) and sodium lauryl sulphate (SLS) have been used for decontamination, for removing these fast growing bacteria from samples for mycobacteria culture before inoculation. Parashar *et al*., 2004 reported *Mycobacterium* species are not equally resistant to the different decontamination methods. Other studies have also shown that decontamination methods are known to be detrimental to mycobacteria, depending on concentration and length of treatment (Brooks *et al*., 1984; Jaramillo and McCarthy 1986). In a study, two decontamination methods and five media were compared for the isolation of mycobacteria from brook waters of different physical, chemical and bacteriological characteristics. Sodium hydroxide in combination with oxalic acid and sulfuric acid-cycloheximide methods were used. The sodium hydroxide in combination with oxalic acid method was found to be better than the sulfuric acid - cycloheximide method (Livanainen *et al*. 1997). In another study where pure cultures were tested, mycobacteria tolerated sulphuric acid better than sodium hydroxide (Jaramillo and McCarthy 1986). Palomino and Portaels in 1998 used the BACTEC system to evaluate the effects of several decontamination methods (Petroff, reversed Petroff, oxalic acid, and mild hydrochloric acid) treatments and antibiotics on the viability of *Mycobacterium ulcerans*. From their results they concluded that, the decontamination methods used for isolation of *M. ulcerans* affected the viability of the bacteria and mild hydrochloric acid gave the best results.

To further reduce contamination in cultures, antibiotics (example, PANTA is a cocktail of antibiotics containing polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin), anti-fungal example cycloheximide and other chemicals (such as malachite green in

L-J) are added to culture media for isolating mycobacteria. While mycobacteria are resistant to the antimicrobials and decontaminating solutions, there seems to be variability among them in the level of resistance.

It is therefore important to evaluate and standardize decontamination methods and selective media that will maximize the recovery of mycobacteria species of interest and at the same time reduce contamination usually found in mycobacteria cultures. This will enable further studies on the isolates from the environmental and the comparison of environmental and clinical isolates which will lead to knowing their mode of transmission and ecology for disease control

1.2 Problem Statement

Although *Mycobacterium tuberculosis* complex (MTC) are responsible for most mycobacterial infections worldwide, other infections due to NTMs are also increasingly becoming more of public health importance (Kankya *et al*., 2011). This is due to the increase in the number of immunocompromised individuals such as people living with HIV/AIDS, recipients of organ transplants (Zumla and Grange, 2002; Mok *et al*., 2007). Some of the NTMs such as *M. xenopi*, has been increasingly been identified as a cause of pulmonary infections among those with impaired immunity (Mangione *et al*., 2001; van Ingen *et al*., 2008; American Thoracic Society ATS, 1997; Zumla and Grange,2002; Ostroff *et al*., 1993; O'Brien *et al*., 1987). In addition human environmental degradation activities have been shown to be a risk for emergence of some disease caused by NTMs, a typical example is Buruli ulcer (BU). Moreover NTMs infections are emerge unrecognized settings with new clinical manifestations (Griffith *et al*., 2007).

While NTMs have been recognized in recent times as important pathogen, large gaps still exist in our knowledge of the ecology and mode of transmission or how humans acquire infections. Thus more studies are needed in different geographic settings to understand the ecology and ultimately how humans acquire infection. Human disease is suspected to be acquired from the environmental exposures, although the specific source of infection cannot be identified (von Reyn *et al*., 2002). Thus cultured mycobacteria are needed for molecular epidemiology analysis, since environmental specimens do not always contain sufficient bacilli to perform direct finger printing analysis. More importantly cultures prove the viability of the bacteria in the source.

However, isolating mycobacteria from the environment is particularly very difficult for several reasons (1) environmental samples are contaminated by other microorganisms causing over growth in the culture (2) the most pathogenic NTMs have very slow growth rate. Thus removing the unwanted microbes from environmental samples before isolation culture is paramount.

1.3 Justification

Mycobacteria species that were previously regarded largely as saprophyte, non- pathogen and environmental are increasingly becoming important human pathogens, not only in immune-compromised individuals but can affect immune-competent individuals. Globally, the picture of NTMs has changed drastically, affects also human and animal population in Africa. A study conducted in Uganda identified NTM from humans suffering from cervical lymphadenitis and cattle with lesion consistent with bovine TB (Kankya *et al*., 2011). Also a study by Asante-Poku *et al* in Ghana (personal communication) identified NTMs from lesions of carcass from cattle with macroscopic appearance of bovine TB. Thus in addition to the main mycobacterial diseases (TB and leprosy) efforts must be put in controlling disease caused by the NTMs. This

identification of risk factors for disease spread is crucial which requires a good understanding of the ecology of the causative agent.

Moreover an important aspect in the control of infectious disease is identification of the risk factors that increase the chance that host will come into contact with the pathogen. Thus the ability to isolate viable bacteria, indicating reservoir makes it possible to identify preventable risk and implement public health measures. Currently there is very limited data on the environmental sources of NTMs in Ghana with few PCR-based studies looking at *M. ulcerans*.

Thus the isolates that will be obtained from the study will be linked in future molecular epidemiological study involving both clinical and environmental study to allow inference about the real sources of NTM infection in the district studied.

1.4 Objective of study

The purpose of the study is to compare different decontamination methods for the isolation of *Mycobacterium* species from the environment.

Specific objectives

The specific objectives of this study are;

I. To directly detect acid-fast bacilli from environmental samples.

II. To detect IS*2404* positive mycobacteria in environmental samples.

III. To evaluate different in house selective media for isolating mycobacteria.

IV. To evaluate different methods for decontaminating environmental samples for isolating mycobacteria.

V. To identify to the species level mycobacteria isolates from the environment.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Mycobacterium*

The genus *Mycobacterium* is a member of the phylum Actinobacteria, in the order Actinomycetales, with its own family Mycobacteriaceae (Ventura *et al*., 2007). The genus comprise of pathogens known to cause important human diseases, notably; tuberculosis (*M. tuberculosis*), leprosy (*M. leprae*) and Buruli ulcer (*M. ulcerans*). The DNA of species of the genus *Mycobacterium* usually has a high guanine and cytosine (G+C) content in the range of 61 to 71 % (except *Mycobacterium* leprae with G+C content of 54 to 57 %).

Mycobacteria are generally aerobic, non-motile rods about 1-10 µm long, non-spore forming and they are able to enter into dormant states. Mycobacteria are usually considered gram positive, not necessarily as a result of gram reaction but the absence of an outer lipid membrane. The mycobacterial cell wall has unique characteristics which make them hydrophobic and retain dyes after acid or alcohol decolourization, this is known as acid fastness. The nature of the cell wall also makes them resistant to many hydrophilic compounds, disinfectants and common antibiotics. Optimum growth temperatures vary widely according to the species and range from 25 °C to over 50 °C; thus some species can survive in hot water heaters and hot water pipes at temperatures of 50 to 55°C (Schulze-Röbbecke and Bucholtz 1992; Santos *et al*., 2007; Falkinham, 2009).

2.1.1 *Mycobacterium* Cell Wall

Mycobacterium cell wall is characteristically thicker than the cell wall of many other bacteria. It is made up of two segments denoted upper (outermost) and lower (cell wall core). Directly attached to the membrane is the peptidoglycan, covalently attached to an arabinogalactan layer which is then linked to a thick layer of mycolic acids. These three layers make the cell wall core, known as Mycolyl arabinogalactan–peptidoglycan. The upper segment composed of free lipids interspersed such that the cell wall process lipoarabinomannan and other lipids. The components give the cell wall a hydrophobic nature wich contributes substantially to the hardiness of the genus, their resistance to common disinfectants and high intrinsic resistance of mycobacteria to many drugs (Hoffmann *et al*., 2008).

The plasma membrane is the first layer from the inner side which also may contain substances such as carotenoids; which produce yellow to orange pigmentation in some non-tuberculous mycobacteria example, *M. gordonae* and *M. kansasii* (Brennan and Nikaido 1995 and Rastogi, 1991).When the cell wall is disrupted, for instance extracted with various solvents, the free lipids, proteins, LAM, and PIMs are solubilized, and the mycolic acid–arabinogalactan peptidoglycan complex remains as the insoluble residue.

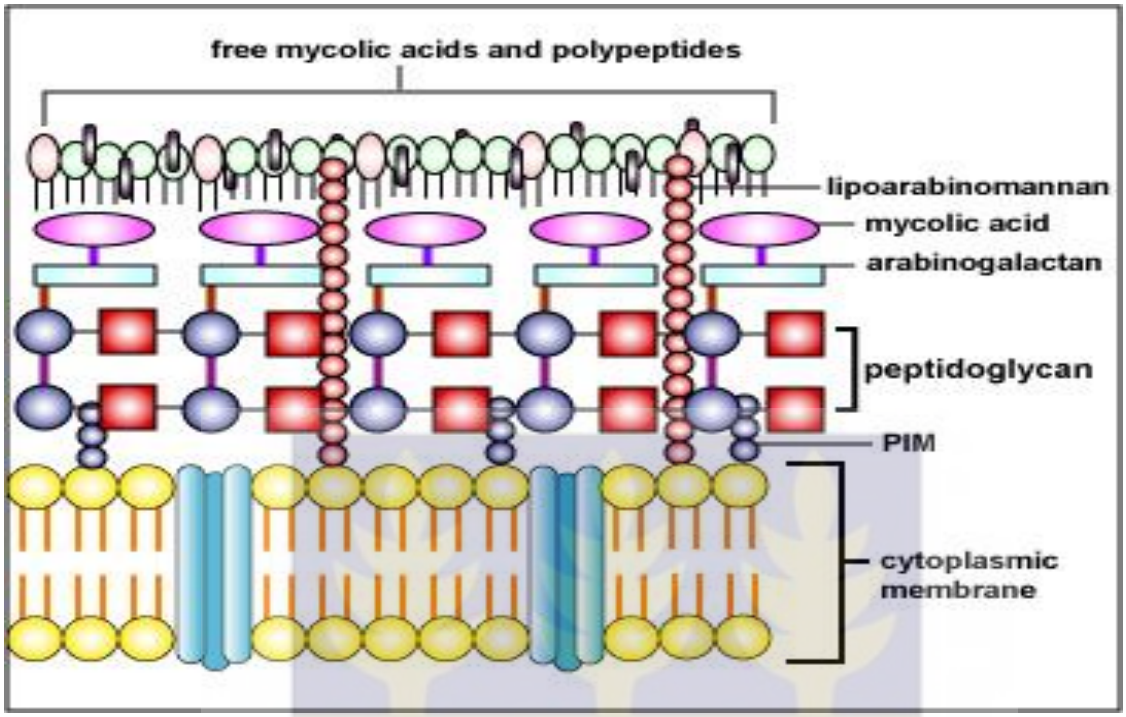


Figure 2. 1: The Mycobacterium cell wall

Source:http://faculty.ccbcmd.edu/courses/bio141/labmanua/lab12/diseases/mtuberculosis/images

2.1.2 Classification of *Mycobacterium*

Mycobacteria can be classified into several groups by different criteria such as growth rates, pigmentation and pathogenicity. Medically mycobacteria are classified as tuberculous and non tuberculous mycobacteria (NTM). The NTMs are also referred to as mycobacteria other than tuberculolosis (MOTT) and atypical mycobacteria (ATM). The tuberculous mycobacteria include members of the *M. tuberculosis* complex and *M. leprae* (van Ingen, 2013). The NTMs were grouped into 4 broad groups by the Runyon classification in 1959. This classification was based on growth rates, colony morphology and pigment production. Some mycobacteria produce pigments without light while others require photo activation for pigment production. Mycobacteria that produce yellow – orange pigment after being exposed to light are photochromogens. Those that produce a pigment without exposure to light are also known as scotochromogens. Those that do not produce pigment are termed as non chromogens.

The rate of growth of mycobacteria that is time to produce visible growth on standard solid media is also used to classify them; those that produce visible colonies within seven days are known as rapid growers and those that produce macroscopic growth after 7 days are termed slow growers.

According to the Runyon classification, Groups I, II and III are slow-growing NTM and group IV are rapid growers. Group I organisms are the photochromogens, group II organisms are the scotochromogens, the group III are the non-chromogenic (Butler and Guthertz, 2001; Jarzembowski and Young, 2008) (This is illustrated on Table 2.1 below). This classification system however could not define species within the *Mycobacterium* genus. Although the Runyon classification system is out-dated, it provided laboratories with guidelines to identify individual species of NTM, resulting in better characterization of distinct diseases.

With advancement in molecular biology and availability of the genomes of most Mycobacterial species, specific probes or biomarkers have been identified and are now being used in mycobacteria laboratories for species identification. Yet these classification and specific biochemical assays are still in use for some mycobacteria species that research has still not advanced in them.

Table 2. 1: This is illustrated on the Runyon classification of non tuberculous mycobacteria

Runyon Class	Description	Growth	Pigment production	Examples
I	Photochromogens	Slow growing	Yellow-orange pigment production when exposed to light	*M. Kansasii, M. marinum*
II	Scorochromogens	Slow growing	Yellow-orange pigment production with or without light	*M. scrofulaceum, M. gordonae, M. szulgai*
III	Non-chromogens	Slow growing	None	*M. avium-intracellulare, M. xenopi, M. terrae, M. ulcerans*
IV	Rapid growth	Rapid growth (produces mature colonies in agar ≤ 7 days)	Some do not produce pigment and others produce late pigmentation	*M. fortuitm, M. pereginum, M. abscessus, M. chelonae*

(Source: Jarzembowski and Young, 2008).

2.2 Non-Tuberculous Mycobacteria (NTM) and Disease

The NTMs cause various disease resembling tuberculosis, lymphadenitis, skin disease, or disseminated disease as described by O'Brien *et al*., (1987). Pulmonary disease is most frequent followed by lymphadenitis in children, skin disease and disseminated infections in severely immunocompromised patients (Wolinsky, 1979). They also cause different diseases including nosocomial infections associated with outbreaks related to insufficient sterilization and disinfection of medical device. About 50 species of NTMs have been reported to be human pathogens (Wagner and Young, 2004) and some examples are described below;

2.2.1 Mycobacterium chelonae

Mycobacterium chelonae is a rapidly growing and ubiquitous NTM, classified as Runyon group IV organism. *Mycobacterium chelonae* has been found in natural and artificial sources including soil, medical instruments, foot baths in clinics and beauty salons, dust, sewage and water especially in tap water and water tanks (Levine *et al*., 1991; Larson *et al*., 2008; Sniezak *et al*., 2003; Khan *et al*, 2005; Santos *et al*, 2005; Hay, 2009).

Infections caused by *M. chelonae* clinically manifested as skin, bone and soft tissue disease which cause several different types of clinical syndromes. These include: lung disease, local cutaneous inflammations, osteomyelitis, joint infections, and ocular disease such as; keratitis or corneal ulcers. *Mycobacterium chelonae* is also involved in several different types of community-acquired infections (Brown-Elliott *et al*., 2002). It has also been implicated in eye infection cases associated with ophthalmologic procedures (Liu *et al*., 2007) and cosmetic surgeries and tattoos (Saha *et al*., 2006, Rajini *et al*., 2007; Munayco *et al*., 2008; Kennedy *et al*., 2012).

Mycobacterium chelonae rarely cause chronic lung disease, this was demonstrated in a study by Griffith *et al*., 1993, which involved 154 patients with chronic lung disease due to rapidly growing mycobacteria (RGM), only 1 out of 146 isolates was identified to species level as *M. chelonae*. Diseases caused by *M. chelonae* are grouped into three basic types. The most common type is disseminated cutaneous disease which usually occurs in patients who are chronically immunocompromised such as AIDS patients (Azadian *et al*., 1981; Hassan *et al*., 2007). In 1992, a research carried out by Wallace *et al* reported that 53% of 100 clinical isolates of *M. chelonae* were from patients with disseminated cutaneous infections. Furthermore, these infections were seen in patients receiving long-term corticosteroids and/or chemotherapy, primarily because of underlying organ transplantation, rheumatoid arthritis, or other autoimmune disorders (Wallace *et al*., 1993).

The second type of infection is acquired localized infections. These infections range from localized cellulitis, or abscess, to osteomyelitis. They are Health care-associated diseases, sporadic localized wound infections following medical or surgical procedures. They have been observed only in injection with contaminated syringes or needles, the implantation of contaminated porcine heart valves and the use of liposuction (Metcalf *et al*., 1981; Wallace *et al*., 1999; Brown-Elliott *et al*., 2002).

The third, and least common, type of infection caused by *M. chelonae* is the most common type of health care-associated disease, and is that of catheter-related infections. In 1992, Wallace and others reported that 8 out of 100 clinical isolates of *M. chelonae* were associated with intravenous catheters, an additional 3 involved chronic peritoneal dialysis catheters, and 1 involved a haemodialysis shunt. They observed that both the use of corticosteroids and renal failure were risk factors for these catheter-related infections (Wallace *et al*., 1992).

Mycobacterium chelonae has been identified as the cause of approximately 10% of nosocomial outbreaks attributed to rapidly growing mycobacteria (Wallace *et al*., 1992). Figure 2.6 is an infection cause by *M. chelonae*.

Figure 2. 2: *Mycobacterium chelonae* lesion on the right lower limb
(Source: Ivan *et al*., 2008)

2.2.2 *Mycobacterium avium- intracellulare*

Mycobacterium avium and *Mycobacterium intracellulare* (MAI) are genetically closely related mycobacterial species referred together as *M. avium* complex (MAC) or *M. avium- intracellulare* complex (MAI) (Tortoli *et al*., 2004; Murcia *et al*., 2006; Guirado *et al*., 2012). *Mycobacterium avium - intracellulare* are slow growing bacilli that produce a yellow pigment in the absence of light (Inderlied *et al*., 1993 and Han *et al*., 2005). *Mycobacterium avium- intracellulare* are ubiquitous in nature and they are found in different environmental sources such as natural sources of water-salt and fresh water, pools, plants and bedding material, and dust and vegetation

(Ichiyama *et al*., 1988). *Mycobacterium avium- intracellulare* have also been known as opportunistic pathogens of humans and are the most frequently isolated NTM worldwide capable of causing disease in both humans and animals (Turenne *et al*., 2007; Thoen *et al*., 1981; Iseman *et al*., 1985). They cause pulmonary disease, mostly in patients with pre-existent pulmonary diseases, followed by lymphadenitis in immunocompetent children and disseminated disease in systemically immunocompromised patients (Griffith *et al*., 2007).

The first case of human disease due to *M. avium* was reported in 1943 in a middle-aged underground miner from the Mesabi Iron Range of Minnesota in what became a classic description of pulmonary disease due to this organism (Feldman *et al*., 1943). Pulmonary disease due to *M. avium* predominantly involves white males 45 to 65 years of age with pre-existing pulmonary disease (Engbaek *et al*., 1981; Etzkorn *et al*., 1986; Rosenzweig and Schlueter, 1981) but there has been tremendous variation in the sex, age, and race of these patients. Predisposing conditions such as chronic obstructive pulmonary disease, bronchiectasis, chronic aspiration or recurrent pneumonia, inactive or active tuberculosis, pneumoconiosis, and bronchogenic carcinoma are present in 54% to 77% of patients with pulmonary MAI disease (Engbaek *et al*., 1981). Differentiation of infection from the coexistent pulmonary disease may be difficult, and the clinical and radiographic presentation may be indistinguishable from tuberculosis (Ortbals and Marr, 1978). A positive tuberculin skin test may be helpful in differentiating the two processes; however, co-infection of *M. tuberculosis* and *M. avium* has been demonstrated (Tsukamura *et al*., 1981). *Mycobacterium avium* causes 95% of AIDS related MAI infections while *M. intracellulare* causes 40% of MAI infections in the immunocompetent patients (Koirala, 2010). Other reported MAI infections among patients with AIDS include mastitis, pyomyositis, cutaneous abscess and brain abscess. The symptoms vary and are nonspecific,

commonly including chronic productive cough, dyspnea, sweats, malaise, fatigue, and, less commonly, hemoptysis. Fever and weight loss are not common but may occur. Figure 2.3 shows M. avuim leision on a patient's foot.

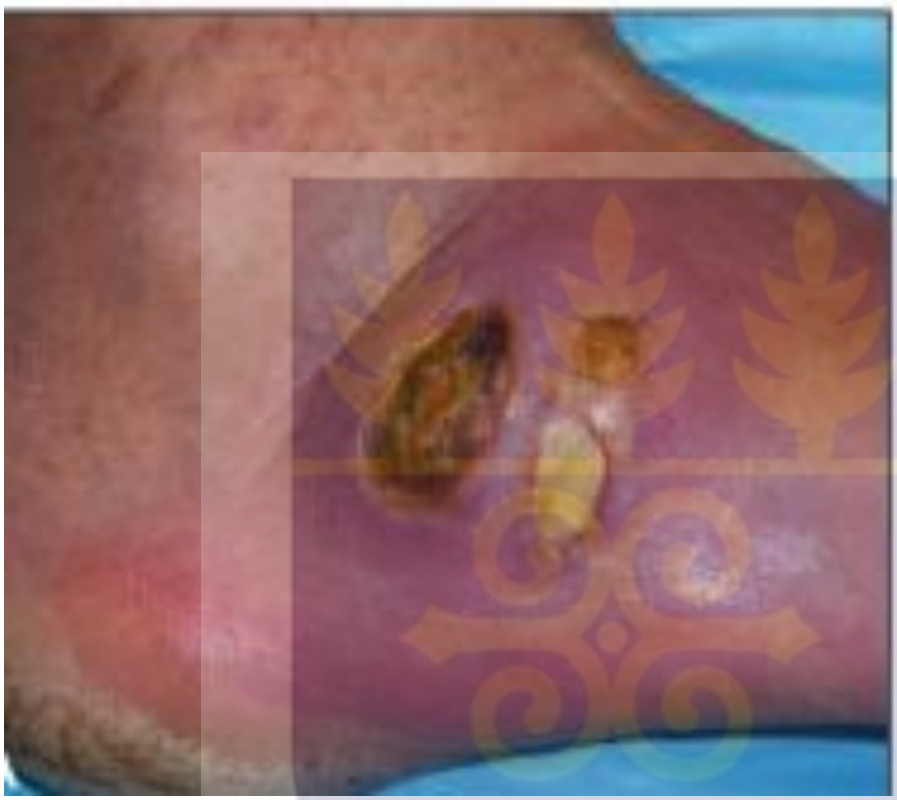


Figure 2. 3: ***Mycobacterium avuim*** **leision on a patient's foot**
Source: http://www.theaidsreader.com/binary_content_servlet The AIDS Reader. Vol. 18 No. 10

2.2.3 *Mycobacterium marinum*

Mycobacterium marinum is a slow-growing environmental *Mycobacterium* classified as group one by the Runyon classification. It is a photochromogenic and saprophytic mycobacteria that cause soft tissue infection in humans, usually acquired by inoculation with the bacterium through broken skin or by scratches or puncture wounds from fish, shrimp, and fins in an aquatic environment (Falkinham, 1996; Lewis *et al*., 2003), usually during swimming and in individuals employed in the fisheries industry (Zeligman, 1972). It most often affects elbows, knees, feet,

knuckles or fingers (Figure 2.4) (Adams *et al*., 1970; Barrow and Hewitt 1971; Ries *et al*., 1990) *Mycobacterium marinum* infect not only humans can infect fish and amphibians worldwide (Clark *et al*., 1963; Laussucq *et al*., 1988). Infections in humans result occasionally, in most cases as a granulomatous infection localized in the skin. Infection in immunosuppressed individuals can lead to chronic disease as the pathogen invades and colonise internal organs.

In fish, the usual course of the disease is the appearance of weak and emaciated fish that eventually die over a long period (Connolly *et al*., 1985). Although sudden mass deaths can sometimes occur after new fish have been purchased, especially in aquaria (Bercovier and Vincent, 2001). In amphibians, example, frogs and toads, *M. marinum* infection can be localised as an ulcerative dermatitis and cellulitis (Done *et al*., 1993; Bercovier and Vincent, 2001) or can be invasive and fatal with the presence of disseminated granulomata (Alavi and Affronti, 1994).

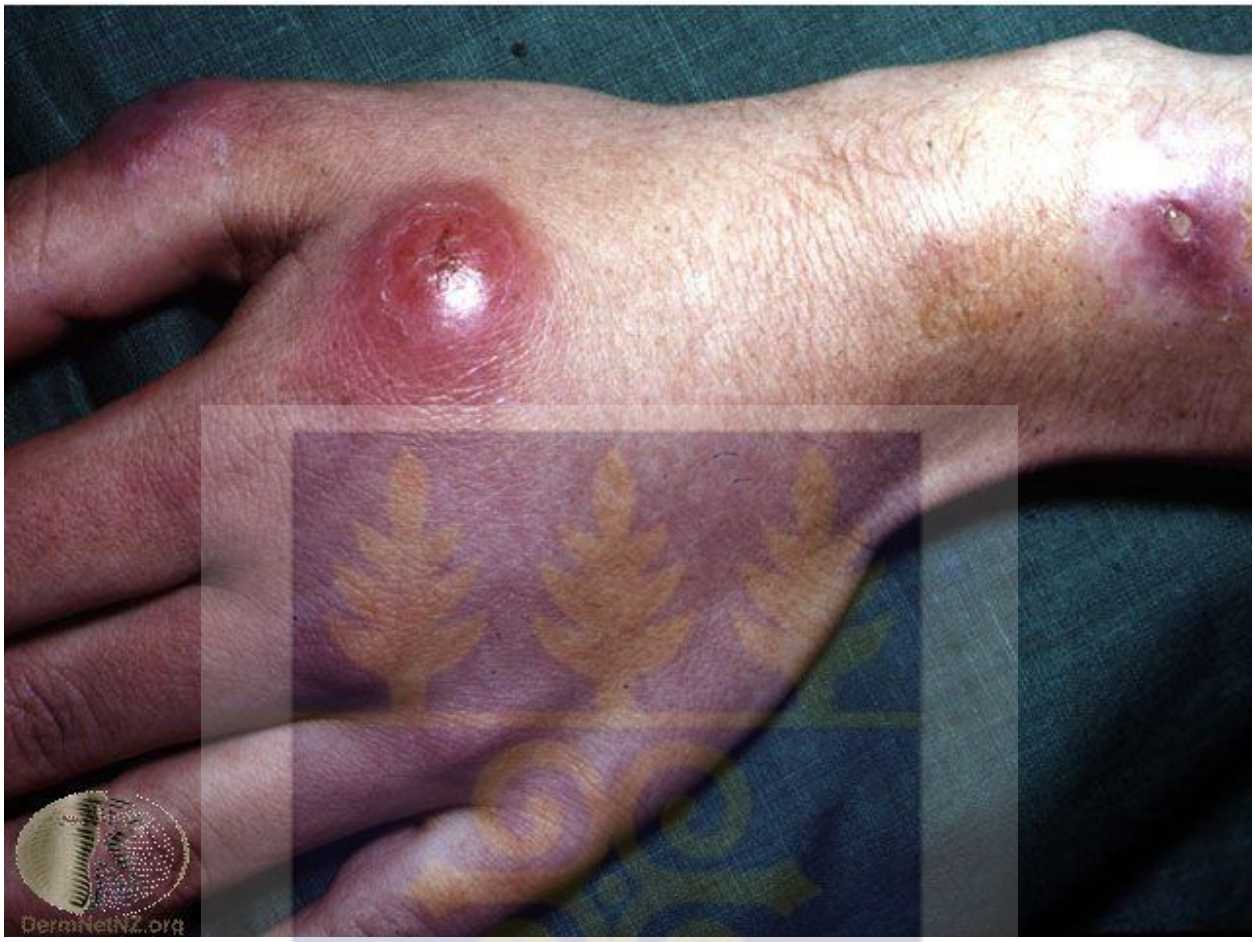


Figure 2. 4: *M. marinum* lesion on patient's left hand
Source: http://dermnetnz.org/common/image.php?path=/bacterial/img/atyp-mb2.jpg
http://www.dermnetnz.org/bacterial/atypical-mycobacteria.html

2.2.4. *Mycobacterium ulcerans*

Mycobacterium ulcerans (MU) is an extremely slow growing *Mycobacterium* which causes mainly infections of soft tissues of the skin. It is an extracellular pathogen and the only mycobacteria that harbours plasmid called pMUM001 with a molecular size of 174-kb. Over half of the plasmid consists of genes that encode the enzymes required for synthesis of mycolactone, the cytotoxic lipid produced by *M. ulcerans* (Stinear *et al*., 2004). It is the only mycobacteria species that uses a toxin as the main virulent factor, which play an important role in the pathogenesis of the disease. In vivo studies, using a guinea pig model of infection suggested that

mycolactone is responsible for both the extensive tissue damage and immunosuppression which accompanies Buruli ulcer. A characteristic of an active lesion is the absence of inflammatory cells which is caused by cytotoxic activity of mycolactone (George *et al*., 1998). The cytoxicity of mycolactone has been linked to the activation of apoptosis; this was observed when several cell types were exposed in vitro to purified mycolactone.

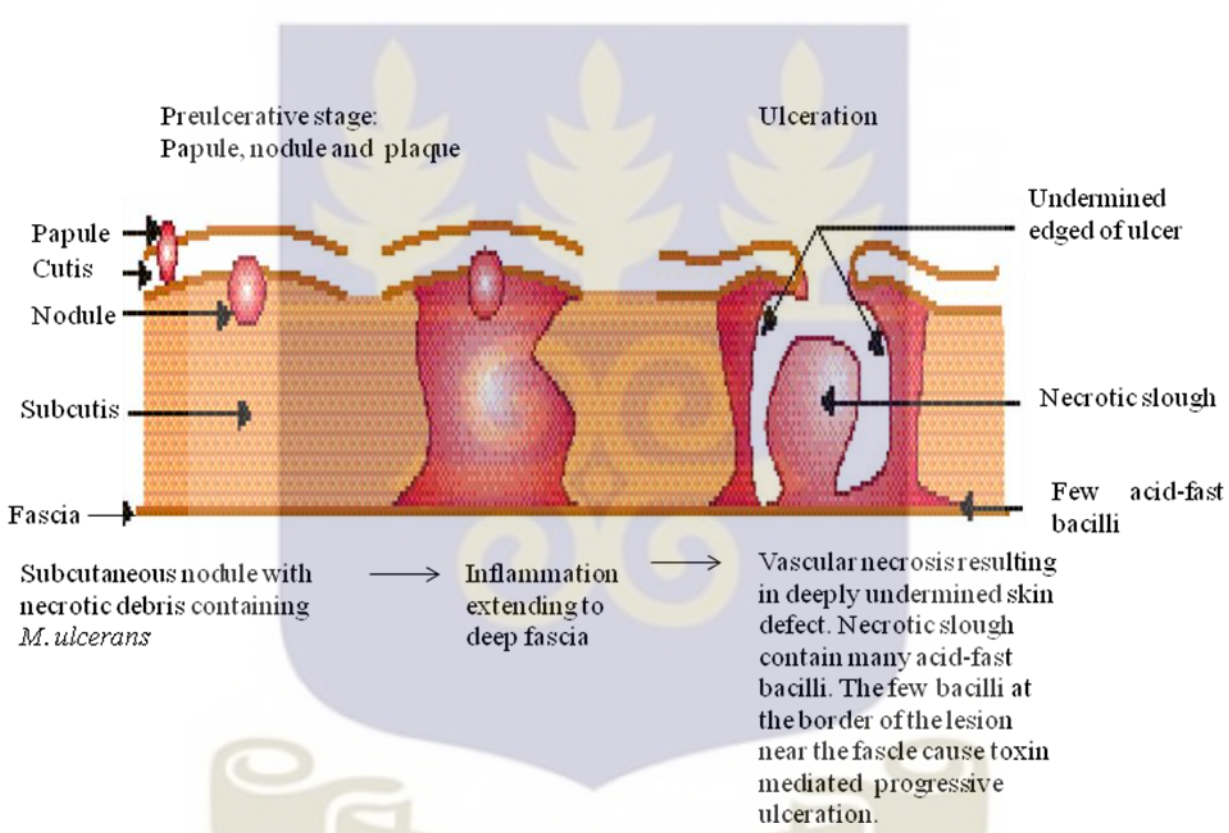


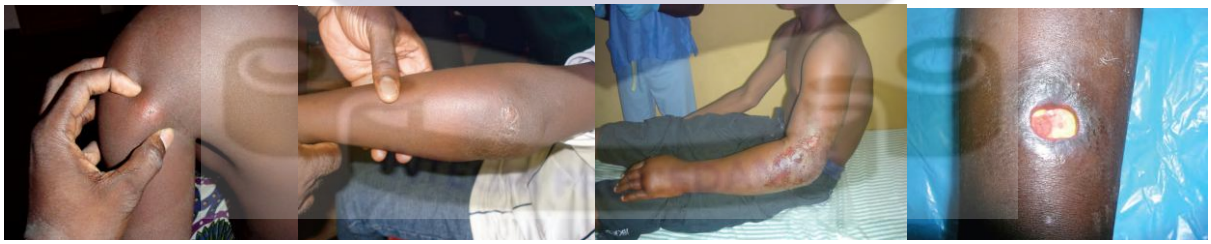
Figure 2. 5: An illustration of changes in the skin caused by Buruli ulcer infection (Van der Werf *et al*., 1999).

There are theories on the mode of entry of *M. ulcerans* into the human body; the actual mode of transmission into the skin is not known (Duker *et al*., 2006). After a successful entry of the organism it confines under the skin. The incubation period may be 2-3 months, during which proliferation takes place within the dermis and produce mycolactone which then destroys the

adipocytes and cause necrosis that extends beyond the localized region to the lower dermis and subcutaneous fat (Guarner, 2003).

In Australia, the presentation of Buruli ulcer disease differs from that in Africa. The first stage of *M. ulcerans* infection is a papule or pimple in the skin, while in African patients it usually starts as a firm nodule. Other early forms include plaque and the more serious form an oedema (figure 2.3). When these early forms are not treated, extensive necrosis leads to a well demarcated ulcers with extensive undermined edges that often extend 15 cm or more which sometimes affects the underlying bones (figure 2.7).

The extensive necrosis of the dermis result into sloughing off of the skin covering the pre-ulcerative region (George *et al*., 1999). Ulcers may remain small and heal without treatment, or may spread rapidly, undermining the skin over large areas, even an entire leg, thigh, or arm. Important structures such as the eye, breast, or genitalia are sometimes severely damaged. Most lesions heal spontaneously but without appropriate therapy frequently leave extensive scarring, and deformity.



Nodule Plaque Oedema Ulcer

Figure 2. 6: Different clinical presentations of Buruli ulcer
(WHO, 2012)

2.3.1 Epidemiology of some Non-Tuberculous Mycobacteria

Non-tuberculous mycobacteria are important human pathogens, yet little is known about their disease epidemiology and the true ecology of the pathogens. Non-tuberculous mycobacteria

cause infections of varying severity in both sporadic and epidemic form (Cassidy *et al*., 2009). There are numerous previously identified species of NTM the recent advancement in molecular methods more are being identified (Tortoli, 2003). Non-tuberculous mycobacteria diseases have been seen in most industrialized countries and the incidence rates vary from 1.0 to 1.8 cases per 100,000 persons (Horsburgh *et al*., 1996; Griffith *et al*., 2007). The incidence of NTMs in the United States was 14.1 per 100,000 in 2003 (Marras *et al*., 2007; von Reyn *et al*,. 1993; Horsburgh *et al*., 1996; Falkinham, 2002). There have been several reports suggesting that the incidence of NTM diseases has increased over the past years. However, there has not been conclusively established due to the lack of comprehensive surveillance data. Moreover they are non-communicable, that is they are not suspected to be transmitted by person to person contact and therefore has not received much attention. Therefore there are no substantially more or better information about NTM disease epidemiology than that which was published in the 1997 by American thoracic society (ATS) statement on NTM. Therefore the epidemiology of some of the species is not known. Although there are variation in species isolation (Griffith *et al*., 2007), the NTM most frequently isolated and associated with disease are the *Mycobacterium avium* and *Mycobacterium intracellulare* (MAI). Other important human pathogens include *Mycobacterium abscessus*, *Mycobacterium chelonae*, *Mycobacterium fortuitum*, *Mycobacterium marinum*, *Mycobacterium kansasii*, *Mycobacterium gordonae*, *Mycobacterium malmoense*, *Mycobacterium xenopi*, *Mycobacterium scrofulaceum* and *Mycobacterium ulcerans* (Griffith *et al*., 2007). Below is epidemiology of some species.

2.3.1.1 *Mycobacterium avium – intracellulare* complex

Mycobacterium avium and *M. intracellulare* (MAI) are the most common and significant NTM species that are associated with human diseases, causing disseminated infection in patients with

AIDS, nodular bronchiectasis and other pulmonary infections, lymphadenitis, and skin infection (Inderlied *et al*., 1993; Han *et al*., 2005).

The actual source of infection is unknown; however water and soil have been implicated as possible environmental source of MAI. Thus, they have been found in different environmental sources (Brooks *et al*., 1984; Falkinham *et al*., 1980). *Mycobacterium avium* and *M. intracellulare* may be normal inhabitants of natural waters and drinking water, water droplets and soil (Falkinham *et al*., 1980; 2001; Brooks *et al*., 1984; Wendt *et al*., 1980; von Reyn *et al*., 1994). In a study where DNA fingerprinting method was used, it was shown that isolates of M. avium obtained from AIDS patients were the same for those obtained from water consumed by the patients (von Reyn *et al*., 1994). This suggests a possible link between disease occurrence and drinking water source. *Mycobacterium avium - intracellulare* are responsible for pulmonary disease similar to tuberculosis in elderly patients (Wolinsky, 1979; Falkinham, 1996) and in immunocompetent individuals with predisposing lung diseases such as silicosis and black lung (Wolinsky, 1979), cervical lymphadenitis in children (Wolinsky, 1995) and disseminated infection in AIDS and immunosuppressed patients (Zakowski *et al*., 1982; Kiehn *et al*., 1985).

Thus, they have been found in different environmental sources (Brooks *et al*., 1984; Falkinham *et al*., 1980). *Mycobacterium avium - intracellulare* are normal inhabitants of natural waters and drinking water, water droplets, soil (Falkinham *et al*., 1980; 2001; Brooks *et al*., 1984; Wendt *et al*., 1980; von Reyn *et al*., 1994).

Mycobacterium avium - intracellulare reportedly occurs worldwide but have been predominant in certain Northern temperate geographic areas, including the United States (Good and Snider, 1982), Canada (Gill *et al*., 1987), Great Britain (Hunter *et al*., 1981), Europe (Debrunner *et al*.,

1992), The Netherlands (Engbaek *et al*., 1981) and Japan (Miyachi *et al*., 1988). The disease also occurs in Australia (De Lalla *et al*., 1992) and South Africa (Nel, 1981). This may not be the true reflectionof endemicity but depicts countries with good laboratories to diagnose them. The incidence of laboratory isolation of MAI in the United States, based on a 1979 survey of 44 state public health laboratories, is estimated to be 3.2 cases per 100,000 population and was greatest for Hawaii (10.8 cases), Connecticut (8.9 cases), Florida (8.4 cases), Kansas (6.8 cases), North Carolina, Maryland, Rhode Island, and Arizona (Good and Snider, 1982) .

In the United States, 40 to 50% of the clinical MAI infections in non-AIDS patients are caused by *M. intracellulare*, whereas in western Germany, 81% of the human infections are due to M. avium and only 19% are due to *M. intracellulare* (Meissner and Anz, 1977). In addition, serovar analyses suggest a shift in the proportion of human disease caused by *M. avium* relative to that caused by *M. intracellulare* in certain geographic areas (Miyachi *et al*., 1988).

2.3.1.2 *Mycobacterium marinum*

Mycobacterium marinum was first isolated from dead fish in a Philadelphia aquarium in 1926 by Arsonson and was first recognized to cause human disease in 1951 after isolation from granulomatous skin lesions in patients from Sweden (Huminer *et al*., 1986). It was also first described as a pathogen of fish under the names *M. marinum*, *M. platypoecilus and M. balnei*, before these species were recognised as synonyms and named *M. marinum* (Wolinsky, 1985). *Mycobacterium marinum* is distributed widely in aquatic environments especially stagnant water, such as in fish tanks and swimming pools, and in natural (fresh or salt) water bodies (Huminer *et al*., 1986; Hautmann *et al*., 1994; Falkinham *et al*., 2001 Gluckman, 1995).

Mycobacterium marinum infection was therefore called swimming pool granulomaas it was found to be an occupational hazard for aquarium cleaners and fishermen. Most often occur in people with recreational or occupational exposure to contaminated fresh or salt water. Temperature and water quality have demonstrated to be important factors for the development of *M. marinum* infection (Clark and Shepard, 1965). Infections in humans have been reported in coastal areas of the Middle East and the Far East (Evan-Paz *et al*., 1976; Iredell *et al*., 1992), in several countries in Europe (Collins *et al*., 1984) and the United States (Zeligman, 1972).

2.3.1.3 *Mycobacterium chelonae*

Mycobacterium chelonae was also described as *M. chelonei* or *M. abscessus* until 1972 (Bercovier and Vincent, 2001). It is widely distributed in the environment in fresh water sources such as rivers, ponds, lakes, drinking water and aquaria. It is principally a pathogen of fish, the bacterium was isolated in epidemics of fish tuberculosis (chronic inflammatory granulomatous disease) in freshwater fish such as the yellow perch (Perca flavescens), marine species such as the Atlantic salmon (Salmo salar) and ornamental fish (Daoust *et al*., 1989; Lansdell *et al*., 1993; McCormick *et al*., 1995; Bruno *et al*., 1998). Due to ubiquitous distribution of *M. chelonae*, infections in pigs, cats and dogs have been reported (Gross and Connelly, 1983; Thorel and Boisvert 1974; Thoen and Hime, 1977). Data shown by United State Centers for Disease Control and Prevention (CDC) in between 1993-1996 showed that 0.93-2.64 cases per million populations for *M. chelonae* related infection.

2.3.1.4 *Mycobacterium ulcerans*

Mycobacterium ulcerans is the *Mycobacterium* species that causes Buruli ulcer (BU), the third most common mycobacterial disease after tuberculosis and leprosy (Johnson *et al*., 2005). The disease was first described by Sir Albert Cook, a British physician, in patients from the Buruli County in Uganda; however the causative organism was first isolated by MacCallum and others in the Bairnsdale region of Victoria, Australia in 1948, hence the name the Bainsdale ulcer.

The mode of transmission of *M. ulcerans* is not known and unlike *M. leperae* and *M. tuberculosis* which are transmitted by person-to-person (Merritt *et al*., 2010), it is thought that infection with *M. ulcerans* occurs through contact with the environment. Buruli ulcer cases are usually found in communities near wetlands such as swamps, marshes and slow moving rivers in areas that are prone to flooding. Also, there has been increasing number of cases reported in areas where the environment has been disturbed example, deforestation, eutrophication, dam construction, mining, population expansion, rice farming and construction of irrigation systems (Merritt *et al*., 2005; Aseidu *et al*., 2000; Duker *et al*., 2006; Hayman, 1991)

The disease has been reported from more than 33 countries worldwide, mainly in tropical and subtropical regions (WHO, 2010); figure 2.5 shows the global distribution of Buruli ulcer. The worst affected areas are countries lying along the Gulf of Guinea in West-Africa, where BU prevalence exceeds that of leprosy, making it the second most important mycobacterioses. In West and Central Africa, the disease typically affects impoverished communities primarily children of remote areas where medical services are unavailable or too expensive. It is estimated that more than 7000 people develop BU annually, with the West African countries like Benin, Côte d'Ivoire and Ghana having the highest incidence rates (WHO, 2008). Globally, 4,907 new cases of Buruli ulcer were reported in 2010 and of these Africa alone reported 4,846 cases. Re-

emergence of cases over the last two decade and the increasing incidence of BU in certain parts of the world, as well as limited knowledge of the disease led the global Buruli ulcer initiative and the Fifty-Seventh World Health Assembly resolved to accelerate research to develop better tools for diagnosis, pathogenesis, and effective treatment (Walsh *et al*., 2008; Wansbrough-Jones *et al*., 2006).

The first case of Buruli ulcer in Ghana was first reported in the Greater Accra in 1971 by Bayleyfrom patients living along the tributaries of the Densu River. In 1989, 96 cases were reported in Asante Akim North District of the Ashanti Region (Van der Werf., 1989). This was followed by the discovery of a major endemic focus in Amansie West in the same region (Amofah *et al*., 1993). Cases of the disease have been reported in all the ten regions of the country with the Ashanti Region reporting the highest number of cases of about 60 % of all cases. The overall national prevalence is 20.7 cases per every 100,000 population making BU the second most important mycobacterial disease after tuberculosis (Amofah *et al*., 2002). Globally, Ghana is the second most endemic country for Buruli ulcer after Cote d'ivoire (WHO, 2012).

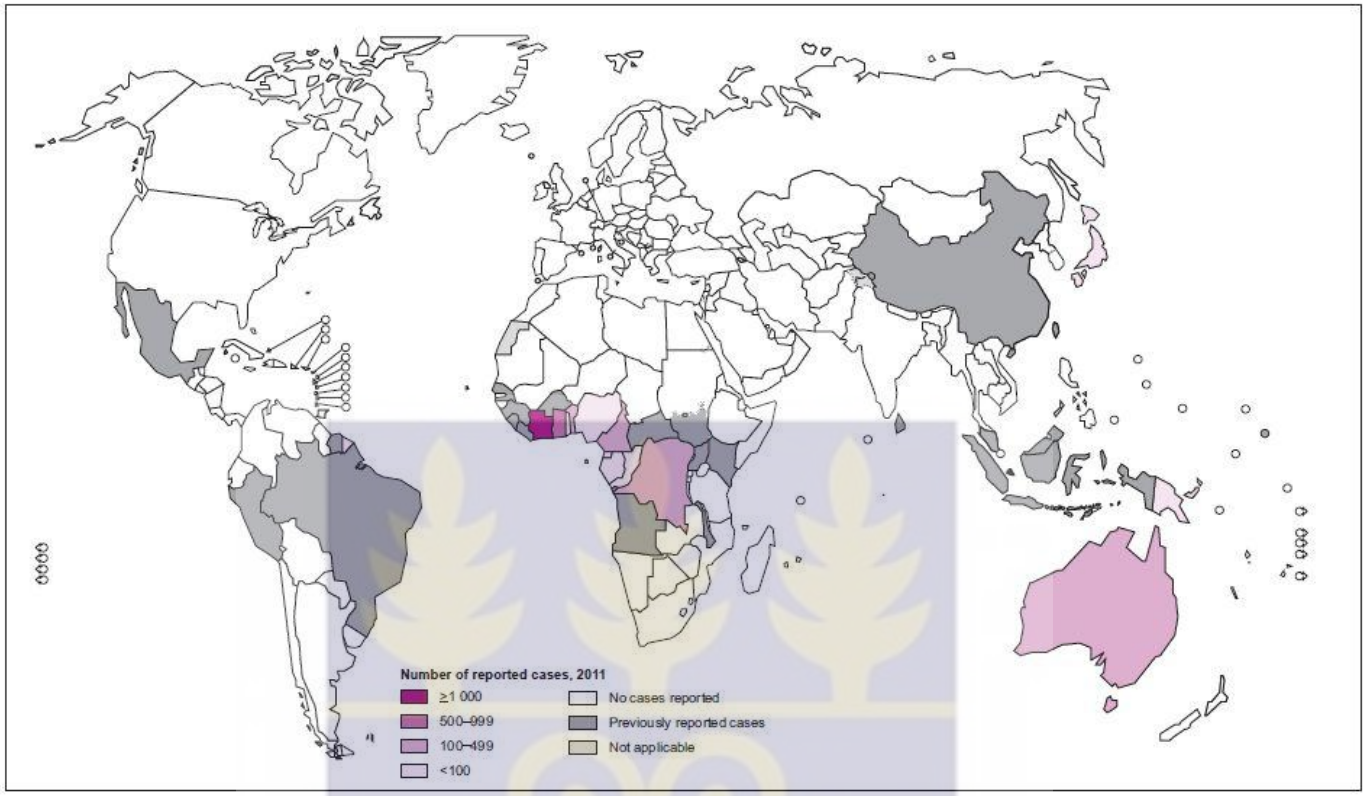


Figure 2. 7: A world map showing the global distribution of Buruli ulcer worldwide in 2011 (WHO, 2011)

2.3.2 Laboratory Diagnosis

The importance of NTM in human pathology has increased due to the epidemic of AIDS (Bercovier and Vincent, 2001). Currently, with the inception of antibiotic treatment more cases are encouraged to be laboratory confirmed before initiation of therapy so that individuals are not unduly put on antibiotics. Despite advances in clinical and laboratory diagnosis of the different NTM diseases, diagnosing NTM diseases remains complicated (van Ingen, 2013). Smear microscopy, culture, polymerase chain reaction (PCR) and histopathology are usually the methods of diagnosis (Zakham *et al*., 2012; Wright *et al*., 1998). Smear microscopy mostly done in a two-step procedure by fluorochrome (auramine) and Ziehl-Neelsen staining. Microscopy is simple and less expensive method. Currently, it is the most extensive laboratory test used in

many developing countries. However, the sensitivity in confirming NTMs by microscopy is low; it is approximately between 20-60 per cent. More importantly acid-fast bacilli detection by micorsocopy is not specific as it cannot distinguish the different species within the NTMs. Culture, though is the final proof procedure; is a slow procedure for some of the species and like histopathology requires elaborate infrastructure and expertise (http://www.oie.int). The polymerase chain reaction has recently been used to detect and differentiate between members of the *Mycobacterium tuberculosis* complex and NTMs. The PCR assays are easily applicable and more rapid than culture (Kox *et al*., 1995; Kox *et al*., 1997; Sanguinetti *et al*., 1998). In addition it is very sensitive and primers can be designed to make it very specific, however PCR is expensive, required elaborate infrastructure and expertise to prevent contamination.

Figure 2. 8: Ziehl- Neelsen (ZN) stained smears of *M. ulcerans* observed under oil immersion x100
(http://www.pathologyoutlines.com/topic/stainsacidfast.html)

Therefore PCR is widely used for the microbiological confirmation of diseases caused by NTMs; for example the gold standard method for laboratory confirmation of clinical diagnosis of Buruli ulcer is done by the detection of *M. ulcerans* specific insertion sequence IS*2404*.

2.3.3 Treatment

Treatment of NTMs infections has been a major problem because of the resistance of NTMs to a wide range of antibiotic. There have been several studies on new and more effective antimycobacterials and new targets for antimycobacterial therapy (Falkinham, 1996). Furthermore, because most mycobacteria are intracellular pathogens and the mammalian host cells serve as a barrier to the delivery of drug to the intracellular environment. There are a number of published guidelines for prophylaxis and treatment of NTMs infections, more especially on *M. avium* (Griffith *et al*., 2007).

Newer antibiotics example; macrolide antibiotics such as, clarithromycin are the most effective agents against NTMs and the ATS guidelines recommend that macrolides be part of all regimens for NTMs infections. Although effective, these agents should not be used as monotherapy to help prevent the risk of resistance. Although no specific macrolide has been shown to be better than the other, it is generally considered that clarithromycin may be more effective whereas azithromycin is usually better tolerated thus have been shown to be effective against *Mycobacterium avium* complex infection), and are more effective against intracellular mycobacteria than standard anti-tuberculosis drugs http://crohn.ie/archive/primer/mycodrug.htm.

Patients with cavitary/fibronodular disease or severe symptomatic infection often require more aggressive treatment and recommended regimen for such patients includes clarithromycin 1000 mg daily (or 500 mg twice a day) or azithromycin 250 mg daily, plus rifabutin 150 to 300 mg daily or rifampin 10 mg/kg/day (maximum 600 mg/day) plus ethambutol 15 mg/kg/day. Intravenous amikacin or streptomycin for 2 to 3 months should be considered in severe or refractory cases (Griffith *et al*., 2007).

Surgery has been shown to improve outcomes in some patients with NTMs infection and is considered as an adjuvant or alternative treatment (Nelson *et al*., 1998; Shiraishi *et al*., 2002). Surgery, in combination with multidrug regimens, has been shown to be better treatment option than drugs alone in patients infected with macrolide-resistant organisms (Shiraishi *et al*., 2002).

Mycobacterium chelonae is one of the most antibiotic- resistant species of the pathogenic rapidly growing mycobacteria. Treating *M. chelonae* infection is challenging because the organism is resistant or only partially susceptible to many antibiotics. Based on sensitivities, and to avoid the emergence of resistance, dual treatment with clarithromycin and another antibiotic that is effective against *M. chelonae* (e.g., amikacin, imipenem, or tobramycin) is recommended. Resistance to mono-therapy with clarithromycin has been reported (Nathan *et al*., 2000). Therefore treatment is continued for >6 months, which is difficult in terms of patient compliance (Kullavanijaya, 1999; Grandinetti *et al*., 2007). In serious disseminated infections involving *M. chelonae*, the injectable agents such as tobramycin plus imipenem are used for the first two to six weeks in combination with clarithromycin to prevent the development of drug resistance to the macrolide (Hassan *et al*., 2007).

Generally, MAI infections are treated with 2 or 3 antimicrobials for at least 12 months. Commonly used first-line drugs include macrolides (clarithromycin or azithromycin), ethambutol, and rifamycins (rifampin, rifabutin). Aminoglycosides, such as streptomycin and amikacin, are also used as additional agents. In children MAI lymphadenitis is treated with surgical excision of the affected lymph nodes.

Surgical debridement followed by skin grafting was the standard treatment for Buruli ulcer even though all bacilli were not completely removed (Rondini *et al*., 2006). Recurrence was common

with varying report rates between 6% and 47% (Amofah *et al*., 1998; Debacker *et al*., 2005). This method was not accessible to poor patients in rural areas of Africa.

In order to limit surgical excision, other treatment options were explored which included the use of topical treatments such as Nitrites, Phenytoin powder, Clay and Heat, hyperbaric oxygen therapy, and antibiotics treatment (Sizaire *et al*., 2006). Many antimicrobial agents were tested as mono-therapy or combination therapy in search of effective antibiotic for the treatment of Buruli ulcer disease (Sizaire *et al*., 2006). Several of the antibiotics that demonstrated activity against *M. ulcerans* in vitro but their clinical efficacy for treatment of *M. ulcerans* disease was not proven (Chauty *et al*., 2007; Thangaraj *et al*., 2000; Portaels *et al*., 1998). A controlled trial was done with Clofazimine, an anti-leprosy drug and the result showed no improvement in healing process, no reduction in the number of surgical excisions and as well as no decrease in disease recurrences (Revill *et al*., 1973). In Côte d'Ivoire where different antibiotics were used in monotherapy for 1 month in a trial the results showed that the use of streptomycin had showed no significant effect on BU lesions (Darie *et al*., 1994).

Studies with animal models showed that aminoglycosides (example, streptomycin and amikacin) and rifampicin had a strong bactericidal activity against *M. ulcerans* when used alone (Dega *et al*., 2000; Bentoucha *et al*., 2001). Following a successful pilot study from Ghana that confirmed that human lesions can be sterilized with antibiotics (Etuaful *et al*., 2005; WHO, 2004).

This made WHO to issue a provisional guidance in 2004 recommending the combination of rifampicin and streptomycin for 8 weeks. The current recommendations for treatment are, a combination of rifampicin and streptomycin/Amikacin for eight weeks as a first-line treatment for all forms of the active disease; Nodules or uncomplicated cases can be treated without

hospitalization. Surgery to remove necrotic tissue, cover skin defects and correct deformities (WHO, 2003).

The treatment protocol was supported by extremely encouraging reports of success, with this protocol in a case series from Benin where 47% of patients treated were completely healed after a year (Chauty *et al*., 2007)

2.3.4 Isolation of Non-Tuberculous Mycobacteria

Isolation of non-tuberculous mycobacteria involves cultivation on selective media after decontamination to remove other bacteria present in the sample being analysed.

Cultivation of mycobacteria is considered to be the "gold standard" for detection of mycobacteria in a sample. Culture of mycobacteria is done on solid or liquid media or both however solid media allow the observation of colony morphology. The recommended solid media include either egg based media such as Löwenstein-Jensen medium (L-J) and agar-based media such as Middlebrook 7H10 and 7H11 agar. Löwenstein-Jensen (L-J) media, containing malachite green dye to inhibit growth of contaminating organisms, is the traditional solid media for culture of mycobacteria. Newer media requires incubation between 2-8 weeks and even more than 6 months for the isolation of slow growing NTMs and by culture (Hosek *et al*., 2006; Sharp *et al*., 2000). However, with liquid media and modern culture systems such as the BACTEC AFB or Mycobacteria Growth Indicator Tubes, growth can typically be seen in approximately 2 weeks. Many *Mycobacterium* species are also able to grow on very simple substrate, using ammonia as nitrogen source and glycerol as carbon source in the presence of mineral salts.

Isolation of these organisms will enhance the progress of the molecular techniques for detection of mycobacteria from the environment. Since the development of techniques also depends on

isolates present, culture is necessary for achieving the highest possible isolation efficiency from the environment.

The recovery of non-tuberculous mycobacteria from the environment is influenced by several factors including decontamination methods, climate conditions and their slow growth (Chilima *et al*., 2006; Ghaemi *et al*., 2006; Falkinham, 2002). The major problem encountered when isolating mycobacteria from the environment is the presence of high numbers of other fast growing microorganisms and fungi in the samples that overgrow on the media before the growth of mycobacteria. This leads to contamination of the medium by other fast growing bacteria which hinder the isolation of mycobacteria.

Enrichment of culture media is needed to allow growth of mycobacteria. *Mycobacterium haemophilum* needs an iron source (ferric ammonium citrate or hemin) in the medium and is best incubated at 30°C (Saubolle *et al*., 1996) while for *M. genavense* it has been reported that media composed of blood, charcoal, caseine, yeast extracts and acidified to pH 6.0, is successful in its isolation (Realini *et al*., 1999). Antibiotic supplements are added to the media for the isolation of some of species, for example PANTA (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin) and cycloheximide can be added to the media for isolation of species such as *M. ulcerans* to reduce contamination. Most mycobacteria grow optimally between 28°C and 37°C. *Mycobacterium marinum*, *M. ulcerans*, *M. chelonae* and *M haemophilum* thrive better between 25 °C and 33°C.

Although there have been many comparisons of different culture isolation methods for environmental samples, general guide for isolation cannot be deduced as a result of differences in sample type , culture media for primary isolation, differences in geographic distribution of

mycobacteria species as well as differences in susceptibility to decontamination (Palomino *et al*., 1998). There is the need for studies into selective media and decontamination methods that will a minimize contamination at the same time increases recovery of NTMs from different geographic regions.

2.3.5 Decontamination Methods

Most samples for environmental mycobacteria culture contain various amounts of other bacteria which usually grow faster. Therefore, recovery of mycobacteria is aided by a chemical decontamination process that effectively kills the contaminant to allow only the growth of mycobacteria of intrest. Decontamination process is a balance between maximizing recovery of mycobacteria, and minimizing contamination by other bacteria and fungi. A wide range of decontamination methods and culture conditions have been used by a variety of researchers to selectively isolate mycobacteria from the environment. However research has shown that, mycobacteria counts are found to be hundred times more in samples before treating with decontamination (Livanainen *et al*., 1997). Their isolation from environmental sample requires both selective decontamination of samples and cultivation on selective media due to the threat of overgrowth by more rapidly growing microbes (Falkinham, 1996). More also decontamination treatment should be at lower concentration. The stronger the alkali, the higher its temperature during the time it acts on the specimen, and the longer it is allowed to act, the greater will be the killing action on both contaminants and *Mycobacterium*. It is also important to note that a laboratory with no experience of contamination is probably using a method that kills too many of the bacilli. Strict adherence to the timed killing period is necessary to maximize recovery (Pfyffer *et al*., 2003).

Sodium hydroxide, oxalic acid, malachite green, benzalkolnium chloride and cetylpyridinium chloride (CPC) are the most commonly used decontaminants. In a study by Kamala *et al*., 1994, decontamination procedures for isolation of non-tuberculous mycobacteria from soil and water were evaluated, six decontamination methods were evaluated and it was found that treatment with 3% Soduim dodecyl sulfate (SDS) in combination with 1% sodium hydroxide (NaOH) was the most effective decontamination method for soil as well as water samples. When this procedure was used by Parashar *et al*., in a study reported in 2004, the decontamination method could not remove any contaminants. In another study, Engbaek *et al*., 1967 evaluated five methods for decontamination, and the 3% sodium lauryl sulfate with 1% Sodium hydroxide method was reported as most suitable. Thus decontamination methods that have been used in the isolation of mycobacteria from the environment and clinical samples, none has been universally accepted for the cultivation mycobacteria.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Equipments and Reagents

3.1.1 Equipments

The following equipments were used: fast prep homogenizer (MP Biomedicals, U.S.A), mortar and pestle (CA Scientific Co., Inc, U.S. A.), eppendorf centrifuge 5415D (Marshall Scientific, U.S.A), 2720 thermal cycler (Applied Biosystems, Singapore), vortex (GENIE, U.S.A), twincubator (Hain Lifescience GmbH, Germany), water bath (Thermostat Io Shaking Water Bath, Thomas Kagaku Co. Ltd, Japan), incubator (Yamato, Japan), digital coagulator (Te-Her, Japan), class II microbiological safety cabinet (BioMAT2, U.S.A)

3.1.2 Reagents

The following reagents were used: sodium dodecyl sulphate (Sigma Aldrich, U.S.A), sodium hydroxide Anhydrous Pellets (Sigma Aldrich, U.S.A), oxalic acid dehydrate (Reagent Plus® >99% (Sigma, U.S.A)), malachite green (Sigma Aldrich, U.S.A), cycloheximide (Sigma, China), Genotype CM version 1.0 (Hain Lifesciences Nehren, Germany), fast DNA spin kit (MP Biomedical, U.S.A), l-Asparagine anhydrous (Fluka Biochemika, Italy), magnesium sulphate (Sigma, U.S.A.), magnesium citrate 14 hydrate (BDH (GPR), U.K.), potassium phosphate monobasic (KANTO chemical co. INC, Japan), agarose (Sigma, U.S.A.), immersion oil (Fluka analytical, Switzerland), glycerol (Riedel-de Haen, Sigma-Aldrich, U.S.A), sulphuric acid (Sigma-Aldrich, U.S.A), phosphate buffer saline (dulbecco A, OXOID, U.K), methylene blue (Sigma, U.S.A.), phenol crystals (Sigma Aldrich, U.S.A.), PANTA (a mixture of antibiotics, polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin) (Becton Dickinson,

U.S.A), mycobactin J (Allied Monitor, U.S.A), isoniazid (Sigma, India), cycloheximide (Sigma, China), ethambutol ihydrochloride (Sigma, India).

3.1.3 Materials

Scalpel blade (Swann-Morton, U.K), 50 ml centrifuge tubes (BD Falcon, U.S.A.), pipetting aid (Gilson, U.K), sterile pipette tips (Gilson, U.K), micropipettes (20µl, 200µl 1000µl) (Gilson, U.K), conical flask (Pyrex, corning life sciences, U.S.A), measuring cylinders(Pyrex, corning life sciences, U.S.A), petri dishes (Pyrex, corning life sciences, U.S.A), pipetteman (Gilson, U.K), glass beads 3mm (MERCK, Germany), ground edge 90° frosted end microscope slides (Thermo scientific, U.S.A), 9 inch sterile pipette (Alpha laboratories, U.K.), serological pipettes (SARSTEDT, Germany), timer (Fisher scientific, U.S.A), DNA strip marker (Hain Lifesciences Nehren, Germany), waste biohazard bag (SARSTEDT, Germany), gloves (Nitrile Bodyguard, U.K.), permanent tube marker (Stabilo, Germany).

3.2 Study Design

The study was an experimental study; samples were collected from three villages. Two of the communities were Buruli ulcer (BU) non endemic while one was endemic. Samples collected include, snail, soil, insects, water and vegetation. The water samples were placed in 50 ml falcon tubes while dried samples were placed in zip-lock bags and transported to the laboratory. Deoxyribonucleic acid extraction and PCR were performed on all collected samples for detection of IS*2404* containing mycobacteria. Smears were then prepared directly and stained by Ziehl-Neelsen procedure. Each of the IS*2404* positive samples were decontaminated with three different decontamination methods and cultured on different in-house egg based selective media.

Acid-fast bacilli positive growths were further identified using line probe assay (HAIN hybridization). Figure 3.1 is the flowchart of the study design.

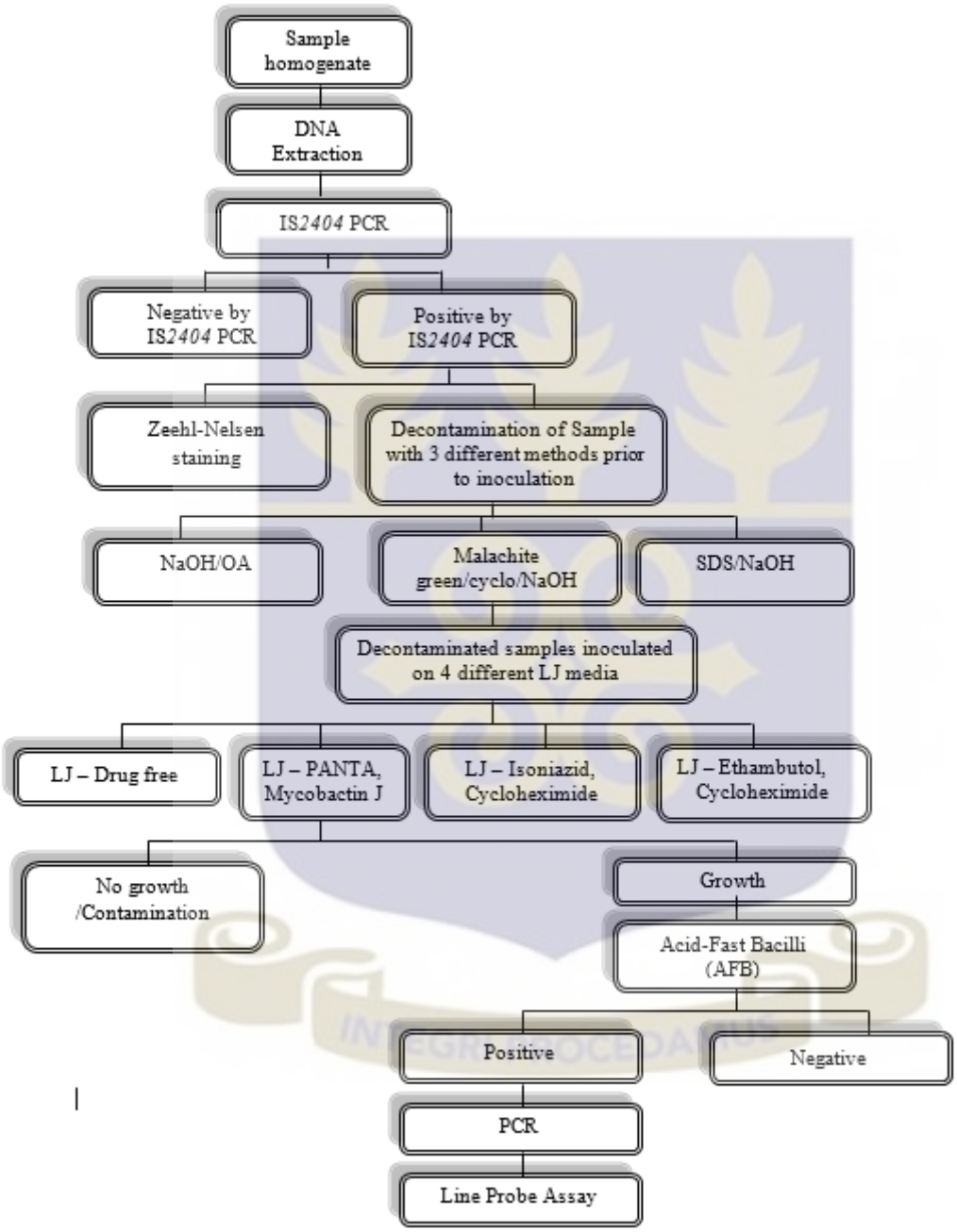


Figure 3. 1: Flowchart of the Study Design

3.3 Study Site

Three villages, Ntabea I, Ntabea II and Ashongkrom in the Eastern Region of Ghana were the sites for the study. These villages are located along the Densu River where most of the communities are known to be Buruli ulcer endemic. Ashongkrom is an endemic community located in the Akuapim South municipality (latitude: 5.83364, longitude: -0.30879) with a population of about 200 projected from the 2010 population census (Yeboah-Manu *et al*., 2012; National Buruli ulcer Control Programme (NBUCP)). The common disease conditions in this community are skin disease, malaria, Buruli ulcer and diarrhoea (Akuapim South municipality health directorate)

Ntabea I is located in Suhum- Kraboa-Coaltar municipality under Obretema Suhum sub-municipality (latitude: 6.12285, longitude: -0.42583). Ntabea I is a small community with a population of 215 projected from 2010 population census. The common disease conditions in this community are skin disease, malaria and diarrhoea (Suhum sub-municipal health directorate).

Ntabea II is located in East-Akim municipal under Asafo sub-district on the coordinates, (latitude: 6.13825, longitude: -0.41788) with a population of 627 projected from 2010 population census. Their main occupation is farming and the common disease condition in this community is malaria (East-Akim municipal health directorate).

However, inhabitants of these two non-endemic communities in the previous study showed exposure to *M. ulcerans* (Yeboah-Manu *et al*., 2012). This was evident by sero-positive reaction to the 18 kDa *M. ulcerans* specific protein as the endemic community. Map of the study site is shown below in Figure 3.2.

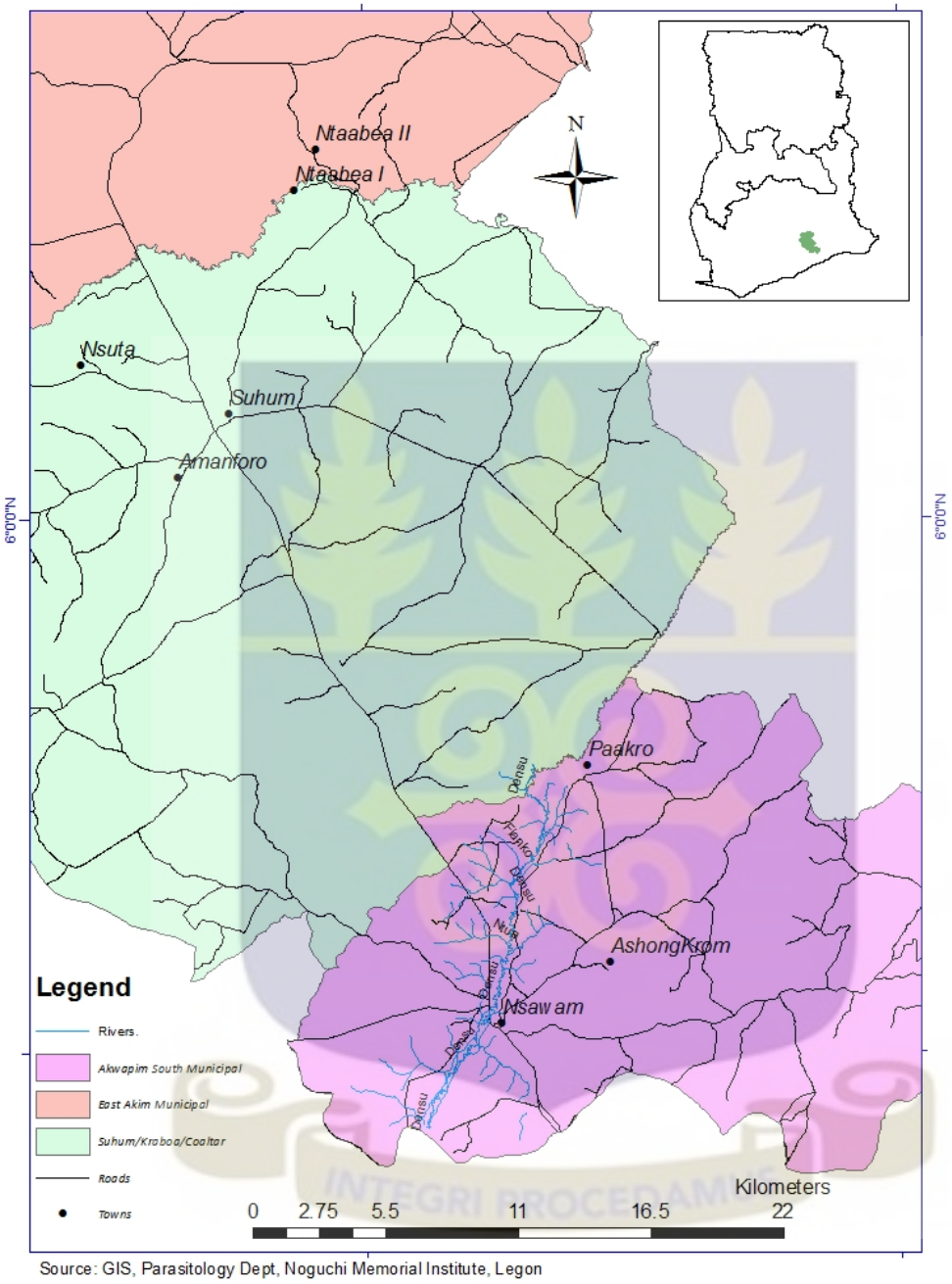


Figure 3. 2: Map showing the district of the villages selected for the study

3.3 Sample Collection

Sixty-five samples were collected randomly from locations such as along footpaths; small water bodies, compounds of houses, farms and along the river bank in all three communities. Twenty

(20) and seventeen (17) samples were collected from Ntabea I and Ntabea II respectively, and 28 from Ashongkrom. The samples collected included water, animal droppings, snails, vegetation (Both living and dead) and soil samples. Table3.1 below shows all the samples analyzed. Solid samples were put in individual zip lock bags and liquid samples were kept in tightly closed 50 mL falcon tubes. Each sample was labelled with sample number, name of community, type of sample and date of collection. The name of the community, the type and identification number of sample was also recorded in a sample collection book. All the collected samples were kept in a transport bag and transported to the laboratory of the Noguchi Memorial Institute for Medical Research the same day. The samples were stored at 4 °C until they were analyzed within a week of collection.

Table 3. 1: Samples analyzed in the study

Sample type	**Number (%)**
Millipede	2(3.08 %)
Caterpillar	1(1.54%)
Termite mount	1(1.54%)
Snail	5(7.69%)
Moss	3(4.62%)
Web	1(1.54%)
Algae	2(3.08 %)
Insect	2(3.08 %)
Vegetation	17(26.15%)
Snail shell	1(1.54%)
Animal dropping	9(13.85%)
Soil	13(20.00%)
Water	6(9.23%)
Fungi	2(3.08 %)
Total	**65(100%)**

3.4 Direct Detection of IS*2404* Containing *Mycobacterium* from Environmental Samples

3.4.1 DNA extractions by FastDNA spin kit for soil protocol

Deoxyribonucleic acid was extracted from about 500mg portions of solid samples and about 500 µL of liquid samples using the FastDNA spin kit according to the manufacturer's instruction. Samples were transferred into lysing matrix E tubes provided in the kit. Lysis buffer (800 µl) and 200 µl of protein precipitation solution (PPS) were added to the sample and vortexed full speed for two minutes. The suspension was further homogenized to break the bacterial cell wall in the fast prep machine for 40 seconds at a speed of 6 m/s and centrifuged at 1400 x g for 10 minutes to sediment the soil, cell wall and other debris. Two hundred microlitres of the supernatant was transferred into 2ml microcentrifuge tube; 500 µl of binding matrix was added and vortexed briefly to bind the released DNA. The mixture was then transferred into a spin column provided in the kit using transfer pipette. It was then centrifuged for 1400 x g for 1 minute to wash away excess diluent. Five hundred microlitres of SEWS-M (contains ethanol) was added gently to the bound DNA in the column for further DNA purification and centrifuged at 1400 x g for 1 minute. The bound DNA in the column was centrifuged at 1400 x g for 2 minutes again without any solvent to dry the matrix after which it was air – dried for 5 minutes. Bound DNA was gently re-suspended in 100 µl of Dnase/Pyrogen-free water (DES) by flicking the sides and incubated for 5minutes. Centrifugation was done for 1 minute at 1400 x g to elute the bound DNA suspension into a sterile catch tube. The DNA was stored at -20 °C until ready for PCR.

3.5 Polymerase Chain Reaction (PCR)

3.5.1 Precaution to prevent contamination

Four rooms were used for all the PCR assays to prevent contamination. The master mix and the addition of template were all done in sterilized safety cabinets. The function of each room is as follows.

First room:	Preparation of Master mix
Second room:	Addition of DNA template
Third room:	Amplification of DNA
Fourth room:	Electrophoresis and Visualization of amplified Products

3.5.2 Polymerase chain reaction procedure

Polymerase chain reaction (PCR) was done on the extracted DNA to detect IS*2404* containing *Mycobacterium* species in the samples. Two primers used initiating amplification were forward MU1New (5′-GAT CAA GCG TTC ACG AGT GA-3′) and reverse MU2 (5′-GGC AGT TAC TTC ACT GCA CA-3′) (Fyfe *et al*., 2007). The reaction mixture consisted of 1 µl MU1and 1 µl MU2, 2 µl of 10x buffer, 4 µl of 5X Q-solution, 1 µl of 25mM MgCl2, 0.12 µl of 5units/ µl hot star tag polymerase, 0.4 µl of 10mM deoxynucleoside triphosphates (dNTPs), 6.48 µl nuclease free water and 4 µl of DNA, in a total volume of 20 µl (WHO, 2001).

The amplification was done at initial denaturing temperature of 95 °C for 15 minutes; 30 cyles of denaturing at 95 °C for 30 seconds, annealing 60°C for 30 seconds, extension at 72 °C for 1 minute; and followed by final extension at 72°C for 10 minutes. Each PCR run contained extraction negative control and IS*2404* positive and negative controls.

3.5.3 Electrophoresis of PCR Product on 2% Agarose Gel

3.5.3.1 Gel Preparation and Electrophoresis

Clean gel trays well were placed on a flat table and combs used for creating wells placed at a centimeter from one end of the tray. Agarose gel was prepared by weighing 2g of agarose powder (Sigma, U.S.A) into a 500 ml sterile flask containing 100 ml 1X Tris-Boric acid-EDTA (TBE) buffer (see appendix). The mixture was dissolved by heating in a microwave, 2 µl of ethidium bromide (100mg/ml) was added and mixed well by swirling. The agarose gel was poured gently into the gel tray and was allowed to set. The comb was carefully removed and the solidified gel was placed in an electrophoresis tank. Electrophoresis buffer (1X TBE) was poured into the tank until the gel was completely covered. Amplified products (7µl) were loaded into the wells and run at 100 volts for 30 minutes.

3.5.3.2 Visualization of Amplified Product

In order to view the amplified product (band), the gel was placed in a gel logic system apparatus (UV transillumination) connected to a computer and viewed under UV light. Images were captured on the computer and saved for further analysis.

3.7 Microscopy

3.7.1 Sample Processing

Hammer was used to break the shell of snails and samples such as leaves were diced with sterile disposable scalpels. They were further homogenized with a sterile porcelain and pestle and suspended in phosphate buffered saline (PBS). Soil samples were shaken vigorously in sterile

distilled water and centrifuged at 600rpm for 5 minutes to sediment soil particles. Samples such as animal droppings were homogenized with a sterile porcelain and pestle and suspended in phosphate buffered saline (PBS). Water samples were vortexed to mix homogenously and centrifuged at 4,000 rpm for 30 minutes to sediment all suspended bacteria. The supernatant was decanted and the resulting pellet was suspended in PBS.

3.7.2 Direct Smear Microscopy

Frosted glass slides were labelled with sample identification number and date. Smear was prepared by transferring 100 µl each of processed onto respective labelled slide. The smear was air-dried in a level 2 biosafety cabinet to prevent risk of infection. The air-dried slides were heat fixed by passing the slide over flame 3 times. Heat fixed slides were arranged on a staining rack, leaving enough space between each slide to prevent cross contamination during the staining process. Slides were stained by Ziehl-Neelsen procedure as below:

Each slide was flooded with filtered carbol fuchsin which contains phenol and heated underside to steam but not to boil; this was to break open the cell wall for the stain to penetrate the lipid rich thick cell wall of mycobacteria. The slides were left at room temperature for 5 minutes. After rinsing off the excess carbol fuchsin stain with tap water, the smears were decolorized by flooding with 20% H_2SO_4 solution to remove the carbol fuchsin from the non acid-fast bacilli cell wall for 5 minutes and then gently rinsed in tap water. The slides were then counter stained with 0.1% methylene blue solution for 1 minute. The slides were air dried before observing under the microscope. Mycobacteria appeared bright reddish-pink in colour while other cells stained blue. The slides were observed using a light microscope under oil immersion and were graded using the International Union against Tuberculosis and Lung Diseases (IUALTD) grading scale (Table 3.2). A smear was declared negative only after reading at least 100 microscopic

visual fields and confirmed by a second reader.

Table 3. 2: The quantitative scale (IUALTD) for grading AFB in Smears

Report	**Number of fields to screen**
(Negative)	No AFB found in at least 100 fields
SCANTY (EXACT NO):	1-9 AFB found in 100 fields
1+	10-99 AFB found in 100 fields
2+	1-10 AFB found per field in at least 50 fields
3+	More than 10 AFB per field in at least 20 fields

3.8 Isolation of Mycobacteria by Culture

Two millilitres each of the processed samples was transferred into separate 50 ml centrifuge tubes for decontamination. Figure 3.1 is a flow chart of isolation method employed within this project.

3.8.1 Decontamination Procedures

3.8.1.1 Sodium hydroxide/Oxalic acid method

Decontamination was performed by treatment of the suspensions for 20 minutes with an equal volume of aqueous 4% sodium hydroxide (NaOH). It was incubated at room temperature for 20 minutes; the suspension was vortexed intermittently. The reaction was neutralized by adding Phosphate buffered saline (PBS) to the 45 ml mark and centrifuged at 3800 rpm for 30 minutes. The supernatant was carefully decanted, and the sediment was re-suspended in 2 ml of PBS and equal volume of 5% oxalic acid was added. It was incubated at room temperature for 30 minutes

and neutralized with PBS to the 45 ml mark. This was then centrifuged at 3800rpm for 30 minutes, the supernatant was decanted carefully. One milliliter PBS was added to the sediment and vortexed to mix thoroughly. Hundred microlitres of the concentrated sediment was inoculated onto respective growth medium (Yeboah-Manu *et al*., 2004).

3.8.1.2 Malachite green/Cycloheximide/Sodium hydroxide method

Two and a half milliliter (2.5 ml) each of (0.3% of Malachite green, 0.075 g /50 ml of cycloheximide and 4% Sodium Hydroxide (NaOH)) solutions was added to 3 ml of the processed sample. It was incubated at room temperature for 30 minutes with intermittent vortexing. One normal hydrochloric acid (1N HCL) was used to neutralize the mixture. The mixture was then centrifuge at 3800 rpm for 30 minutes. Supernatant was carefully decanted leaving the sediment. One milliliter PBS was added to the sediment and vortexed to mix thoroughly. Hundred microliters of the concentrated sediment was inoculated onto respective growth medium (Portaels *et al*., 1988).

3.8.2.3 Sodium dodecyl sulphate (SDS) / Sodium hydroxide (NaOH)

Decontamination was performed by treatment of the suspensions for 30 minutes with an equal volume of 3% Sodium dodecyl sulphate and processed sample. It was incubated at room temperature for 30 minutes; the suspension was vortexed intermittently. The reaction was neutralised by adding PBS to the 45 ml mark and centrifuged at 3800 rpm for 30 minutes. The supernatant was carefully decanted, and the sediment was re-suspended in 2 ml of PBS and equal volume of 4% NaOH was added and incubated at room temperature for 30 minutes with intermittent vortexing. The mixture was then centrifuge at 3800rpm for 30 minutes and carefully decanted. One millitre PBS was added to the sediment and vortexed to mix thoroughly. Hundred

microliters of the concentrated sediment was inoculated onto respective growth medium (Parashar *et al*., 2004).

3.8.3 Cultivation of Mycobacteria

3.8.3.1 Growth medium and Incubation

Four different growth media were evaluated. The media used for isolation were egg based; drug free Lowenstein-Jensen (L-J) and 3 L-J media containing drugs. The drug containing media were 1) PANTA plus (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin) and mycobactin J (PM), 2) ethambutol plus cycloheximide (EB+ cyclo) and 3) Isoniazid plus cycloheximide (INH+ cyclo).

Inoculated media tubes were incubated at 31°C, observed daily for the first week and weekly thereafter until six months. The colonial morphology and length of time before visible colonies appeared were recorded during culture reading. A culture was said to be positive if at least one tube from the same treatment had confirmed AFB growth; culture was said to be negative if none has microbial growth and contaminated when all tubes had more than half of the tube with other bacterial growth or liquefied.

3.8.3.2 Purification and Amplification of Microbial Growth by Sub-Culture

An inoculating loop was used to transfer a loopful of colonies into a sterile tissue culture tube containing six of three millimeter (3mm) glass beads and 2 drops of PBS. The mixture was vortexed for about two minutes until the colonies are completely emulsified. The tube was left for 5 minutes to settle all created aerosol and 500 µl PBS added. A transfer pipette was used to transfer 100 µl of the inoculum onto 2 Lowenstein-Jensen (L-J) slants, incubated at 31 ºC and observed weekly until confluent growth was achieved. A drop of the prepared suspension was

also used to prepare a smear for ZN staining to confirm the acid-fastness of the microbial growth.

3.8.4 Species Identification

Acid-fast bacilli isolates were identified using the GenoType CM version 1.0. (Hian Lifesciences) identification kit. The GenoType CM version 1.0 permits the identification *of M. tuberculosis* and 24 most common NTMs such as the following species: *M. avium, M chelonae, M. abscessus, M. fortuitum M. gordonae, M. peregrinum, M. marinum/M. ulcerans* and *M. xenopi*. This technology is a line probe assay and follows three steps; DNA extraction, PCR amplification of specific genomic regions of the DNA and reverse hybridization of the amplicons. DNA extraction was done by the boiling method.

3.8.4.1 PCR Amplification

The GenoType *Mycobacterium* CM line probe assay was performed as recommended by the manufacturer. The reaction mixture consisted of 2 µl of $MgCl_2$, 5 µl of 10 X buffer, 35 µl of primer (biotinylated) - nucleotide mix (PNM) (provided in the kit), 0.2 µl HotStarTaq polymerase (QIAGEN, Hilden, Germany), 2.8 µl of nuclease free water and 5µL of purified DNA, in a total volume of 50 µl.

The amplification protocol was initial denaturation and enzyme activation of 95 ºC for 15 minutes, 10 cycles performed as follows: 95ºC for 30 seconds, 58°C for 2 minutes, followed by additional 20 cycles of 95°C for 25 seconds, 53°C for 40 seconds and 70°C for 40 seconds and final extension at 70ºC for 8 minutes.

3.8.4.2 Hybridization

The hybridization step was done by following the manufacturer's instruction. Hybridization and detection were performed in an automated washing and shaking machine known as the Twincubator.

Amplicon denaturation was achieved by adding 20 µl of the amplicon and 20 µl of provided denaturing solution (DEN) (contains< 2% NaOH and dye) and mixed. It was incubated at room temperature for 5 minutes.

One millitre of pre-warmed hybridization buffer (HYB) (contains 8-10% anionic tenside and dye) was then added to the trough containing the denatured amplicon. The solution was mixed by gentle tilting mixture up and down until a homogenous colour was developed. The strip with immobilized oligonucleotide probe was labeled and placed in the trough. To make sure the strip was carefully covered by the solusion. The hybridization procedure was performed at 45 °C for 30 minutes at 300 rpm using the twincubator. The strips were then washed twice to remove unbound DNA with RIN solution (< 1% anionic tenside and < 1%NaCl) (provided with the kit). One millitre pre-warmed stringent solution (STR) (contains >25% of quaternary ammonium compound, < 1% anionic tenside and dye) was added to each trough containing the strip incubated for 15 minutes to remove the non-specific bounded DNA. Excess stringent solution was removed completely by two washing steps using 1 ml rinsing solution (RIN) to and incubation at room temperature for 1 minute in each of the washing step. One millitre diluted conjugate solution (contains streptavidin-conjugated alkaline phosphate, 1% blocking reagent, 1%NaCl) was then added to each strip and incubated for 30 minutes on the twincubator.

After rinsing twice for 1 minute each, one millitre of diluted Substrate solution (contains substrate concentrate (SUB-C) which contains dimethyl sulfoxide and (SUB-D) <1% MgCl2, < 1%NaCl) was added to each and incubated for 5 minutes. The substrate was washed away twice with distilled water without shaking, this was done to stop the reaction and the DNA strips were removed and dried between two layers of absorbent paper.

3.8.4.2.2 Evaluation and Interpretation

The developed strips were pasted in the designated fields by aligning the conjugate control (CC) and universal control (UC) and genus control (GC) bands with the respective lines on the evaluation sheet.

The Conjugate control (CC); this band must develop to show the efficiency of the conjugate binding and the substrate reaction.

The universal control (UC) band detect all known *Mycobacterium* species and members of the group of Gram positive bacterium with high G+C content. If the universal control band, the conjugate control band and other bands are positive but pattern that cannot be assigned to a specific *Mycobacterium* species. Only those bands whose intensities are about as strong as or stronger than that of the universal control are to be considered.

The genus control (G-C) band confirms the organism is a member of the genus *Mycobacterium*, the intensity of the band varies depending on the *Mycobacterium* species.

Interpretation of the results was done based on the presence and absence of bands compared with reference provided in the kit. The organisms were identified based on the number and position of bands formed on the strip (Figure 3.4).

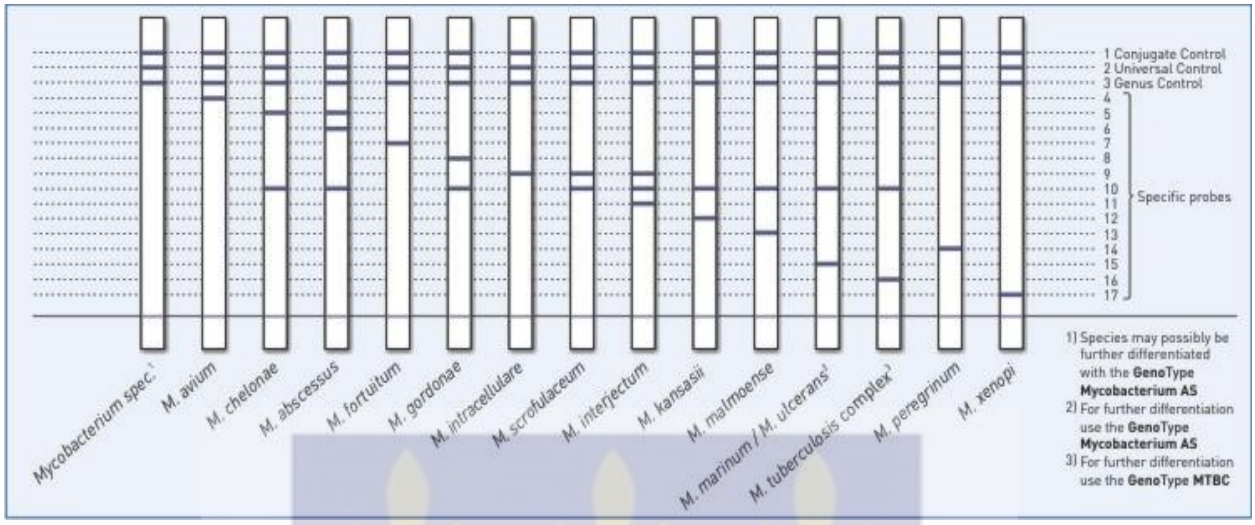


Figure 3. 3: Evaluation chart for identifying Common Mycobacterial species

CHAPTER FOUR

RESULTS

4.1 Samples

Sixty-five samples were collected from three communities namely: Ntabea I (20 samples), Ntabea II (17 samples) and Ashongkrom (28 samples) in the Eastern region of Ghana. The samples collected included, soil 13 (20.00%), water 6 (9.23%), millipede 2 (3.08%), caterpillar 1(1.54%), termitarium 1 (1.54%), snail 5 (7.69%), moss 3 (4.62%), spider web, 1 (1.54%), fungi 2 (3.08%), algae 2 (3.08%), insect 2 (3.08%), vegetation other than moss 17 (26.15%), snail shell 1 (1.54%) and animal droppings 9 (13.85%).

4.2 Direct Microbiological Analysis

4.2.1 Detection of IS*2404*-positve *Mycobacterium* species

The presence of IS*2404* containing *Mycobacterium* species was confirmed in 37 (56.9%) of the samples by direct PCR analysis. This was evident by the detection of a 450bp amplification band after gel electrophoresis (Figure 4.1). The sample type and proportion that were IS*2404* PCR positive is indicated in Table 4.1.

Table 4. 1: Polymerase chain reaction positivity of respective samples analysed

Sample type	Sample positivity
Moss	1/3(33.3%)
Spider web	1/1(100%)
Insect	2/2(100%)
Snail shell	1/1(100%)
Fungi	2/2(100%)
Snail	5/5 (100%)
Vegetation	12/17 (70.6%)
Water	4/6 (66.7%)
Millipede	1/2(50.0%)
Animal dropping	4/9 (44.4%)
Soil	4/13(30.8%)

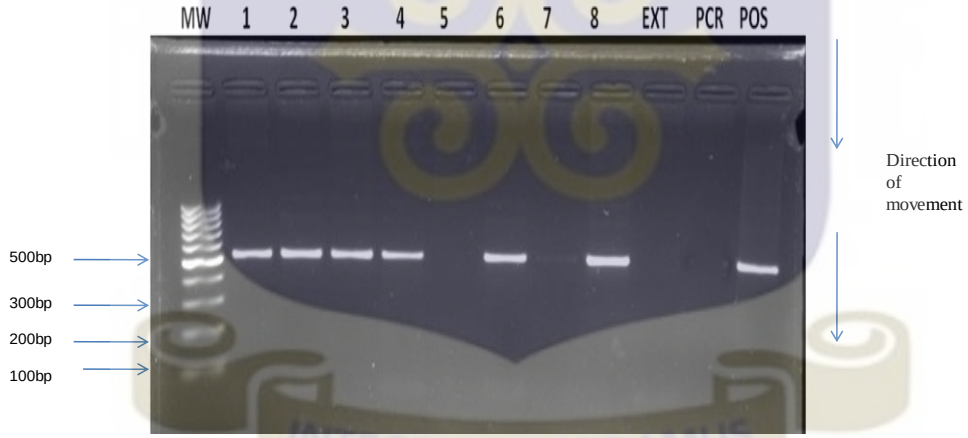


Figure 4. 1: Gel electrophoresis analyses of some environmental samples

MW is molecular weight ladder (100bp), Lanes 1-8 are analyzed environmental samples, EXT is extraction negative control, PCR is PCR negative control and POS is positive control.

4.2.2 Direct Smear Microscopy

Smears were prepared from the thirty – seven samples that were IS*2404* PCR positive. Acid- fast bacilli (AFB) were detected in 5 (13.51%) samples while thirty-two (82.49%) of the smears were negative as shown in table 4.2

Table 4. 2: Acid-fast bacilli positivity of IS*2404* positive samples by direct smear analysis

Sample type	Positivity for acid-fast bacilli (Number of positive / Total number of samples)
Moss	1/1 (100%)
Snail	1/5 (20.0%)
Animal dropping	1/4 (25.0%)
Soil	1/4 (20.0%)
Vegetation	1/12 (8.3%)

4.2.3 Isolation of *Mycobacterium* species

4.2.3.1 General Results

Thirty-seven (37) IS*2404* PCR positive samples were cultured on 3 different drug-containing media and 1 drug–free media. The test was duplicated for the four media types (Drug free (DF), Isoniazid (INH), PANTA-Mycobactin-J (PM), Ethambutol (EB)) and the three different decontamination methods (sodium hydroxide/oxalic acid (NaOH/OA), malachite green/cycloheximide/sodium hydroxide (Malachite) and Sodium dodecyl sulphate/Sodium hydroxide (SDS/NaOH)). Thus 24 media tubes were inoculated per sample giving a total of 888 inoculated tubes. Out of the 888 tubes inoculated, 91 (10.25%) had mycobacterial growth (acid-fast bacilli), 700 (78.82%) had no bacterial growth and 97 (10.92%) were observed to be contaminated. Nine (24.32%) samples had macroscopic AFB growth after six months of

incubation while the remaining 28 (75.68%) samples had no mycobacterial growth. None of the 37 inoculated samples had all 24 tubes contaminated.

Some of the mycobacterial colonial morphologies observed were, smooth yellow dysgonic, smooth white dysgonic, rough buff dysgonic and smooth orange dysgenic (Figure 4.2). Acid-fast bacilli positive cultures were obtained from soil, 3/4 (75.0%), vegetation, 3/12 (25.0%), water, 2/6 (33.3 %) and snail, 1/5 (20.0%).

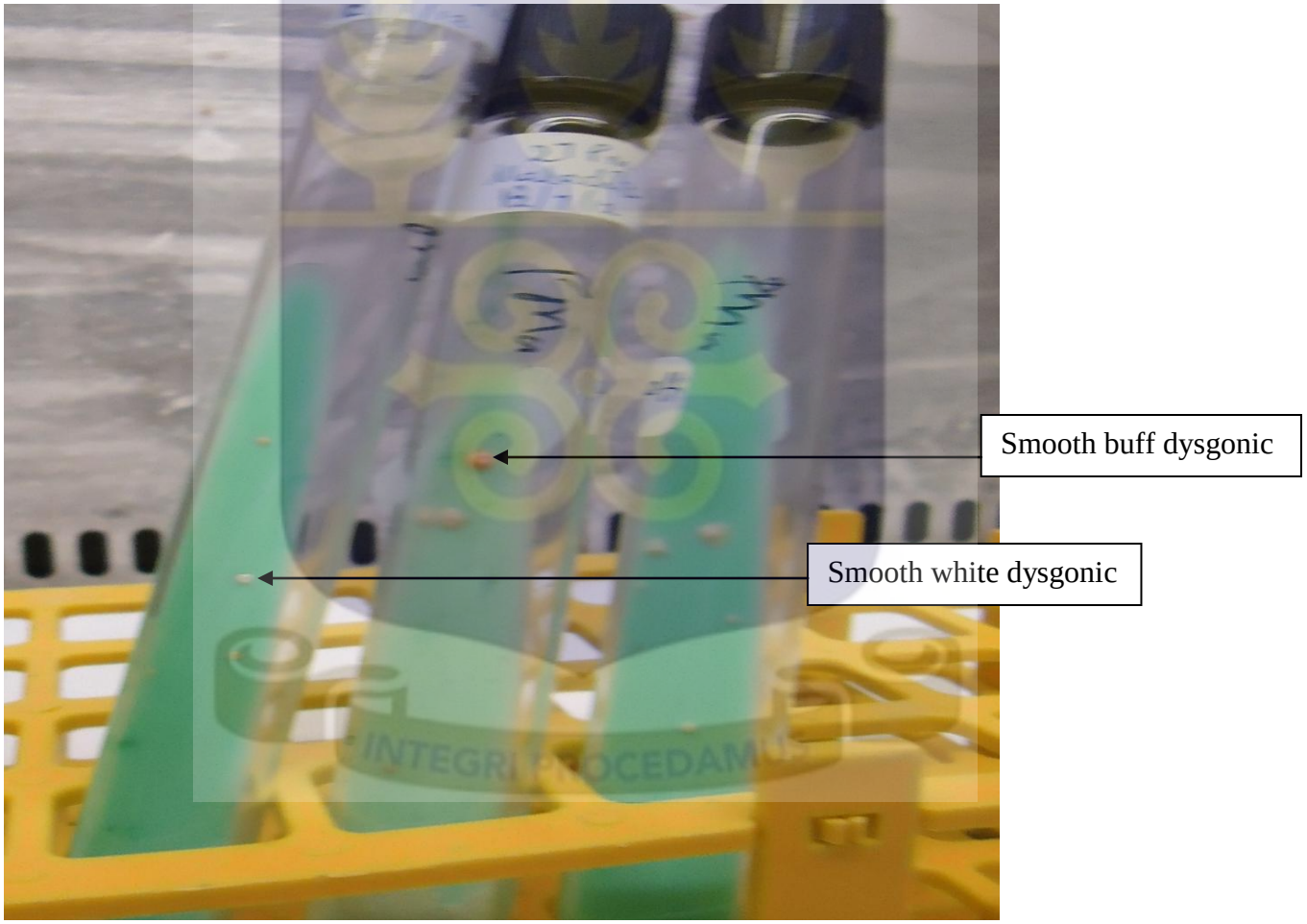


Figure 4. 2: Culture tubes with AFB positive isolates: note the different colonial morphologies

4.2.3.5 Combined Performance of the Decontamination Methods and In-House Formulated Media.

Three different decontamination procedures and four different media either with or without antibiotic supplementation were evaluated. The decontamination procedures evaluated were sodium hydroxide/oxalic acid (NaOH/OA), malachite green/cycloheximide/sodium hydroxide (Malachite) and sodium dodecyl sulphate/sodium hydroxide (SDS/NaOH). For each of the three decontamination procedures 296 tubes were inoculated. The four different media evaluated were L-J-media with no antibiotic (drug free (DF), isoniazid +cycloheximide (INH+ Cyclo), PANTA-mycobactin-J (PM) and ethambutol + cycloheximide (EB + Cyclo)). A total of 222 tubes of each media type were inoculated after sample processing.

Table 4.3 shows the combined performance of the decontamination methods and the four in-house media types used for this study. The results of the analysis using ANOVA revealed that the three decontamination methods (NaOH/OA, SDS/NaOH and malachite) had a statistically significant effect on the performance of in-house selective media studied ($P<0.05$) (Appendix E) at 5% level of significance. In the case of NaOH/OA decontamination methods there was a significant difference in the mycobacteria growth ($P=0.0001$, $F=355.667$) and those that got contaminated ($P=0.02$, $F=39.444$) in relation to the four in-house selective media. The media with the highest number of mycobacterial growth was PM media whilst least was recorded in EB + Cyclo.

Using the SDS/NaOH decontamination methods, the results showed that there was a statistically significant difference in the mycobacterial growth ($P=0.030$, $F=11.33$) and the contamination ($P=0.001$, $F=78$) (Appendix E) with respect to the media. Drug free media was the highest in

terms of contamination whilst PM was least contaminated. Similarly, in the case of malachite green decontamination methods, the results showed that there was significant difference in the mycobacaterial growth (P=0.010, F=16.33) and those contaminated (P=0.010, F=16.3) at 5% level of significance.

With the performance of in-house selective media used in the isolation of *Mycobacterium* species, PM recorded the highest mycobacterial growth value of 27 and the least value of 15 recorded for EB+Cyclo. Isoniazid +cycloheximide and DF also recorded a mycobacterial growth value of 26 and 23 respectively. Statistical analysis using single factor Analysis of variance (ANOVA) at 95% confidence level (5% level of significant) showed that there were no significant differences in the performance of the four in-house selective media examined (F=8.056, P=0.008) in the growth of mycobacteria (Appendix G). However, with respect to contamination of the media, PM was the least contaminated and DF, the highest contaminated. Analysis of variance (ANOVA) at 95% confidence level (5% level of significant) showed that the contamination of the selected media differed significantly (P=0.0001) (Appendix G).When a post hoc analysis was conducted using the Least Significant Difference (LSD), it revealed that there were significant differences in contamination among the four media studied (Appendix F). The performance of the media in terms of contamination in a decreasing order of ranking are as follows; DF > INH + Cyclo > EB + Cyclo > PM.

Table 4. 3: Performance of in-house formulated media and the decontamination methods

Media	Decontamination Method								
	NaOH/OA			SDS/NaOH			Malachite		
	P	N	C	P	N	C	P	N	C
DF	10	56	8	6	55	13	7	57	10
INH+cyclo	13	57	4	8	55	11	5	58	11
EB+cyclo	4	65	5	8	56	10	3	62	9
PM	15	57	2	8	60	6	4	62	8
Total	42	235	19	30	226	40	19	239	38

P= Positive mycobacterial growth; N= No bacteria growth; C= contamination: DF =Drug free; INH + Cyclo = Isoniazid + cycloheximide; PM = PANTA-Mycobactin-J; EB + Cyclo = Ethambutol + cycloheximide.

4.2.3.6 Species Identification

Based on colony morphology (Figure 4.2), media and decontamination method as well as sample type, a total of 80 isolates were obtained from the nine positive cultured samples; 52 (65.0%) of the isolates were from the NaOH/OA decontamination method, 15 (18.75%) were from the SDS/NaOH decontamination method and 13 (16.25%) were obtained from the malachite green decontamination method. Figure 4.3 shows some of the line probe hybridization results of the isolates identified. Out of the 80 isolates 76 were identified as *Mycobacterium* species, 1 isolate identified as bacterium with high G-C content and 3 could not be amplified. As indicated in table 4.4.

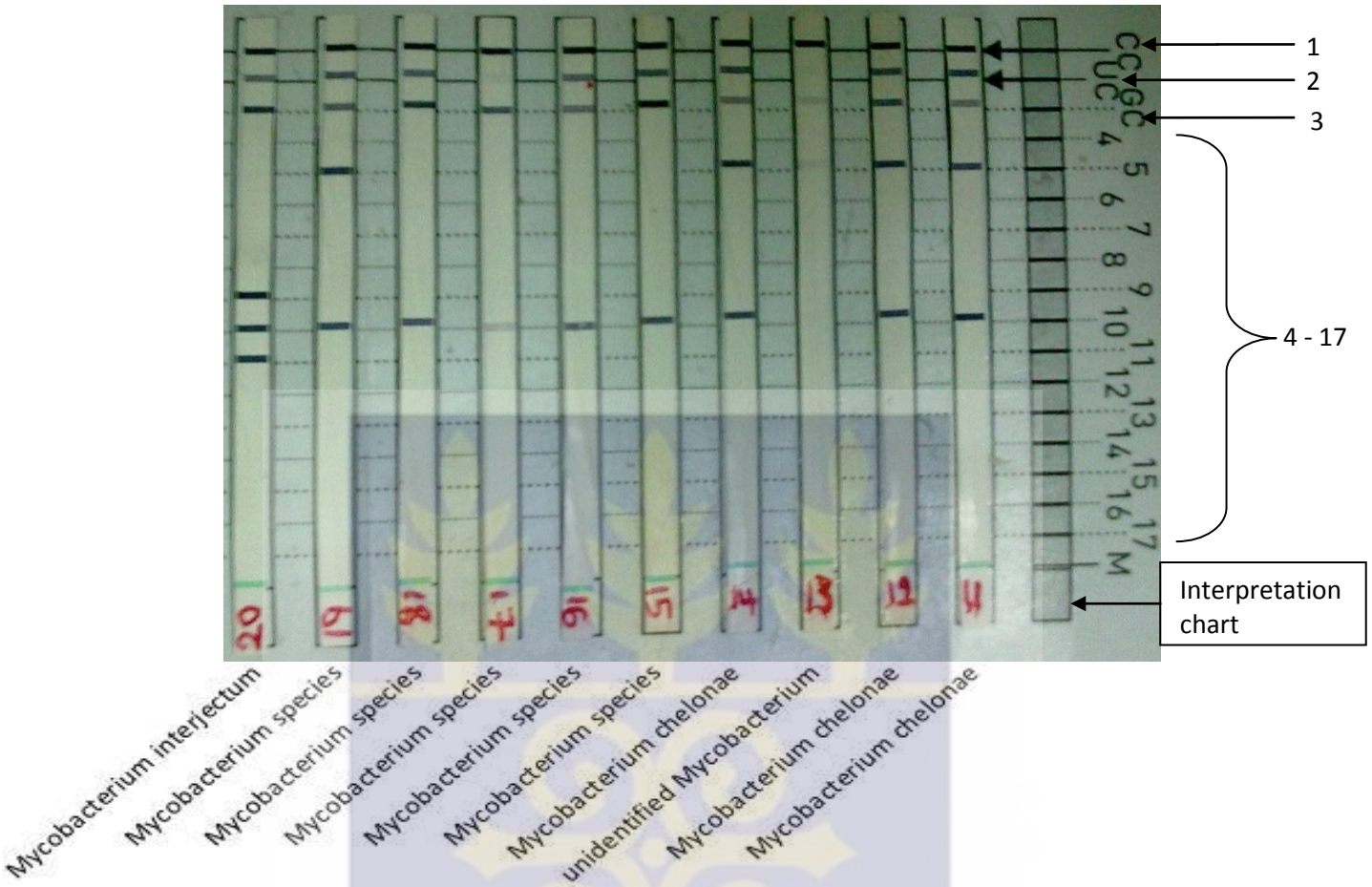


Figure 4. 3: Line probe hybridization analysis of isolates used for identification Bands 1-3 are Conjugate control, Universal control and Genus control respectively. The remaining 4-17 are species specific bands.

Table 4. 4: List of Identified isolates obtained

Organism	Number of organisms isolated
Mycobacterium species	32
Mycobacterium chelonae	16
Mycobacterium interjectum	7
Mycobacterium fortuitum	6
Mycobacterium avium	6
Mycobacterium abscessus	4
Mycobacterium malmoense	3
Mycobacterium gordonae	1
Mycobacterium peregrinum	1
high G-C content bacterium	1
Total	77

4.2.3.5 The Sample Type and *Mycobacterium* species isolated

As indicated in table 4.5 *Mycobacterium chelonae* was isolated from 3 soil, 1 vegetation and 1 water samples. *Mycobacterium avium* was obtained from 3 soil samples, *Mycobacterium fortuitum* from 2 soil, *Mycobacterium malmoense* from 2 soil samples and *Mycobacterium gordonae* from 1 soil sample. Whereas *Mycobacterium avium*, *Mycobacterium fortuitum*, *Mycobacterium malmoense* and *Mycobacterium gordonae* were isolated from only soil, *Mycobacterium abscessus* was isolated from both soil and vegetation. On the other hand, *Mycobacterium interjectum* was isolated from only water. Other *Mycobacterium* species isolated from various samples are all indicated in Table 4.5. Some of the isolated mycobacteria were fast-growing *Mycobacterium* species Table 4.6 and were isolated from soil, vegetation and water

samples where as others were slow growers (Table 4.7.) and were from isolated from only soil samples.

Table 4. 5: Type of sample and *Mycobacterium* species identified

Organism	Soil	Vegetation	Snail	Water	Total
Mycobacterium species	1	13	3	15	32
Mycobacterium avium	6	-	-	-	6
Mycobacterium abscessus	1	3	-	-	4
Mycobacterium chelonae	10	5	-	1	16
Mycobactrium peregrinum	-	1	-	-	1
Mycobacterium gordonae	1	-	-	-	1
Mycobacterium malmoense	3	-	-	-	3
Mycobacterium interjectum	-	-	-	7	7
Mycobacterium fortuitum	6	-	-	-	6
Total	28	22	3	23	76

Table 4. 6: Fast-growing mycobacteria isolate and sample type

Organism	Soil	Vegetation	Snail	Water	Total
Mycobacterium abscessus	1	3	-	-	4
Mycobacterium chelonae	10	5	-	1	16
Mycobactrium peregrinum	-	1	-	-	1
Mycobacterium interjectum	-	-	-	7	7
Mycobacterium fortuitum	6	-	-	-	6
Total	17	9	0	8	34

Table 4. 7: Slow-growing mycobacteria identified and sample type

Organism	Soil	Vegetation	Snail	Water	Total
Mycobacterium avium	6	-	-	-	6
Mycobacterium gordonae	1	-	-	-	1
Mycobacterium malmoense	3	-	-	-	3
Total	10	0	0	0	10

4.2.3.6 Organism Isolated and Decontamination Method

Out of the 44 identified isolates, 25 were obtained from the NaOH/OA decontamination method, followed by 10 from malachite green decontamination method and 9 from the SDS/NaOH decontamination method. *Mycobacterium chelonae* was the most isolated species from NaOH/OA. Out of the 8 species identified, 7 were isolated using NaOH/OA. Table 4.8 shows *Mycobacterium* species isolated and the decontamination methods employed. Table 4.9 and Table 4.9 shows the slow-growing and the fast-growing *Mycobacterium* species identified from the three decontamination methods respectively. Analysis of variance (ANOVA) at 95% confidence level (5% level of significant) showed that NaOH/OA, SDS/NaOH and Malachite green decontamination methods did not have any significant effect on the slow growing mycobacteria ($P>0.05$) (Appendix H) However, on the fast-growing mycobacteria, NaOH/OA ($P=0.0001$, $F=148.5$) and malachite green ($P=0.002$, $F=26.167$) were the decontamination methods that were found to have a significant effect ($P<0.05$) (Appendix I). NaOH/OA decontamination method was found to have the strongest effect on the fast-growing mycobacteria. SDS/NaOH decontamination method however did not have any significant effect on fast-growing mycobacteria ($P=0.148$, $F=2.750$).

Table 4. 8: *Mycobacterium* species identified from the three decontamination methods

Organism	NaOH/OA	SDS/NaOH	Malachite green	Total
Mycobacterium avium	4	1	1	6
Mycobacterium abscessus	1	2	1	4
Mycobacterium chelonae	14	2	-	16
Mycobacterium peregrinum	-	1	-	1
Mycobacterium gordonae	1	-	-	1
Mycobacterium malmoense	3	-	-	3
Mycobacterium interjectum	2	2	3	7
Mycobacterium fortuitum	1	-	5	6
Total	35	9	11	44

Table 4. 9: Slow-growing mycobacteria obtained from the decontamination methods

Decontamination methods	Organism isolated			Total	P-value
	Mycobacterium avium	***Mycobacterium gordonae***	***Mycobacterium malmoense***		
NaOH/OA	4	1	3	8	0.142
SDS/NaOH	1	-	-	1	0.650
Malachite green	1	-	-	1	0.164
Total	6	1	3	10	

Table 4. 10: Fast-growing mycobacteria obtained from the decontamination methods

Decontamination methods	Organism isolated					Total	P-value
	M. abscessus	*M. chelonae*	*M. peregrinum*	*M. interjectum*	*M. fortuitum*		
NaOH/OA	1	14	-	2	1	18	0.000
SDS/NaOH	2	2	1	2	-	7	0.142
Malachite green	1	-	-	3	5	8	0.002
Total	4	16	1	7	6	34	

4.2.3.7 *Mycobacterium* Species Identified and Type of Media

In the case of in-house formulated media used, PM (P=0.001) and INH+ Cyclo (P=0.023) were found to be the only media that significantly influenced the growth of fast-growing mycobacteria (Appendix J). None of the four in-house selective media studied however had a significant effect on the slow-growing mycobacteria (P>0.05) at 5% level of significance (Appendix K).

Seven different species were isolated from PM media. *Mycobacterium chelonae* was the only species isolated by all four media. Table 4.11 shows the *Mycobacterium* species identified from the in-house media used. Table 4.12 and Table 4.13 show the fast-growing and the slow-growing *Mycobacterium* species identified from the in- house media respectively.

Table 4. 11: *Mycobacterium* species identified and in- house selective media used

Organism	DF	INH	EB	PM	Total
Mycobacterium avium	3	1	-	2	6
Mycobacterium abscessus	1	-	1	2	4
Mycobacterium chelonae	1	4	2	9	16
Mycobactrium peregrinum	-	-	1	-	1
Mycobacterium gordonae	-	-	-	1	1
Mycobacterium malmoense	1	-	1	1	3
Mycobacterium interjectum	2	2	-	3	7
Mycobacterium fortuitum	-	1	-	5	6
Total	8	8	5	23	44

Table 4. 12: Effects of in-house formulated media on fast-growing mycobacteria

Decontamination methods	Organism isolated					Total	P-value
	M. abscessus	***M. chelonae***	***M. peregrinum***	***M. interjectum***	***M. fortuitum***		
DF	1	1	-	2	-	4	0.101
INH+ Cyclo	-	4	-	2	1	7	0.023
EB+ Cyclo	1	2	1	-	-	4	0.398
PM	2	9	-	3	5	19	0.001
Total	4	16	1	7	6	34	

Table 4. 13: Effects of in-house formulated media on slow-growing mycobacteria

Decontamination methods	Organism isolated			Total	P-value
	Mycobacterium avium	*Mycobacterium gordonae*	*Mycobacterium malmoense*		
DF	3	-	1	4	0.142
INH+ Cyclo	1	-	-	1	0.385
EB+ Cyclo	-	-	1	1	0.385
PM	2	1	1	4	0.650
Total	6	1	3	10	

CHAPTER FIVE

DISCUSSION

The aim of the study was to compare decontamination methods for the isolation of *Mycobacterium* species from the environment. Many mycobacterial species that may be the cause of important diseases may escape detection and characterization as a result of inappropriate decontamination and growth conditions. Contamination of cultures by undesirable fast growing bacteria and fungi may hinder the isolation of desirable slow growing mycobacteria. More over infections due to NTMs are increasingly becoming more of public health importance (Kankya *et al*., 2011) in both immune-compromised individuals and immune-competent individuals. This makes it important to optimize decontamination methods for the isolation of non-tuberculous mycobacterial species from clinical and the environment.

While most studies use genus specific biomarkers such as the heat shock protein for the detection of the species of the genus *Mycobacterium*, this study employed PCR to detect the biomarker IS*2404* as a first screening procedure. This was done to give an assurance of the possibility of the sample containing *M. ulcerans*.

The study was bias for *M. ulcerans* which causes BU, the most important non-tuberculous mycobacterial in Ghana and in West Africa. For the past decade, an average of 1,000 BU cases are diagnosed in various health facilities mainly in 6 of the 10 regions in Ghana, making BU the second most important mycobacterial disease and Ghana the second most BU endemic country after Ivory- Coast. At the same time the mode of transmission and the ecology of *M. ulcerans* is not known, a knowledge of which is greatly needed for the design of preventive strategies.

Thirty-seven of 65 (representing 56.9%) of the total samples collected were confirmed as IS*2404* positive. This was suggestive of the presence of IS*2404*-containing mycobacteria such as *Mycobacterium ulcerans* (Stinear *et al*., 1999; Stragier *et al*., 2007). While IS*2404* is used in clinical samples for confirmation of *M. ulcerans*, this is not the case for environmental samples as other mycobacterial species such as *Mycobacterium liflandii* found in the environment contain the biomarker IS*2404*.

Of the IS*2404* positive samples, 17/28 (60.7%) were from the endemic community while 20/37 (54.1%) were from the non-endemic communities. This suggests that IS*2404* containing mycobacteria are ubiquitous and may be found in a BU non- endemic community in Ghana. In similar studies where environmental samples from both the endemic and non-endemic communities were analyzed using IS*2404*, samples from both BU endemic and non-endemic communities tested positive, however some studies recorded low positivity when compared with this study (de Vandelannoote *et al*., (2010); Williamson *et al*., 2012; Yeboah-Manu *et al*., 2012). The differences in IS*2404* positivity rates may be due to differences in sample sources and communities sampled. In a sero-epidemiology study by Yeboah-Manu *et al*. (2012) conducted in the same villages sampled in this study; it was found that the individuals in both endemic and non-endemic communities along the Densu River were exposed to *M. ulcerans*. Their findings and the findings from this study suggest that other factors such as host genetics, behavioral and nutrition may be important in converting sub-clinical infections to overt diseases.

In this study all the five snail samples were IS*2404* positive as shown in Table 4.1. Similarly, Marsollier *et al*. (2002) in a study collected ten snails from *M. ulcerans* endemic community and

found that 2 out of the 10 were IS*2404* PCR positive. The findings of this study support the finding that certain aquatic snails harbor *Mycobacterium* species such as *M. ulcerans* after consuming aquatic macrophytes. Aquatic snails are the hosts of many organisms responsible for human infections such as schistosomiasis. In schistosomiasis snails are considered to be intermediate hosts because humans harbour the sexual stages of the parasites and the snails harbour the asexual stages. However humans serve as vectors by contaminating the environment and in transferring of the infection requires no direct contact between snails and human (Madsen, 1992). However, more work needs to be done to confirm the role of snail in *M. ulcerans* transmission and ecology. Many of the vegetation samples 12/17 (70.6%) were positive for IS*2404* PCR in this study (Table 4.1) and this compared with a study by Kazda *et al*., that isolated mycobacteria from plants (Kazda *et al*., 2009).

Direct smears analysis of the 37 IS*2404* PCR positive samples found only 5 (13.5%) samples as acid-fast bacilli positive by direct microscopy. The low percentage confirms the low sensitivity of Ziehl-Neelsen method compared to PCR (Table 4.1). Polymerase chain reaction has the added advantage of multiplying initial copy numbers (DNA) thereby increasing ability to detect mycobacteria. Thus, even though mycobacterial load may be low, mycobacteria can be detected by PCR. Although PCR has greater sensitivity, it is limiting in viability testing since it detect the presence of DNA of an organism which can either be alive or dead. For a sample to be microscopy positive, the mycobacterial load must be approximately 10^4/ ml, thus the low recorded positivity rate implies the mycobacterial load in the analyzed samples was low. Real time-PCR analysis confirmed that the samples had cycle threshold (CT) values higher than 30 which implied that the mycobacterial load was low (communication with Mr. Samuel Yaw

Aboagye) (unpublished data). This finding probably suggests that while mycobacterial species may be ubiquitous in the environment, the load is quite low Three out of five smear positive samples were from the non-endemic communities and the other two from the endemic community. Mycobacteria were isolated from three out of the five the smear positive samples and there were no mycobacterial growth in the other two samples. Similar results were found in the previous studies conducted in Ghana, Benin and Ivory-Coast (Marsollier *et al*., 2002; Eddyani *et al*., 2004; Ngazoa-Kakou *et al*., 2011).

Many decontamination methods have been used in the isolation of mycobacteria from both environmental and clinical samples but none of them have been universally accepted as the standardized method. The most important factors in isolation of mycobacteria from the environment are: (1) the decontamination technique and (2) recovery rate of viable mycobacteria bacilli. In this study, malachite green/cycloheximide/NaOH, sodium hydroxide/ oxalic acid and sodium dodecyl sulphate/sodium hydroxide decontamination methods were evaluated. A good decontamination agent is that which effectively remove unwanted microorganisms (contaminants) and maximize recovery of wanted mycobacteria. Decontamination by 4% NaOH followed by a simplified 5% oxalic acid (Yeboah- Manu *et al*., 2004) gave the highest number of total tubes that confirmed mycobacterial growth (42/91, 46.1 %) (Table 4.3). This method also gave the least number of contaminated culture tubes (19/97) (Table 4.3).

In another study, 4% NaOH / 5% OA and H_2SO_4 were used to decontaminate natural water and the results obtained showed that NaOH/OA was the best (Livanainen *et al*., 1997). In contrast, a study by Livanainnen (1995) showed that decontamination with sodium hydroxide - malachite green-cycloheximide yielded the highest counts of mycobacteria and a low rate of contamination than decontamination with NaOH followed by oxalic acid. A similar result was obtained by

Portaels *et al*. (1988) when soil samples were pre-incubation in tryptic soy broth (TSB), followed by decontamination with malachite green, cycloheximide, and NaOH. The present study showed that sodium hydroxide/malachite green/cyclohiximide/ decontamination however had the least confirmed mycobacterial growth culture positivity rate 19/91(%). Sodium dodecyl sulphate / sodium hydroxide decontamination had the highest number of contaminated cultures 40/97 (13.5%) (Table 4.3) and least number of *Mycobacterium* species (Table 4.8). The differences in the results may be due to the differences in sample sources.

Almost all slow growers were obtained from the NaOH/OA. If more samples are decontaminated using NaOH/OA, other important slow-growing mycobacteria may be isolated. Furthermore, using the data obtained from culture, characterization of the isolates with the available molecular tools and the use of ecological data may aid in elucidating the actual source and mode of transmission of some NTMs.

Among the in-house selective media used, L-J containing PANTA and Mycobatin J (PM) was the most efficient in supporting the growth of both rapid (Table 4.12) and slow growing mycobacteria (Table 4.13). The present study showed that addition of antifungal (mycobactin J) and the antibiotic PANTA to the medium was effective in preventing contamination by fungi and fast growing bacteria. This was the first time both antifungal and antibiotic were used in a medium to isolate mycobacteria from different environmental samples. However, in another study on soil samples, it was shown that addition of mycobactin did not significantly enhance positivity (Portaels *et al*., 1988).

Our observation was however supported by another study where the addition of mycobactin for the isolation of mycobacteria from specimens of clinical or animals significantly enhanced the

positivity and allowed the isolation of some strains which were missed when other media were used (Thoen *et al*., 1979; Portaels *et al*., 1982, 1985; Portaels *et al*., 1988). In addition, the addition of cycloheximide and INH did also have effect in reducing contamination as well as maximizing isolation rate (Table 4.3).

Isoniazid (INH) and ethambutol (EB) were incorporated in selective media because some studies have shown that some NTMs are resistant to isoniazid (INH) and ethambutol (EB) (Portaels., 1996; Portaels., 1998; Makarova and Freĭman 2009). Therefore addition of these drugs was to enhance isolation of such mycobacteria from the environment. From the results obtained, medium containing isoniazid supported the growth of four different species of *Mycobacterium*, namely: *Mycobacterium chelonae*, *Mycobacterium interjectum*, *Mycobacterium fortuitum* and *Mycobacterium avium*. Four different species of *Mycobacterium* were isolated from ethambutol. They include: *Mycobacterium chelonae*, *Mycobacterium peregrinum*, *Mycobacterium abscessus* and *Mycobacterium malmoense* (Table 4.11). This confirms our findings (not published) and that of other workers that some mycobacteria are resistant to both Isoniazid and ethambutol.

Hian GenoType *Mycobacterium* CM assay was used to identify the isolates obtained from the study. Forty-four (55.0%) mycobacteria isolates could be identified to the species level, but 32/80 (40%) confirmed as belonging to the genus *Mycobacterium*, the species could not be by Hian Genotype *Mycobacterium* CM assay. The assay has been reported to be 100% sensitive and 94.4% -100% specific for identification of common mycobacteria (Padilla *et al*., 2004; Tortoli *et al*., 2003 Sarkola *et al*., 2004; makinen *et al*., 2002). However, in this study the specificity was 76/80 (95.0%). The assay was not discriminatory enough in the identification of some of the obtained isolates. Nevertheless *Mycobacterium* species of clinical importance such as *Mycobacterium chelonae*, *Mycobacterium fortuitum*, *Mycobacterium abscesscus*,

Mycobacterium avium, *Mycobacterium malmoense* and *Mycobacterium gordonae* were identified. However, the assay is rapid, reliable, and easy to perform, it will be best for clinical samples. Future work on identification of isolates from the environment needs a more comprehensive assay such as sequencing of biomarkers including heat shock protein and 16SrRNA.

The data obtained from this study revealed that both fast growing and the slowly growing *Mycobacterium* species can be isolated from the environment. The most frequently occurring *Mycobacterium* species was *Mycobacterium chelonae* and was isolated from soil and vegetation samples (Table 4.5).

The study also identified some NTM species that are common cause of skin and soft tissue infections in humans. These include *M. fortuitum*, *M. chelonae*, *M. abscessus* and *M. avium* (Figure 4.4). The NTMs are increasingly becoming important especially because infections can be either community acquired or nosocomial infections. Mostly, infection occurs following an exposure of cut or abraded skin to organisms present in aquariums, pools, natural water supplies, vegetation and soil (Feldman, 1974; Wolinsky, 1979; ATS, 1997). A similar study was conducted by Thorel *et al*., (2004) where soil, peat, humus, tufa, sphagnum, and wood were collected in alpine and subalpine habitats and *M. fortuitum, M. chelonae* and *Mycobacterium malmoense* were the most isolated species from soil. The presence of NTM species may be influenced by levels of organic matter in soil and surface water contributing to the mycobacterial flora (Parashar *et al*., 2009). Precaution on protection while working on or with the soil or ground by people should be encouraged. Children who play on the ground should be worn long-sleeved shirts and trousers when outdoors. Cuts and abrasions on people who are always in contact with the soil or ground should clean and covered all the time till it heals.

Mycobacterium malmoense is one of the most clinically relevant NTM globally and a difficult species to isolate because of its exceptionally slow rate of growth and therefore need for special culture conditions. However, in this study three isolates were obtained from 2 different soil samples with NaOH/OA decontamination method. This confirms that our simplified NaOH/OA could be used to isolate *Mycobacterium* species that are difficult to isolate even though they are clinically relevant. *Mycobacterium malmoense* is the most common NTM isolated from suspected TB cases at the bacteriology department of Noguchi memorial institute for medical research (communication with Miss. Adwoa Asante-Poku). The increasing isolation from clinical cases and also the environment implicates *M. malmoense* as an important NTM in Ghana.

Mycobacterium avium which was isolated from soil samples in this study also have been reported to be isolated from HIV/AIDS patients. It is one of the most significant NTMs associated with human diseases, causing disseminated infection in patients with AIDS and other pulmonary infections and skin infection (Han *et al*., 2005). Because NTM diseases in immune-compromised individuals are mostly disseminated in many organs, concerns about the portal of entry of these mycobacteria have been raised as there is no evidence of person-to-person transmission of NTM. The environment has been considered a likely source of NTMs (O'Brien, 1989; Covert *et al*., 1999) and findings from this study support this hypothesis. Although there is evidence that the environment could be the source of NTMs that infect patients, further studies are needed to correlate the relatedness of patients' isolates to that of the environment from the same community.

The major finding from this study is that decontamination with NaOH/OA may increase the odds of isolating slow growing *Mycobacterium* species such as *M. ulcerans* from the environment. This will further be enhanced by inoculation on medium containing INH or PANTA and mycobactin J.

CHAPTER SIX

CONCLUSION

This study confirmed the presence of acid-fast bacilli and IS*2404* containing mycobacteria in both aquatic and non-aquatic sources. The study identified NTMs including *Mycobacterium chelonae*, *Mycobacterium fortuitum*, *Mycobacterium abscesscus*, *Mycobacterium avium*, *Mycobacterium malmoense*, *Mycobacterium gordonae*, *Mycobacterium interjectum* and *Mycobacterium peregrinum* in the environment. Decontamination with 4% sodium hydroxide and 5% oxalic acid was shown to be the best decontamination method for the isolation of environmental mycobacteria. The modified Lowenstein-Jensen media containing isoniazid and cycloheximide or PANTA and mycobactin J media when used for isolating mycobacteria from the environment can further inhibit the growth of fungi. Four percent sodium hydroxide and five percent oxalic acid decontamination method with Lowenstein-Jensen media containing PANTA and mycobactin J media may enhance the recovery of slow growing *Mycobacterium* species.

6.1 Limitation of the Study

The initial screening process for this study was done using only IS*2404*-specific biomarker for mycobacteria. This however is biased for only IS*2404*-containing mycobacteria. The use of heat shock protein (Hsp) specific for the genus mycobacteria is recommended to increase the isolation of mycobacteria from environmental samples.

The line probe hybridization assay was not discriminatory enough to identify all the isolates. Therefore, a more comprehensive assay such as sequencing of biomarkers including heat shock

protein (hsp 65) and 16S rRNA should be used to obtain isolates identified to their species-specific level.

6.2 Recommendation

Environmental samples of diverse heterogeneity should be considered to test the robustness and efficiency of the modified NaOH/OA method.

Two or more identification methods should be used in order to identify new mycobacteria species.

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APPENDIX A

Samples collected from Ntabea I

Sample Number	Type of Sample
1	Soil from community centre
2	Sheep dropping
3	Coconut shell
4	Lizard dropping
5	Algae from a house compound
6	Mud at the bank of river Densu
7	Decayed leaves at the bank of river Densu
8	Water skates from river Densu
9	Soil close to Densu
10	Moss close to Densu
11	Leaves in the river
12	Vegetation along river path
13	Soil under plantain tree
14	Web on a plant
15	Chicken dropping
16	Fungi
17	Snail shell
18	Cocoyam leaves
19	Millipede on a leaf
20	Snail from a farm

Samples collected from Ntabea II

Sample Number	Type of Sample
21	Water from Densu river
22	Dead leaves from Densu river
23	Dark soil around Densu river
24	Cocoa pod husk
25	Insects on decayed cocoa husk
26	Caterpillar
27	Mud from the Densu river bed
28	Submerged leaves from river Densu
28	Soil from farm
30	Algae from the ground in a house
31	Lizard dropping
32	Snail
33	Millipede
34	Goat dropping
35	Water in a bowl from a house
36	Sheep dropping mixed with palm kernel shell
37	Sand from school compound

Table 3: Samples collected from Ashongkrom

Sample Number	Type of Sample
38	Sheep dropping
39	Vegetation along footpath
40	Dried leaves along footpath
41	Soil along footpath
42	Cocoyam leaves
43	Rotten plantain sucker
44	Sand from house
45	Lizard dropping
46	Snails
47	Water on a path
48	Palm husk
49	Mushrooms
50	Moss from palm fronts
51	Bigger snails
52	Dead leaves in a stream
53	Stagnant water on the road
54	Dead leaves along the road
55	Community swimming water
56	Green vegetation along road
57	Community swimming water and sand from the river
58	Moss from community swimming water and underground water
59	Snails from community swimming water

60	Dead leaves from community swimming water
61	Termites mounts along the road
62	Sand along the road
63	Sand from community square
64	Lizard dropping from community
65	Soil from foot path in palm plantation

APPENDIX B

Reagents for Ziehl-Neelsen Staining Method

Stock Alcoholic Fuchsin Solution; the stock solution was prepared by dissolving 3g of basic fuchsin in a 100 ml of a 95% ethanol.

Carbol fuchsin solution; this was prepared by dissolving 10 ml of alcoholic fuchsin solution in a 90 ml of 5% phenol solution. The resultant solution was filtered to remove fuchsin crystals before use.

20% Sulphuric acid solution; 20 ml of concentrated sulphuric acid was slowly added to 80 ml of distilled water in a 100 ml volumetric flask.

0.3% Methylene blue solution; 0.3g of Methylene blue was initially dissolved in a 50 ml distilled water contained in a 100 ml volumetric flask. The resultant solution was then topped to the mark with distilled water. Again the solution was filtered before use.

APPENDIX C

Reagents for Decontamination

4% Sodium hydroxide (NaOH); Weigh 4grams of sodium hydroxide and dissolve in 100ml of distilled water

2% Sodium hydroxide; Weigh 2 grams and dissolve in 100 ml of distilled water

0.3% Malachite green; Weigh 0.3 grams and dissolve in 100 ml of distilled water

5% Oxalic acid (OA); Weigh 5 grams of oxalic acid and dissolve in 100 ml of distilled water.

3% Sodium dodecyl sulfate (SDS) + 4% NaOH, 100ml

Weigh 3 grams of SDS and weigh 4 grams of NaOH, dissolved in 100 ml of distilled water.

1Normal HCL; Add 8.6 ml of concentrate hydrochloric acid to 91.4 ml of distilled water contained in a 100 ml volumetric flask.

Note: sterilize all the above solutions at 121 degree Celsius for 15min before use.

Cycloheximide for decontamination; Weigh 0.075grams and dissolve in 50 ml of absolute ethanol and filter, dispense into 15ml Falcon tubes.

APPENDIX D

Antibiotics for Media Preparation

Cycloheximide for media; Weigh 150 mg or 0.15 grams +1ml ethanol and filter (For 150 ml media, add 0.5ml of cycloheximide)

PANTA (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin); Add 3ml of sterile distilled water aseptically to PANTA (1 bottle). PANTA containing media; (For 1000ml of prepared media, add 20ml of PANTA)

Mycobactin J; Dissolve mycobactin J by adding 0.5 ml of absolute ethanol (For 1000 ml of prepared media add all the 0.5ml dissolved mycobactin J)

APPENDIX E

ANOVA for performance of in-house formulation media and the decontamination methods.

		Sum of Squares	df	Mean Square	F-ratio	P-value
NaOH Positive culture	Between Groups	133.375	3	44.458	355.667	0.000
	Within Groups	.500	4	.125		
	Total	133.875	7			
NaOH No bacterial growth	Between Groups	94.375	3	31.458	83.889	0.000
	Within Groups	1.500	4	.375		
	Total	95.875	7			
NaOH Contamination	Between Groups	44.375	3	14.792	39.444	0.002
	Within Groups	1.500	4	.375		
	Total	45.875	7			
SDS/NaOH Positive culture	Between Groups	8.500	3	2.833	11.333	0.020
	Within Groups	1.000	4	.250		
	Total	9.500	7			
SDS/NaOH No bacterial growth	Between Groups	34.000	3	11.333	22.667	0.006
	Within Groups	2.000	4	.500		
	Total	36.000	7			
SDS/NaOH Contamination	Between Groups	58.500	3	19.500	78.000	0.001
	Within Groups	1.000	4	.250		
	Total	59.500	7			
Malachite Positive culture	Between Groups	18.375	3	6.125	16.333	0.010
	Within Groups	1.500	4	.375		
	Total	19.875	7			
Malachite No bacterial growth	Between Groups	36.375	3	12.125	32.333	0.003
	Within Groups	1.500	4	.375		
	Total	37.875	7			
Malachite Contamination	Between Groups	18.375	3	6.125	16.333	0.010
	Within Groups	1.500	4	.375		
	Total	19.875	7			

APPENDIX F

Multiple Comparisons of the performance of in- house media used in the isolation of mycobacterium species.

Dependent Variable	(I) Medium	(J) Medium	Mean Difference (I-J)	Std. Error	P-value.	95% Confidence Interval	
						Lower Bound	Upper Bound
Culture positive	DF	INH + Cyclo	1.00000	2.50555	.700	-4.7778	6.7778
		EB + Cyclo	8.33333*	2.50555	.010	2.5555	14.1111
		PM	-3.66667	2.50555	.182	-9.4445	2.1111
	INH + Cyclo	DF	-1.00000	2.50555	.700	-6.7778	4.7778
		EB + Cyclo	7.33333*	2.50555	.019	1.5555	13.1111
		PM	-4.66667	2.50555	.100	-10.4445	1.1111
	EB + Cyclo	DF	-8.33333*	2.50555	.010	-14.1111	-2.5555
		INH + Cyclo	-7.33333*	2.50555	.019	-13.1111	-1.5555
		PM	-12.00000*	2.50555	.001	-17.7778	-6.2222
	PM	DF	3.66667	2.50555	.182	-2.1111	9.4445
		INH + Cyclo	4.66667	2.50555	.100	-1.1111	10.4445
		EB + Cyclo	12.00000*	2.50555	.001	6.2222	17.7778
No bacterial growth	DF	INH + Cyclo	-2.00000*	.47140	.003	-3.0871	-.9129
		EB + Cyclo	-15.00000*	.47140	.000	-16.0871	-13.9129
		PM	-11.00000*	.47140	.000	-12.0871	-9.9129
	INH + Cyclo	DF	2.00000*	.47140	.003	.9129	3.0871
		EB + Cyclo	-13.00000*	.47140	.000	-14.0871	-11.9129
		PM	-9.00000*	.47140	.000	-10.0871	-7.9129
	EB + Cyclo	DF	15.00000*	.47140	.000	13.9129	16.0871
		INH + Cyclo	13.00000*	.47140	.000	11.9129	14.0871
		PM	4.00000*	.47140	.000	2.9129	5.0871
	PM	DF	11.00000*	.47140	.000	9.9129	12.0871
		INH + Cyclo	9.00000*	.47140	.000	7.9129	10.0871
		EB + Cyclo	-4.00000*	.47140	.000	-5.0871	-2.9129
Contamination	DF	INH + Cyclo	5.00000*	.47140	.000	3.9129	6.0871
		EB + Cyclo	7.00000*	.47140	.000	5.9129	8.0871
		PM	15.00000*	.47140	.000	13.9129	16.0871
	INH + Cyclo	DF	-5.00000*	.47140	.000	-6.0871	-3.9129
		EB + Cyclo	2.00000*	.47140	.003	.9129	3.0871
		PM	10.00000*	.47140	.000	8.9129	11.0871
	EB + Cyclo	DF	-7.00000*	.47140	.000	-8.0871	-5.9129
		INH + Cyclo	-2.00000*	.47140	.003	-3.0871	-.9129
		PM	8.00000*	.47140	.000	6.9129	9.0871
	PM	DF	-15.00000*	.47140	.000	-16.0871	-13.9129
		INH + Cyclo	-10.00000*	.47140	.000	-11.0871	-8.9129
		EB + Cyclo	-8.00000*	.47140	.000	-9.0871	-6.9129

*. The mean difference is significant at the 0.05 level.

APPENDIX G

Performance of in-house media for isolation of mycobacteria

Source of Variation		Sum of Squares	df	Mean Square	F-ratio	P-Values
Culture positive	Between Groups	227.583	3	75.861	8.056	0.008
	Within Groups	75.333	8	9.417		
	Total	302.917	11			
No bacterial growth	Between Groups	462.000	3	154.000	462.000	0.00001
	Within Groups	2.667	8	.333		
	Total	464.667	11			
Contamination	Between Groups	350.250	3	116.750	350.250	0.00001
	Within Groups	2.667	8	.333		
	Total	352.917	11			

APPENDIX H

ANOVA showing the effects of decontamination methods on slow growing mycobacteria.

Source of variation (Decontamination methods)		Sum of Squares	df	Mean Square	F-ratio	P-value
NaOH/OA	Between Groups	4.000	2	2.000	4.000	0.142
	Within Groups	1.500	3	.500		
	Total	5.500	5			
SDS/NaOH	Between Groups	.333	2	.167	1.500	0.650
	Within Groups	1.000	3	.333		
	Total	1.333	5			
Malachite green	Between Groups	2.333	2	1.167	3.500	0.164
	Within Groups	1.000	3	.333		
	Total	3.333	5			

APPENDIX I

ANOVA showing the effects of decontamination methods on fast growing mycobacteria

Source of variation (Decontamination methods)		Sum of Squares	Df	Mean Square	F-ratio	P-value
NaOH/OA	Between Groups	237.600	4	59.400	148.500	0.000
	Within Groups	2.000	5	.400		
	Total	239.600	9			
SDS/NaOH	Between Groups	4.400	4	1.100	2.750	0.148
	Within Groups	2.000	5	.400		
	Total	6.400	9			
Malachite green	Between Groups	31.400	4	7.850	26.167	0.002
	Within Groups	1.500	5	.300		
	Total	32.900	9			

APPENDIX J

Effects Of In -House Selective Media on Fast Growing Mycobacteria

Source of variation Selective Medium		Sum of Squares	Df	Mean Square	F-ratio	P-value
	Between Groups	5.600	4	1.400	3.500	0.101
DF	Within Groups	2.000	5	.400		
	Total	7.600	9			
	Between Groups	12.400	4	3.100	7.750	0.023
INH+Cyclo	Within Groups	2.000	5	.400		
	Total	14.400	9			
	Between Groups	2.000	4	.500	1.250	0.398
EB+Cyclo	Within Groups	2.000	5	.400		
	Total	4.000	9			
	Between Groups	80.000	4	20.000	40.000	0.001
PM	Within Groups	2.500	5	.500		
	Total	82.500	9			

APPENDIX K

Effects of In-House Selective Media on Slow Growing Mycobacteria

Source of variation Selective medium		Sum of Squares	df	Mean Square	F-ratio	P-value
DF	Between Groups	4.000	2	2.000	4.000	0.142
	Within Groups	1.500	3	.500		
	Total	5.500	5			
INH+Cyclo	Between Groups	1.333	2	.667	1.333	0.385
	Within Groups	1.500	3	.500		
	Total	2.833	5			
EB+Cyclo	Between Groups	1.333	2	.667	1.333	0.385
	Within Groups	1.500	3	.500		
	Total	2.833	5			
PM	Between Groups	.333	2	.167	.500	0.650
	Within Groups	1.000	3	.333		
	Total	1.333	5			