Dissertation By

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VARIABLE NUMBER TANDEM REPEAT TYPING OF NON-TUBERCULOUS MYCOBACTERIA ISOLATES IN HUMANS AND ENVIRONMENTS SAMPLES; TRANSMISSION STUDIES IN A BURULI ULCER ENDEMIC DISTRICT IN THE ASHANTI REGION, GHANA.

June, 2013

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By

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A Thesis submitted to the Department of Clinical Microbiology, Kwame Nkrumah University of Science and Technology in partial fulfilment of the requirements for the degree of

MASTER OF PHILOSOPHY

School of Medical Sciences, College of Health Sciences

June, 2013

DECLARATION

I hereby assert that this submission is my own work. To the best of my knowledge, it contains no material neither previously published by another person nor accepted for the award of any other degree of the University. All cited references from other works/publications have been duly acknowledged.

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DEDICATION

This work is preeminently dedicated to God Almighty, who arms me with strength and makes my way perfect. This work is also dedicated to my graceful mother, Grace Narh and beloved cousins.

ACKNOWLEDGEMENT

Sir Isaac Newton, once said, “We have come this far because we stood on the shoulders of others”. Indeed, I am indebted to Dr. Lydia Mosi, my supervisor, for giving me this wonderful opportunity. I enjoyed the wonderful relationship we had, remembering how sometimes she would say, “You call me as if I am your girlfriend”. Equally important, for teaching me the arts of successful grant writing, she made supervision as an interaction between colleagues and yet professional.

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ABSTRACT

Non-Tuberculous Mycobacteria (NTM) also known as Environmental Mycobacteria (EM) are opportunistic and ubiquitous mycobacteria. Their mode of transmission appears to be dependent on the close proximity of humans with contaminated environments. One subset of NTMs, the Mycolactone Producing Mycobacteria (MPM); *Mycobacterium pseudoshottsii*, *Mycobacterium liflandii*, *Mycobacterium xenopi and Mycobacterium ulcerans* (MU), are of immense importance because of their pathogenicity in causing debilitating ulcers in both animals and man. Possible transmission routes will involve the interplay of the environment and humans.

This study aimed to determine and compare strain distribution of selected NTMs (focus on *Mycobacterium ulcerans*) from selected MU-infected environments and humans within four Buruli ulcer (BU) endemic communities in Ghana. Environmental samples (N=140) including biofilms, soil, detritus and water filtrates were obtained from 10 water bodies within four study areas. Following screening for *Mycobacterium spp* and MPMs using primers detecting the mycobacterial 16S rRNA and IS2404 genes, MU VNTR loci; locus 6, locus 19, ST1 and MIRU1, were used to genotype human (n=14) and environmental (n=20) isolates. Length polymorphism was validated using a reference strain, *M. marinum* DL and sequencing.

Twenty-seven percent (27%) of samples, mostly biofilms, from 9 water bodies were positive for *Mycobacterium spp*, of which 65.8% were MPMs, mostly *M. ulcerans*. Four MU genotypes, designated, W, X, Y and Z, which represented human isolates, matched those obtained in the environment, suggesting water bodies as major sources of infection. Additionally, previously reported genotypes, A and B (MU), MLF (*Mycobacterium liflandii*) and MMDL (*Mycobacterium*

marinum DL) were found only in water bodies. Genetic comparisons showed that for each community, at least one MU genotype is found both in humans and water bodies within it. Genotype Y was shared by all human and environmental isolates from all four communities, suggesting wide dissemination within the Offin River. All repeats were confirmed with sequencing and phylogenetic analysis clustered isolates in this study with reference MU sequences from GenBank.

These findings support the hypothesis that transmission of NTMs, particularly MU, is dependent on the overlapping habitats of the pathogen and man. Further, it showed VNTR typing as a comparably useful tool for differentiating MU strains in BU endemic communities.

CHAPTER ONE

INTRODUCTION

1.1 Introduction

Non-Tuberculous Mycobacteria (NTM) also known as Environmental Mycobacteria (EM) are ubiquitous mycobacteria distributed in a wide variety of environments and reservoirs including soil, water, aerosols, protozoa, etc. (van Ingen *et al.*, 2009) . In contrast, the causative agents of tuberculosis (TB) and leprosy (*Mycobacterium tuberculosis* and *M. leprae*, respectively) and *M. bovis*, the origin of the attenuated Bacillus Calmette Guerin (BCG) vaccine used against TB, are obligate parasites, as well as human and animal pathogens (Chilima *et al.*, 2006). Of the 95 known species of Mycobacteria, nearly one third have been observed to be associated with disease in humans (Katoch, 2004). NTM including *Mycobacterium avium* complex (MAC), *M. gordonae*, *M. fortuitum*, *M. marinum*, etc cause diseases in both healthy and immunocompromised humans (MAC causes opportunistic infections in HIV patients) and animals (*M. marinum* causes fish granuloma), just to mention a few. These diseases result in profound effects on the morbidity and mortality of both humans and animals that is directly related to the socio-economic profile of affected communities (Asiedu and Etuaful, 1998). A confounding factor affecting the control and prevention of infection with NTMs is their opportunistic nature and the fact that they have a wide distribution in possible reservoirs including soil, water, aerosols, insects, protozoans and small animals. More importantly, it has been demonstrated in a number of classical studies that the mode of transmission of NTMs is dependent on close proximity of humans with contaminated environments and infected animals (Primm *et al.*, 2004, van Ingen *et al.*, 2009). Thus, several

salient questions remain to be addressed with respect to the epidemiology of NTMs. It is essential to gain understanding of the environmental and reservoir species distribution of NTMs in relation to the human disease burden. Advances in molecular biology have made it possible to study the epidemiology of a few species of NTM which were previously not cultivable under standard bacteriological conditions. However, the epidemiology of certain NTMs of public health importance in relation to disease burden has not been adequately addressed. Consequently, it is difficult to understand their possible modes of transmission and, therefore, it is difficult to employ pragmatic control and preventive strategies. One subset of NTMs, the Mycolactone Producing Mycobacteria (MPM) is of immense importance because of their pathogenicity in causing debilitating ulcers in both animals and man. *Mycobacterium pseudoshottsii*, *M. liflandii*, *M. xenopi* and *M. ulcerans*, which have been isolated from fish, frogs, and humans respectively (Ranger *et al.*, 2006a, Rhodes *et al.*, 2001, Rhodes *et al.*, 2005). Other related studies have isolated strains of MPMs from small mammals; possum, koala (Fyfe *et al.*, 2010, Mitchell *et al.*, 1984) and other domestic animals including cats and horses (Elsner *et al.*, 2008, van Zyl *et al.*, 2010). In relation to the latter, the issue of possible zoonosis has not received considerable attention and hence defining the host specificity and natural ecology of MPMs is also a research priority. It may be that there are many different MPMs but the only strains isolated are those producing mycolactones with sufficient potency to cause disease in humans, fish, frogs, possums, and koalas. A better understanding of distribution of MPMs in the environment will be crucial to halting the spread of the diseases they cause, in particular, Buruli ulcer (Yip *et al.*, 2007).

Buruli ulcer (BU) is a flesh eating disease that has enormous socioeconomic effects on affected individuals and on their families and communities. The disease is sometimes described as the ‘mysterious disease’, ‘the flesh eater’, ‘the rural disease’ and ‘the social stigma’. These depictions

are attributable to the perceived myths about the disease, the debilitating effects and its associated disabilities, the populations affected most and the social scars it leaves on the individuals and their families. Buruli ulcer is tropical and subtropical in distribution, endemic in over thirty countries mostly in Africa, including Ghana, Togo, Cote d'Ivoire and Cameroon with affected populations mostly rural. Over 70% of cases are reported in children below 15 years of age, though all sexes are affected equally (WHO/CDS/CPE/GBUI, 2001).

The disease has been described as a neglected but treatable tropical disease. The causative organism, *Mycobacterium ulcerans* (MU), a mycolactone producing mycobacteria (MPM), belongs to the same family of bacteria which causes leprosy and tuberculosis but has received less attention (Stienstra *et al.*, 2002). *Mycobacterium ulcerans* infection, another name for BU, manifests as a painless nodule (pimple like) in the skin, a plaque, or a more extensive oedema all of which may finally progress to an ulcer (WHO/CDS/CPE/GBUI, 2001). Most stages of the disease are painless due to the immunosuppressive nature of a toxin, mycolactone, released by the bacteria during active infection. WHO recommended treatment regimen is a combination therapy of daily injection of streptomycin and rifampicin (oral) for 56 days, with surgery only for extensive ulcers (WHO/CDS/CPE/GBUI, 2001). Though treatment is free in many endemic countries, including Ghana, most BU patients find it difficult to commute between their communities and the Health Centers due to poor condition of roads, lack of transportation, and stigmatisation (Asiedu and Etuaful, 1998). With the mode of transmission still not known, it is difficult to implement preventive and control strategies. Public health control efforts have, therefore, focused on early case detection and treatment through education and active case searches in endemic communities (Stienstra *et al.*, 2002). Activities around water bodies and farming near slow flowing rivers in endemic areas have been identified as risk factors for infection (Asiedu and Etuaful, 1998). Risk

factors which have been suggested to predispose people to BU infection in endemic communities were either by personal communication with BU patients or anecdotal reports with no statistical association to disease burden (Stienstra *et al.,* 2002). Consequently, the factors that predispose one to infection cannot be generalized for all endemic communities due to variation in socioeconomic and cultural practices. More comprehensive assessment tools are therefore needed to address these concerns.

Owing to the difficulty in culturing MU from environmental samples (Portaels *et al.*, 2008), studies have relied on finding the genomic material of *M. ulcerans* in several aquatic environments and correlating it with disease burden. (Williamson *et al.*, 2012, Williamson *et al.*, 2008). Finer molecular tools such as Variable Number Tandem Repeats (VNTR) and Single Nucleotide Polymorphism (SNP) typing have also been developed to specifically differentiate *M. ulcerans* from other MPMs due to the closely shared genome and plasmid sequence similarity (Yip *et al.*, 2007).

This study posits that NTM transmission is dependent on the overlapping environmental habitats of the pathogen and humans. Elucidation of possible transmission routes will therefore involve the interplay of the environment and humans.

1.2 Study rationale

Considerable research on the ecology of *Mycobacterium ulcerans* (MU) with focus on possible reservoirs, vectors and habitats have associated the bacterium with slow flowing water bodies, ponds and wetlands. Additionally, increase in Buruli ulcer (BU) cases in endemic communities has been associated with frequent contact with infected water bodies and wetlands. These studies which detected the bacterial DNA, have correlated its abundance with increase BU cases

(Williamson *et al.*, 2012). It therefore, follows that if patients are infected from MU-contaminated water bodies, then bacterial isolates from these patients should be genetically identical to isolates from the water bodies they are frequently exposed to. Much of research efforts have focused on comparing and differentiating human isolates within and from different geographical origins (Kaser *et al.*, 2009a, Roltgen *et al.*, 2010). This has left a knowledge gap, the similarity of environmental isolates to human isolates and source tracking of infection to infected environments. It is well documented that NTM including MU are environmental but their transmission to humans is still not well understood though various hypotheses have been proposed (Eddyani *et al.*, 2004, Fyfe *et al.*, 2010, Merritt *et al.*, 2010, Williamson *et al.*, 2008). Therefore, to understand how these bacteria are transmitted to humans, it is imperative to compare environmental isolates with human isolates, "footprint" infections to infected environments and identify possible reservoirs or vectors of transmission. Additionally, a few authors have reported an apparent homogeneity of MU strains within different geographical regions. Hilty *et al.* (2006) using two VNTR loci; ST1 and MIRU1, identified two strains of MU circulating within Amansie West, now Amansie Central district (study area). This suggests that addition of other polymorphic loci could increase the discrimination power revealing more genotypes (Williamson *et al.*, 2008). Thus, this study will increase the discrimination power and uncover additional genotypes in the environmental and humans, to help elucidate possible modes of transmission from the environment to humans.

This study has one important leap in BU research, by adopting the 'OneHealth' concept, which seeks to define, manage and prevent diseases using a holistic approach of human, animal and environmental importance. By achieving its objectives, this study will establish plausible causal relationships between infections in humans and infected environments in BU endemic

communities. Ultimately, it will help inform policy on effective control and preventive measures in fighting Buruli ulcer.

1.2.1 Aims

This study aims to determine and compare strain distribution of selected NTMs (focus on *Mycobacterium ulcerans*) from selected MU-contaminated environments and humans within four BU endemic communities, to help elucidate possible routes of transmission of MU.

1.2.2 Specific objectives

1) To identify specific risky and MU-contaminated environments that inhabitants are exposed to within each endemic community as a relation to BU cases.
2) To genotype and compare selected NTM isolates in humans and specific water bodies.
3) To source track MU infections in humans to contaminated water bodies and elucidate possible transmission routes.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 General mycobacteria

The genus *Mycobacterium*, family Mycobacteriaceae and phylum Actinobacteria (Skerman *et al.*, 1980), has over 120 identified species (Tortoli, 2006) , which can be grouped into four based on growth rate and pigmentation (Runyon, 1959). Runyon I, are slow growers (more than seven days to see visible colonies from subculture) and photochromogenic (colonies acquire pigment in the presence of light only); *Mycobacterium kansasii*, *M. marinum, M. simiae, M. pseudoshottsii* (Runyon, 1959), etc. Runyon II are slow growers and scotochromogenic (acquire pigmentation both in the dark or light) and they include *M. scrofulaceum, M. gordonae* (Runyon, 1959), etc. Runyon III are slow growers but non-chromogenic (non-pigmented), e.g. *M. xenopi, M. avium, M. ulcerans, M. haemophilium* (Runyon, 1959), etc. and finally, Runyon IV are the rapid growers (less than seven days to see visible colonies); e.g. *M. fortuitum, M. chelonae, M. abscessus*, etc. (Grange, 1996). Mycobacteria species belonging to these groups and other related classifications have been documented and reviewed by various authors (Falkinham, 1996, Grange, 1982, Grange, 1996, Kankya *et al.*, 2011, Owens, 1978, Rastogi *et al.*, 2001, Runyon, 1959). Tortoli (2006) has extensively reviewed the phenotypic and genotypic characteristics of some selected mycobacteria with their clinical importance. The classification systems described above are based on phenotypic characteristics, which cannot give precise identification of certain species. More recently, advances in molecular microbiology have afforded mycobacteriologist a more informative approach on the taxonomy of the genus. Through sequencing and phylogenetic analyses of the 16S

rDNA, encodes the 16S rRNA, (Chilima *et al.*, 2006), DNA hybridization of species-specific sequences (Alvarado-Esquivel *et al.*, 2009) and Restriction Fragment Length Polymorphism (RFLP) of amplified mycobacterial genes (Oloya *et al.*, 2007) have resolved ambiguities in identification of closely related species. These were previously described as similar strains as result of the phenotypic classifications. It has reshuffled the taxonomy of species within the genus, identified and characterized new strains and has improved the diagnosis on mycobacterial infections.

The genus contains both strict and opportunistic pathogens that infect both humans and animals. Among the strict human pathogens, *M. tuberculous* and *M, leprae*, the causative agents of tuberculosis and leprosy respectively, cause serious morbidity and mortality especially in developing countries (Stienstra *et al.*, 2002). Opportunistic pathogens comprising *M. avium*, *M. kansasii*, *M. simiae*, and *M. haemophilium*, just to mention a few, cause disease in immunocompromised patients (Rastogi *et al.*, 2001). Other pathogenic species which cause disease include *M. ulcerans*, the causative agent of Buruli ulcer and *M. marinum* which causes fish-tank granuloma (Broussard and Ennis, 2007, Mve-Obiang *et al.*, 2005). *M. scrofulaceum* has also been implicated both in cervical lymphadenitis in children and pulmonary tuberculosis in adults (Jogi and Tyring, 2004). Equally important are *M. fortuitum*, *M. chelonae* and *M. abscessus*, which have all been associated with traumatic and surgical wound infections, skin and soft tissue infections and pulmonary disease (Rastogi *et al.*, 2001). Several other mycobacterial diseases have been well documented, including *M. bovis*, the causative agent of bovine tuberculosis, *M. paratuberculosis*, which causes Johne's disease and *M. avium*, which causes disease in pig and poultry (Rastogi *et al.*, 2001).

Bacteriologically, most mycobacteria can grow on Lowenstein's media and most standard media (Yeboah-Manu *et al.*, 2004). They can be identified by observing their growth rates, pigment formation, pathogenicity for animals, niacin reaction and drug susceptibility (Owens, 1978). Most laboratory protocols for culturing mycobacteria from clinical samples include initial decontamination with biocides, e.g. sodium hydroxide (to inhibit or eliminate majority of microorganisms under conditions that most mycobacteria will survive) (Chilima *et al.*, 2006, Kankya *et al.*, 2011). In contrast, culturing of mycobacteria from environmental samples is very challenging and introduces a lot of contaminations from other fast growing microorganisms (Neumann *et al.*, 1997). Comparable to clinical samples, soil has wider and variable pH and contains both organic and inorganic substances, including cell free enzymes, trace elements, as well as many non-mycobacterial organisms that can withstand the selective decontamination and culture procedures and therefore can degrade the culture medium or inhibit PCR detection (Chilima *et al.*, 2006). Recent advances in molecular biology have provided a myriad of alternatives to circumvent some of these barriers encountered with culturing and phenotypic identification of mycobacteria species (Shamputa *et al.*, 2004).

Mycobacteria are non-motile, slender, acid-fast bacilli and weakly gram-positive (Hett and Rubin, 2008). The mycobacterium cell is just like most bacteria, with all the cell inclusions, e.g. *E. coli*, it additionally has a thick cell wall which gives it a distinct morphology from most Gram positive bacterium (Hett and Rubin, 2008). Much of the cell wall is composed of mycosides and mycolic acids which make the cell wall waxy and hydrophobic (Hett and Rubin, 2008). Due to the waxy nature of the cell wall, mycobacteria are found at air-water interface where organic matter is concentrated (Wendt *et al.*, 1980), readily dispersed in aerosols (Falkinham, 2003a), form biofilms

(Islam *et al.*, 2012), virulent and resistant to some antimicrobials (Grange, 1996) and are acid-fast (Oloya *et al.*, 2007).

2.1.1 Non-Tuberculous Mycobacteria (NTM)

Historically, for clinical purposes, mycobacteria were classified as 'tuberculous mycobacteria'; those species causing tubercle-like infections in man and animals (Owens, 1978). These include *M. tuberculosis, M. africanum* (epidemiologically linked to Africa), *M. bovis*, *M. canettii,* and *M. microti* which cause tuberculosis in man, cattle, and other ruminant and rodents respectively (Grange, 1996). The rest were described as atypical or non-tuberculous mycobacteria (Owens, 1978). Unlike the former, which are mostly obligate pathogens in humans and animals, NTMs are ubiquitous, found in a wide range of environmental habitats and reservoirs including water and soil, protozoa, tap water, hospital waste water, house hood plumbing systems, waste water, etc.(Primm *et al.*, 2004) . They live harmlessly in the environment, though the specific role they play is not clear, and only cause disease when they enter a susceptible host (Primm *et al.*, 2004). Compared to the *M. tuberculosis* complex, most NTM are less virulent and are rarely transmitted from human to human (Tortoli, 2006). However, they are a major cause of infection in both immunocompetent and immunocompromised humans and animals. Over 90 species (Katoch, 2004, Runyon, 1959, Runyon, 1960) have been recognized with just a few not implicated in disease (Tortoli, 2006). Several authors have reviewed some NTMs of public health importance as previously underlined above (Tortoli, 2006). However, NTMs of research priority in this study include *M. marinum*, *M. ulcerans*, *M. pseudoshottsii*, *M. liflandii*, *M. avium* complex, *M. gordonae*, *M. fortuitum*, *M. abscessus*, *M. chelonae* and other clinically importance species.

2.1.2 Epidemiology and transmission

NTMs or environmental mycobacteria, unlike members of the *Mycobacterium tuberculosis* complex, are ubiquitous saprophytes found in almost all environmental habits including ground and natural water (Falkinham, 2009), as well as, potable water (Tortoli *et al.*, 2001), hospital waste water and household plumbing systems (Wallace *et al.*, 1998), food (Yoder *et al.*, 1999), etc. Hence, human exposure to mycobacterial infections include contaminated potable water, natural water and soil, and food as stated above. However, there is a major overlap in water where humans are exposed to mycobacteria in water through drinking, swimming, and bathing (Primm *et al.*, 2004). Aerosols generated during these activities can also lead to human exposure (Primm *et al.*, 2004). In water bodies, NTMs are found in the air-water interface where organic matter is concentrated, forming biofilms and survive extremes of temperature, disinfectants and biocides (Falkinham, 2010). Subsequently, this has resulted in the emergence and persistence of resistant strains which are disseminated in municipal water supplies (Tortoli *et al.*, 2001). Reports of human-to-human transmission of NTMs are rare, suggesting that water is a major vehicle for the dissemination of NTMs (Tortoli, 2006). Frequently, *M. avium* has been isolated from water bodies and has been implicated as a source of infection in humans (Primm *et al.*, 2004). Tortoli *et al*. (2001) showed by sequence analysis of the hypervariable region of the mycobacterial 16S rRNA gene that *M. avium* isolates from HIV-AIDS patients were similar to isolates from the water sources they were exposed to. Similar results were obtained by Mansfield and Lackner (1997) using experimental monkeys. These results were consistent with work by Yoder *et al*. (1999) who also showed strain similarities, *M. avium*, between isolates from HIV-AIDS patients and their food. Although several studies have reported high prevalence of NTM infection in immunocompromised patients (Cassidy *et al.*, 2009, Tortoli *et al.*, 2001, Yoder *et al.*, 1999), all ages and sexes, especially

HIV-AIDS patients, a study by Cassidy *et al*.(2009), showed high prevalence of *M. avium* infection in women than in men, and highest in patients aged > 50 years.

2.1.3 Pathogenesis, diagnosis and treatment of NTM infections

NTM have been implicated in a myriad of infections in both immunocompetent and immunocompromised host, mostly HIV-infected patients throughout the world. Several studies suggest that pathogenic mycobacteria are intracellular pathogens capable of growing inside phagosomes and phagolysosomes and are able to inhibit the cidal activity of lysosomes upon macrophage infection, e.g. in *M. avium* (Frehel *et al.*, 1986a, Frehel *et al.*, 1986b). This allows mycobacteria to modulate host immune system by mediating cytokine secretion and macrophage effector functions (Rastogi and Barrow, 1994). Some components of the mycobacterial cell wall like, lipoarabinomannan (LAM), mycolic acid-containing glycolipid, mycosides of the phenoglycolipid type, Wax-D, cord factor, mycobactins and exochelins are all associated with virulence and pathogenicity, as reviewed by Rastogi *et al*. (2001). Mycobacterial infections range from pulmonary and extrapulmonary diseases (Falkinham, 2003b), cutaneous and skin infections (Bartralot *et al.*, 2000) to disseminated infections in both immunocompromised and immunocompetent hosts both humans and animals (Chetchotisakd *et al.*, 2007).

Diagnosis of mycobacterial infections include, acid-fast bacilli using the Ziehl-Neelsen stain (Bartralot *et al.*, 2000), growth rate and pigmentation on Lowenstein-Jensen media or Middlebrook 7H9 (Radomski *et al.*, 2010), biochemical test including, niacin reaction, catalase activity, etc. (Rastogi *et al.*, 2001) and antigen skin tests (Falkinham, 1996) can be used to diagnose most mycobacterial infections. It is worth noting that, though these tests are widely used to diagnose

clinical cases, their application to studying epidemiology of outbreaks and epidemics is limited. In epidemiological studies where identification and characterization of the precise causative organism is necessary, molecular methods provide more informative approaches and overcome the limitations of phenotypic identifications (Rastogi *et al.*, 2001). PCR detection of specific mycobacterial DNA sequences, 16S rRNA gene, insertion sequences (IS), heat shock protein (hsp 65), etc. are currently supplanting phenotypic identifications as they are more sensitive, specific, rapid and less laborious (Rastogi *et al.*, 2001).

Treatment of infections due to NTMs does not follow any standardized guideline. It involves a combination of different antimicrobials depending, in part, on the susceptibility pattern of the pathogenic species (Brown-Elliott *et al.*, 2012, van Ingen *et al.*, 2012). Various macrolide antimicrobials (halts protein synthesis by inhibiting peptidyltransferase activity of the 50S ribosome of mycobacteria) have been used successfully in treating different mycobacterial infections (Tartaglione, 1997). Clarithromycin, azithromycin, ethambutol, rifampin, ciprofloxacin, isoniazid, erythromycin, etc. have been used in combination with other antibacterial drugs like streptomycin and other sulfonamides to treat NTM infections in both immunocompetent and immunocompromised hosts (Tartaglione, 1997). Treatment regimens could be as long as 6-9 months (Tartaglione, 1997). In other cases where infection involves the skin and soft tissue, ulcer, as in *Mycobacterium ulcerans* infection, Buruli ulcer, surgery (skin grafting) is normally performed (Asiedu and Etuaful, 1998).

2.1.4 Mycolactone Producing Mycobacteria (MPM)

Mycolactone Producing Mycobacteria (MPM) is an emerging group of NTMs with increasing public health importance because of the devastating diseases they cause in both humans and animals (Broussard and Ennis, 2007, Mve-Obiang *et al.*, 2005, Rhodes *et al.*, 2001, Stinear *et al.*, 2007). One distinguishing feature of MPM from other NTMs is the production of mycolactone, a molecule with both cytotoxic and immunosuppressive properties, both *in vitro* and *in vivo* (Dobos *et al.*, 2001, George *et al.*, 2000, Hong *et al.*, 2008). Mycolactone is encoded by a plasmid within the mycobacterium cell and a few mycobacteria, *Mycobacterium ulcerans*, *M. pseudoshottsii*, *M. liflandii* and *M. marinum* DL, have been confirmed as harbouring copies of this plasmid (Pidot *et al.*, 2008). It is thought that MPM may have descended from a *M. marinum* ancestor by acquiring a large circular plasmid that conferred the ability to make mycolactone, and then spread throughout the world occupying different hosts (Pidot *et al.*, 2008). Sequence comparison (Figure 1) of plasmids from these mycobacteria showed over 98% sequence similarity (Pidot *et al.*, 2008). Based on this sequence information, mycobacterial investigators have designed primers targeting species-specific sites on the plasmid to detect MPM from both clinical and environmental sources (Williamson *et al.*, 2008). Primers targeting genes coding the enoyl reductase (ER) and keto reductase (KR) (enzymes involved in mycolactone synthesis) on *M. ulcerans* plasmid, pMUM001, have been used to detect the bacteria in clinical and environmental samples (Williamson *et al.*, 2012, Williamson *et al.*, 2008, Yeboah-Manu *et al.*, 2012). However, successful differentiation of MPM strains relies on a combination of other molecular tools (reviewed in section 2.3).

2.1.5 Mycolactone production and cytotoxicity

Mycolactone is produced via a condensation reaction involving two polykide chains, a core chain (invariant in all congeners of mycolactone) and an acyl side chain (varies among congeners) as portrayed in Figure 1 (Pidot *et al.*, 2008). The former is produced by two polykide snythetases encode by mlsA1 and mlsA2 genes on the plasmid. The fatty acid-acyl chain is produced by a different polykide synthetase encoded by mlsB (Pidot *et al.*, 2008). The various modules (contiguous gene clusters) annotated on the plasmids (Figure 1) encode several enzymes during fatty acid synthesis for mycolactone production. Several *in vivo* studies in different experimental animals (guinea pigs and mice) (George *et al.*, 2000, Torrado *et al.*, 2007a) and *in vitro* studies with different cell lines (adipose and fibroblast cells) (Dobos *et al.*, 2001, Sarfo *et al.*, 2010) have shown the cytotoxic and immunosuppressive activities of mycolactone. In *M. ulcerans* infection, BU, mycolactone is responsible for both the painless (immunosuppressive) and ulceration (necrosis) forms of the disease (Mve-Obiang *et al.*, 2003). However, its role in the survival and colonization of *M. ulcerans* within various environmental niches or some insects implicated in vectoring it to the mammalian host is not clear and has been debated by a few authors (Marsollier *et al.*, 2007, Mosi *et al.*, 2008).

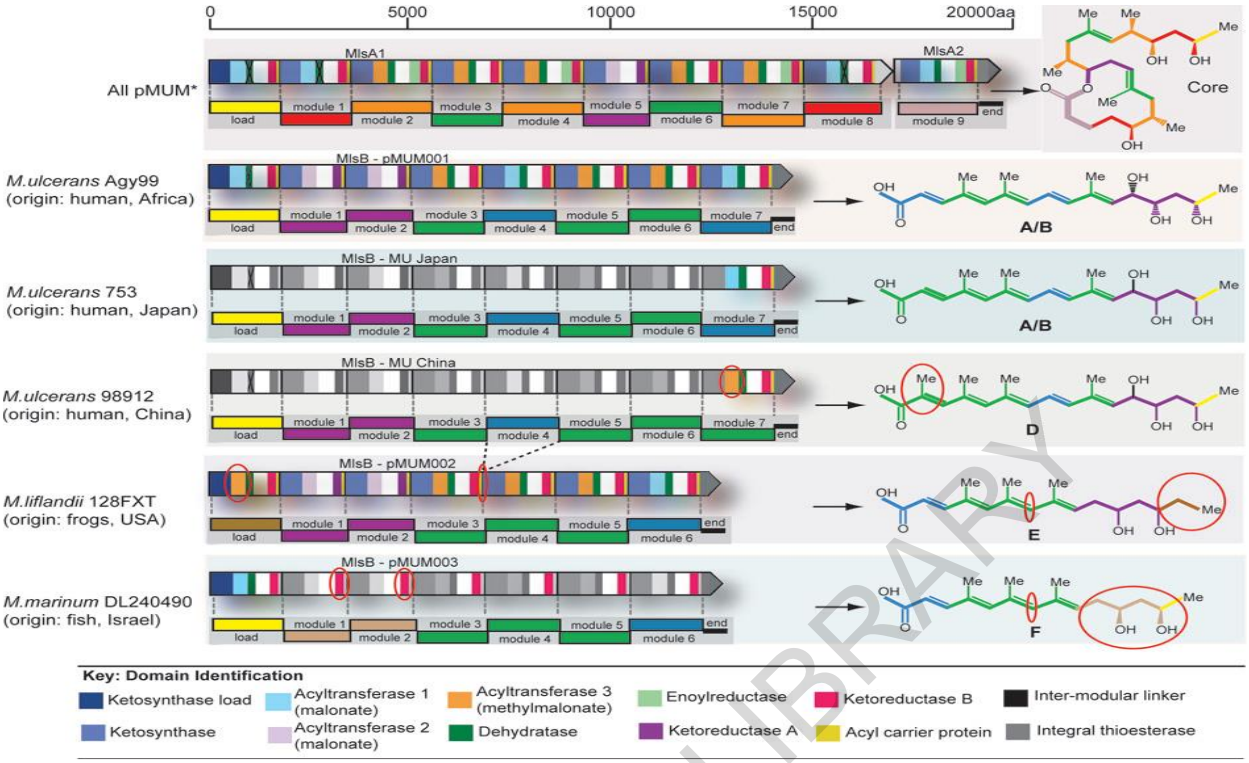

Figure 1 Genetic organization of the mycolactone biosynthetic cluster from some MPM plasmids. (Pidot *et al*., 2008).

2.2 Buruli ulcer (BU)

2.2.1 History, perception and socio-economic impact of BU

Buruli ulcer (BU) is a flesh eating disease that has enormous socioeconomic effects on affected individuals and on their families and communities (Stienstra *et al.*, 2002). The disease is sometimes described as the "mysterious disease", "the flesh eater", "the rural disease" and "the social stigma" (Asiedu and Etuaful, 1998). These depictions are attributable to the perceived myths about the disease (Stienstra *et al.*, 2002), the debilitating effects and its associated disabilities, the populations affected most and the social scars it leaves on the individuals and their families. It is the third most common mycobacterial infection after tuberculosis and leprosy (Walsh *et al.*, 2010).

The causative agent, *Mycobacterium ulcerans*, was first described by MacCallum and colleagues, cited from (Johnson *et al.*, 2005). In the 1980s, BU became a public health problem prompting the World Health Organization (WHO) to establish the Global Buruli Ulcer Initiative (GBUI) in 1998 (Amofah *et al.*, 2002) to raise awareness about the disease, improve access to early diagnosis, treatment and promotion of research to develop better tools for treatment and prevention of BU (WHO, 2012).

The disease is prevalent in the tropics and sub-tropical regions of Africa, with most of the cases from West Africa. It affects populations in the rural areas where the predominant occupation is farming (Asiedu and Etuaful, 1998). In these communities, most people think the disease is caused by witchcraft, punishment for sins, or as a result of a curse (Asiedu and Etuaful, 1998). Consequently, some patients resort to the fetish priest or spiritual healer for treatment (Stienstra *et al.*, 2002). Others believe it is due to personal hygiene and hence do not seek medical treatment. Like leprosy, most BU patients are stigmatized and marginalized (Adamba and Owusu, 2011). Children especially, drop out of school and become burdens on their families. A study by Asiedu and Etuaful (1998), showed that treatment per patient is over $ 900. However, currently, treatment for BU is free in most government hospitals, especially in Ghana. The latter is augmented by some Non-governmental Organizations in carrying out free educational and treatment programmes in endemic communities (Adamba and Owusu, 2011).

2.2.2 Epidemiology

Buruli ulcer is tropical and sub-tropical in distribution (WHO, 2010). It is endemic in over thirty countries in the world, mostly in West Africa including Ghana, Togo, Benin and Côte d'Ivoire. Cases have also been reported in Cameroon, Mexico, Papua New Guinea and in South East Asia

(WHO, 2008, WHO, 2010). In Australia, the disease is predominant in South East (Johnson and Lavender, 2009). In Ghana, the disease is endemic in all regions with national recorded cases of 853, 1005 and 1048 in 2002, 2005 and 2010 respectively (WHO, 2010). There is under-reporting of cases and hence the figures could be much higher (Amofah *et al.*, 2002). The disease affects all sexes and age groups but majority of cases are seen in children below fifteen years of age (Marston *et al.*, 1995, van der Werf *et al.*, 1989). Several reasons why such cohort is affected have been put forward. Frequently, when running errands or playing, they come into contact with wet environments and water bodies, contaminated with *Mycobacterium ulcerans* (MU) (Asiedu and Etuaful, 1998, Stienstra *et al.*, 2002). Though the mode of transmission is still not clear, several hypotheses have been proposed. It is suggested that people are infected via inhalation of aerosols containing the bacteria (Johnson and Lavender, 2009), inoculation into open skin or via bites of some aquatic insects (Portaels *et al.*, 1999). However, a few things stand out as risk factors. Increase in BU incidences has been associated with contact with slow moving water bodies and wet lands (Debacker *et al.*, 2006). The role of aquatic insects in the transmission of BU remains unclear although it is still a research priority (Benbow *et al.*, 2008).

2.2.3 Transmission and ecology of *Mycobacterium ulcerans*

There have been no reports of human-to-human transmission and the mode of transmission of *M. ulcerans* from the environment to humans is still not clear (Johnson *et al.*, 2005). Like all NTMs, *M. ulcerans* is an environmental and saprophytic mycobacteria (Merritt *et al.*, 2010). It has been detected in several aquatic biotopes and its abundance has been correlated to increase disease burden in some studied communities (Williamson *et al.*, 2012, Williamson *et al.*, 2008). Yet how it is transmitted to humans remains unclear. It is worth highlighting that, in these studies, it was the bacterial DNA detected not live bacilli (Merritt *et al.*, 2010). This is because it is quite difficult

to culture *M. ulcerans* from most environmental samples although in one case it was cultured from an aquatic insect (Portaels *et al.*, 2008). Comparative genomic analyses suggest that *M. ulcerans* is probably evolving from a generalist-environmental bacterium to a niche-adapted specialist in the mammalian host (Stinear *et al.*, 2007). Within an aquatic environment, *M. ulcerans* can be found at the air-water interface, form biofilms on surfaces and probably occupy microhabitats not directly exposed to light but aerated (Stinear and Johnson, 2007). Like most NTMs, *M. ulcerans* proliferates in aquatic environments with pH ranging from 5.5-6.5 (Radomski *et al.*, 2010). A few studies suggested that *M. ulcerans* could be transmitted to susceptible hosts via aerosols generated from activities in contaminated water bodies, inoculation via an open skin or even through drinking of water containing bacilli (Ross *et al.*, 1997). However, experimental field studies to test these hypotheses were not fine-tuned to adequately identify specific modes of transmission (Merritt *et al.*, 2010). Such studies would have to rely on advances in molecular biology to identify habitats and reservoirs of *M. ulcerans* persistence and proliferation (Williamson *et al.*, 2012). Advances in molecular biology have been used to source track infections, some infections caused by NTMs, to specific environments (Komijn *et al.*, 1999, Tortoli *et al.*, 2001).

2.2.4 *Mycobacterium ulcerans* infections in animals

M. ulcerans is thought to have evolved from *M. marinum* via acquisition of a mycolactone encoding plasmid. Although *M. ulcerans* infection is largely in humans, there have been reported cases in some animals (Fyfe *et al.*, 2010). *M. ulcerans* infection with clinical presentations similar to those of humans have been observed in cats (Elsner *et al.*, 2008), koalas (Mitchell *et al.*, 1984), possum (Fyfe *et al.*, 2010), horses (van Zyl *et al.*, 2010), turtle (Sakaguchi *et al.*, 2011), armadillo (Walsh *et al.*, 1999), etc. In each case, the isolated *Mycobacterium* was similar to the human pathogen, M. ulcerans (Fyfe *et al.*, 2010). Furthermore, *M. ulcerans* DNA have been detected in

mollusks, crustaceans and mosquitoes (Eddyani *et al.*, 2004, Johnson *et al.*, 2007). Experimental studies by Addo *et al*. in Ghana (2007), showed that indigenous grass-cutters are susceptible to *M. ulcerans* infection. These reports and studies suggest that small mammals, living in close proximity to humans and commonly hunted animals, like grasscutters, rabbits, could be potential reservoirs of *M. ulcerans*, posing future risks of zoonosis (Addo *et al.*, 2007). Hence effective control of the disease would involve, in part, the identification of both feral and domestic animal reservoirs.

2.2.5 Clinical pathology, diagnosis and treatment

M. ulcerans is a slow-growing *mycobacterium*, generation time of about 52 hours, and hence infection progresses slowly with no visible signs of trauma (Hayman, 1985). The skin is a good target for infection as its relatively lower temperature favours the growth of the bacterium, with optimum temperature of 28-32°C (Hayman, 1985, Matsumura *et al.*, 2012). Mycolactone production by *M. ulcerans* during infection is largely responsible for the pathology associated with BU (George *et al.*, 2000). The molecule is both immunosuppressive and cytotoxic, causing patients to feel minimal pains at most stages of the disease (Hong *et al.*, 2008). It usually starts as a nodule (Figure 2a) or papule and could be mistaken for a pimple (WHO, 2012). If this is diagnosed early, a few weeks of antibiotics or surgical excision usually terminates the infection. Progression from this stage leads to a painless plague, oedema and finally ulceration of the skin (Asiedu and Etuaful, 1998). Although BU is rarely fatal, it can lead to permanent disabilities like contractures, scarring and deformation in the limb (WHO, 2012). Most infections are localized, especially to the extremities and cooler parts of the body. Systemic disease or dissemination is rare, intimating that 37°C is unfavourable for growth (Matsumura *et al.*, 2012). Histopathological examinations of stained sections from excised lesions showed that *M. ulcerans* is extracellular. However, some

experimental studies observed intracellular bacteria in macrophages, explaining that it could be a transient stage during infection (Schutte *et al.*, 2009, Torrado *et al.*, 2007b).

In Ghana a few healthcare centers and hospitals, in endemic areas, have been resourced by the government to treat BU cases (personal communications). Furthermore, various educational programmes on the disease have been carried out by stakeholders in the health sector under the aegis of the Ministry of Health, Ghana. Diagnosis involves both physical and clinical examinations. The former simply looks for clinical signs associated with the disease while the latter uses various laboratory tests to confirm the diagnosis. Fine Needle Aspiration (FNA) and swab specimen can be taken from suspected nodules and ulcers respectively for laboratory tests. Ziehl-Neelsen staining for acid fast bacilli, culturing on Lowenstein-Jensen or Middlebrook 7H9 media coupled with specific biochemical tests and histopathological examination of stained sections taken from excised lesions can all be used for diagnosis (Bretzel *et al.*, 2005, Mensah-Quainoo *et al.*, 2008, Yeboah-Manu *et al.*, 2011b). However, a more specific and sensitive test is PCR (Mensah-Quainoo *et al.*, 2008).

Early diagnosis, especially at the nodular stage, facilitates rapid termination of infection and prevents disabilities associated with BU (WHO, 2010, WHO, 2012). WHO recommended therapy include combination of rifampicin and streptomycin/amikacin for eight weeks as first-line treatment for all forms of active cases, surgery to remove necrotic tissue, cover skin defects and correct deformities and interventions to minimize or prevent disabilities (WHO, 2012). Other drugs have been shown to be effective both *in vitro* and *in vivo*; clarithromycin, kanamycin, cycloserine, enviomycin and levofloxacin (Matsumura *et al.*, 2012, Nienhuis *et al.*, 2010). Currently, there is no vaccine for BU although a few studies have identified candidate antigens for vaccine development (Einarsdottir and Huygen, 2011, Tanghe *et al.*, 2008).

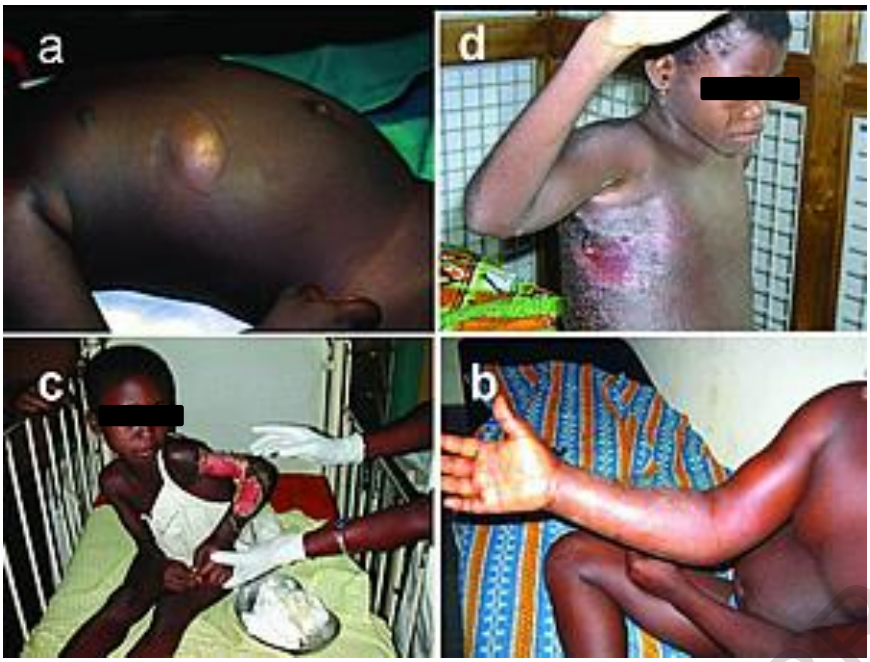

Figure 2 Clinical symptoms of Buruli ulcer disease.

(a) Nodule on abdomen of a young child, (b) non-ulcerative oedematous swelling of child's hand, (c), ulcer covering most of a young child's arm, (d) a plaque. This picture was retrieved from Richard Merritt, Aquatic Entomology Laboratory, Michigan State University.

2.2.6 Control efforts, research priorities and policy implementation

The Global Buruli Ulcer Initiative (GBUI) was established by the WHO in 1998 to raise awareness about the disease, improve access to early diagnosis, treatment and promotion of research to develop better tools for treatment and prevention of BU (WHO, 2012). Most endemic countries including Ghana have adopted WHO guidelines to treat and manage BU patients (WHO, 2012, WHO/CDS/CPE/GBUI, 2001). However, in rural areas, access to healthcare centres is challenging as most patients find it difficult to commute to nearby Centers (Asiedu and Etuaful, 1998). In Ghana, the Ministry of Health in collaboration with some NGOs have carried out programmes

educating residents in endemic areas of various risk factors leading to BU and how to recognize signs of BU. Alternatively, community health volunteers have been trained to do basic wound care services for patients. Research on BU has focused largely on diagnosis and treatment with a few now venturing into ecology and transmission of *M. ulcerans* (Benbow *et al.*, 2008, Mosi *et al.*, 2008, Williamson *et al.*, 2012, Williamson *et al.*, 2008). Transmission studies are taking advantage of tools in molecular biology to elucidate possible routes of transmission (Williamson *et al.*, 2012). Studies on the immunology and host-bacteria interaction are scarce relative to Tuberculosis and other diseases of public health importance. There is therefore the need to raise awareness about the disease especially in endemic communities. More funding is needed to develop molecular epidemiological tools to monitor transmission including improved diagnosis and develop capacity building in academic and research laboratories using multidisciplinary research approaches.

2.3 Molecular Microbiology

2.3.1 Genome and genetic makers for studying transmission of *M. ulcerans*

M. ulcerans has 2 circular replicons, a 5,631,606-bp chromosome, Agy99 and a 174,155-bp plasmid, pMUM001 (Pidot *et al.*, 2008). The plasmid contains 4 copies of IS2404 and 8 copies of IS2606, both are used as genetic markers (Williamson *et al.*, 2008). Also prominent are 81 coding domain sequences (CDS), of which, enoyl reductase (ER) and keto reductase (KR), two keys enzymes involved biosynthesis of mycolactone, are used as genetic markers as well. It has an average G+C content of 62.5% (Stinear and Johnson, 2007). The chromosome has 209 and 83 copies of IS2404 and IS2606 respectively and 4,281 CDS. It has an average G+C content of 65.72% (Stinear and Johnson, 2007, Stinear *et al.*, 2007). There are numerous DNA sequences,

within functional and non-functional genes, which have been employed to differentiate strains of *M. ulcerans* from other MPM (Ablordey *et al.*, 2005a). These include Variable Number Tandem Repeats (VNTRs) like locus 6, locus 19, locus 1, ST1, MIRU1, etc. (Ablordey *et al.*, 2005b, Roltgen *et al.*, 2010) and a few housekeeping genes for Multilocus sequence typing (MLST). The mycobacterial 16S rRNA gene (encodes 16S rRNA), is of great evolutionary importance and has been widely used to trace phylogenies and differentiate strains of NTM (Clarridge, 2004, Janda and Abbott, 2007, Portaels *et al.*, 1996). Despite the currently available tools for studying transmission, it worth highlighting that, there are limited advanced genetic tools, as compared to malaria and tuberculosis studies, for detecting emergence of resistance strains and carrying out molecular epidemiological studies (Merritt *et al.*, 2010).

2.3.2 Genetic tools for studying transmission

Advances in molecular biology, genomics, proteomics and bioinformatics have greatly accelerated and thrust research forward into understanding the molecular mechanisms of infection, pathology and treatment of various mycobacterial diseases, notably TB and BU (Walsh *et al.*, 2010). Sequencing of *M. ulcerans*, strain Agy99 genome has significantly improved studies on diagnosis and transmission (Stinear *et al.*, 2000b). Consequently, various genetic tools for research and clinical purposes have been developed and documented (Merritt *et al.*, 2010).

2.3.2.116S rRNA gene sequencing.

The mycobacterial 16S rRNA (encodes 16S rRNA), is of great evolutionary importance and has been widely used to trace phylogenies, differentiate strains of NTM (Clarridge, 2004, Janda and Abbott, 2007, Portaels *et al.*, 1996) and to resolve ambiguities in bacterial nomenclature (Janda and Abbott, 2007). The 16S rRNA gene is about 1.5 kbp and has both conserved and variable

regions across different taxa (Clarridge, 2004). Universal or species-specific mycobacterial primers complementary to conserved regions are used in a PCR reaction to amplify a 500bp or 1,500bp segment of the 16S rRNA gene (Clarridge, 2004). Sequence comparisons of either the 500bp or 1,500bp to published sequences can be used to identify isolates to the species level and generate phylogenetic trees with similar topologies (Clarridge, 2004). This tool has been used in various fields for accurate disease confirmation and study transmission (Janda and Abbott, 2007, Portaels *et al.*, 1996).

2.3.2.2 IS2404 and IS2606 PCR and RFLP analyses

Sequence analyses of the *M. ulcerans* reference genome, Agy99, have revealed that both IS2404 and IS2606 are abundantly present on both the chromosome and plasmid (Pidot *et al.*, 2008). Consequently, primers targeting these sequences have been designed for use in PCR detection of *M. ulcerans* in clinical, veterinary and environmental isolates (Phillips *et al.*, 2005, Ranger *et al.*, 2006b, Stinear *et al.*, 2000a, Yeboah-Manu *et al.*, 2011a). RFLP of amplified IS2404 products have been used to genotype *M. ulcerans* from different geographical isolates (Chemlal *et al.*, 2001). Based on RFLP-IS2404, Chemlal *et al*. (2001) showed that six distinct genotypes of *M. ulcerans* can be differentiated corresponding to the different geographical origins. However, recently, other MPMs have been shown to harbour copies of IS2404 (Suykerbuyk *et al.*, 2007) and hence definite diagnosis of *M. ulcerans* infection for research purposes employs additional markers.

2.3.2.3 Multilocus sequence typing (MLST)

In Multilocus sequence typing (MLST), sequences from different housekeeping genes are compared simultaneously (Stinear *et al.*, 2000b). MLST analysis of *M. ulcerans* isolates from

different geographical areas yielded six different genotypes related to the six geographical areas of Surinam, Papua New Guinea, Mexico, China, Africa and Australia (Stinear *et al.*, 2000b). This was consistent with findings by Chemlal *et al*. (2001) using RFLP-IS2404. Similarly, comparative genomics studies of *M. ulcerans* and *M. marinum* genomes, showed recent divergence of the former from *M. marinum* by the acquisition of a plasmid (Stinear *et al.*, 2000b).

2.3.2.4 Variable Number Tandem Repeats (VNTR-typing)

Variable Number Tandem Repeats (VNTR) is a location in the genome where short sequences of DNA occur in tandem repeats (Ablordey *et al.*, 2005b). These repeats which vary in number per genome can be used to differentiate between related species. In *M. ulcerans* and other MPMs, numerous VNTRs both within functional and non-functional genes have been identified at specific loci in the reference genome, Agy99 (Ablordey *et al.*, 2005a, Ablordey *et al.*, 2005b). PCR reactions targeting loci like, locus 6, locus19, MIRU1 and ST1, all with variable repeats, have successfully been used to differentiate *M. ulcerans* from other MPMs harbouring IS2404 (Stragier *et al.*, 2007). It has been used to resolve the apparent genetic homogeneity within/between geographical isolates. A study in Ghana, by Hilty *et al*. (2006) using a combination of two polymorphic VNTR loci, ST1 and MIRU1, on 72 African isolates, including 57 *M. ulcerans* isolates from Ghana, revealed three different genotypes with clonal clustering, suggesting genetic diversity of *M. ulcerans* in Ghana. In this study, the authors reported two strains of MU in the Amansie West, now Amansie Central District of Ghana. One strain had a repeat of 2 for ST1 and 1 for MIRU1, genotype (2, 1) and the other had (1, 3). This suggests that addition of other polymorphic loci could increase the discrimination power revealing more genotypes.

2.3.2.5 Single Nucleotide Polymorphisms (SNPs) and microarray analysis

Single Nucleotide Polymorphism (SNP typing), detects a single base pair mutation at a specific locus, revealing genetic variations between members of a species. Using this tool, various techniques from hybridization to enzyme-based methods and sequencing have been employed. Owing to the existing lack of genetic variability among *M. ulcerans* geographical isolates, Kaser *et al*. (2009b) employed SNP analysis to genotype 83 *M. ulcerans* isolates from African countries. They identified SNPs that differentiated between regional strains (Kaser *et al*., 2009b). Following their success, Roltgen *et al*. (2010) developed a real-time PCR SNP typing method to genotype *M. ulcerans* patient isolates collected from different parts of Ghana. In one case, the authors observed dominance of one local complex and its clustering along the Densu River, suggesting possible focal transmission. Large scale application of SNP typing for epidemiological studies would involve the use of DNA microarrays on chips (Kaser and Pluschke, 2008). This technique has been used to differentiate *M. tuberculosis* from *M. bovis* BCG through the identification of 18 regions of diversity (RD1-RD18) (Behr *et al*., 1999, Gordon *et al*., 1999).

2.3.2.6 Databases and bioinformatics tools for sequence alignments

VNTR profiles of a few MPM have been elucidated and published. This can be used as reference strains to correctly assign repeat numbers based on PCR product sizes (Lavender *et al*., 2008, Williamson *et al*., 2008). Several databases are currently available with partial and complete sequences of numerous mycobacterial genomes. Publicly available ones include the National Center for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov/), the European Molecular Biology Laboratory (EMBL) database (www.ebi.ac.uk/embl/) and the DNA database of Japan (DDBJ) database (http://www.ddbj.nig.ac.jp/). Notably, the genomes of *M. tuberculosis*,

M. leprae and *M. ulcerans* have been deposited in public databases like GenBank, TubercuList and BuruList. Other databases like RIDOM, Microseq and RDP-II contain sequences of some NTM. Information from these databases is freely accessible. Software packages like Clustal X2, MEGA 5.1, PAUP, BLAST, Phylip and BIBI (Clarridge, 2004) and many others can be used for sequence comparisons and phylogeny studies. Stinear and colleagues, in a comparative genome analysis of *M. ulcerans* and *M. marinum* showed significant differences in genome size, insertion sequences, and pseudogenes, and inferred the function of putative genes from the well-studied *M. tuberculosis* genome (Stinear *et al.*, 2000b). By aligning *M. ulcerans* genome, Agy99 sequence, to closely related genomes, *M. marinum*, *M. leprae* and *M. tuberculosis*, several genetic markers have been identified and developed for accurate diagnosis and transmission studies (Huber *et al.*, 2008).

CHAPTER THREE

3.0 Materials and methods

3.1 Description of selected sub-district and communities

The Amansie Central District, with Jacobu as its capital has five Sub-districts: Fiankoma, Numereso, Jacobu, Twapease and Fenehia. All four communities in which the questionnaires were administered are in the Numereso sub district. The Numereso sub-district has about 26 communities with Numereso as the capital. The health center also collaborates with governmental or non-governmental organizations in the screening and treatment of many diseases of public health importance in the sub district. This sub-district is endemic for Buruli ulcer (BU). The four communities selected from this sub district were Bepotenten, Sukuumu, Monia-Gyaman and Wromanso. BU prevalence in this communities were quite high with rates of 12.1, 7.8, 6.6 and 6.4 (per 1000 of population) for Bepotenten, Sukuumu, Monia-Gyaman and Wromanso respectively. Majority of the inhabitants in all four communities are farmers but some youth are into illegal mining along the Offin River which runs through all four communities. There is at least one working borehole with pump in each community but inhabitants still fetch water from water bodies for domestic and agricultural activities. This study is one facet of a multi-faceted project encompassing a "OneHealth" approach to studying NTM transmission (focus on BU).

Table 1 Buruli ulcer prevalence in some communities within the Amansie Central District.

District	Community	Population	BU cases	Prevalence (%)
Numereso	**Sukuumu**	1406	11	0.78
	Wromanso	312	2	0.64
	Bepotenten	247	3	1.21
	Monia Gyaman	454	3	0.66
Fenehia	Fenaso	578	2	0.35
	Mile 14	1411	2	0.14
Nyankoma	Abuakwaa	1181	2	0.17
	Abradei	201	2	1.00

Studied communities are in bold.

3.2 Study approval

3.2.1 Ethical clearance and community entry

Ethical approval (Appendix 2) was obtained from the Institutional Review Board of the Noguchi Memorial Institute for Medical Research, University of Ghana. Study was explained in the local language by the Assembly man or woman to all inhabitants within each community. Permission was sought from respective chiefs and opinion leaders. All patients and parents on behalf of their children, signed a consent form (Appendix 2) before enrolling into the study. Patients who were confirmed positive for BU were put on 52 days of antibiotic treatment by the Amansie Central Health Directorate.

3.2.2 Description of questionnaire

The questionnaire bordered on perceived transmission of BU, treatment modalities, information on water sources, agricultural activities and many others (Appendix 2). The number of questionnaires administered in each community was calculated using the sample size calculator for questionnaire survey Epi Info V7. Settings for alpha, power and expected frequency was 0.05,

80% and 50% respectively. Sampling for community respondents was randomized. A copy of the questionnaire is provided in Appendix 2. Data from this informed my choice of specific water contact sites to sample (Table 2).

Table 2 Water bodies most contacted by community members.

community	Sukuumu	Wromanso	Monia-Gyaman	Bepotenten
Water bodies	Offin River Akotia stream Twingum Pond 1 Twingum Pond 2	Offin River Bebonu pond	Offin River Akotia stream Ampomaa pond	Oda River Offin River[a]

a means not sampled

Figure 3 Water bodies that were sampled.

A) Twingun 2 pond at Sukuumu, B) illegal mining activities on the Offin River at Monia-Gyaman, C) Nkotia stream at Sukuumu/Yawkrakrom, D) Bebonu pond at Wromanso.

3.3 Specimen collection

3.3.1 Environmental sampling

Sampling of each water body was performed following the procedures described by Williamson *et al*. (2008) with slight modifications. At a water body, four matrices (water filtrate, biofilm, detritus and soil) were collected. Soil, water filtrate and detritus were collected in triplicates and biofilm was collected in quintuplicates. For soil, a sterile scalpel was used to pick about 4000mg (3X), one from the water floor and two from the riparian zone 5m apart. The soil samples were preserved in a 15ml falcon tube (BD Biosciences) containing 10ml of absolute ethanol (Pharmacos). Detritus consisted of dead plant leaves, stems and grass blades within the water body. Portions, about 10cm long or appreciable sizes were cut and put in a 15ml falcon tube containing 13ml of absolute ethanol. In the case of biofilms, stems and leaves of some dominant aquatic plants were cut and put into a ziplock bag containing 100ml of double distilled water. The bag was sealed air-tight and biofilm dislodged by shaking and robbing the bag vigorously several times. Fifty milliliters (50ml) of resulting mixture was then poured into a 50ml falcon tube. For water filtrates, first, a clean bowl was used to scope the surface water covering an area of about $1m^2$. Fifty milliliters of this was poured into a 50ml syringe fitted with a 0.6 micron fiberglass filter (Whatman Inc), lined with a 0.45µm nitrocellulose filter (Whatman Inc). The water was then pumped through until built resistance could not be overcome. The nitrocellulose filter was then removed and covered completely with aluminum foil. All matrices were well labeled and preserved on ice packs, in a cooler, in the field. Samples were later transported to the lab and preserved at 4 °C until processed for DNA extraction (described below). For each site, all materials were washed and decontaminated with 70% bleach and, sterilized with 90% ethanol and DNA away (Molecular BioProducts).

Figure 4 Environmental sampling of different matrices.

A) Filter, B) Biofilm, C) Filter on aluminum foil, D) Soil preserved in ethanol.

3.3.2 Sample collection from patients

There was a case search for people with signs of BU, nodule, oedema and ulcer. Fine Needle Aspiration (FNA), from nodules and swabs from ulcers were taken by a well-trained Laboratory Technician from the St. Peters Hospital, Jacobu, Amansie Central District. FNA was kept in sterile 2ml screw-cap (STARSTEDT) tubes and swabs in 15ml conical tube. All samples were kept on ice packs in a cooler. Table 5 (section 4.3.1) shows the clinical history of all patients from whom specimen were taken.

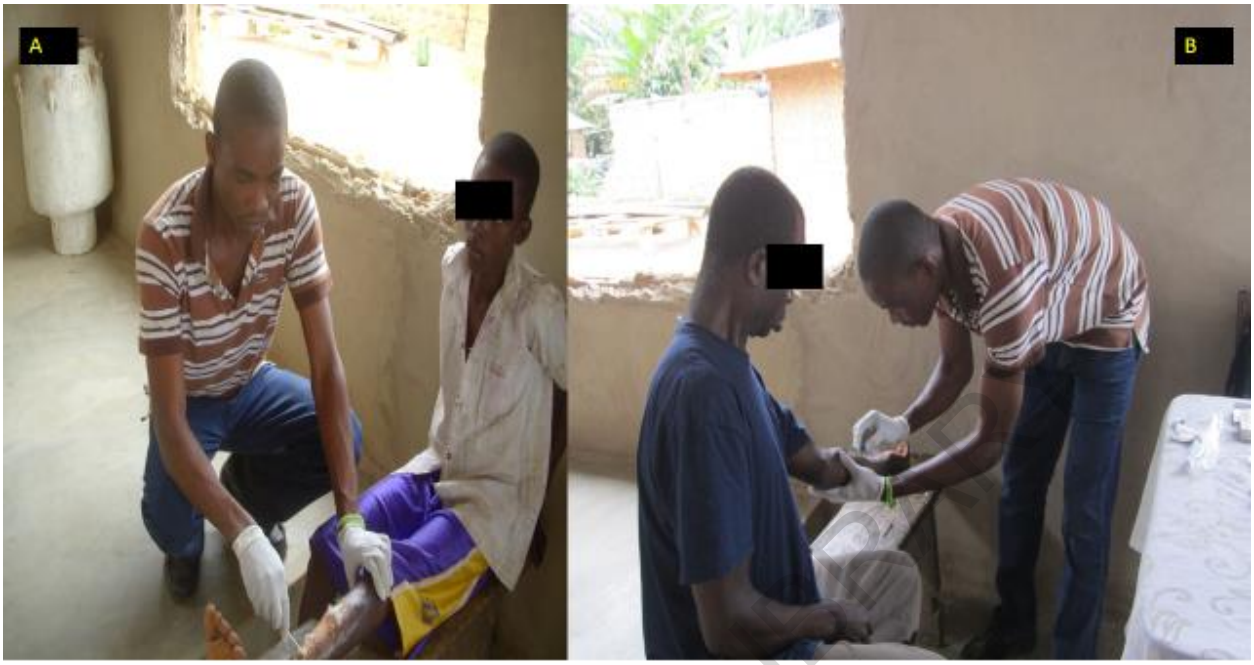

Figure 5 Specimen from patients.

A) Swabbing of ulcer, B) FNA

3.4 Sample processing for laboratory analyses

All samples were processed prior to microscopy and DNA extraction.

3.4.1 Biofilm

Biofilm was concentrated based on an optimised protocol developed in the laboratory. Briefly, the 50ml falcon tube containing the biofilm was spun at 12,000 rpm for 5 minutes in a High Speed Refrigerated Centrifuge at 4°C (Suprema 21, TOMY). Twenty milliliters of this was slowly decanted and the remaining spun again at 13,000 rpm for 5 minutes. Fifteen milliliters of this was decanted and the rest spun at 14,000 rpm for 5 minutes. Five milliliters of this was pipetted off and the remaining 10ml was preserved at 4°C. For DNA extraction, 1000µl was centrifuged in a 2ml

screw-cap tube at 14,000 rpm in a mini Centrifuge (Centrifuge 5415D, Eppendorf) for 5 minutes, 700µl of the supernatant was pipetted off and the remaining 300µl with pellet was used for DNA extraction.

3.4.2 Detritus

A piece (about 700mg) of the detritus (preserved in absolute ethanol) was cut with scissors into a 2ml screw-cap tube and 400µl of the used ethanol to preserve it was added. The tube was then centrifuged at 14,000 rpm for 5 minutes, 300µl of ethanol was pipetted off and the remaining, with detritus, used for DNA extraction (described below).

3.4.3 Soil

The Falcon tube containing soil sample, preserved in ethanol, was vortexed vigorously and 500ml was pipetted into a 2ml screw-cap tube. The tube was then spun at 14,000 rpm for 5 minutes, all the supernatant, with just enough to cover soil surface, was pipetted off and the remaining was used for DNA extraction.

3.4.4 FNA and swabs

Two milliliters of 1X Phosphate Buffer Saline (PBS) was added to 300µl of FNA in a 2ml screw-cap tube. For swabs, 2ml of 1X PBS was pipetted into the 15ml conical tube. It was then vortexed for 5 minutes to dislodge bacterial cells and later transferred to a 2ml screw-cap tube. For both samples, 1ml was pipetted into a new 2ml screw-cap tube. The tube was then vortexed briefly and spun at 14,000 rpm for 5mins. Later, 800ul of supernatant was pipetted off and the remaining 200µl (plus pellet) was used for DNA extraction. The remaining 1ml was for preserved at 4ºC for later use.

3.5 Ziehl-Neelsen staining for acid fast bacilli (AFB)

AFB was performed following the method describe by Yeboah-Manu *et al*. (2004) with slight modifications. Briefly, about two drops of specimen was smeared on a glass slide (Micro slide glass, MATSUNAMI) and gently passed over flame until smear was fixed. The slide was then flooded with carbol fuchsin and left for 5 mins. The carbol fuchsin was later washed off with distilled water and stain was decolorized with acid alcohol (3% hydrochloric acid). The slide was then flooded with methylene blue and left for 2 mins. Stain was washed off completely and the slide was air-dried. Slide was viewed under a light microscope (Olympus, CX21FS1), in oil immersion at a magnification of X1000. Picture was taken using a camera (WRAY CAM G500).

3.6 Molecular microbiology

3.6.1 DNA extraction

DNA extraction for human and animal specimen were performed with the Qiagen Dneasy blood and tissue kit (QIAGEN) following the manufacturer's protocol. Briefly, 850ul of 1X PBS was added to 150ul of FNA. From this, 500ul was aliquoted into a 2ml screw cap tube, centrifuged at 14,000 rpm for 15mins to pellet bacterial cells and 300ul of supernatant was pipetted off. To the remaining, 180ul of buffer ATL and 20ul of proteinase K were added. The mixture was vortexed briefly and incubated at 56°C for 3 hours. Later, 200ul of buffer AL was added. The mixture was vortexed briefly and incubated at 70°C for 30 mins. To this, 250ul of 100% ethanol was added. The mixture was vortexed briefly and transferred to a spin column. The column was then centrifuged at 8,000 rpm for 1 min and flow-through was discarded. It was then washed by adding 500ul of buffer AW1, centrifuged at 8,000 rpm for 1 min and flow-through discarded. Washing was repeated but with buffer AW2. The column was dried by spinning at 13,000 rpm for 3mins. It

was then put into freshly labelled 1.5ml tubes, 150ul of buffer AE added and centrifuged at 8,000 rpm to elute DNA. The DNA was stored at -40°C until further use.

For environmental samples, DNA was extracted following the protocol described by Williamson *et al*. (2008) with slight modifications. For each processed matrix (biofilm, soil, detritus or filtrate), 450µl of Lysis buffer (0.1M Tris (pH 8.0), 0.05M EDTA, 0.5M NaCl, 1.3% SDS and 50µg/ml RNase A) and 1g of glass beads (Sigma-Aldrich) were added in a 2ml screw-cap tube. This was followed by bead-beating on a vortexer (Mini vortexer, VMR Scientific Products) for 10 minutes. The sample was then incubated at 65°C in a water bath, spun at 5,600 rpm for 2 minutes and 400ul of supernatant was transferred to a new 1.5ml tube (Posi-click tube, Denville). To this, 150µl of 5M potassium chloride was added and incubated at -40°C for 3 hours. The Sample was later centrifuged at 5,600 rpm for 30 minutes and 400µl of supernatant transferred to a new 1.5ml tube containing 600µl of binding buffer (1M Guanidium hydrochloride and 63% V/V absolute ethanol). The tube was vortexed briefly, 5 seconds, and 700µl resulting mixture transferred to a spin column (MOBIO). This was spun at 5,600 rpm for 2 minutes and the flow-through discarded. The spin column was washed by adding 500µl of washing buffer (100mM Tris (pH 8.0), 1mM EDTA, 500mM NaCl and 67% V/V absolute ethanol) and spun at 5,600 rpm for 2 minutes and the flow-through discarded. The procedure was repeated again but with 100% ethanol. The column was spun at 5,600 rpm for 5 minutes to dry. To elute DNA, the column was placed in a new 1.5ml tube, 200µl of elution buffer (10mM Tris (pH 8.0)) was added to the column and incubate at room temperature for 5 minutes. It was then spun at 5,600 rpm for 2 minutes to elute DNA. The DNA was stored at -40°C until further use.

3.6.2 Polymerase chain reaction (PCR)

For each PCR run, both negative and positive controls were included. Additionally, for environmental samples, spiked samples (2.5µl each of positive control and an environmental extract) were included to check for inhibitions. Subsequently, for environmental samples, Bovine Serum Albumin (BSA, Promega) was added to relieve PCR inhibition in the amplification of all studied loci. A 2720 Thermal Cycler (Applied Biosystems) was used for all PCR reactions. All primer sequences are listed in Table 3 below.

Table 3 List of primers used for PCR amplifications.

Primer	Forward and reverse sequences	expected sizes	References
IS2404 (nest 1)	pGp1: 5'-AGGGCAGCGCGGTGATACGG-3' pGp2: 5'-CAGTGGATTGGTGCCGATCGAG-3'	400	(Ablordey *et al.*, 2012)
IS2404 (nest 2)	pGp3: 5'-GGCGCAGATCAACTTCGCGGT-3' pGp4: 5'-CTGCGTGGTGCTTTACGCGC-3	200	(Ablordey *et al.*, 2012)
16S rRNA	PA: 5'AGAGTTTGATCCTGGCTCAG 3' MSHA: 5'AAAAAGCGACAAACCTACGAG 3'	600	(Hughes *et al.*, 1993)
ER	F-5' GAGATCGGTCCCGACGTCTAC 3' R-5' GGCTTGACTCATGTCACGTAAG 3'	719	(Williamson *et al.*, 2008)
Locus 6	F-5' GACCGTCATGTCGTTCGATCCTAGT 3' R-5' GACATCGAAGAGGTGTGCCGTCT 3'	variable	(Williamson *et al.*, 2008)
Locus 19	F-5' CCGACGGATGAATCTGTAGGT 3' R-5' TGGCGACGATCGAGTCTC 3'	variable	(Williamson *et al.*, 2008)
ST1	F-5' CTGAGGGGATTTCACGACCAG 3' R-5' CGCCACCCGCGGACACAGTCG 3'	variable	(Williamson *et al.*, 2008)
MIRU1	F-5' GCTGGTTCATGCGTGGAAG 3' R-5' GCCCTCGGGAATGTGGTT 3'	variable	(Williamson *et al.*, 2008)

3.6.2.1 IS2404 detection

Amplification for IS2404 loci was performed in a nested PCR, adapted from Ablordey *et al*. (2012) with slight modifications. The first reaction was done in a 25µl reaction containing 1X PCR buffer

(Promega), 1mM $MgCl_2$ (Thermo Scientific), 300µM each of deoxyribonucleotide (Thermo Scientific), 700nM of each primer, Pg1 and Pg2, 1U GoTaq Polymerase (Promega) and 5ul of genomic DNA. In nest 2, 1µl of PCR product from nest 1 was used as template in a 25µl reaction containing similar concentrations of reagents as in nest 1 except for the primers; 500nM each of, Pg3 and Pg4. Preheating was done at 95°C for 2mins, followed by 40cycles each of, denaturation at 94°C for 30 seconds, annealing at 64°C for 1min and extension at 72°C for 1.5min. Final extension was at 72°C for 10mins and reaction held at 4°C.

3.6.2.2 PCR amplification of ER and 16S rRNA

Both ER and 16S rRNA genes were amplified separately as described by (Williamson *et al.*, 2008) with slight modifications, using similar master mix concentration. This was performed in a 25µl reaction containing 1X PCR buffer, 1.5mM $MgCl_2$, 400µM each of deoxyribonucleotide, 160nM each of forward and reverse primers, 1U GoTaq polymerase and 5µl of genomic DNA. For environmental samples, 300ng/µl BSA was added to relieve inhibition. Cycling for ER was done at 94°C for 3mins, followed by 35 cycles each of, denaturation at 94°C for 1 min, annealing at 58°C for 45s and extension at 72°C for 1 min. Final extension was at 72°C for 10mins and reaction held at 4°C. For 16S rRNA, it was done at 95°C for 5mins, followed by 34 cycles each of, denaturation at 94°C for 45s, annealing at 56°C for 45s and extension at 72°C for 45s. Final extension was at 72°C for 10mins and reaction held at 4°C.

3.6.2.3 PCR for VNTR loci

PCR for VNTRs was adapted from (Hilty *et al.*, 2006, Williamson *et al.*, 2012) with slight modifications. Four loci were amplified in separate reactions but with similar master mix concentrations as described above for ER and 16S rRNA amplifications. Thermal cycling, 95°C

for 2mins, 40 cycles each of, 94°C for 1 min, 58°C for 1 min and 72°C for 1 min, 72°C for 10mins and held at 4°C, was same for locus 6, locus 19 and MIRU1 loci. That of ST1 was cycled for 95°C for 2mins, 40 cycles each of, 94°C for 30s, 65°C for 30s and 72°C for 1 min, 72°C for 10mins and held at 4°C.

3.6.2.4 Gel electrophoresis and UV visualisation

For electrophoresis, 7µl of PCR products were run on a 2% agarose (Sigma-Aldrich) gel, stained with ethidium bromide (Sigma-Aldrich), using 1X Tris Acetate EDTA (0.8mM Tris, 0.8mM glacial acetic acid, 10mM EDTA) as running buffer, at 100V (Power PAC 3000, BIO RAD) for 50mins. The gel was visualised under a UV transilluminator (ClearView, Cleaver Scientific Ltd). For each run, 100bp ladder was loaded alongside. The gel picture was taken using a Gel Logic Imaging System (Kodak) and band sizes were independently scored by two Laboratory Assistants.

3.6.3 VNTR Analysis and strain designation

Repeat numbers for all VNTR loci were calculated based on published data (Ablordey *et al.*, 2005a, Hilty *et al.*, 2007, Hilty *et al.*, 2006, Lavender *et al.*, 2008, Stragier *et al.*, 2005, Williamson *et al.*, 2008). Table 4 shows PCR product sizes and corresponding repeats for each VNTR locus.

Table 4 PCR product (band) sizes and corresponding repeats.

VNTR Loci	Bands (bp) (Repeat)				Repeat length (bp)
MIRU1	404 (1)	457 (2)	510 (3)	563 (4)	53
Locus 6	454 (1)	509 (2)	565 (3)	621 (4)	56
ST1	369 (1)	423 (2)	477 (3)	531 (4)	54
Locus 19	288 (1)	344 (2)	400 (3)	456 (4)	56

3.6.4 Sequence and data analyses

PCR products (40ml), two for each locus, were sent for sequencing (Macrogen Inc, Netherlands). Forward primers for all loci were used for sequencing. Sequences were aligned using NCBI basic local alignment of sequences tool (BLAST) (Altschul *et al.*, 1990). Sequence similarity to database sequences were based on expect value (E), maximum identity and score, query coverage and total score. Multi sequence alignments and phylogenetic analyses were performed with MEGA V5 (Tamura *et al.*, 2011). Tandem repeats were analysed using Tandem Repeat Finder (Benson, 1999). Data from questionnaire was analyzed with Epi Info version 7. The map of study communities was drawn with Arc GIS. Flow of Offin River was sketched with the aid of satellite images and GPS coordinates from Google Earth. The graphs were drawn in Microsoft Excel.

CHAPTER FOUR

4.0 Results

4.1.1 Questionnaire

Although entire questionnaire was analyzed, only relevant data was extracted for this study was extracted. Majority of respondents, 95% (Appendix 1) were engaged in agricultural activities, particularly farming. Activities around water bodies was mainly fishing (55%) and (bathing/swimming). Each community had at least one working borehole with pump, hence the lower numbers (26%) who drank from these water bodies.

4.1.2 Acid-fast bacilli staining

Figure 6 shows AFB for two biofilm samples. A shows a cord (clump of bacilli) of acid fast bacilli in a biofilm sample and B shows individual bacilli (detritus sample) as shown by the arrow.

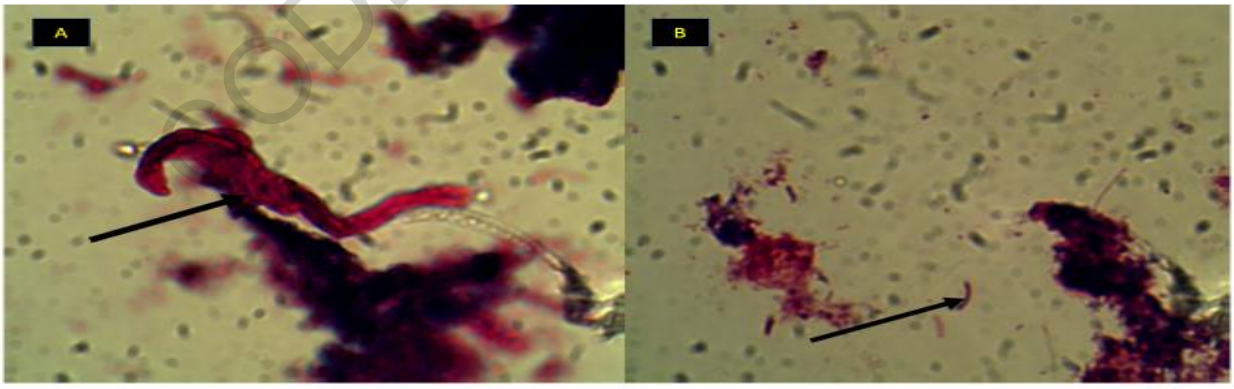

Figure 6 Comparison of AFB in two environmental samples.

4.2 Presumptive diagnosis

4.2.1 ER and IS2404 for Patient samples

Table 5 below shows the clinical history and test results of 15 patients who were enrolled in this study. Briefly, 5 were females and the rest males. Out of these 15 patients, 14 were positive for IS2404 (this was considered definite diagnosis of MU). ER PCR was successful for only 5 patient samples.

4.2.2 16S and IS2404 for environmental samples

Table 6 (section 4.3.2) shows the type and total number of environmental samples collected from each water body within the four studied communities. All samples (N=140) were first screened for *Mycobacterium spp* using the 16S rRNA primer. Figure 7 shows both 16S and IS2404 positivity in all 10 water bodies. As expected, 16S positivity was higher (38/140) in all water bodies, NTM present in most except in the Offin contact site at Monia-Gyaman. The IS2404 discriminated MPM from these NTM and hence positivity was lowered (25/38), in all water bodies, as compared to the 16S. Figure 8 shows 16S and IS2404 positivity in sample matrices. Biofilms were the most positive, (22/38) and (14/25) for 16S and IS2404 respectively. Detritus samples were the least positive, (2/38) and (1/25) for 16S and IS2404 respectively.

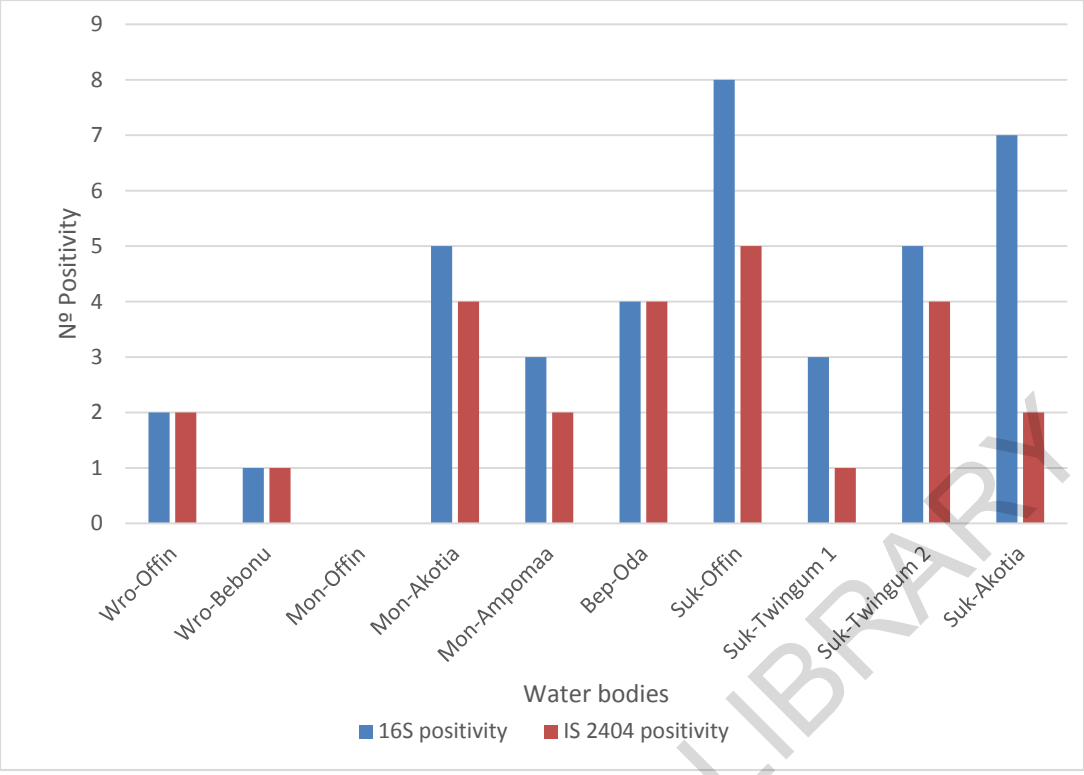

Figure 7 16S and IS2404 positivity in 10 water bodies.

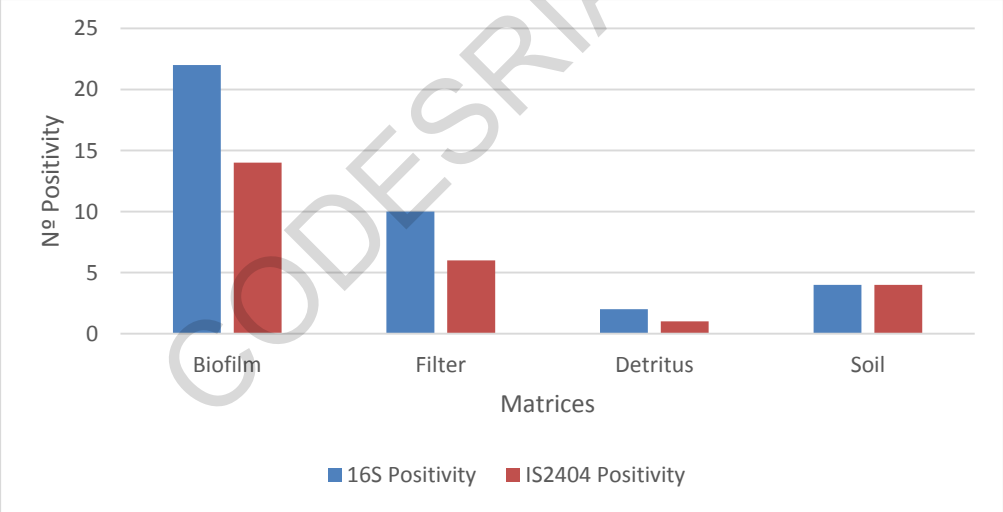

Figure 8 16S and IS2404 positivity in sample matrices.

4.3 Definite diagnosis and genotyping

4.3.1 VNTR analysis of human isolates

VNTR-PCR was performed for IS2404 positive (14/15) human samples. Separate reactions were performed for each of the four VNTR loci described in section 3.6.2.3. PCR was repeated for each locus to reproduce results. Repeat sizes were calculated as described in section 3.6.3. Table 4.1 below shows the VNTR profiles and designated genotypes of human MU isolates. Four genotypes, designated, W, X, Y and Z, were observed for human isolates (Tables 5 & 7). Genotype Y and W were the predominant genotypes (9/14 patients). Three patients, each had MU infection with two genotypes. Figure 9 shows a gel picture of VNTR profile of two patients. Patient FS5 had two isolates (X and W). Band sizes at 280bp (1) and 340bp (2) for locus 19 differentiated the two genotypes.

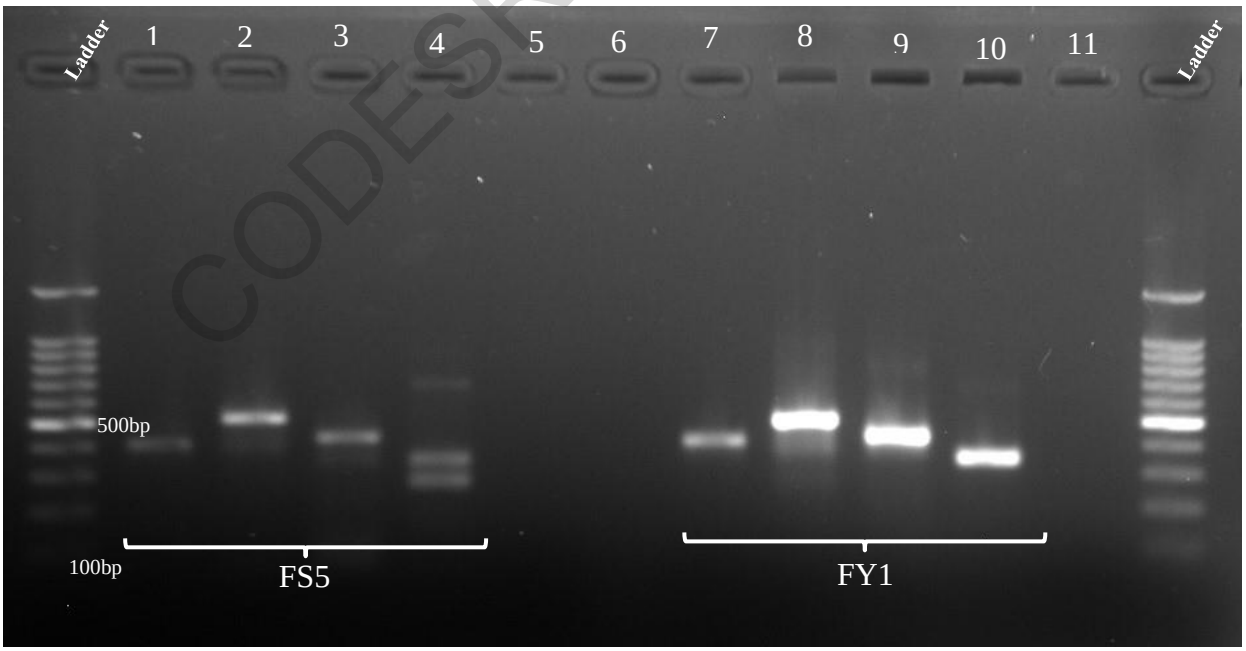

Figure 9 Gel picture showing VNTR profiles of two patient MU isolates.

Lanes 1 and 7 = MIRU1, Lanes 2 and 8 = Locus 6, Lanes 3 and 9 = ST1, Lanes 4 and 10 = Locus 19, Lanes 5 and 11 = Negative controls, Lane 6 = empty well. Patient Profiles, FS5 = **(1, 1, 2, 1)/ (1, 1, 2, 2)** and FY1 = **(1, 2, 2, 2)**.

Table 5 Clinical history of patients and VNTR profile of human MU isolates

Amansie Central		Clinical history			Tests						
					IS2404/ER		VNTR allelic profiles				genotypes
Numeresu Communities	Test ID	sex	Lesion	Specimen	IS2404	ER	MIRU1	Locus 6	ST1	Locus 19	
Wromanso	FW1	female	nodule	FNA	Pos		1	2	2	2	Z
	FW2	male	nodule	FNA	Pos	Pos	1	2	2		Z/Y
Monia-Gyaman	SM1	male	ulcer	swab	Pos	Pos	1		2	1	Y/W
							1		2	2	Y/W
	SM2	male	ulcer	swab	Pos	Pos	1	1	2	1	W
	FM3	female	nodule	FNA	Pos		1	2	2	1	Y
							1	2	2	2	Z
	FM4	male	nodule	FNA	Pos		1	1	2	2	X
Bepotenten	FB1	male	nodule	FNA	Pos		1	2	2	1	Y
	FB2	male	nodule	FNA	Neg						
Sukuumu	FS1	female	nodule	FNA	Pos			1	2	2	X-
	FS2	male	nodule	FNA	Pos			2	2	1	Y-
	FS3	male	nodule	FNA	Pos		1	1	2		X/W
	FS4	male	nodule	FNA	Pos	Pos	1	2	2	2	Z
	FS5	female	nodule	FNA	Pos		1	1	2	2	X
							1	1	2	1	W
	FS6	male	nodule	FNA	Pos		1	1	2		X/W
Sukuumu/Yawkrakrom	FY1	female	nodule	FNA	Pos	Pos	1	2	2	2	Z

Empty cells means amplification was unsuccessful. Genotypes without one locus (absence did not affect type of genotype) were indicated by (-) sign.

4.3.2 VNTR analysis of environmental isolates

Table 6 below shows the VNTR profiles and designated genotypes of environmental MU and other MPM isolates. All MU genotypes (W, X, Y and Z) for human isolates were identical to environmental isolates. Additionally, four previously reported MU genotypes A and B, MMDL and MLF (Table 7) were identified as well. MU genotype Y was identified in all water bodies. Figure 10 shows a gel picture of VNTR profiles of two MU isolates, FB1 and BAB-4 and a reference *M. marinum* strain, *M. marinum* DL. FB1 (patient from Bepotenten) and BAB-4 (biofilm

from Oda River, Bepotenten) had identical profiles **(1, 2, 2, 1)**. Estimation of band sizes (all VNTR loci) was validated by the *M. marinum* DL reference strain **(1, 4, 2, 2)** and confirmed with sequencing. For BAB-4, lane 7, additional bands, suggest presence of other MPM (observed in some other environmental samples). There was band streaking in some lanes. Figure 11 shows the allelic frequencies of VNTR loci. Loci 6 and 19 were polymorphic (2 repeats each) for human isolates and were the determinants in strain discrimination. Conversely, MIRU1 and ST1 were observed to give about five different repeat lengths for environmental samples. Additionally, repeats of 4, 6 and 7 were obtained for ST1 and repeats of 2 and 4 for MIRU1, suggesting presence of other MPMs. These were confirmed with sequencing as previously underlined.

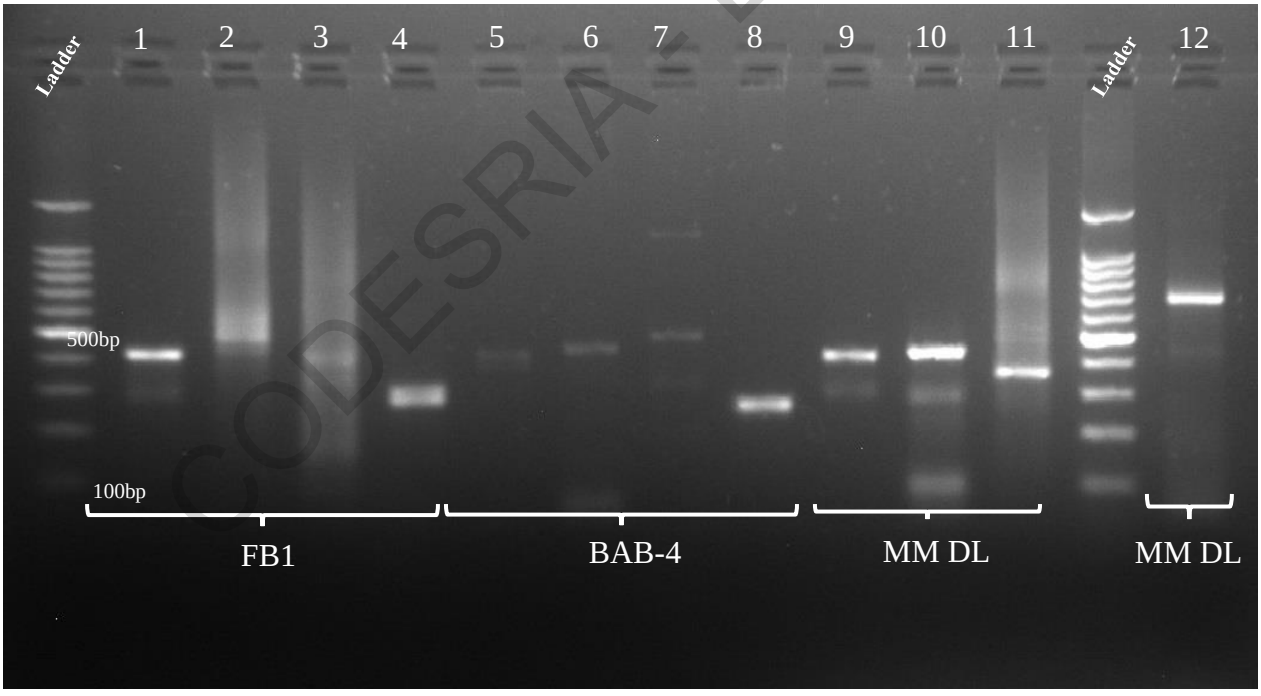

Figure 10 Gel picture of two MU isolates.

FB1and BAB-4, and a reference strain, M. marinum DL. Lanes 1, 5 and 9 = MIRU1, Lanes 2, 6 and 12 = Locus 6, Lanes 3, 7 and 10 = ST1, Lanes 4, 8 and 11 = Locus 19. BAB-4 = **Y (1, 2, 2, 1)**, FB1 = **Y (1, 2, 2, 1)**, MMDL **(1, 4, 2, 2)**.

Table 6 16S rRNA, IS2404 and VNTR analyses of environmental samples.

				Tests						
Community	**Water body**	Matrices	Replicates	Positivity		**VNTR allelic profiles (Repeats)**				**genotypes**
				16S rRNA	IS 2404	MIRU1	Locus 6	ST1	Locus 19	
Wromanso	**Offin**	Biofilm	5	1	1					
		Filter	3	1	1					
		Detritus	3	0	0					
		Soil	3	0	0					
	Bebonu	Biofilm	5	1	1	7	2	2,4,6	1,2	MU(Z,Y), MLF
		Filter	3	0	0					
		Detritus	3	0	0					
		Soil	3	0	0					
Monia-Gyaman	**Offin**	Biofilm	5	0	0					
		Filter	3	0	0					
		Detritus	3	0	0					
		Soil	3	0	0					
	Akotia	Biofilm	5	3	2	1	2	1,2,4,6	1	MU(Y), MLF
		Filter	3	0	0					
		Detritus	3	0	0					
		Soil	3	2	2		2			
	Ampomaa	Biofilm	5	2	1	1	2	6		
		Filter	3	0	0					
		Detritus	3	1	1	1	2	1,2,7	1	MU(Y),MLF
		Soil	3	0	0					
Bepotenten	**Oda**	Biofilm	5	1	1	1	2	2	1,2	MU(Z,Y),MLF
		Filter	3	2	2		2	2		
		Detritus	3	0	0					
		Soil	3	1	1		2			
Sukuumu	**Offin**	Biofilm	5	5	2	1	2	1,2,7	10	MU(X-/Y-), MLF-
		Filter	3	2	2	1	2	7		
		Detritus	3	0	0					
		Soil	3	1	1	1	2	2,7	10	
	Twingum 1	Biofilm	5	1	0					
		Filter	3	2	1	1	0	1		
		Detritus	3	0	0					
		Soil	3	0	0					
	Twingum 2	Biofilm	5	4	4	1,2,3,4	2	1,4	1,2,11	MU(A-/B-,Z/Y),MM(DL),
		Filter	3	1	0					
		Detritus	3	0	0					
		Soil	3	0	0					
	Akotia	Biofilm	5	4	2	1	0	2,4	1,11	MU(W-,X-)
		Filter	3	2	0					
		Detritus	3	1	0		1			
		Soil	3	0	0					
	Total Number of samp		**140**	**38**	**25**					

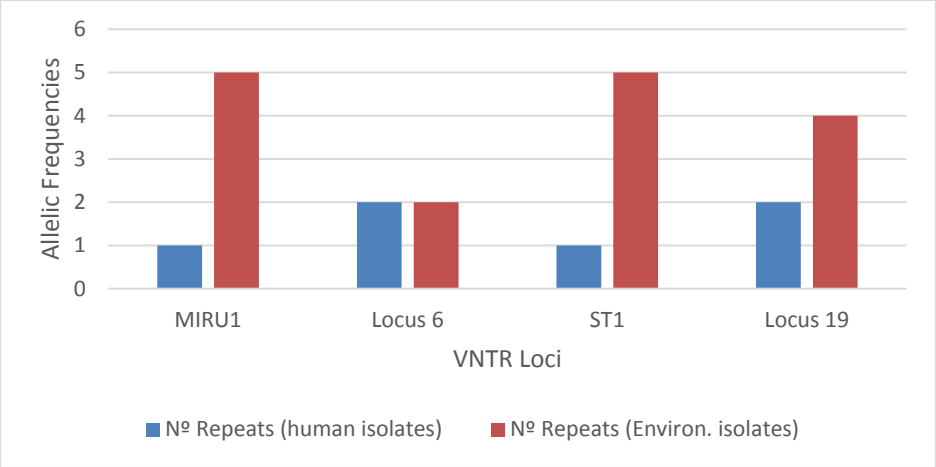

Figure 11 Allelic frequency of VNTR loci for MPM human and environmental isolates.

4.3.3 Isolated MPM and strain designation of genotypes.

Table 7 shows the VNTR profiles and MPM strain designated genotypes of this study and from published data. It was observed that repeat variation for Locus 6 and Locus 19 were similar to published genotypes except for MIRU1. For MIRU1 and ST1, a common repeat of 1 and 2 respectively, were present in all human isolates and was similar to the Amansie West strain 2 were the current study was conducted. Table 8 shows designated genotypes for MPM isolated from humans and the environment. Genotype Y was shared by all human and environmental isolates from all four communities. Genotypes W, X, Y, and Z were identified in humans and water bodies in Sukuumu.

Table 7 VNTR profiles and MPM strain designated genotypes of current study and from published data.

Designated genotype	VNTR Profiles				Reference
Current study	MIRU1	Locus 6	ST1	Locus 19	
W	1	1	2	1	Current study
X[a]	1	1	2	2	
Y	1	2	2	1	
Z	1	2	2	2	
Published data					
A	1	1	1		(Williamson *et al.*, 2008)
B	3	1	1		
C	3	1	2		
D[a] (unpublished strain)	1	1	2	2	
MMDL	1	4	2	2	
	1	2	1	2	
MLF	1	2	2	1	
MPS	1	4	2	2	
Amansie West MU strain 1	1	ND	2	ND	(Hilty *et al.*, 2006)
Amansie West MU strain 2	3	ND	1	ND	
Ghana sequence MU strain	ND	1	ND	2	(Ablordey *et al.*, 2005a)

W, X, Y and X are *M. ulcerans* designated genotypes from current study. A, B, C, and D are *M. ulcerans* designated genotypes from literature. MMDL is *M. marinum* DL, MPS is *M. pseudoshottsii* and MLF is *M. liflandii*. [a] means identical. ND, not done.

Table 8 Designated genotypes for MPM isolated from humans and the environment.

MPM genotypes		
Community	Human isolates	Environmental isolates
Wromanso	Y, Z	Y,Z
		MLF
Monia-Gyaman	W, X, Y, Z	Y,Z
		MLF
Bepotenten	Y	Y,Z
		MLF
Sukuumu	W,X,Y,Z	W,X,Y.Z, A-, B-
		MLF, MMDL

W, X, Y and X are *M. ulcerans* designated genotypes from current study. A- **(1, 2, 1, 2)** and B- **(3, 2, 1, 2)** are designated genotypes identical (except at locus 6 with addition of Locus 19, this work) to those published by Williamson *et al*.(2008). MMDL is *M. marinum* DL and MLF is *M. liflandii*.

4.3.4 Sequence analysis of VNTR loci

Figure 12 shows the sequences of some selected VNTR repeats, Locus 19 (A), ST1 (B) and Locus 6 (C), each with a repeat of 2 (shown by different colour shades). There was a partial repeat for locus 19, shaded in light blue. All repeats for corresponding isolates confirmed repeat typing using band sizes. During sequencing, some sequences are trimmed at both ends and hence sequence lengths do not always correspond exactly with band sizes on agarose gel. Figure 12 (D) shows the phylogenetic tree of representative human and environmental isolates. MSA was done using their MIRU1 sequences (Appendix 3). As shown, there was clustering of both human (FW1, FY1 and FW2) and environmental (BAB4 and SKB5) isolates with reference *M. ulcerans* ScoA, suggesting they are *M. ulcerans* isolates as predicted with the VNTR profiles.

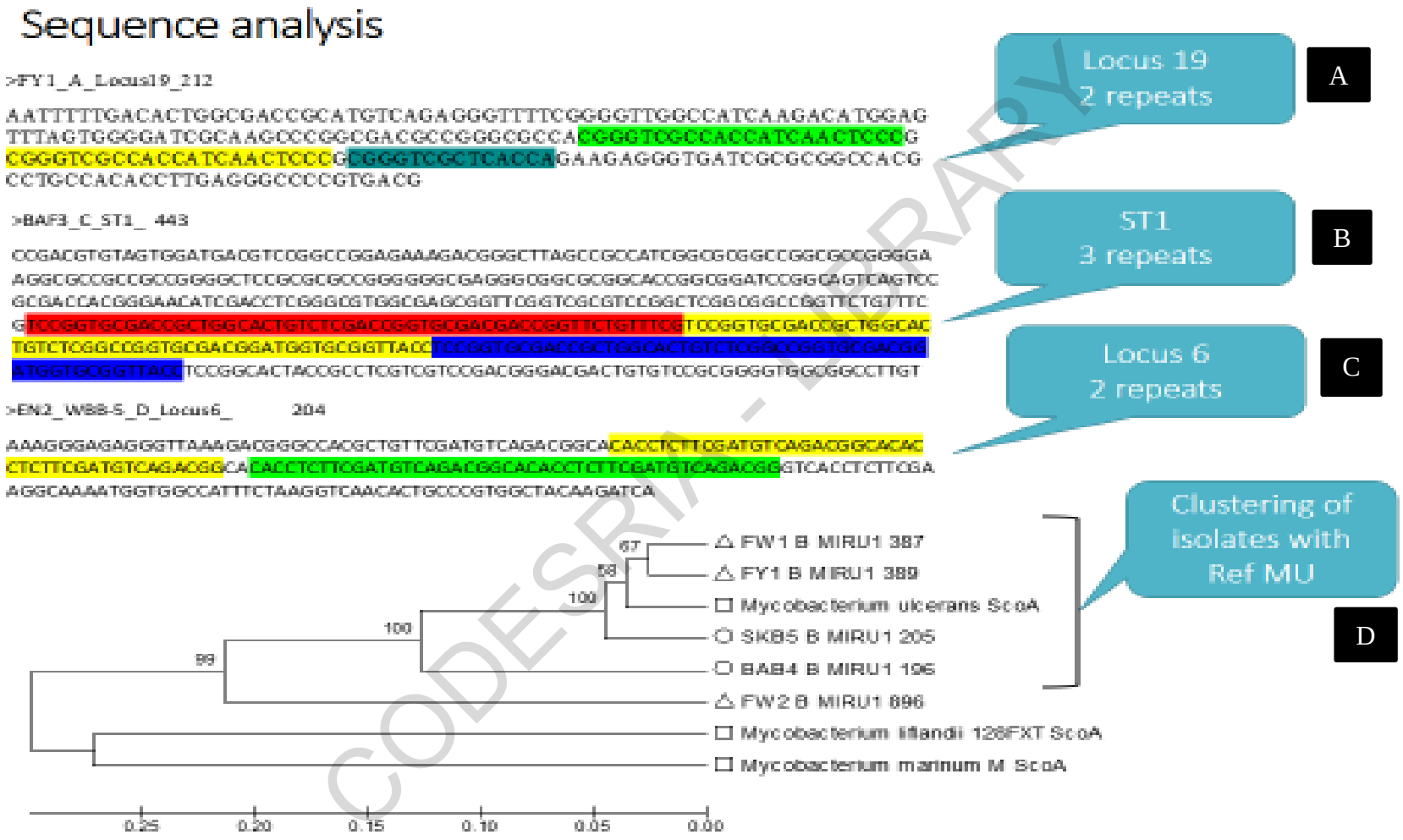

Figure 12 Sequence confirmation of VNTR repeats and phylogeny of MU isolates.

The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973). The optimal tree with the sum of branch length = 1.31639434 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000) and are in the units of the number of base differences per site. The analysis involved 8 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 153 positions in the final dataset. Evolutionary analyses were conducted in MEGA V5 (Tamura *et al.*, 2011). The reference sequences (represented with square) of the MIRU1 (ScoA gene) orthologs, *M. marinum*, *M. liflandii* and *M. ulcerans* were retrieved from GenBank with accession numbers CP000854.1, CP003899.1 and DQ397533.1 respectively. Sequences of human and environmental isolates are represented with triangles and circles respectively.

4.4 Geographical distribution of genotypes in communities

Figure 13 shows a scaled map of the four studied communities within the Amansie Central District. The Offin River meanders through all the four communities. For each community, identified genotypes from humans and the environment (water bodies) are shown in coloured callouts and genotypes common to the sources (humans and water bodies) are shown at the intersection of the callouts. Community settlements were represented by small brown houses. Figure 14 shows a diagram of possible models of transmission of NTMs, particularly, MU. One critical route of infection which could be a key player in transmission of MU, is the cut hypothesis.

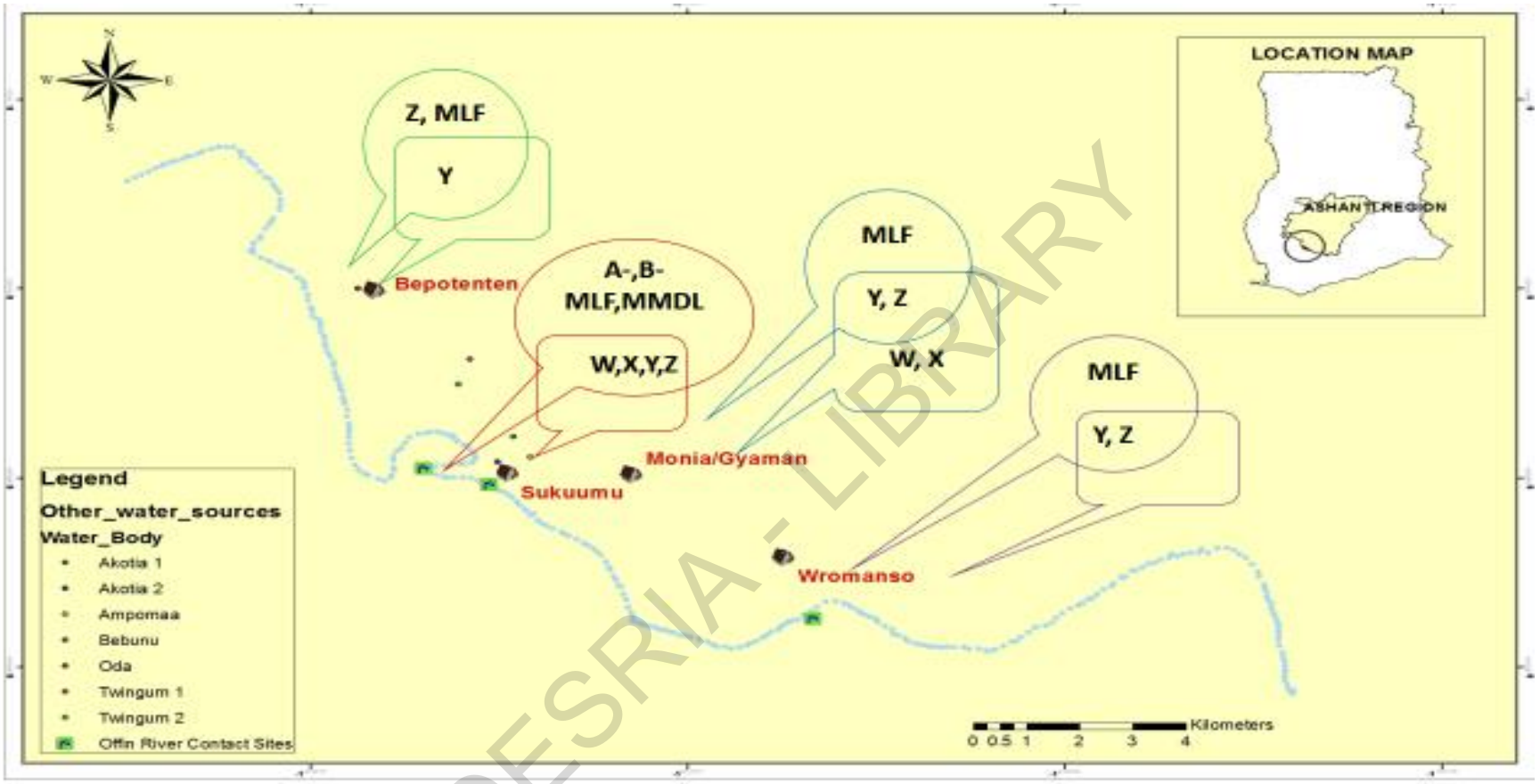

Figure 13 Community-based geographical distribution of MPM genotypes from humans and water bodies.

Map was drawn to scale. Flow of Offin River was sketched with the aid of satellite images and GPS coordinates from Google Earth. Rectangular and circular callouts contain genotypes of isolates detected in humans and the environment respectively. Intersection of callouts contain genotypes common to both

4.5 Model of overlaps and possible transmission routes

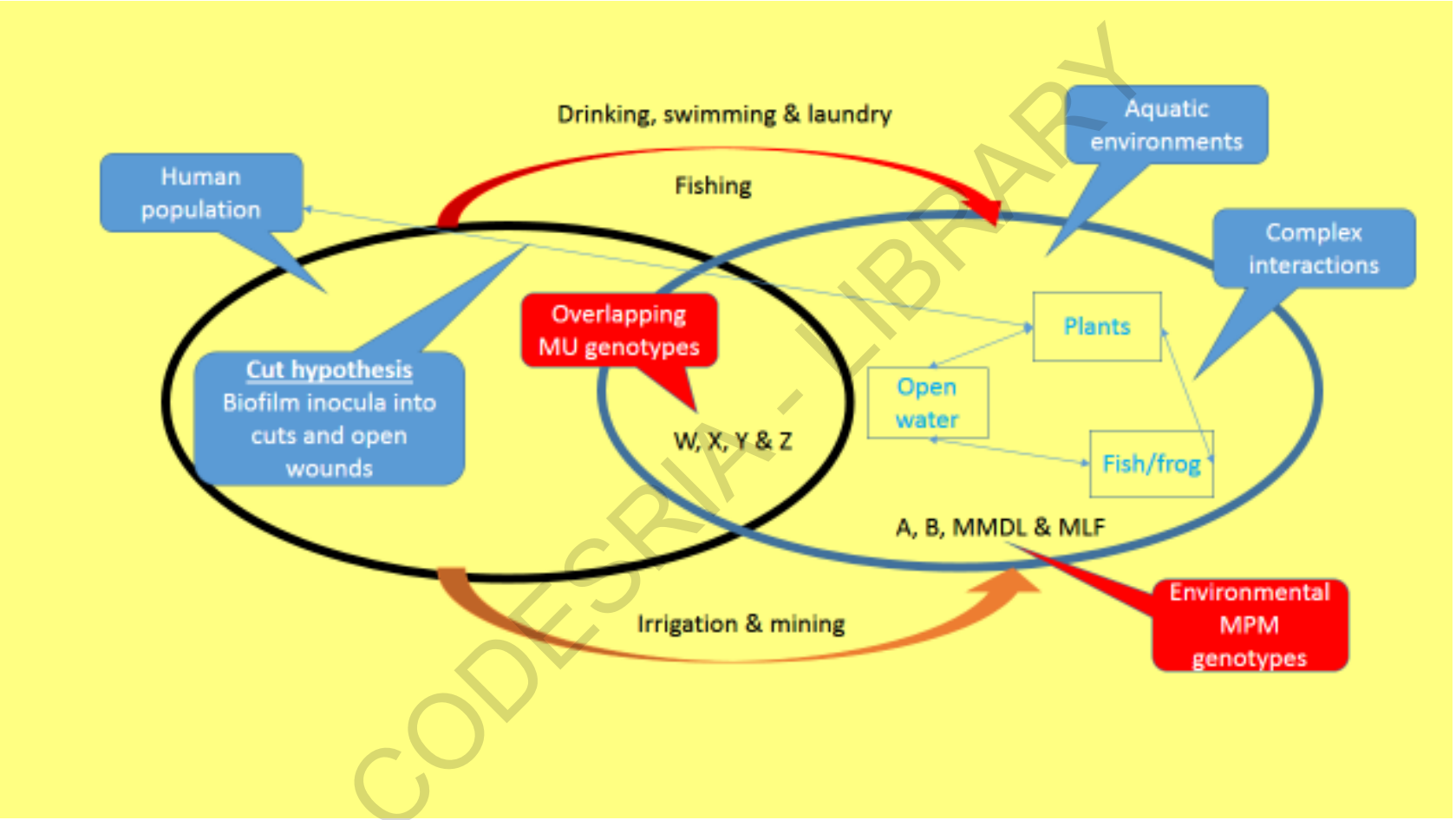

Figure 14 Interaction of ecosystems, potential routes of NTM transmission.

CHAPTER FIVE

5.0 Discussion and conclusion

5.1 Discussion

NTMs, particularly MPMs, are of great importance because of their pathogenicity in causing debilitating ulcers in both animals and man (Fyfe *et al.*, 2010). Hence a better understanding of their epidemiology and ecology, especially MPMs, will be crucial to controlling the spread of the diseases they cause, in particular *M. ulcerans* infection.. Owing to the challenges in obtaining pure cultures from the environment, most studies relied on detecting the genomic material of *M. ulcerans* in several aquatic environments. Other studies have associated these genomic material to disease burden intimating that slow moving water bodies were potential reservoirs of MU (Marsollier *et al.*, 2004, Williamson *et al.*, 2008). However, due to the broader distribution of these environmental pathogens against observed focal infections in human populations (Roltgen *et al.*, 2010, Williamson *et al.*, 2012), source tracking of infections to infected environments ought to begin with systematic sampling of suspected risk environments. Comparing strains from the latter to those causing human infections is vital in elucidating possible transmission routes. This study addressed three key objectives; identification of MPM contaminated water bodies, MPM genotypes in these environments and their similarity to those causing BU in humans. This section discusses data from the study to support the assertion that NTM (using MU as a model) transmission is dependent on the overlapping environmental habitats of the pathogen and humans.

Major overlap occurs in water where humans are exposed to mycobacteria through drinking, swimming, and bathing (Primm *et al.*, 2004). Although each community had at least one working

borehole with pump, over 55, 44 and 26% of respondents fished, bathed and swam respectively in all sampled water bodies (Appendix 1). Separately, 0ver 95% of respondents were into farming and other agricultural activities (Appendix 1). These results reflect the large number of the populations that were exposed to water bodies contaminated with MU (Table 6). One female patient in Sukuumu, who went to the farm daily crossing the Offin River on foot, narrated having the infection thrice. Frequencies to these contact sites were higher during the morning and evening hours of the day (data not shown) suggesting prolonged exposure. The Offin River passed through all four communities (Figure 13) and therefore presented a good model to compare MPM distribution among the studied communities. It plays an indispensable role in the domestic and economic activities in each community. However, illegal mining activities on the river (Figure 3B) have made it unsafe for drinking. Most of the remaining water bodies were smaller streams and ponds (personal observation)

In order to increase bacilli yield, samples were collected in triplicates except biofilms, which were collected in quintuplicates. This increased the chance of recovering bacilli (Figure 6) and/or DNA and to assure positivity for genomic DNA which was representative of the entire water body (Williamson *et al.*, 2012, Williamson *et al.*, 2008). Initial screening for NTM identified 38 (27.1%) samples as positive (Table 6). Biofilms were the most positive (57.9%) and detrita were the least positive (2.6%) (Figure 8). This pattern is consistent with that reported by Williamson and colleagues (Williamson *et al.*, 2012, 2008). Similar observation was made (Figure 8) after screening 16S rRNA-PCR positive samples (NTM) for IS2404, albeit the numbers were incomparable. The reduction in IS2404-PCR positive samples (65.8%), which were previously positive for 16S rRNA suggested that the remaining 34.2% were non-MPM *Mycobacterium spp*. IS2404 has been reported in only MPMs and not all NTMs (Yip *et al.*, 2007). Although this study

had limited data on ER positivity for environmental samples, one can infer the relative abundance of MPMs, including MU, in biofilms (figure 6) as reported by Williamson and colleagues (2012). Moderate positivity of filters (Figure 8) suggests presence of bacilli at the air-water interface (Wendt *et al.*, 1980). Some biofilms were collected from blades of aquatic grasses and therefore suggests that cuts from these grasses (as a result of wading in water or walking across wet foot paths) or open wounds could easily be inoculated with MU contaminated biofilms (Marsollier *et al.*, 2004). Again, Marsollier and colleagues (2004) showed that crude extracts from some aquatic plants halved the doubling time of MU and promoted biofilm formation. Although the exact role of biofilm formation for MU survival and proliferation has not been investigated, studies on bacteria harbouring plasmids have shown that transfer of genetic material among coexisting species is not unusual (Becq *et al.*, 2007, Doig *et al.*, 2012). *M. ulcerans* itself is thought to have evolved from *M. marinum* via acquisition of the 174kbp plasmid (Stinear *et al.*, 2007). Thus, as evolution continues within populations of organisms, it is possible that non-MPM mycobacteria, which form biofilms with other MPMs could acquire this pathogenic trait (Ranger *et al.*, 2006a). Studies in this seminal field could help better understand the role of biofilms in enhancing MPM proliferation and dissemination within aquatic ecosystems.

Additional data in Figure 7 shows 16S rRNA and IS2404 positivity for water bodies that were sampled. All water bodies were positive for at least the two tests except for the Offin contact site at Monia-Gyaman. Accounting for the apparent absence of genomic DNA; possibly, samples from this water body had higher concentrations of inhibitors to out compete BSA in relieving inhibition. This was evident from the high turbidity observed during sampling, which could be due to the observed high level of illegal mining activities on the River (Figure 3B), perturbing the river bed. This was however, not observed at other Offin contact points. In general, positivity for both

genomic markers was higher for ponds than Offin and Oda Rivers except at the Offin contact site in Sukuumu which had a higher value than the rest. The Offin contact point at Sukuumu had an inland-like extension of the river, forming a quasi-pond, and therefore was relatively slow moving. This data suggest a higher positivity of NTM, especially MPM in slow moving water bodies (ponds) than swiftly flowing rivers. This implies that, slow water currents passively facilitates attachment of (do not readily dislodge) biofilms on biotopes and consequently favour MU survival, proliferation and persistence. Rivers (fast currents) may better facilitate the dissemination of MPM by dislodging biofilms from places of attachment. Thus, this data shows the relative distribution of NTMs, especially MPMs within water bodies in the four endemic communities.

Although IS2404 positivity was used to infer MPM distribution in the water bodies, its applicability to human isolates was considered as presumptive. Definite diagnosis for BU was confirmed using positivity for at least one VNTR marker (Table 5). Additionally, ER-PCR was successful for only five patients (Table 5). Possibly, the copy numbers for ER in the remaining nine patient samples might have been insufficient to sustain complete PCR cycling. For the five successful amplifications, PCR products had to be reamplified in a separate reaction (data not shown) to detect ER amplicons. This however slightly contrasts reports by Williamson and colleagues (2012) who achieved successful amplification in a single reaction. This data suggest that a negative ER-PCR for previously identified IS2404 positive samples should be reamplified (using 1µl of PCR product) to rule out false negatives. Or IS2404-PCR could be complemented with a VNTR marker to confirm BU in humans.

One major challenge in detecting MU in human specimens (FNA and swab) was the varying bacilli load in lesions (band brightness was used as an indicator of bacilli load); a sharp contrast in band intensity and brightness is observed in Figure 9, showing patients with different VNTR profiles

for MU isolates. To improve amplification, PCR products were reamplified as previously stated or slight increase in thermal cycling, especially for environmental samples were adapted for VNTR typing. Correct repeat numbers for band sizes of each VNTR locus was calculated from their corresponding sequences (figure 12) and other published data (Table 4). Loci 6 and 19, and ST1 and MIRU1 were the most polymorphic for typing human and environmental MPM isolates respectively (Figure 11). Using four loci, this study increased the discrimination power of VNTR typing to obtain four MU genotypes as against two and three separately reported by Hilty and colleagues (2006) and Williamson and colleagues (2008) respectively. Loci 6 and 19 were the main determinants in specifying MU genotypes (Table 7). Thus this study partly resolved the apparent intra-species homogeneity within MU isolates reported in some Ghanaian studies (Ablordey *et al.,* 2005a, Hilty *et al.,* 2006, Stragier *et al.,* 2005) and lends credence to the use of VNTR in differentiating MU from other MPMs (Williamson *et al.*, 2008). Repeats of 1 and 2, for MIRU1 and ST1 respectively, were constant for all human isolates but varied for environmental isolates. This is consistent with that reported by Hilty and colleagues (2006) for MU strains in the Amansie West District (Table 7), the area of this study. Further, this data corroborates findings by Ablordey and colleagues (2005a), who had repeats of 1 and 2 for locus 6 and 19 respectively (Table 7) for a Ghanaian MU strain. It is however, partly, at variance with that reported by Williamson and colleagues (2008) (Table 7). Explaining the latter, their study areas, Ga South District, among others in the Ashanti region, are different from those of this study. Additionally, their study was randomized and used three VNTR loci to type, mostly environmental isolates (Williamson *et al.*, 2008). Data from this study (Table 6) show that VNTR typing for environmental samples could give varying allelic combinations, suggesting the presence of other MPMs. Thus, this study first genotyped MU isolates from humans; currently MU is the only reported MPM causing BU in

humans except a few reported cases in some mammals (Fyfe *et al.*, 2010), and then compared the profiles to isolates obtained from water bodies frequently used by inhabitants, including patients. This helped to properly match genotypes from the two sources and establish a source of infection within each community. The four MU genotypes; W, X, Y and Z, from humans were identical to those obtained in the environment. Additionally, genotypes A and B (MU), MLF (*M. liflandii*) and MMDL (*M. marinum* DL), are similar to those of Williamson and colleagues (2008) which were found in some water bodies. This data suggest that while it is sufficient to use loci 6 and 19, and, ST1 and MIRU1 to type/discriminate MU isolates from humans and the environment respectively, a combination of all four are necessary to match human isolates to environmental isolates.

In comparing MPM genotypes, a community-based geographical map (Figure 13) was drawn to show the relative distribution of genotypes between human populations and their water bodies, within each community. Genotype Y was the predominant genotype found in humans and was also found in all water bodies. This was also observed for genotype Z except at Bepotenten. The Offin passes through all communities (Figure 13) and could possibly be disseminating this strain among communities. Thus, water appears to be a major vehicle for disseminating NTM in the environment. Genotypes W, X, MLF and MMDL appear to be limited to specific water bodies, mostly, the ponds (Table 6 & Figure 13). Again, genotypes W and X appear to be restricted to two close communities, Sukuumu and Monia-Gyaman (isolates from these communities cluster together on the phylogenetic tree). These two share a common stream, Akotia. This suggests a common source of infection, the Akotia stream. Genotypes A and B were found only in Twingum 2 pond in Sukuumu (Table 6). This further consolidates my earlier assertion that slow moving water bodies, particularly ponds, though favour MU survival and persistence, do not readily disseminate bacilli, giving support to the hypothesis of clonal clustering of MU strains and focal

epidemiology of BU cases (Roltgen *et al.*, 2010, WHO, 2008). For each community, at least one MU genotype was common (intersection of callouts) between humans and water bodies (Table 8 and Figure 13). In Figure 10, an isolate from a biofilm sample, BAB-4, collected from the Oda River in Bepotenten had identical profile, Y (1, 2, 2, 1) to a patient (FB1) isolate from the same community. Alongside, is a reference strain, *M. marinum* DL (Pam Small lab), which was included in this study to validate VNTR repeat numbers. Its profile, MMDL (1, 4, 2, 2) was consistent with published data (Williamson *et al.*, 2008). Sukuumu, which had the highest number of cases in this study, had all four MU genotypes found both in humans and its water bodies. This data further suggest that water bodies are reservoirs of MU and humans are infected from these water bodies. Also, one can infer from this genetic "footprints" that contaminated water bodies are major sources of MU infection in endemic communities. Genotypes MMDL and MLF were not detect in humans although MLF profile (1, 2, 2, 1) was identical to the human MU X, profile (1, 2, 2, 1) (Williamson *et al.*, 2008). However, isolates with this profile clustered with MU, Agy99 on the phylogenetic tree (Figure 12D), suggesting they are *M. ulcerans*. Further analyses like culturing, biochemical tests and sequencing for other loci could help bring out more differences. Within the scope of this study, it was assumed they were different, as only MU is known to cause BU in humans, although some animals have been reported to have BU lesions (Fyfe *et al.*, 2010).

Sequence analysis (figure 12) confirmed VNTR repeats as predicted using band sizes of PCR products. It however revealed subtle differences like partial repeats in locus 19 (figure 12A) and single nucleotide mutations in the consensus repeat for some loci, as reflected in multi sequence alignments (data not shown). Thus, although band sizes (length polymorphism) is comparable to sequencing (sequence polymorphism) in typing MU isolates, it does not reveal SNPs between isolates with the same VNTR profile. Inferences from the phylogenetic tree shows that both human

and environmental isolates used for the analysis are indeed *M. ulcerans*, as they cluster with the reference MU (MIRU1 ortholog). This was predicted with the VNTR typing (Tables 5 and 6). The topology of the tree also show that *M. marinum* is more of an ancestor and the isolates in this study are descendants. This supports the hypothesis that *M. ulcerans* evolved from *M. marinum* (Stinear *et al.*, 2000b).

This study has addressed its three key objectives; identification of MPM contaminated water bodies, MPM genotypes in these environments and their similarity to those causing BU in humans. Using this information, a transmission model for NTM (with MU as model mycobacteria) was proposed (Figure 14). One distinct feature in this model is the overlapping habitats of the pathogen and human (via activities). A possible critical transmission route in this proposed model is cuts from contaminated grass blades and inoculation of open wounds from these water bodies. Children appear to be the most susceptible, as they come into contact (swimming, bathing and fetching water) more frequently (Asiedu and Etuaful, 1998). Conversely, these rivers can be "reinfected", that is, shedding of MU from open BU lesions through similar human activities, and hence finding common genotypes in different communities is possible (Figure 13). Ponds appear to be more contaminated than rivers as previously explained, and thus more risky of MU infections.

5.2 Conclusion

VNTR typing has confirmed repeats previously reported by some studies and has resolved the apparent homogeneity in MU isolates. In this study, VNTR profiling at four loci has revealed four *M. ulcerans* genotypes (W, X, Y, & Z) both in humans and sampled water bodies in the Amansie Central District of the Ashanti region, Ghana. However, previously reported *M. ulcerans* genotypes (A & B) were found only in one water body. Other MPM genotypes, MLF and MMDL,

identical to *M. liflandii* and *M. marinum* respectively were found only in water bodies. All repeats were confirmed with sequencing and phylogenetic analysis clusters isolates in this study with reference MU sequences from GenBank. Genetic comparisons showed that for each community, at least one MU genotype is found both in humans and water bodies, suggesting that patients were infected from these water bodies. Thus, these data substantiates the hypothesis that transmission of NTMs, particularly, *M. ulcerans*, is dependent on the overlapping habitats of the pathogen and humans.

5.3 Recommendations

Similar studies can be done in other endemic communities, e.g. those in the Ga South District to see if findings will corroborate those of this study. Furthermore, culturing of the bacteria from environmental samples and establishing infection in a mouse model would help in understanding how it is transmitted to humans. This study has shown that there is genetic variation among MPMs, particularly *M. ulcerans*, and hence additional polymorphic markers can be scanned from the *M. ulcerans* genome, Agy99, to differentiate strains. Indeed, related studies have shown subtle differences in MU genotypes (six geographical strains) as specific to a geographical area. That is, African isolates are distinct from Australian isolates. However, given the wide environmental distribution of MPMs, it is possible that other geographical isolates, such as Australian/Mexican types may be present in Africa environments but are not causing infection in Africa populations. In agreement, this study has shown that previously reported genotypes in Ga South District of Ghana, can be found in certain water bodies in Amansie Central District although not detected among the genotypes identified in humans. Thus, replicating this studies in other endemic communities within the different geographical regions could help verify this hypothesis and address existing evolutionary postulates on MPMs. It is important to culture MU from water bodies

contaminated in endemic communities and test if those strains can cause infection in animal models.

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APPENDICES

Appendix 1: Some data from questionnaire (water sources section).

Table 9 Livelihood strategies

livelihoodstrategies	Frequency	Percent	Actual percentage
Agriculture	215	77.90%	95.98214286
Commerce	40	14.49%	17.85714286
Fishing	8	2.90%	3.571428571
Hunting	7	2.54%	3.125
Animal Husbandry	6	2.17%	2.678571429
TOTAL	**276**	**100.00%**	

Imported from Epi Info

Table 10 Activities around water bodies

Activitiesriverpond	Frequency	Percent	Actual frquency
Fishing	125	38.34%	55.80357143
Bathing/swimming	100	30.67%	44.64285714
Drinking	59	18.10%	26.33928571
Laundary/dishwashing	42	12.88%	18.75
TOTAL	**326**	**100.00%**	

Imported from Epi Info

Appendix 2: Information on ethical and administrative issues

Ethical clearance for Study

NOGUCHI MEMORIAL INSTITUTE FOR MEDICAL RESEARCH
Established 1979
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University of Ghana

INSTITUTIONAL REVIEW BOARD

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

6th July, 2011

ETHICAL CLEARANCE

FEDERALWIDE ASSURANCE FWA 00001824 **IRB 00001276**

NMIMR-IRB CPN 041/10-11 **IORG 0000908**

On 6th July 2011, the Noguchi Memorial Institute for Medical Research (NMIMR) Institutional Review Board (IRB) at a full board meeting, reviewed and approved your protocol titled:

TITLE OF PROTOCOL : **Zoonotic risks of non-tuberculous mycobacteria between humans and small mammals (Potential transmission of Buruli Ulcer) in Cote D'Ivoire and Ghana**

PRINCIPAL INVESTIGATOR : **Lydia Mosi, PhD**

CO-INVESTIGATORS : **Charles Quaye & Heather Williamson Jordan**

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

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

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

This certificate is valid till 5th July, 2012. You are to submit annual reports for continuing review.

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

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

CONSENT FORM

Title: Zoonotic risks of non-tuberculous mycobacteria between humans and small mammals (potential transmission of BU) in Côte d'Ivoire.

Principal Investigator: Lydia Mosi

Noguchi Memorial Institute for Medical Research,University of Ghana, P.O.Box LG 581, Legon-Accra

Introduction: This consent form informs you about the background, aims and the method of this study. In addition it explains the anticipated benefits, potential risk of the study and the discomfort it may entail. Finally it informs you of inclusion criteria and your rights regarding participating in this study.

Purpose of the research: Non-tuberculous mycobacteria are notorious opportunistic pathogens with a wide range of possible environmental reservoirs and a variety of host comprising fishes, mammals and humans. This proposal posits that NTM transmission is dependent on the overlapping environmental habitats of the pathogen and humans.

The work for this proposal is divided into three specific aims that will independently address distribution of the select NTMs with respect to active case surveillance of human and small animal disease burden; zoonotic risk analysis between animals and humans living in close proximity; and molecular characterization of NTMs in described study areas. We believe there is considerable under reporting and misdiagnosis of infections caused by NTMs in both humans and small animals.

In human disease, NTM infections represent a major global health problem particularly in developing countries. The mortality due to animal disease still comprises one of the highest losses in revenue in agriculture and local wildlife conservation. Particular emphasis will be placed on identifying foci of *Mycobacterium ulcerans* in the environment and in humans in within the selected study sites. This is because of the high importance of Buruli ulcer as an emerging neglected tropical disease in Côte d'Ivoire. It is also unknown whether the pathogen can be transferred from animals to humans. Data gathered from this study will be essential in the formulation of prevention and eradication policies.

Procedure: Samples will be taken from patients with suspicious lesions and ulcers by trained individuals. The samples to be collected will be swabs or biopsy samples from the above mentioned areas using the recommended protocol according to WHO guidelines. Samples obtained will be stored appropriately and transported to CSRS for further processing. All samples will undergo case diagnosis and confirmation.

Risks and Discomfort: There is no major risk associated with your participation in this study apart from the slight discomfort you may get from parts of the wound being cleaned for sample collection. Samples will be taken only once.

Benefits: Buruli ulcer treatment is free of charge in hospitals. There will be no direct benefit for subjects' participation in this study. Patients diagnosed with other mycobacterial infections will be counseled accordingly on the treatment options. However, the main benefit of your participation is indirect as you will help us identify the prevalence of environmental mycobacterial diseases in Côte d'Ivoire. At the end of the study, you will be contributing significantly to preventing the horrible effects of the disease in your community.

Incentives**:** Aan incentive, patients identified with Buruli ulcer as well as other lesions will be counseled on treatment choices and the appropriate treatments shall be meted out free of charge..

Confidentiality: Your records will be kept in a secure location at CSRS. All information collected during the study will be stored in a file which will not have your name on it, but a study number assigned to it. Only the research team will have access to the names associated with the study numbers and for special reasons such as treatment. It is likely that data obtained from tests done on you may be published in medical journals; however, your identity will not be disclosed.

Your Right to refuse or withdraw: You have the right not to take part in the study if you do not want to, and this will not affect you or your ward in any way. Refusal to participate in or withdraw from this study will not have any penalties or loss of benefits that you may be entitled to. Your position in the community will also not be affected in any way, even if you decide not to participate in the study.

Contact information: You may ask any questions about the study now or later. For any further information you may contact any of the following:

(Chairman, Institutional Review Board/Ethics Committee)

NMIMR

Volunteer Agreement

I have read this consent form. I have received satisfactory answers to my questions. I understand that my participation is voluntary. I know about the purpose, methods, risks and possible benefits of the research study to judge that I want to participate. I consent voluntarily to allow my ward to participate in this study. I understand that I have the right to withdraw from the study at any time, and I know that I can call on any member of the study team if I have any questions or concerns.

Name of participant_____

Parent's Name _____ Signature of participant/parent _____

Witness Name _____ Signature of witness _____

Primary Investigator Signature _____

Date _____ Place _____

(Study ID number assigned to this participation) _____

If volunteers cannot read the form themselves, a witness must sign here**:**

I was present while the benefits, risks and procedures were read to the volunteer. All questions were answered and the volunteer has agreed to take part in the research.

Date_____ Signature of Witness_____

I certify that the nature and purpose, the potential benefits, and possible risks associated with participating in this research have been explained to the above individual.

Date_____

Signature of Person Who Obtained Consent _____

AFRIQUEONE BURULI ULCER PROJECT

DATE:……/……../……..

PART ONE: SOCIOLOGY ON BURULI ULCER

SECTION A

IDENTIFICATION OF THE INFORMANT (head of household or his representative)

1. Name and surname……..
2. Age…….
3. Status in the community……
4. BU case or non-case……
5. Profession of the informant……..
6. Marital status……
7. Ethnic group (Optional)…….
8. Level of education Primary ☐ Secondary ☐ University ☐
9. Sex : M ☐ F ☐
10. Duration of stay in the community……..……
11. Some prevalent diseases in the community……..……
12. Have you ever suffered from buruli ulcer? Yes ☐ No ☐
13. If yes, how do you think you contracted it?
    ……
    ……

SECTION B

BACKGROUND INFORMATION ON THE VILLAGE

B1. Is the village increasing or decreasing in population? *If population size is changing, note the reasons below.*

☐ Increasing ☐ Decreasing ☐ No change

_____
_____

B2. Does the population in this village fluctuate significantly during the year? ☐ Yes ☐ No If yes, during what seasons does it increase and decrease, and for what reasons?

_____
_____

B3. Do all people in this village use the same water sources? ☐ Yes ☐ No

If not, explain:

B4. Do all people in this village have equal access to all water sources? ☐ Yes ☐ No *If not, explain:*_____
_____

SECTION C

HISTORY OF LESION/ ULCER PRESENTATION

C1.What is the name of BU in your local language? _____

C2a. When did the patient or caretaker first notice the lesion/ ulcer? *Establish approximate date through seasonal calendar.* (__/__/____)

_____
_____

C2b. Describe the behavioural activities of the patient when the lesion/ulcer was first noticed *(Be as specific as possible.)*

_____
_____

C3. Where was the lesion/ ulcer located?

☐ Arm ☐ Leg ☐ Torso ☐ Head ☐ Genitals ☐ Other_____

C4a. How did the lesion present during its development? *Check all that apply.*

☐ Nodule ☐ Plaque ☐ Oedema ☐ Ulcer ☐ Osteomyelitis

C4b. How did the lesion present during its development? *Provide a brief narrative of the order of development stages. Note whether the ulcer first appeared from trauma.*

_____
_____

C5a. Are you undergoing any treatment? ☐ Yes ☐ No

C5b. If Yes, Where? _____

SECTION D

IDENTIFICATION AND ANALYSIS OF SOCIAL FACTORS RELATED TO TRANSMISSION AND TREASTEMENT OF BURULI ULCER

Knowledge of Buruli ulcer

D1. Do you know a disease called Buruli ulcer? Yes ☐ No ☐

D2. If yes, could you give the name of this disease in your local language? …….

D3. Does this disease exist in your village? Yes ☐ No☐

D4. If yes do you know people suffering from it? Yes ☐ No ☐

D5. What are some symptoms of this disease that you know?

- Fever ☐
- Headache ☐
- Cough☐
- Itching ☐
- Wound (open wounds) ☐
- Other (precise) ☐……

D6. Which part of the body is particularly affected?

- Arm ☐
- Body ☐
- Hands ☐
- Leg ☐
- Foot ☐
- Eye☐
- Genitals ☐
- Other (precise) ☐……

D7. Do you know the consequences of this disease? Yes ☐a No ☐b

D8. If yes, cite at least three (3) of them

- ……
- ……
- ……
- ……..
- ……..

D9. Do you know if it is treatable?
Yes ☐a No ☐b

D10. If yes, How?
……
……..

PART TWO : TRANSMISSION OF BURULI ULCER

SECTION E

Transmission of Buruli ulcer

E1. For you, what are the causes of the disease?

- Malediction ☐
- Hereditary ☐
- Insect biting ☐
- Poor hygiene ☐
- Working in water ☐

- Other (Precise) ☐……..

E2. What is the main cause (for those who propose many answers)?

……
……

E3. Who are the most affected people in this area?

- Women ☐
- Men ☐
- Young boys ☐
- Young girls ☐
- Fishermen ☐
- Hunters ☐
- Farmers ☐
- Others (precise) ☐

E4. Do you think this disease is linked to water?

Yes ☐ No ☐

E5. If yes, which type of water?

.....
.....

E6. At which season are people mostly affected?

- Rainy season ☐
- Dry season ☐
- Any season ☐

E7. What are your sources of water supply in the area (village)?

- Pipe-borne water ☐
- Stream/river/lake… ☐
- Well ☐
- Other (precise) ☐……

E8. What is the main source of water ?.....

E9. What are different activities people from the village have at the river?

- Bathing/swimming ☐
- Drinking water ☐
- Fishing ☐
- Laundry and dish washing ☐
- Other (precise)☐……..

E10. Who is generally responsible for those activities?

- Women ☐
- Men ☐
- Children ☐

E11. At which season do they carry those activities?

- Dry season ☐
- Rainy season ☐
- Any season ☐

E12. At what time of the day do they go to the river?

- Morning ☐
- Afternoon ☐
- Evenning ☐

SECTION F
Intervention Perceptions

F1. Where and How are people affected by buruli ulcer treated here?

- Hospital/ health centre ☐
- Traditional healer ☐
- Marabout ☐
- Self medication (traditional) ☐
- Self medication (drugs) ☐
- Other (precise) ☐…….

F2. What is the most preferred mode of treatment system in your area?

……

F3. How do you find the treatment cost of this disease at the hospital?

- Affordable ☐
- Not affordable ☐
- Other (precise) ☐……

F4. Are there sensitization programmes on this disease in this area? Yes ☐ No ☐

F5. If yes, have you ever attended one of those programmes?

. Yes ☐ No ☐

What did you learn ?.....

F6. If No, why?

……
……..

F7. Who were the targeted groups through this programme?

……
……

SECTION G

Preventive Practices

G1. What are your preventive measures to avoid Buruli ulcer?

- I avoid going to the stream/river ☐
- I take drugs ☐
- I use medicinal herbs ☐
- Other (precise) ☐……..

G2. How do you personally fight BU?

……
……

G3. Do you sensitize your relatives on the consequences of Buruli ulcer?

- Yes ☐ No ☐

G4. If yes, what do you recommend them precisely?

- To have a good hygiene ☐
- To avoid bathing/swimming in stream/river/lake ☐
- To wear appropriate clothes while working in water ☐
- Other (precise) ☐

SECTION H

Background information on living areas

H1. Where have you being residing in the last three (3) months?

_____

H2. If patient, where have you being residing in the last three (3) months before lesion appeared?

_____

H3. What time of the year does the informant lives in this house? *Interviewer:*

_____

H4. If the patient spends significant (one month and above) periods of the year in another residence, note the duration above and identify the location(s) below:

_____
_____

_____

H5. When was the house built? *Use seasonal calendar* (___/___/_____)

H6. What is the house made of? *Check all that apply. If Mud is not checked, continue to question D6.*

☐ Cement ☐ Mud ☐ Wood or bamboo ☐ Other: _____

H8. What type of soil was used in the construction of the house?

☐ Sandy ☐ Clay ☐ Loamy ☐ Other: _____

H9. What are the structures in the compound where the house is found? *Check all that apply and indicate the number.*

☐ Residences ____ How many rooms/doors? __/__

☐ Storage rooms ____

☐ Cooking areas ____

☐ Grain silos ____

☐ Animal enclosures ____

☐ Kitchen gardens adjacent ____

☐ Other: _____

H10. What are the most common methods of waste and sewage disposal? List below

_____
_____

H11. What kind of latrine is used in the house? _____

H12. Describe the bath house. _____

H13. What animals are kept in the compound where the house is found? *Check all that apply.*

☐ Goats ☐ Dogs

☐ Sheep ☐ Cats

☐ Chickens ☐ Pigs

☐ Guinea fowl ☐ Cows

☐ Donkeys ☐ Other _____

H14. Does the household keep any other animals in areas outside of the compound? ☐ Yes ☐ No, if *yes, identify them.*

☐ Goats ☐ Dogs

☐ Sheep ☐ Cats

☐ Chickens ☐ Pigs

☐ Guinea fowl ☐ Cows

☐ Donkeys ☐ Other _____

H15. Has household composition changed in the past five years? ☐ Yes ☐ No *If yes, check all that apply and explain below.*

☐ Some members live in field houses during part of each year

☐ Members of the current household lived in other villages

☐ Members of the current household lived elsewhere in this village

☐ Other: _____

SECTION I

<u>Water sources</u>

I1. Where does the household get their water for **drinking and cooking** during the dry season and the rainy season? *Check all that apply and indicate on map.*

Dry season	*Rainy season*	
☐	☐	Pump
☐	☐	Covered well
☐	☐	Open well
☐	☐	Cistern
☐	☐	River (name of river) _____

☐ ☐ Pond, swamp or low-lying area, _____

☐ ☐ Other _____

I2. Where does the household get their water for **showering and washing clothes** during the dry season and the rainy season? *Check all that apply and indicate on map.*

Dry season *Rainy season*

☐ ☐ Pump

☐ ☐ Covered well

☐ ☐ Open well

☐ ☐ Cistern

☐ ☐ River (name of river) _____

☐ ☐ Pond, swamp or low-lying area, _____

☐ ☐ Other _____

I3. Was there a time in the last year that water sources that you normally use became unavailable (broken pump, dry or polluted well, etc.) ☐ Yes ☐ No

Identify below, and describe the alternative water sources the household used.

_____
_____

I4. Does the patient ever get water for the household, or accompany the person who does?

☐ Yes ☐ No *If yes, check all that apply and indicate on map.*

Dry season *Rainy season*

☐ ☐ Pump

☐ ☐ Covered well

☐ ☐ Open well

☐ ☐ Cistern

☐ ☐ River (name of river) _____

☐ ☐ Pond, swamp or low-lying area, _____

☐ ☐ Other _____

I5. Do you have contact with any other water sources? *If a child, include areas where they play. If adult, include areas where they work.*

_____
_____

I6. *Interviewer:* draw on map the paths used by the household to access the water sources indicated in Section E.

SECTION J

<u>Livelihood</u>

J1. List all of the livelihood strategies undertaken by members of the household.

☐ Agriculture

☐ Animal husbandry

☐ Fishing

☐ Hunting

☐ Commerce

☐ Artisan *Identify:* _____

☐ Other: _____

J2. What are the crops grown by the household? *Check all that apply.*

☐ Corn ☐ Tomatoes

☐ Rice ☐ Okra

☐ Palm nut ☐ Yams

☐ Greens ☐ Manioc

☐ Peanuts ☐ Beans

☐ Onions ☐ Other _____

J3a. What kinds of fields are used for cultivation? *Check all that apply.*

☐ Inundated fields

☐ Irrigated fields

☐ Dry fields

☐ Fields tilled each year

☐ Other, _____

J3b. Did the informant spend time around fields, which one and how long?

☐ Yes ☐ No

_____

J3c. *Interviewer:* draw the paths on the map used by the household to access fields, hunting grounds, forests, or bush.

J4. Are there any livelihood strategies or other activities undertaken by the household that expose family members to the environment (forest/bush)?

☐ Yes ☐ No *If yes, describe and explain below using F1 as guide.*

_____
_____

J5a. Do members of this household or friends engage in hunting, or catch wild animals while in their fields? ☐ Yes ☐ No.

J5b. Does the informant engage in these activities, or accompany those who do? ☐ Yes ☐ No

J5c. Does hunting occur ☐ seasonally, or ☐ throughout the year?_____

J5d. What are the animals most frequently caught?

☐ Grasscutter ☐ Rat

☐ Squirrel ☐ Rabbit

☐ Bush chicken ☐ Snake

☐ Pangolin ☐ Deer

☐ Porcupine ☐ Monkey

☐ Other _____ ☐ Other _____

☐ Other _____ ☐ Other _____

SECTION K

Other family members

K1. Are there any other members of the household that have had BU infections? ☐ Yes ☐ No, *If No, continue to G3. If yes, identify their relationship to the informant.*

☐ Mother

☐ Father

☐ Child_____

☐ Sibling (same mother) _____

☐ Sibling (same father) _____

☐ Other genetic relationship _____

☐ Non-genetic relationship _____

K2a. Do the afflicted members engage in similar work activities as the patient? ☐ Yes ☐ No

K2b. Do the afflicted members work in the same location as the patient? ☐ Yes ☐ No

K3. Does the patient have any close relatives (child, parent, or sibling) who do not live in the compound, but who have had BU infections? ☐ Yes ☐ No, *If No, continue to Section H. If yes, check all that apply and indicate number if multiple cases exist.*

☐ Mother

☐ Father

☐ Child_____

☐ Sibling (same mother) _____

☐ Sibling (same father) _____

SECTION L

Mapping (supplementary section)

L1. If a map of the village is available, locate the house on the map and any other places where the patient resides during the year. Mark the areas in the village where the patient spends significant periods of time.

L2. Locate all of the village water sources for drinking, cooking, washing, playing etc. on the map. Note which of these sources are used by the household, and for what purpose.

L3. Mark the locations where the household gets soil for house construction.

Appendix 3: Sequence data

>FW1_B_MIRU1_387

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CGATGCAGAGCGAAGCGATGAGGAGGAGCGGCGCCAGATGAGCTGGAGCAGAGAC
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>FW2_B_MIRU1_896

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>SKB5_B_MIRU1_205

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>BAB4_B_MIRU1_196

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>FY1_B_MIRU1_389

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