UNIVERSITY OF GHANA

COLLEGE OF BASIC AND APPLIED SCIENCES

GENETIC DIVERSITY OF MYCOLACTONE PRODUCING MYCOBACTERIA CAUSING BURULI ULCER IN GHANA AND CÔTE D'IVOIRE

BY

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DEPARTMENT OF BIOCHEMISTRY, CELL AND MOLECULAR BIOLOGY

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DECLARATION

I, Magdalene Dogbe, do certify and declare that this project aside other cited works is a product of my own research undertaken at the Department of Biochemistry, Cell, and Molecular Biology, University of Ghana, Legon and Department of Biological science, Mississippi State University by me under the supervision of Dr. Lydia Mosi and Dr. Heather Jordan. Reference made to the works of others have been duly acknowledged. I certify that no part of this dissertation has been previously submitted for a degree or any other qualification.

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DEDICATION

I dedicate this work to the memory of my late father, Boniface Dogbe who was keen on providing the best education for me and my siblings. I also dedicate this work to my lovely family (Ms. Mary Nyame (Mum), Mrs. Patricia Appiah (sister), Gloria Dogbe (sister), and Kelvin Dogbe (brother), Roland Dogbe (brother) and Divine Kwabena Appiah Nyame (nephew) for being my greatest inspiration.

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LIST OF ABBREVIATIONS

BU – Buruli ulcer

CI - Côte d'Ivoire

MIRU – Mycobacterial Interspersed Repetitive Units

VNTR – Variable Number Tandem Repeat

MPM - Mycolactone Producing Mycobacteria

MTC - Mycobacterium tuberculosis complex

NTM - Non-Tuberculous Mycobacteria

MU – *Mycobacterium ulcerans*

MLF - *Mycobacterium liflandii*

MHB - *Mycobacterium marinum hybrid*

MDL - *Mycobacterium marinum DL*

MCL - *Mycobacterium marinum CL*

MSA - *Mycobacterium marinum SA*

MPS - *Mycobacterium pseudoshottsii*

PCR - Polymerase Chain Reaction

BCG - Bacille Calmette–Guérin

gDNA – genomic DNA

ABSTRACT

Buruli ulcer remains a neglected tropical disease endemic in Africa especially Ghana and Côte d'Ivoire. It is a necrotizing skin and soft tissue infection caused by *Mycobacterium ulcerans* which produces mycolactone which causes adverse effects associated with disease in humans. Other mycolactone producing bacteria (MPMs) have been identified to cause granulomas in fish and frog. There are limited advanced genetic tools for studying transmission of the diseases and for carrying out molecular epidemiological studies. The mycolactone producing mycobacteria (MPM), *M. shinshuense* has been reported to cause Buruli ulcer and other studies have revealed several MPM infections other than *M. ulcerans* in clinical samples. It is therefore imperative to know the exact MPMs that are in circulation by genotypically distinguishing them using molecular tools. Patients with Buruli ulcer-like disease presentation were recruited from endemic communities in Ghana and Côte d'Ivoire. Swabs and FNA samples were screened using primers detecting the insertion sequence *2404* gene and the Enoyl reductase gene. Genotyping was achieved at 16 MU VNTR loci and length polymorphisms arising from differences in tandem repeats for samples were validated using six (6) controls and sequencing.

A total one hundred and fifty-nine (159) of the 189 samples were confirmed as Buruli ulcer positive. Genotyping was successful for all controls (100%) and most samples (69%) at all sixteen (16) VNTR loci. Seven (7) MU genotypes designated, A, B, C, D, E, F and G and five MPM genotypes MLF (*Mycobacterium liflandii*), MHB (*Mycobacterium marinum hybrid*), MDL (*Mycobacterium marinum DL*), MCL (*Mycobacterium marinum CL*) and MSA (*Mycobacterium marinum SA*) were generated samples were assigned genotypes based on their VNTR profiles. Genotypes C, D and F were present in both Ghana and Côte d'Ivoire. However, genotypes E and MLF were only found in Ghana whiles Genotype A and G were found in only Côte d'Ivoire.

Genotype D has persisted over the years from 2008 to 2019 comparing this study to published data. These findings support the hypothesis that Mycolactone producing mycobacteria causing Buruli ulcer in Ghana and Côte d'Ivoire are diverse and affirms VNTR typing as a comparably useful tool for differentiating MU strains as well as other MPMs in Buruli ulcer endemic communities.

CHAPTER ONE

1.0. INTRODUCTION

Non-Tuberculous Mycobacteria (NTM) are environmental pathogens that are usually found in water, aerosols insects, soil, protozoans and small animals (van Ingen et al., 2009; Falkinham, 2011) and are not members of the Mycobacterium Tuberculosis Complex or *Mycobacterium leprae* (Falkinham et al., 1996). NTMs can cause infections in humans, other mammals and birds and usually affects the socio-economic situation of the affected community (Asiedu and Etuaful, 1998; Falkinham, 2011). Nearly thirty-three percent (33%) of all mycobacteria infections causing diseases occur in humans (Katoch, 2004). Infection with NTMs is difficult to control and ultimately prevent due to their opportunist nature and their wide distribution in soil, aerosols, water, insects, protozoans and small animals (Primm et al., 2004; van Ingen et al., 2009). Most NTM infections such as *Mycobacterium kansasii*, *Mycobacterium avium* complex (MAC) and *Mycobacterium intracellulare* cause pulmonary disorders (Ahn et al., 1979) whiles NTMs such as *Mycobacterium marinum*, *Mycobacterium abscessus*, *Mycobacterium fortuitum* and *Mycobacterium chelonae* cause cutaneous infections (Lamb and Dawn, 2014).

The epidemiology of certain NTMs of public health importance have not been adequately addressed. Mycolactone Producing Mycobacteria (MPM) are a subset of NTMs and these MPMs are of enormous importance due to the debilitating cutaneous disease-causing ability in both humans and animals. *Mycobacterium pseudoshotsii* and mycolactone producing *Mycobacterium marinum* have been isolated from fish, *Mycobacterium liflandii* from frogs and *Mycobacterium ulcerans as well as Mycobacterium shinshuense* from humans (Rhodes et al., 2001; Rhodes et al., 2005; Ranger et al., 2006; Funakoshi et al., 2009). Similar studies have also isolated different

strains of MPMs from small mammals such as possum and koala (Mitchell et al., 1984; Fyfe et al., 2010) and other domestic animals including cats and horses (Elsner et al., 2008, van Zyl et al., 2010). The infection of domestic animals raises concern of possible zoonosis; however this field of research has not received substantial attention. There is the possibility of other unidentified MPMs besides the isolated strains that produce mycolactone with sufficient potency to cause infections. A better understanding of distribution of MPMs causing infections would be essential to accurate disease diagnosis and to a larger extent curtailing the spread of the diseases they cause, in particular, Buruli ulcer (Yip et al., 2007).

The debilitating and extensive loss of the cutaneous and sub-cutaneous tissue is the hallmark of the disease, Buruli ulcer (Mosi et al., 2008). It was named after a town in Uganda that had levels of disease occurrence in the 1960s (Baker et al., 1972). Buruli ulcer is caused by *Mycobacterium ulcerans*. It usually affects all body parts indiscriminately, however, there are more infections on the extremities (lower and upper limbs) and rarely on the genitalia and trunk areas (Zingue et al., 2018). This disease is a World Health Organization (WHO) reportable disease geographically present in over thirty-three (33) countries in Southern parts of America, Southeastern parts of Asia, Africa and Western Pacific and is highly endemic in Australia, some West African countries such as Côte d'Ivoire, Togo and Ghana (Röltgen et al., 2012; Narh et al., 2014; Dassi et al., 2017; Zingue et al., 2018). The World Health Organization reported that the total number of Buruli ulcer cases recorded globally was 5,076 in the year 2012 with Africa being the worst affected continent and Ghana, the second most endemic African country after Côte d'Ivoire (WHO, 2012). In Ghana, the Ashanti region usually represents sixty percent (60%) or more of all total cases with the most affected district, Amansie Central having a prevalence of 151 cases per 100,000 inhabitants (Amofah et al., 2002).

Disease pathogenesis is associated with a lipid toxin, mycolactone produced from a naturally acquired 174kb plasmid (Stinear et al., 2004; (Adusumilli et al., 2005). This lipid toxin synthesized by similar plasmids with varying sizes (174kb – 180kb) has also been identified in other NTM such as *M. liflandii*, *M. pseudoshottsii* and *M. marinum DL* which also cause debilitating ulcers in both fish and frog (Ranger et al., 2006). These NTM are collectively known as Mycolactone Producing Mycobacteria. Genetic analysis indicates the emergence of *M. ulcerans* from a progenitor, *M. marinum* following a series of insertion and deletion events and the acquisition of a virulence plasmid pMUM001 through horizontal gene transfer (Pidot et al., 2008). The acquisition of insertion sequences IS*2404* (213 copies) and IS*2606* (91 copies) leading to the creation of seven hundred and seventy-one (771) pseudogenes, loss of twenty- eight (28) PE-PPE genes and approximately 1Mb of genome decay differentiates *M. ulcerans* from its progenitor, *M. marinum*. This makes *M. ulcerans* genome (5.8 MB), 1MB smaller than that of classical *M. marinum* (6.6 MB) (Yip et al., 2007).

The mycobacterial 16S rRNA gene has been employed to differentiate NTM strains (Janda and Abbott, 2007) while SNP typing has been employed to differentiate *M. ulcerans* globally (Käser et al., 2009). Enoyl reductase (ER) and keto reductase (KR) are two enzymes encoded by the virulence plasmid and are involved in biosynthesis of mycolactone (Pidot et al., 2008). These are usually used in combination with the insertion sequence, IS*2404* as genetic markers for the diagnosis of Buruli ulcer (Johnson et al., 2005). MPMs can be differentiated from one another using variable number of short tandem repeats located in certain loci of the genome (Ablordey et al., 2005). The most typed and explored loci for Variable Number Tandem Repeats (VNTR) include ST1, locus 6, locus 19, and Miru1 (Röltgen et al., 2010; Williamson et al., 2014; Narh et al., 2015; Dassi et al., 2017).

1.1. STUDY RATIONALE

Currently, Buruli ulcer is a key public health concern in Africa. As the third most common form of mycobacteriosis after tuberculosis and leprosy, it is the most poorly understood (Amofah et al., 2002). There have been reported cases of bone deformities and in rare cases death if left untreated (WHO, 2012). There is also limited advanced genetic tools for studying transmission of the diseases and for carrying out molecular epidemiological studies (Narh et al., 2015). Most studies have relied on the detection of genomic material of *M. ulcerans* as well as other MPMs in the environment (Stinear and Johnson, 2008) due to the difficulty in culturing environmental samples in relation to disease burden (Williamson et al., 2008; Merritt et al., 2012). All of these MPMs also share about ninety-eight percent (98%) nucleotide similarity therefore hindering proper typing which is reliant on subtle genomic differences (Yip et al., 2007). Advanced molecular tools such as Variable Number Tandem Repeats (VNTR) and Single Nucleotide Polymorphism (SNP) typing has been developed to specifically differentiate *M. ulcerans* from other MPMs due to the closely shared genome and plasmid sequence similarity (Yip et al., 2007). Some studies have however been unsuccessful at genotyping both clinical (Dassi et al., 2017) and environmental samples (Dassi et al., 2017; Tano et al., 2017) due to unsuccessful amplification at target loci making it prudent to optimize and increase the number of loci to distinctively genotype MPMs.

Recently, a presumptive Buruli ulcer lesion from a young patient tested negative for *M. ulcerans*. The MPM, *M. shinshuense* was rather identified as the cause of the ulceration (Funakoshi et al., 2009). Most of the clinical and environmental samples typed by Dassi et al., (2017) using phylogenetic analysis for IS*2404* sequences revealed ninety-six (96) to ninety-nine (99) percent similarity to MPMs, *M. pseudoshottsii* and *M. liflandii* rather than to *M. ulcerans*. Also, using

two VNTR loci; ST1 and MIRU1, Hilty et al., (2006) identified two strains of *M. ulcerans* in circulation within the Amansie Central district (then Amansie West). This suggests that increasing the loci required for typing by the addition of polymorphic loci would increase the discriminatory power of this tool and reveal more hidden genotypes masked by not including such loci. It is therefore necessary to know the exact MPMs that are causing infection by genotypically distinguishing these isolates using VNTR Tying at 16 Miru VNTR Loci to increase the discriminatory power and reveal more genotypes in this study.

1.2. AIM OF STUDY

This study aims to assess the genetic diversity of Mycolactone Producing Mycobacteria infections in, within and between selected communities in Ghana and Côte d'Ivoire.

1.3. SPECIFIC OBJECTIVES

Specifically, this study seeks to,

1. Confirm MPM infection using microscopy and Polymerase Chain Reaction (PCR) assays
2. Genotype human MPM isolates at a panel of 16 VNTR loci present in *M. ulcerans* Agy99 genome
3. Assess the genetic diversity in the MPM populations from Ghana and Côte d'Ivoire

CHAPTER TWO

2.0. LITURATURE REVIEW

2.1. Mycobacteria

Mycobacteria taxonomically belongs to the kingdom Bacteria and the phylum, Actinobacteria. Mycobacteria are also grouped under the Order actinomycetales, belongs to the sub-order Corynebacterineae and ultimately part of the family Mycobacteriaceae (Skerman *et al.*, 1980; Wayne, 1984; Rastogi et al., 2001;). Presently, Mycobacteria are the only genus in the Mycobacteriaceae family with about 170 species (Forbes, 2017). Members in this genus can be grouped into four major categories; Mycobacterium tuberculosis complex (MTC), *Mycobacterium leprae*, *Mycobacterium ulcerans*, and other Non-Tuberculous Mycobacteria (NTM) based on growth in vitro, epidemiology and disease association (Forbes et al., 2018). Runyon, (1959) and Tortoli (2006) also groups' Mycobacteria into four based on growth rate and pigmentation.

The first group of mycobacteria are typically slow growers and photochromogenic thus they usually take longer to produce visible colonies which produces pigment only in the presence of light. Examples include *M. kansasii*, *M. marinum*, *M. simiae* and *M. pseudoshottsii* (Grange, 2008; Runyon, 1959). The second group of mycobacteria are also slow to grow but produces pigmentation with or without light (scotochromogenic). Examples in this category also include *M. scrofulaceum* and *M. gordonae* (Runyon, 1959). The third group of mycobacteria are slow growers but do not produce pigmentation (non-chromogenic). *M. ulcerans* as well as *M. xenopi*, *M. avium* and *M. haemophilium* belong to this category (Runyon, 1959). The last group of mycobacteria unlike the first three groups are fast growers and as such produce visible colonies

in less than one week. Examples include *M. fortuitum*, *M. chelonae* and *M. abscessus* (Grange, 2008; Runyon, 1959).

Mycobacteria have a hydrophobic nature with their hydrophobicity attributed to the possession of a highly complex cell wall which is essentially rich in lipids. This feature gives them the innate ability to proliferate on the surfaces of liquid media as mould pellicles (Grange, 2008). The cell wall has four layers that surrounds a lipid bilayer membrane which contains carotenoids and usually impart the yellow color formation to bacterial colonies (Grange, 2008). The possession of a cell wall rich in mycolic acids makes them acid fast and thus resistant to acid alcohol decolorization. Characteristics of this group of bacteria include non-motility, non-spore-forming, gram-positive straight or curved to an extent and aerobic growth requirements. They are characterized by their slow growth as compared to other bacterial groups and some species show pigmentation (Pfyffer, 2015). Phylogenetic analysis shows that a trade-off between faster growth rate and their pathogenicity in humans has been made even though the genus encompasses obligate pathogen, opportunistic and non-pathogenic forms (Pfyffer, 2015; Forbes et al., 2018).

2.1.1. Mycobacterium Tuberculosis Complex (MTC)

These are similar bacteria causing tuberculosis in a range of animals, including humans. The tubercle bacilli can either be eliminated by the host immune system or the establishment of an active or latent infection may occur. Though very similar, members of the group vary and can be differentiated using genotypic assays (Vernon A., 2013; Forbes et al., 2018). Among this group is *M. tuberculosis*, which has caused substantial infections with high mortality in the last decade though numbers, have decreased over the years. Highly resistant forms have also been recorded

which raises concerns about the disease. M. *bovis* affect animals and also humans (Forbes et al., 2018).

2.1.2. Mycobacteria leprae

Mycobacterium leprae cause the most common mycobacterial disease after tuberculosis and Buruli ulcer (Cambau et al., 2012). Leprosy is a skin disease with chronic granulomatous, which may be mild or systemic (Cambau et al., 2012). The bacteria cannot be cultivated in vitro in the lab and is therefore usually diagnosed using observed clinical symptoms (Alotaibi et al., 2016). With its low prevalence and most cases recorded in India, leprosy cases have decreased (Cambau et al., 2012).

2.1.3. Non-Tuberculosis Mycobacteria (NTM)

Non-Tuberculosis Mycobacteria (NTM) has been classified in to either slow or fast growing groups (Runyon, 1959; Forbes et al., 2018). Members of this group include *M. abscessus, M. arcueilense, M. bourgelatii, M. celeriflavum,* and *M. europaeum* having other species and complexes as close relatives (Runyon, 1959; Tortoli, 2006). They may isolated from humans but may not necessarily cause disease in humans (Forbes et al., 2018). They are present on a wide range of niches in the environment from animals to inorganic inanimate surfaces and fomites due to their cell wall structure and biofilm formation, which serves as protection. This may lead to pseudoinfections during diagnosis (Griffith et al., 2007; Falkinham et al., 2015).

2.1.4. Mycobacterium ulcerans

Mycobacterium ulcerans causes Buruli ulcer, the disease highly characterized by the debilitating and extensive loss of the cutaneous and sub-cutaneous tissue (Williamson et al., 2008). This mycobacterium was first isolated in the year, 1948 by Maccallum et al., (1948) in Bairnsdale,

Australia thus known as Bairnsdale ulcer in some parts of Australia (Zingue et al., 2018). *M. ulcerans* infection is however widely known as Buruli ulcer named after a county in Uganda, Buruli which is now referred to as Nakosongola where high prevalence of disease was observed (Lunn et al., 1965).

Most studies that looked entirely at the bacterium's whole genome as well as specific coding and non-coding genes for phylogenetic analysis suggests that *M. ulcerans diverged* from a progenitor, *M. marinum* (Stinear et al., 2000). One of the two major genomic events that occurred to cause divergence of *M. ulcerans* from *M. marinum* is the acquisition of a 174kb, pMUM001 which encodes two enzymes, enoyl reductase and keto reductase collectively referred to as the polyketide synthase required for mycolactone synthesis (Stinear et al., 2004). Secondly, the acquisition and proliferation of insertion sequences (IS), *2404* and *2606* by *M. ulcerans* has contributed to its divergence from *M. marinum*. There are two hundred and thirteen (213) copies of IS*2404* (205 copies on the circular chromosome and 8 copies on the pMUM001 plasmid) and ninety-one (91) copies of IS 2606 (88 copies on the circular chromosome and 4 copies on the pMUM001 plasmid) (Stinear et al., 2007).

Worthy of mention are notable differences that exist between classical, non-mycolactone producing *M. marinum* and *M. ulcerans* due to these genomic events that occurred over time. Phenotypically, classical *M. marinum* grows faster (four hours doubling time) than *M. ulcerans* (seventy-two hours doubling time) and produces yellow pigmented colonies when exposed to light (photochromogenic) (Stinear et al., 2007; Yip et al., 2007). In relation to metabolism, *M. marinum* unlike *M. ulcerans* can make use of carbohydrates such as glucose, succinate and pyruvate as the main carbohydrate source (Stinear et al., 2007). Deletions and genomic

rearrangement events as well as acquisition of these insertion sequences have led to the formation of seven hundred and six (706) more pseudogenes in *M. ulcerans* than classical *M. marinum* which has only sixty-five (65) pseudogenes and loss of twenty-two (22) to twenty-eight (28) more PE-PPE genes. These pseudogene formations have interrupted certain activities and processes such as anaerobic respiration, formation of pigment and expression of potent T-cell antigens (Stinear et al., 2007). Studies have also shown that the PE-PPE genes present in classical *M. marinum* but absent in *M. ulcerans* supports the latter's survival in phagocytes (Stinear et al., 2007; Zingue et al., 2018). These activities can thus confer niche-specific adaptation and increased pathogenicity for the bacterium.

2.1.4.1. Mycobacterium ulcerans strain Agy 99

Mycobacterium ulcerans strain Agy 99 is a Ghanaian isolated strain which has been extensively studied and whole genome sequenced (Zingue et al., 2018) and hence serves as a good control strain for studies in Ghana and surrounding countries. This sequence is available on National Center for Biotechnology Information (NCBI) (uid62939, accession numbers: NC_005916, NC_008611). *M. ulcerans* strain Agy 99 was completely sequenced and readily available on the April 6, 2006 at the Unite de Genetique Moleculaire Bacterienne, Institut Pasteur, Paris using Sanger sequencing. This strain was isolated from an ulcer on the right elbow of a female patient in the Ga district of Ghana, West Africa in the year 1999 (Stinear et al., 2007). It has a circular genome of a 5.6 Mb chromosome and a 174,155bp plasmid. The chromosome contains four thousand, one hundred and sixty (4160) coding sequences and seven hundred and seventy-one (771) pseudogenes and it shelters two (2) prophages. It also has 302 copies of insertion sequences as well as multiple gene deletions and rearrangements. Studies show that this strain contains two prophages, phiMU01, a 18kb prophage which encodes an eighteen (18) coding sequence and

phiMU02, a 24kb prophage which encodes a seventeen (17) coding sequence (Stinear et al., 2007; Yip et al., 2007). The latter's function is possibly disturbed due to the acquisition and proliferation of IS 2606 (Stinear et al., 2007). The plasmid pMUM001 encodes eighty-one (81) coding sequences out of which six (6) code for proteins required for mycolactone synthesis. All these events have led to the loss of approximately 1Mb of *M. ulcerans* DNA causing a reduction in genomic size (5.8Mb) than that of *M. marinum* (6.6 Mb).

2.1.5. Genetic Classification of Mycobacterium ulcerans

Reports indicate geographic diversity of *M. ulcerans* into African and Australian subtypes revealed by partial gene sequencing of the 16S rRNA (Portaels et al., 1996; Huys et al., 2000). Käser et al., (2007) accessed polymorphic regions contained in twelve (12) region of differences (RD) in a study involving thirty (30) *M. ulcerans* strains from different geographical locations. He reported five (5) haplotypes based on insertion and deletion events and classified isolates as of ancestral lineage (fairly and closely similar to the progenitor, *M. marinum* and includes isolates from South America, Mexico and Asia, specifically China and Japan) and classical lineage (greatly different from the progenitor, *M. marinum* and involves the most pathogenic MPM isolates from South East Asia, Australia and Africa) (Käser et al., 2007; Zingue et al., 2018) (Figure 1). Stinear et al., (2000) also affirms a similar geographic diversity pattern of isolates using probe hybridization of IS*2404* after restriction fragment length polymorphism (RFLP).

These two lineages have been estimated to have diverged about 250,000 to 400,000 years ago; Classical African lineages may have emerged in the past 18,000 years (Qi et al., 2009). Molecular epidemiological studies on classical African isolates also indicate West African isolates (Benin, Togo, Côte d'Ivoire and Ghana, Togo) and Central African isolates (Angola, Gabon, Congo-

Brazzaville, Democratic Republic of the Congo and Cameroon) shared similar mycobacterial interspersed repetitive unit-variable number of tandem repeat (MIRU-VNTR) profiles and have only a small number of single nucleotide polymorphisms (SNPs) separating them genome-wise (Stragier et al., 2006; Bolz et al., 2015).

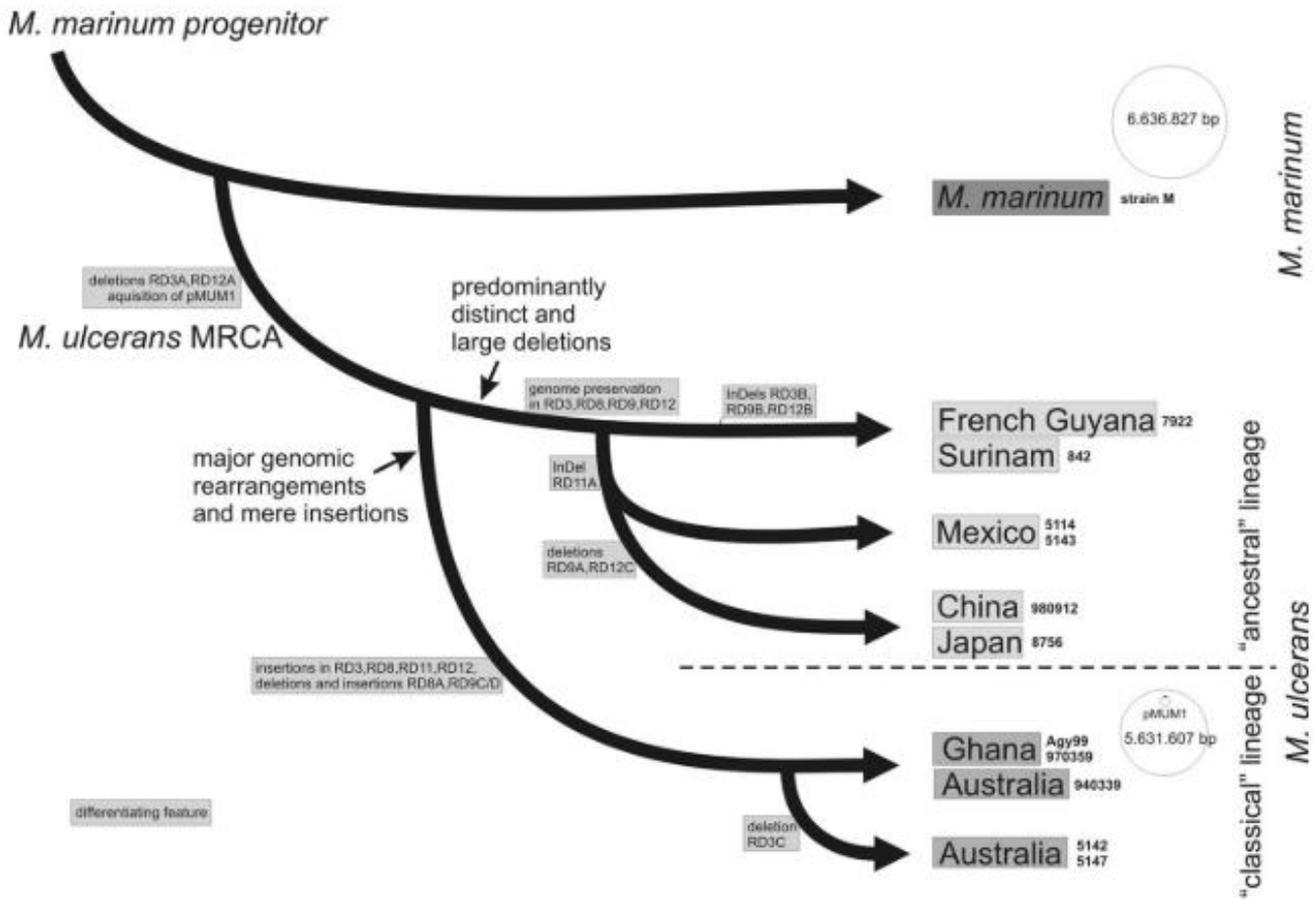


Figure 1: *Divergence of Mycobacterium ulcerans from Mycobacterium marinum progenitor into two distinct lineages analyzed based on region of differences of isolates from diverse geographic locations. Adapted from Käser et al., (2007)*

2.1.6. Mycolactone and Mycolactone Producing Mycobacteria (MPMs)

Mycolactone is a diffusible polyketide molecule responsible for lesions observed in the Buruli ulcer disease (Boulkroun et al., 2009; Bolz et al., 2015). Mycolactone production is a main distinguishing factor between *M. ulcerans* and its progenitor, *M. marinum*, produced by a 174kb plasmid pMUM001, acquired by *M. ulcerans* (Farrar et. al., 2014). The chemical structure of mycolactone shows three regions labelled as the Northern chain (NC), a lactone core (LC) and a Southern chain (SC) (Sarfo et al., 2016) (Figure 2). Changes based on the SC generates congeners that are produced by the bacteria, also with differing geographic distribution (Fred Stephen Sarfo et al., 2016) (Figure 2).

The plasmid is responsible for synthesizing mycolactone by the mlsA1, mlsA2 and mlsB genes, which synthesize the major components, notably the NC, LC, and SC. The final molecule is formed through the action of mup045 and mup038 accessory proteins that combine the individual components (Porter et al., 2013). The different types of mycolactone include mycolactone A/B, C, D, E and F, with the congener showing the highest cytotoxicity in vitro being A/B (Pidot et al., 2008). Recent studies show two mechanisms of action of mycolactone; through scaffolding proteins which leads to cell death by anoikis, and through the inhibition of translocation of proteins from the endoplasmic reticulum due to the binding of mycolactone to the Sec61translocon (Hong et al., 2008, Guenin-Macé et al., 2013; B. Hall & Simmonds, 2014; Demangel & High, 2018). This latter effect shows a time-dependent effect on immune cells and others cells, along with a different range of effects observed depending on the type of cell (Fred Stephen Sarfo et al., 2016). Currently, another effect of mycolactone is its ability to activate angiotensin II receptor which causes the production of prostaglandin E2 leading to its analgesic effect (Hong et al., 2008; Marion et al., 2014; Song et al., 2017; Guenin-Macé et al., 2019).

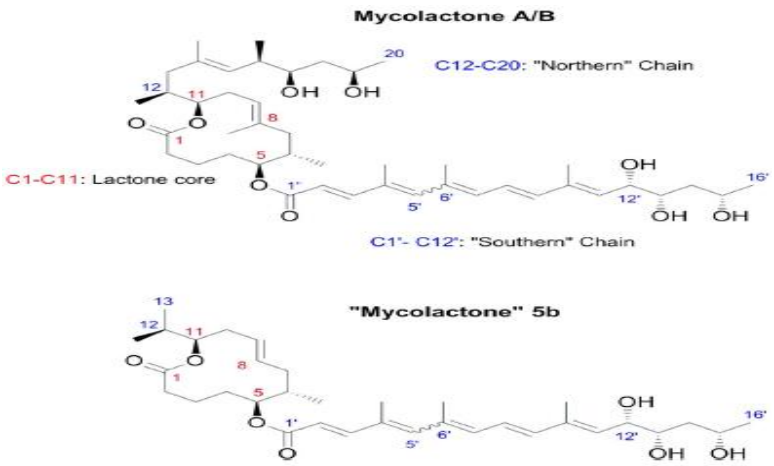


Figure 2: *Structure of mycolactone depicting the various divisions present in the compound. Adapted from Sarfo et al., (2013).*

M. ulcerans strains from diverse geographic locations produce different congeners of mycolactone. Isolates from Africa, Australia and China produce mycolactones A/B, C, and D respectively (Pidot et al., 2008) (Figure 3).

Recently, other mycobacterial species (*M. pseudoshottsii*, *M. liflandii*, *M. marinum* DL, *M. ulcerans* subsp. *shinshuense*) have been identified with this unique toxin production ability and are thus classified as Mycolactone Producing Mycobacteria (MPM) by the possession of similar but larger plasmids (Mve-Obiang et al., 2005; Ranger et al., 2006a; Yip et al., 2007). MPMs have also been categorized under both classical and ancestral lineages. *M. ulcerans* isolated from South East Asia, Australia and Africa are categorized under the classical lineage while *M. ulcerans* strains isolated from Asia (Japan and China), North America and Mexico. *M. pseudoshottsii* and mycolactone producing *M. marinum* isolated from fish and *M. liflandii* from frogs are classified under the ancestral strain (Doig et al., 2012). All of the MPMs share about ninety-eight percent

(98%) nucleotide similarity and contain varying copy numbers of IS*2404* and IS*2606* (Pidot et al., 2008).

The first report of mycobacteriosis by mycolactone producing *M. marinum* was reported in *Dicentrarchus labrax,* a European sea bass fish cultured from the Red sea (Ucko et al., 2002, Colorni, 1992). Subsequently, fish mycobacteriosis have been detected in over twenty (20) fish species as well as sea turtle (Diamant et al., 2000; Ucko et al., 2002). This species has an optimum growth temperature between thirty to thirty-two degrees Celsius (30^{o}C-32^{o}C) and has an incubation period of approximately four (4)weeks and a doubling time of four and a half (4.5) hours (Ranger et al., 2006a). Mycolactone producing *M. marinum* possess a similar 210kb plasmid, pMM23, which is responsible for the production of Mycolactone F (Käser et al., 2007; Pidot et al., 2008).

Rhodes et al., (2005) first isolated *M. pseudoshottsii* from *Morone saxatilis,* a striped bass fish. Imajoh et al., (2013) in Japan also reports successful isolation of this species from two fish species, striped jack *Pseudocaranx dentex* and yellowtail *Seriola quinqueradiata. M. pseudoshottsii* is photochromogenic and retains Ziehl Neelsen stain thus is acid fast and is coccobacilli in shape (Rhodes et al., 2005). *M. pseudoshottsii* also possess a 210kb plasmid, pMUM003 which is the largest sized-plasmid amongst the MPMs besides *M. marinum* DL but produces however, the smallest mycolactone, Mycolactone F (Ranger et al., 2006a; Pidot et al., 2008). It grows at twenty-three to twenty-five degrees Celsius (23^{o}C-25^{o}C) and has an incubation period of approximately four (4) to six (6) weeks (Rhodes et al., 2005; Ranger et al., 2006a). *M. liflandii* is the only species of MPM isolated and associated with mycobacteriosis in frogs (Trott et al., 2004; Suykerbuyk et al., 2007). *M. liflandii* infection was first reported in 2004 by Trott et

al., (2004) in *Xenopus tropicalis,* an African tropical clawed frog. This species has an optimum growth temperature at twenty-eight degrees Celsius (28^{o}C) with 5% carbon dioxide (CO_2) and has an incubation period of approximately four (4) weeks and a doubling time of eighteen (18) hours (Trott et al., 2004; Ranger et al., 2006). It harbors a 190kb plasmid, pMUM002 which produces Mycolactone E (Mve-Obiang et al., 2005; Käser et al., 2007; Pidot et al., 2008).

M. ulcerans subsp. *shinshuense* was first isolated from a nineteen (19) year old Japanese woman (Mikoshiba et al., 1982), and later from the right elbow of a twenty (20) year old woman in Japan (Nakanaga et al., 2011). Notably, all subsequently isolated *M. ulcerans* subsp. *shinshuense* have been isolated from Japan and China (Funakoshi et al., 2009; Nakanaga et al., 2011). It also possesses a 174kb plasmid, pMUM001 and produces mycolactone A/B. The only structural difference between mycolactone produced by *M. ulcerans* and its subspecies *M. ulcerans* subsp. *shinshuense* is the side chain which arises from differences within the respective regions encoding the sidechain (Kim et al., 2005; Nakanaga et al., 2007). Phenotypically, the availability or absence of light does not perturb the formation of yellow colonies of *M. ulcerans* subsp. *shinshuense* (Nakanaga et al., 2011). It has an optimum growth temperature between twenty-five to thirty-two degrees Celsius (25^{o}C-32^{o}C) (Nakanaga et al., 2011).

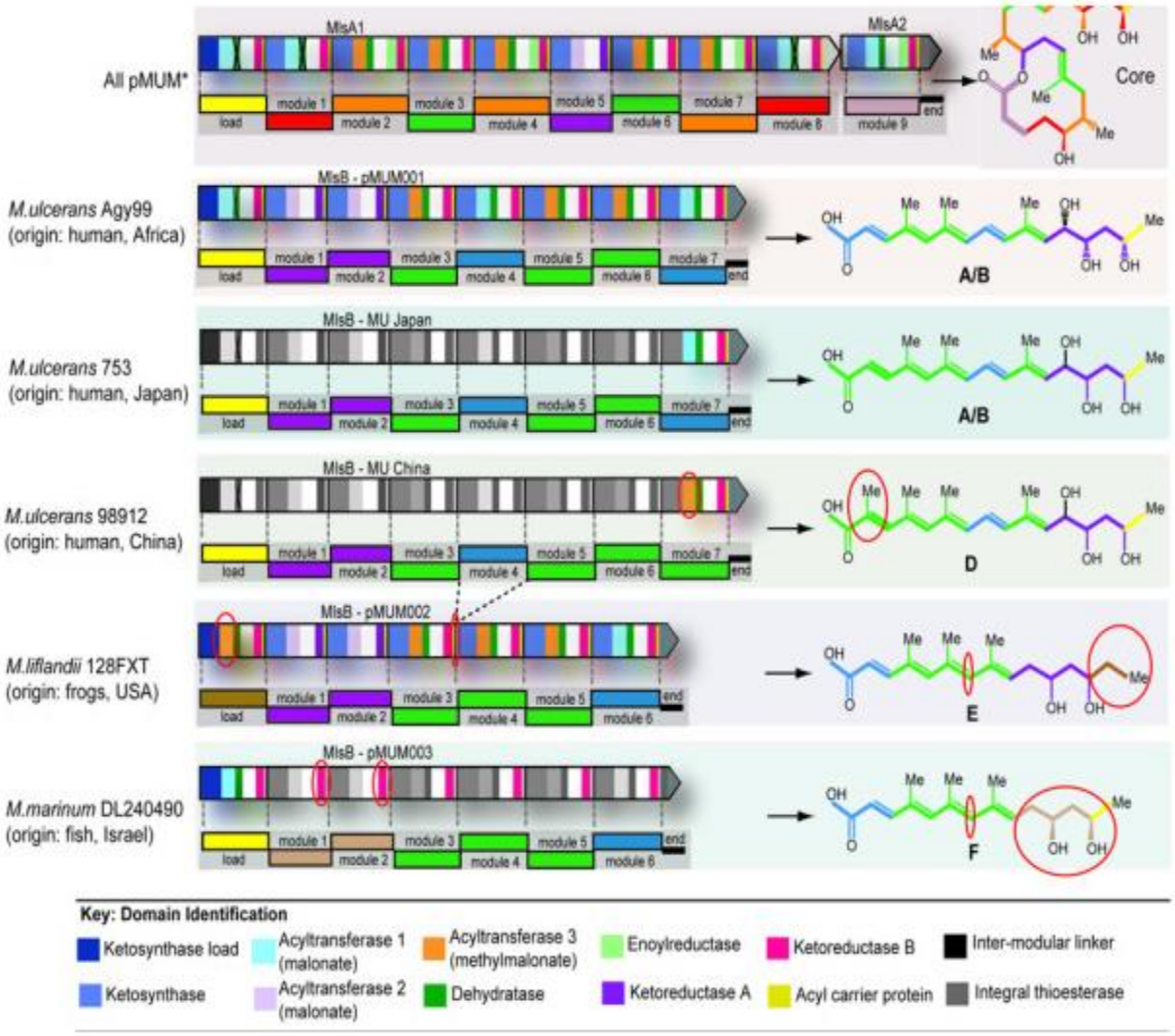


Figure 3: *Genetic organization of the mycolactone biosynthetic cluster from plasmids present in MPMs. Adapted from Pidot et al., (2008).*

2.2. Buruli ulcer disease

Buruli ulcer (BU) is an infection cause by *M. ulcerans* (Williamson et al., 2008). The pathogenesis of this disease is attributed to the production of mycolactone (George et al., 1999). The disease has been attributed to humid areas and affects mostly children in Africa (Johnson et al., 2005) but this might not be the case if there is an active case search which seems to be absent for the disease in endemic areas, especially Africa (Quaye et al., unpublished data). It has a large impact on the socioeconomic status of affected communities and families. To a larger extent, debilitating effect which mostly results in deformities and disability but rarely fatal cases, leads to social stigmatization to the affected individuals (Stienstra et al., 2002).

According to Walsh et al, (2010), Buruli ulcer is the third most common mycobacterial infection after tuberculosis (TB) and leprosy; however reports indicate that the burden of leprosy has diminished making Buruli ulcer second to TB (Cambau et al., 2012; WHO, 2017). In the 1980s, Buruli ulcer became a public health problem, thus prompting Buruli ulcer to be declared a neglected tropical disease, and the initiation of the establishment of Global Buruli Ulcer Initiative (GBUI) by the World Health Organization (WHO) in 1998 (Amofah et al., 2002). GBUI was implemented for raising disease awareness, improving access to early diagnosis, treatment and the promotion of research to develop better tools for treatment and prevention of Buruli ulcer (WHO, 2012). Buruli ulcer mostly affects residents of rural communities who predominantly farm as an occupation (Asiedu and Etuaful, 1998). Residents in these communities associate disease occurrences with curses, witchcraft and punishment of sins of affected individuals in the community (Asiedu and Etuaful, 1998) and as a result prefer to consult and rely upon spiritualists or herbalists for treatment (Stienstra et al., 2002). Another group also likens disease occurrence with personal hygiene and thus forgo treatment (Adamba and Owusu, 2011). Buruli ulcer patients

are usually stigmatized and marginalized just like patients with leprosy (Adamba and Owusu, 2011; Cambau et al., 2012).

2.2.1. Epidemiology

This disease has been geographically present in over thirty-three (33) countries in Southern parts of America, Southeastern parts of Asia, Africa and Western Pacific and is highly endemic in Australia and West African countries namely Côte d'Ivoire, Togo and Ghana but in recent years, Australia has recorded an increase in the number of cases especially in Victoria state (Röltgen et al., 2012; Loftus et al., 2018) (Figure 4) .The disease is also found in the global South East in Australia (Johnson and Lavender, 2009). A number of cases have also been reported in South East Asia, North America, Japan, China and Mexico (Oliveira et al., 2005;WHO, 2008; WHO, 2010; Nakanaga et al., 2011).

The World Health Organization in the year 2012 reported that the total number of Buruli ulcer cases recorded globally was 5,076 with Africa being the worst affected continent and Ghana, the second most endemic African country after Côte d'Ivoire (WHO, 2012). In Ghana, the Ashanti region usually represents 60% or more of all total cases with the most affected district, Amansie Central having a prevalence of 151 cases per 100,000 inhabitants (Amofah et al., 2002). According to the WHO in 2012, cases have dropped, more than fifty percent (50%), for most of the countries that consistently report it, in comparison to their report in 2009. Buruli ulcer usually affects all body parts indiscriminately, however, there are more infections on the extremities (lower and upper limbs) and rarely the genitalia and trunk areas (Zingue et al., 2018). Most cases are diagnosed in the final stage (Category III) of the disease (WHO, 2012). These statistics may be affected by underreporting, especially in African countries where infected individuals do not

report to the hospital and the presence of a poor health system (Farrar et. al., 2014; Quaye et al., unpublished data).

In Africa, the majority of Buruli ulcer cases are observed in children below fifteen (15) years of age and also among individuals in close proximity to slow moving water bodies usually engaging in farming activities (Van Der Werf et al., 1999; Adamba and Owusu, 2011). Asiedu and Etuaful, (1998) and Stienstra et al., (2002) attribute this observance in young age groups to their adamant exposure to aquatic habitats and swamp areas which are contaminated with the bacterium and its debatable vectors.

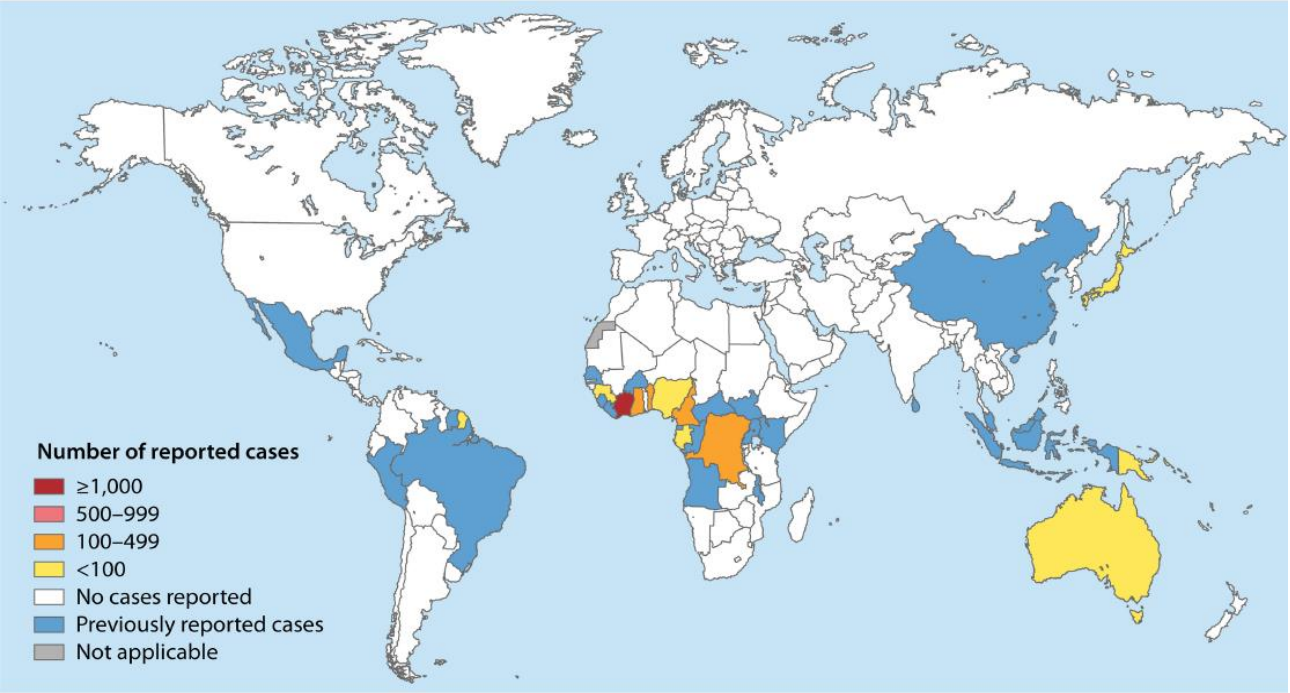


Figure 4: *The distribution of BU cases reported as of 2014. Adapted from Zingue et al., (2018)*

2.2.2. Transmission of Buruli ulcer

Transmission is yet to be fully elucidated as it still remains a mystery to date (Mosi et al., 2008). Inability of the pathogen to cause diseases in dry and vegetative regions of the globe strongly implies that disease occurrence may be limited by environmental constraints as the pathogen has been severally isolated from aquatic ecosystems (Mosi et al., 2008). In addition, rainfall has been associated with transmission but this varies from place to place (Yerramilli et al., 2018). Several organisms including invertebrate insects such as Belostomatidae and Naucoridae have been associated with transmission (Portaels et al., 1999; Mosi et al., 2008). However, the mode of transmission of the pathogen from environments that pose extremely serious threats of infection to humans remains unknown and its reservoirs in the environment still investigated (Merritt et al., 2010; Narh et al., 2015). Johnson et al., (2005, 2007), Quek et al., (2007) and Lavender et al., (2011), all studies from Australia suggests that mosquitoes may be probable vectors of *M. ulcerans* and reservoirs of Buruli ulcer.

A study in Benin, an endemic Buruli ulcer country however suggests that mosquitoes do not play any major role in the transmission of the disease (Zogo et al., 2015). Yerramilli et al., (2017) posits that biting from invertebrate insects is a more probable means of transmission due to the pattern of distribution of the lesions on different parts of the body (Farrar et. al., 2014). Direct human contact as a means of transmission has not been recorded but the probability of recording such cases might be very low as result of the means of infections by the bacteria, which requires subcutaneous access (George et al., 2000, Marsollier et al., 2003; Tanghe et al., 2008). Johnson and Lavender, (2009) loosely links infection through the inhalation of aerosols or droplets with the bacteria. Risk factors of the disease include proximity to wet or swampy, slow moving water bodies (Debacker et al., 2004). Stienstra et al., (2006) correlates susceptibility of the disease to

polymorphisms in NRAMP1 also known as SLC11A1 gene required for the production of the natural resistance-associated macrophage protein and reports a thirteen percent (13%) risk in study population.

2.2.3. Clinical pathology and manifestation

According to the WHO, Buruli ulcer manifests as either a painless nodule (Figure 5A) or plaque (Figure 5B) to an oedema (Figure 5C), which may be painful and finally to a minimally painful or painless ulcerative stage (Figure 5D) (Debacker et al., 2004; WHO,2008). WHO categorizes the ulcerative forms as category I (ulcers less than five (5) cm in diameter), category II (ulcers between five (5) and fifteen (15) cm in diameter) and category III (ulcers greater than 15 cm in diameter, multiple lesions and extreme deformities) (Sizaire et al., 2006). The infection is localized especially to the extremities with rare systemic infections as the body's temperature is unfavorable for *M. ulcerans* growth (Matsumura et al., 2012).

The disease primarily affects fat tissues under the epidermis and spreads. The affected area contains large clusters of the bacteria, which decreases as the distance increases from the focal point due to the action of mycolactone (Zingue et al., 2018). Stained sections of excised tissues that were histopathologically examined showed that *M. ulcerans* is extracellular (Bolz et al., 2015), however, some studies also observed the bacteria intracellularly in macrophages, and attributes that to a transient stage during infection (Schutte et al., 2009, Torrado et al., 2010). Mycolactone is responsible for the death of immune cells and interferes with innate and adaptive immune responses (Sarfo et al., 2011). As the disease advances, bone lesions may develop in addition to the metastatic osteomyelitis (Farrar et. al., 2014). Mortality associated with Buruli

ulcer is very low, however severe disfigurement and permanent disabilities such as contractures are frequently reported (usually on the limbs) (WHO, 2012).

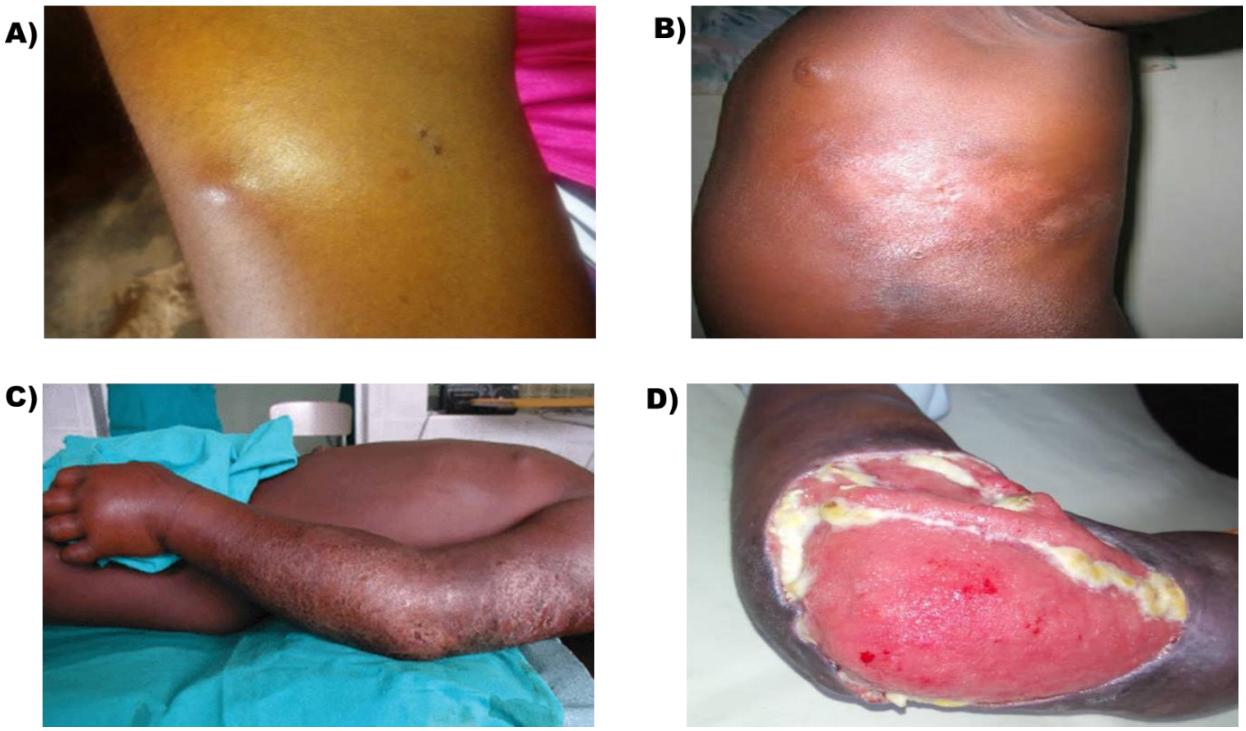


Figure 5: Clinical manifestations of Buruli ulcer. ***A*** *is the nodular form of the disease.* ***B*** *and* ***C*** *are the plaque and oedematous form of the disease. D is an ulcerative from with characteristic cotton-like patches with undermined areas. Images* ***A*** *and* ***D*** *are from this present study while Images* ***B*** *and* ***C*** *were adapted from Dégboé et al., (2019) and Boleira et al., (2010) respectively*

2.2.4. Diagnosis

Diagnosis of the disease involves culturing on supplemented Lowenstein-Jensen (LJ) or Middlebrook 7H9 and Middlebrook 7H10 media with specific biochemical tests, microscopy for acid fast bacilli, polymerase chain reaction (PCR) and histopathological examinations of stained excised tissues (Mensah-Quainoo et al, 2003; Sizaire et al., 2006; Beissner et al., 2010; Yeboah-manu et al., 2011).Fine Needle Aspiration (FNA) and swab specimen are usually taken from suspected nodules and ulcers respectively for laboratory analysis.

Bacterial isolation upon culture has twenty to sixty percent (20–60%) sensitivity and requires more than eight (8) weeks of growth on specific medium with tissue biopsy being the preferred sample (Sizaire et al., 2006). Microscopy which involves bacterial staining using auramine O acid-fast or Zheil-Nelsen stain is the common point-of-care diagnosis with about forty to eighty percent (40- 80%) sensitivity (Sizaire et al., 2006). PCR targeting the IS*2404* on the other hand, has become the most common diagnostic in reference labs due to its specificity and sensitivity (over 90%) (Sizaire et al., 2006). Histopathological examination of excised tissues also have over ninety percent (90%) sensitivity and involves observation of features such as vasculitis, epidermal hyperplasia and coagulative necrosis of epidermal cells (Sizaire et al., 2006; Farrar et. al., 2014). WHO however recommends IS*2404* as the standard method of disease confirmation.

2.2.5. Treatment and Management

Early diagnosis and detection of the disease is treatable and terminable within eight weeks course of antibiotics alone or along surgical excision for more extension ulcers (Asiedu and Etuaful, 1998; WHO, 2012). WHO recommends the use of antibiotic combinations, streptomycin and rifampicin or clarithromycin and rifampicin for eight weeks as first-line treatment for all active

cases of any form (WHO, 2012). The advent of antimycobacterial therapy has helped curb the disease but the long duration and close monitoring of patients may undermine its effectiveness as some antibiotic resistance has been reported in some cases. Surgical excision of necrotic tissue and skin grafting are measures to prevent and reduce disabilities (WHO, 2012). Other antibiotics such as streptomycin, kanamycin cycloserine, enviomycin and levofloxacin have been reported to be effective both *in vitro* and *in vivo* (Bretzel et al., 2010; Matsumura et al., 2012). Thermotherapy is another explored area for BU treatment by the generation and maintenance of a temperature of forty degrees Celsius (40°C) at the site of infection (usually ulcers) for longer periods compared to the drug therapy (WHO, 2012; Junghanss et al., 2014).

According to Junghanss et al. (2014), Buruli ulcer treatment has three different phases. The first phase involves the treatment of the disease, which requires the practice of good nutrition hygiene preceded by an initial reaction known as the paradoxical reaction, whereby drug therapy decreases deteriorating of the ulcer. The next phase involves wound dressing and healing serving as a crucial phase of this treatment regime as co-infection with other bacteria may hinder or slow down the healing process. The last phase of this treatment involves the gradual restoration to normal activities involving the affected part of the body (van der Werf et al., 2010). There is currently no vaccine for Buruli ulcer; however potential antigen candidates have been identifies for vaccine development (Tanghe et al., 2008; Einarsdottir et al., 2011).

Strategies to curb this disease has been marred by the lack of attention given to the disease. The interplay between accessible healthcare and the pursue of healthcare by the diseased has also played a role in the inability to manage the disease (Stienstra et al., 2002).

Prevention of the disease still remains daunting and elusive due to inability to fully understand and elucidate the transmission of the disease. Transient protection can be achieved with the use of Bacille Calmette–Guérin (BCG) vaccine Bacille Calmette–Guérin vaccine has been shown to have transient protection, however prolonged protection is not assured (Junghanss et al.,2014; Guenin-Macé et al., 2019)

2.3. Molecular Diagnosis of Buruli ulcer

2.3.1. IS2404 and IS 2606 PCR and Restriction Fragment Length Polymorphism (RFLP)

IS*2404* and IS*2606* have been identified in the genome of *M. ulcerans* strain, Agy99 on both the circular chromosome and plasmid (Pidot et al., 2008). Primers flanking this region have been designed for the molecular detection of the MPMs in clinical, veterinary and environmental samples (Stinear et al., 2000; Phillips et al., 2005; Ranger et al., 2006b; Yeboah-manu et al., 2011). RFLP of IS*2404* amplicons have been used to genotype *M. ulcerans* from different geographical origin into six (6) distinct genotypes (Portaels et al., 2001), however, the emergence of MPMs with the same insertion sequences require additional markers for definite diagnosis (Suykerbuyk et al., 2007).

2.3.2. Multi-locus sequence typing (MLST)

MLST involves the simultaneous analysis of aligned sequences from different housekeeping genes compared simultaneously (Stinear et al., 2000). Stinear et al., (2000) obtained six (6) different genotypes distributed in six geographical locations of Africa, Australia, China, Surinam, Papua New Guinea, and Mexico which is consistent with Portaels et al., (2001) IS*2404* RFLP analysis.

2.3.3. Variable Number Tandem Repeats (VNTR) Typing

VNTR is a region in the genome where short nucleotide sequences occur in tandem repeats (Ablordey et al., 2005). These tandem repeats lead to differences in length polymorphisms of the gene locus that results in differences in genomes and can thus be used to differentiate related species. Loci for VNTR typing were identified by two approaches. One approach explored by Ablordey et al., 2005 and Hilty et al., 2006 was the screening of the whole genome of classical *M. marinum* for loci containing sequences repeated in tandem. Stragier et al., 2005 used the same approach in addition to BLAST searches to find *M. tuberculosis* MIRU homologs in *M. ulcerans*. The completely assembled genome of *M. ulcerans* for loci containing sequences repeated in tandem for the first time was explored (Hilty et al., 2007) and this revealed forty-five (45) new tandem repeat containing loci that showed genetic diversity among eleven (11) isolates used.

MPMs have a number of VNTRs located within both functional and non-functional genomic regions identified in *M. ulcerans* strain Agy99 (Ablordey et al, 2005; Ablordey et al., 2005). VNTR typing is a highly beneficial typing tool for MPMs due to its ability to differentiate between MPMs based on even four loci as compared to other standard molecular typing tools (Ablordey et al., 2005; Hilty et al., 2006; Stragier et al., 2005; 2006). The most explored VNTR loci targeted by PCR reactions include locus 6, locus19, MIRU1 and ST1 which differentiates *M. ulcerans* from other MPMs harboring IS*2404* and IS*2606*, and also solve the apparent genetic homogeneity within/between *M. ulcerans* geographical isolates (Ablordey et al., 2007). Most studies have relied on VNTR tying to genotype isolates (Stragier et al., 2005; Hilty et al., 2006; Williamson et al., 2014; Narh et al., 2015; Tano et al., 2017). Genetic diversity among African isolates was for the first time achieved using VNTR typing (Hilty et al., 2006; Stragier et al., 2006).VNTR analysis of two polymorphic loci, MIRU1 and ST1 on seventy-two (72) African

isolates (fifty-seven (57) Ghanaian isolates) revealed three different genotypes with clonal clustering, suggesting high genetic diversity of *M. ulcerans* among Ghanaian isolates (Hilty et al., 2006).

CHAPTER THREE

3.0. METHODOLOGY

3.1 Ethical Issues

Ethical clearance was obtained from the Institutional Review Board (IRB) of Noguchi Memorial Institute for Medical Research as well as Ghana Health Service Ethical Review Committee (GHSERC) (Ghana) with reference number GHS-ERC017/07/17 and the "Comite National D'Ethique des Sciences de la Vie et de la Santé (CNESVS)" in Côte d'Ivoire with reference number 112-18/MSHP/CNESVS-km.

3.2. Study Site and Design

This study was a longitudinal study aimed at identifying the genetic diversities among Mycolactone Producing mycobacteria and their ability to cause Buruli ulcer. A total of one hundred and eighteen (118) samples were obtained over a period of six (6) months in Ghana while seventy one (71) samples were obtained over a period of three (3) months were collected from Côte d'Ivoire. Study sites in Ghana included Amasaman in Ga West Municipal district of Greater Accra, Amansie Central district of Ashanti region and Pakro in the Akuapim South Municipal district, in the Eastern Region (Figure 6). The primary wound care facility in these districts were used as sample collection and storage centers; the Pakro Health Facility, the Amasaman District Hospital and the Amansie Central District Health Directorate at Jacobu. In Côte d'Ivoire, samples were collected from Buruli ulcer treatment centers located in seven communities. Due to the endemic nature of this disease, all the hospitals visited had Buruli ulcer treatment centers. The centers visited for the purpose of this study were located in Yamoussoukro, the country's capital, Kongouanou, a small city in Yamoussoukro, Zoukougbeu in the west central, Divo in the south, Bouaké and Sakassou in the central part and Djenedoufla.

3.3. Data Collection and Questionnaires and Sample size Determination

The purpose of the study was elucidated to participants. Participant's written consent and parental consent (in the case of children) were obtained for each patient. The questionnaire touched on the consented patient's perception of Buruli ulcer disease, period and mode of possibly contraction, ethnicity, age, occupation and category and position of lesion as well as treatment options. Participants with confirmed Buruli ulcer infection, but not on treatment, were referred to a nearby approved Buruli clinic for treatment.

The minimum samples size were determined using the equation below:

$$n = Z^2 \frac{(P)(1-P)}{Error^2}$$

[Sample size, n; Prevalence, P; Standard score, Z (95% confidence level)]

[Z=1.96; P= 0.0015 (Amofah *et al.*, 2002) ; E=0.05]

$$n = 3.84 \frac{(0.0015)(1-0.0015)}{0.027}$$

n= 2.13

The minimum sample size was approximately 2. However, 189 samples were obtained in both countries.

3.3.1. Inclusion and Exclusion Criteria

Inclusion: BU patients of all ages and sexes who accept to participate in the study

Exclusion: All non BU patients of all ages and sexes as well as BU patients who refuse to participant in the study.

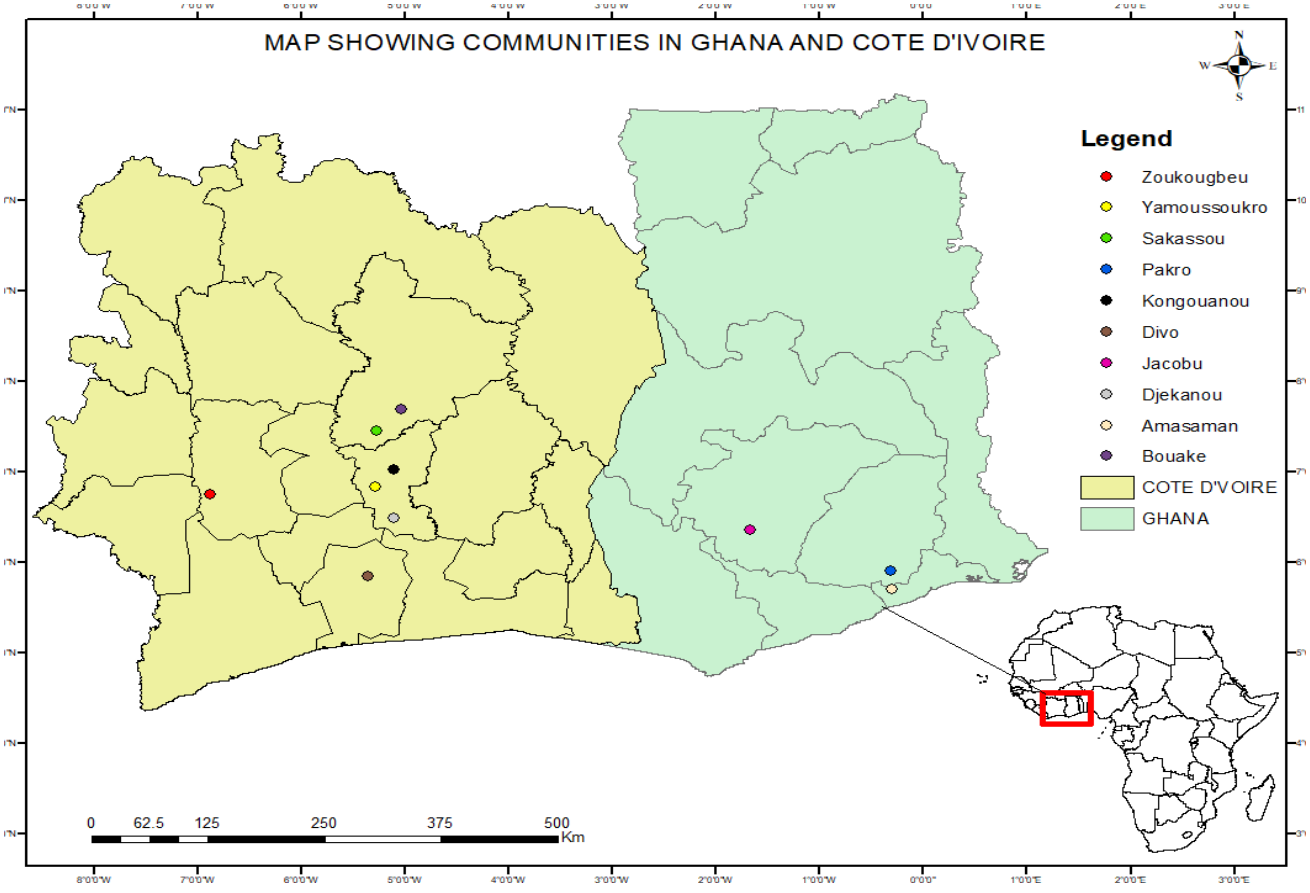


***Figure 6**: Location Map of Sample and Information Collection Centers in Ghana and Côte d'Ivoire.*

3.4. Collection, Storage, Transportation of Samples

Community health workers, nurses and disease control officers were trained in a one day workshop to aid disease identification and sample collection (Figure 7A). Swabs and Fine needle aspirates (FNA) sample**s (**depending on the type of lesion) were taken from patients presenting known clinical presentation and symptoms of Buruli ulcer as specified by WHO (papule, nodule, plaque, edema or an ulcer with undermined edges and cotton wool-like floors from necrotic slough) and other lesions to health facilities or identified in the community. Two swabs were taken per patient and kept in cryotubes on ice each containing 2ml 1x Phosphate buffered saline (PBS) (Figure 7B, 7C and 7D). FNA samples were handled identically. All samples collected in Ghana were immediately sent to the molecular biology lab of the Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Legon while the samples collected in Côte d'Ivoire were sent to Centre Suisse de Recherchés Scientifiques en Côte d'Ivoire (CSRS), Abidjan for storage. All samples were kept at -4^{o}C until further processing. Demographic data of patients were collected alongside sample collection (Figure 7D).

Figure 7*: A section of nurses and community health workers undergoing training on how to interview patients and obtain demographic data (A); proper sampling (B); storage (C) in Buruli ulcer treatment centers in Côte d'Ivoire. In Ghana, patients were interviewed and sampled directly by our team (D).*

3.5. Acid Fast Staining

Bacterial smears from samples were prepared on clean microscopic slides and stained for the presence of Acid Fast Bacilli (AFB) under sterile conditions. Briefly, smears were heat-fixed after air drying and covered with few drops of carbon fuchsin stain. Slides were heated until the stain began to vapourize and were rinsed after five (5) minutes with distilled water. Twenty percent (20%) sulphuric acid (decolourizer) was added for one minute until the slide appeared light pink in color, then rinsed with distilled water. The slide was then flooded with methylene blue stain for two (2) minutes washed off with distilled water, dried and examined under a light microscope in oil immersion at a magnification of x100.

3.6. DNA extraction

Genomic DNA was extracted from samples stored in 1X PBS. Samples stored in M7H9 containing PANTA were decontaminated and used for culturing. DNA from clinical samples were directly extracted using the ZR Quick gDNA MiniPrep kit (Zymo Research Corporation, California, U.S.A and Catalog Number: D3025) while samples from bacterial isolates (positive controls) were extracted using ZR Quick DNA Fungal/Bacterial DNA MiniPrep kit (Zymo Research Corporation, California, U.S.A and Catalog Number: D6005) following the manufacturer's protocol without any modifications.

3.7. Mycobacterial Infection Confirmation of Samples by IS*2404* Amplification

To confirm the BU status of a recruited patient, conventional and nested PCRs targeting the IS*2404* were performed on all clinical samples following Tano *et al.*, (2017) protocol with a few modifications. The IS*2404* oligonucleotide primers used were synthesized by Invitrogen (California, United States). PCR amplification was carried out on the IS*2404* target in a 10 µM PCR

reaction concentration in a total volume of 25µL. Briefly, a 22.5µL PCR master mix consisted of 9 µL of nuclease free PCR water (Thermoscietific, Massachusetts, United States and Catalog number: K1081), DreamTaq Green PCR Master Mix (2X) (Thermo Scientific, Massachusetts, United States and Catalog number: K1081) and 0.5ul each of forward primer and reverse primer, (Invitrogen, California, United States) at a concentration of 10 µM). A 2.5 µL of DNA template was added to the mastermix and was run in an Eppendorf Mastercycler® nexus thermocycler (Eppendorf, New York and Catalog Number: 6331000017). Positive Controls (Bacteria strain from culture) and Negative controls were incorporated in each amplification. Target gene, IS*2404* was initially denatured at 95 °C for 2 minutes. This was further denatured at 95 °C for 1 minute, with primer annealing at 57 °C for 30seconds and target gene elongation at 72 °C for 1 minute. These three steps were repeated 34 times and reaction held at 4 °C. This was followed by a final elongation step at 72 °C for 10 minutes.

Amplicons were separated on 1.5% agarose gels containing 4 µL of gel red and visualized with an Amersham Imager 600UV (29083463) (Wipro GE Healthcare Pvt Ltd, Ahmedabad, India). For the first amplification round of the nested PCR, the same protocol was used with only the primer annealing temperature changed to 60.4 °C and DNA amplicon used as template for the second round of amplification. Positivity of these samples were further confirmed by real time PCR targeting the same insertion sequence. Final PCR volume was 20 µL containing 7 µL nuclease free water (New England Biolabs, Massachusetts, United States and Catalog number: M3003S), 10 µL Luna® Universal qPCR Master Mix (New England Biolabs, Massachusetts, United States and Catalog number: M3003S), 0.5 µL of both forward and reverse primers (Invitrogen, California, United States) and 2µL of template DNA. The Amplification was carried out as described by Fyfe *et al.*, (2007) with slight modifications. Briefly, the IS*2404* gene was denatured at 95 °C for 1 minute.

This was further denatured at 95 °C for 15 seconds, with primer annealing at 60 °C for 30 seconds and target gene elongation at 72 °C for 30 seconds. These three steps were repeated 29 times. This was followed by a final elongation step at 72 °C for 5 minutes.

3.8. Mycolactone Producing Mycobacteria Confirmation by ER Gene Amplification

Samples positive for both nested and quantitative PCR were further confirmed as MPM positive (before VNTR typing) by performing PCR targeting the Enoyl reductase (ER) gene as described by Williamson *et al.*, (2008). ER PCR was also run using the same protocol described for IS*2404* PCR with the annealing temperature modified to 62.1 °C. Briefly, PCR amplification was carried out on the target gene in a 10 µM PCR reaction concentration in a total volume of 25 µL containing 12.5µL DreamTaq Green PCR Master Mix (2X) (Thermo Scientific, Massachusetts, United States and Catalog number: K1081), 9 µL of nuclease free water (Thermo Scientific, Massachusetts, United States and Catalog number: K1081), 0.5 µL of both forward and reverse primer (Invitrogen, California, United States). DNA template (2.5 µL) was added to tubes and amplification carried out in an Eppendorf Mastercycler® nexus thermocycler (Eppendorf, New York and Catalog Number: 6331000017). ER Cycling was performed at 95°C for 2 mins, followed by 34 cycles each of, denaturation at 94°C for 1 minute, annealing at 58°C for 45 seconds and extension at 72°C for 1 minute. A final extension step was included at 72°C for 10minutes and reaction held at 4°C.

3.9. VNTR Typing of Mycolactone Producing Mycobacteria (MPM)

Samples positive for IS*2404* and Enoyl Reductase (ER) were profiled for length polymorphisms at sixteen (16) *M. ulcerans* VNTR loci. All oligonucleotide VNTR primers used were synthesized by Invitrogen (California, United States) and are described in Table 2. PCR amplification on 16 different loci were carried out in a 25 µL reaction volume containing 12.5µL DreamTaq Green PCR

Master Mix (Thermo Scientific, Massachusetts, United States and Catalog number: K1081), 9 µL of nuclease free water (Thermo Scientific, Massachusetts, United States and Catalog number: K1081), 0.5 µL of both forward and reverse primer and 2.5 µL of template DNA in an Eppendorf Mastercycler® nexus thermocycler (Eppendorf, New York and Catalog Number: 6331000017). Cycling conditions for all loci was performed at 95°C for 2mins, followed by 40 cycles each of denaturation at 95°C for 30 seconds, annealing at different temperatures for different loci (Table 2) for 30 seconds and extension at 72°C for 1 minute. A final extension step was included at 72°C for 10 minutes. Amplicon sizes of samples as well as controls (*M. ulcerans* 1615, *M. marinum* SA 200695, *M. marinum* hybrid 270995, *M. marinum* DL 180892, *M. marinum* CL 240299 and *M. pseudoshotsii* L15) were separated on an agarose gel and viewed on a Bio – rad Gel Doc™ XR+ Imaging System (5838) and used to estimate band sizes. PCR amplicons for all controls as well as randomly selected samples with their respective forward and reverse primers for each locus was Sanger sequenced (CLAS DNA Laboratory, Arizona State University). Length polymorphisms at all the VNTR loci were computed based on target loci size and repeat length from published data (Ablordey et al., 2005, Stragier et al., 2005, Hilty et al., 2006, Hilty et al., 2007, Lavender et al., 2008, Williamson et al., 2008, Narh et al., 2015) and confirmed by sanger sequencing. Controls included in the study can be found in Table 1.

Table 1: Positive controls included in the VNTR Typing of MPM isolates**.**

Species	Strain	Source
M. ulcerans	1615	Human Ghanaian Isolate (Ashanti Region)
M. marinum	hybrid 270995	Red seabream Pagrus major (f) x Sparus aurata (m) (Red Sea, Israel)
M. marinum	DL 180892	Sea bass Dicentrarchus labrax (Ein Yahav, Israel)
M. Pseudoshottsii	L15	Sea bass Morone saxatilis
M. marinum	CL 240299	Koi Cyprinus carpio (Ma'agan Michael, Israel)

Table 2: Primer sequences of different Miru – VNTR Loci used for typing

Loci	Forward (5' - 3')	Reverse (5' - 3')	Annealing Temp(°C)	size (BP)
MU 5 & 6	AGCGACCCCAGTGGATTGGT	CGGTGATCAAGCGTTCACGA		400
PG 1 & 2	AGGGCAGCGCGGTGATACGG	CAGTGGATTGGTGCCGATCGAG		400
PG 3 & 4	GGCGCAGATCAACTTCGCGGT	CTGCGTGGTGCTTTACGCGC		210
ER	GAGATCGGTCCCGACGTCTAC	GGCTTGACTGTCACGTAAG		719
Locus 1	GGCAGTGGGTGACGTCTCAGT	TCGAGGCGATCTACACCAAGGATTA	67.9	variable
Locus 4	GCCTTGCTTACCGTCGTGCCAA	CGAGCCAAGTTGGACCGTCAACACAT	70.6	variable
Locus 8	CGGATGACGTCGGAACTCTGA	GGACGCGGTAGCACGTTTTGT	66.6	variable
Locus 9	GGTGGATCTCCGCGTCATTTG	CGACCGCCCTCGAGACAG	66.4	variable
Locus 10	ACAAGCCACGGCGAGATATAG	GCGGGGCTTTTATCTGCTTA	63.5	variable
Locus 13	CAGGTATTCCAGGAGATCAAA	GGCGACAAGGCTCGTT	60.2	variable
Locus 14	CCTTGTATCCGAGTTTCAGTT	GTCGACCAGATATGAGCAAT	60.3	variable
Locus 15	GCCACCGGTCAGGTCAGGTT	TCACCAACTACGACGGCGTTC	67.5	variable
Locus 16	CCAACGCTCCCCCAACCAT	GCTCACAGGCCTTCGCTCAGA	68	variable
Locus 18	CCCGGAATTGCTGATCGTGTA	GGTGCGCAGACTGGGTCTTA	65.4	variable
Locus 33	CAAGACTCCCACCGACAGGC	CGGATCGGCACGGTTCA	65	variable
ST1	CTGAGGGGATTTCACGACCAG	CGCCACCCGCGGACACAGTCG	65.4	variable
MIRU 1	GCTGGTTCATGCGTGGAAG	GCCCTCGGGAATGTGGTT	64.5	variable
MIRU 9	GCCGAAGCCTTGTTGGACG	GGTTTCCCGCAGCATCTCG	66.4	variable
Locus 19	CCGACGGATGAATCTGTAGGT	TGGCGACGATCGAGTCTC	64	variable
Locus 6	GACCGTCATGTCGTTCGATCCTAGT	GACATCGAAGAGGTGTGCCGTCT	68.5	variable

3.10. Genotype Assignment

Genotypes were assigned based on length polymorphisms associated with different tandem repeats copy number calculated based on published data (Narh et al., 2015; Williamson et al.,2014) and confirmed by sequencing. Isolates that could not be differentiated and genotyped using the four standard loci (Miru 1, Locus 6, Locus 19 and ST1) were further differentiated at the remaining 12 loci.

3.11. Population Genetics

Data on VNTR profiles of isolates from Narh et al., 2015 and Williamson et al., 2014 at four loci; ST1, Miru 1, Locus 6 and Locus 19 were included in the study and analyzed for difference in genotypes across years (2008 – 2019).

Genotypes obtained were compared for differences that existed such as:

- Within population (Among communities in a sample site)
- Across communities (Different sampling sites)
- Across countries (Côte d'Ivoire & Ghana)
- Disease stages and categories (for ulcers)

3.12. Data and Sequence Analysis

Questionnaires were analyzed using Epi Info version 7. The map of study communities was drawn with Arc GIS. The graphs were drawn using GraphPad Prism. Sequence data was viewed with SnapGene Viewer and Tandem repeats were analyzed from the sequences using the Tandem Repeat Finder (Benson, 1999). 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 to reveal genetic relatedness among isolates were performed with MEGA V5 (Tamura et al., 2011).

CHAPTER FOUR

4.0. RESULTS

4.1. Sample and Data Collection

A total of one hundred and eighty-nine (189) participants with presumptive Buruli ulcer presentations were recruited [one hundred and eighteen (62.43%) for Ghana from June to November 2018 and seventy-one (37.57%) for Côte d'Ivoire from August to November 2018]. Community health workers, nurses and disease control officers were trained in one-day workshops on case identification and sample collection. Over the period reported for sample collection in Ghana, fifty-six (47.5%), twenty-six (22.03%) and thirty-six (30.51%) suspected cases came from Amansie District, Amasaman District and Pakro respectively. Similarly, for Côte d'Ivoire, nineteen (26.76%), eighteen (25.35%), fourteen (19.72%), nine (12.68%) and eleven (15.49%) suspected cases were sampled from Zoukougbeu, Djekanou, Yamoussoukro, Kongouanou and Bouake respectively.

Overall, there were more female (52.2%) than male (47.8%) suspected cases recruited in this study (Figure 8B), however, this difference was not statistically significant when tested by student t-test ($p= 0.202$) $p < 0.05$ (Appendix 2). This scenario was observed for patients in Ghana but in Côte d'Ivoire the same number of samples were obtained from both males and females. Ages of the recruited patients ranged from 1 to 90 years for both countries and samples were categorized into three sections based on age; children (<18 years), adult (18 – 50) and the aged (> 50). Most of the affected persons were in the adult category (18-50) as supposed to the children and the aged (Figure 8A). The majority of patients were farmers and traders with either primary or no formal education (Appendix 1). Only four recruits (2.02%) had attained tertiary level education. Age and occupation

status did not offer any correlation with the development of Buruli ulcer disease (p = 0.420 and 0.588 respectively) (Appendix 2)

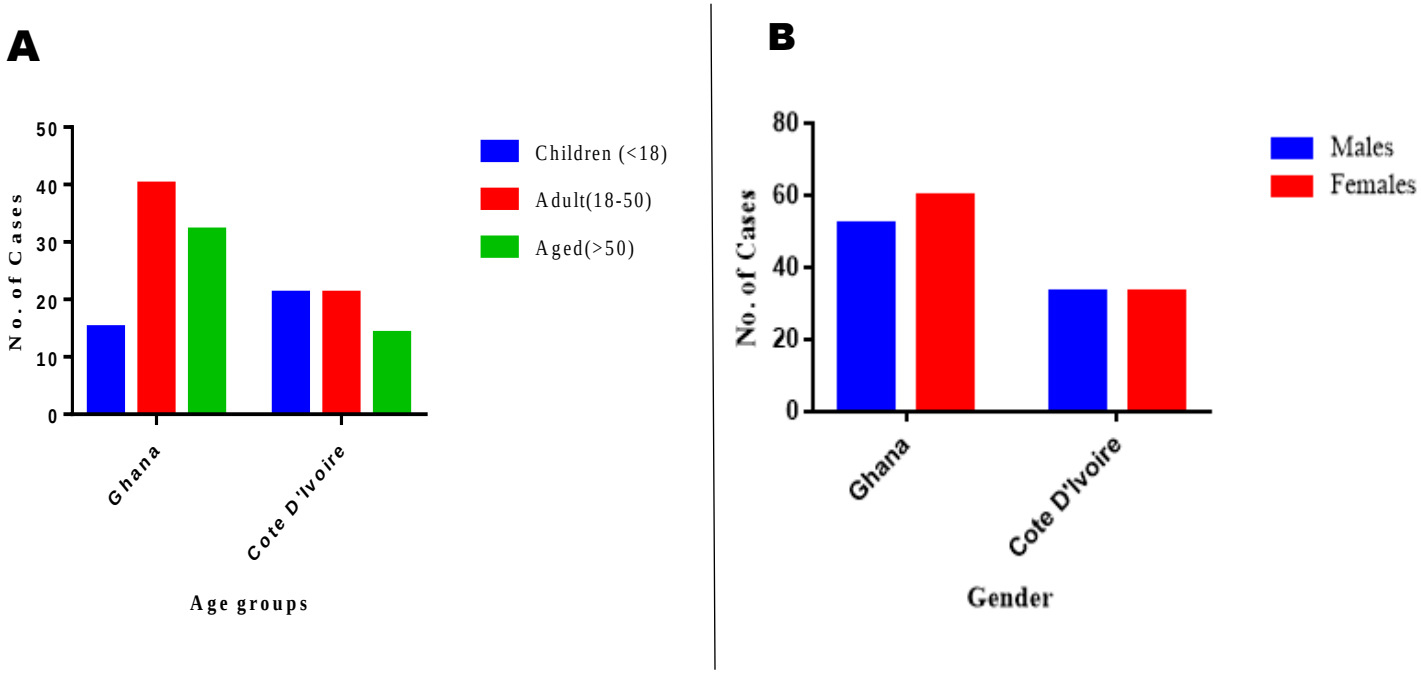


***Figure 8**: Age **(A)** and Gender **(B)** Distribution of suspected BU participants. Participants were grouped into three categories; children (less than 18 years), adult (between 18 – 50 years) and aged (greater than 50 years).*

In Ghana, all presumptive stages of the disease (nodule, plaque, oedema and ulcer) were identified and sampled unlike Côte d'Ivoire where the majority of cases were in the ulcerative stage with only two nodular infections identified. One hundred and eighty (180) of the of the infections across both countries were in the ulcerative stage (95.5%) with only four (4) edematous ulcers (2.1%), four (4) nodules (2.1%) and one (1) plaque (0.5%) (Figure 9). Out of the one hundred and eighty (180) ulcer cases and four (4) edematous ulcers, one hundred and five (105) cases were Category II (57.1%)

lesions, followed by forty-seven (47) Category III lesions (25.5%) with just thirty-two (32) Category I lesions (17.4%)(Figure 10).

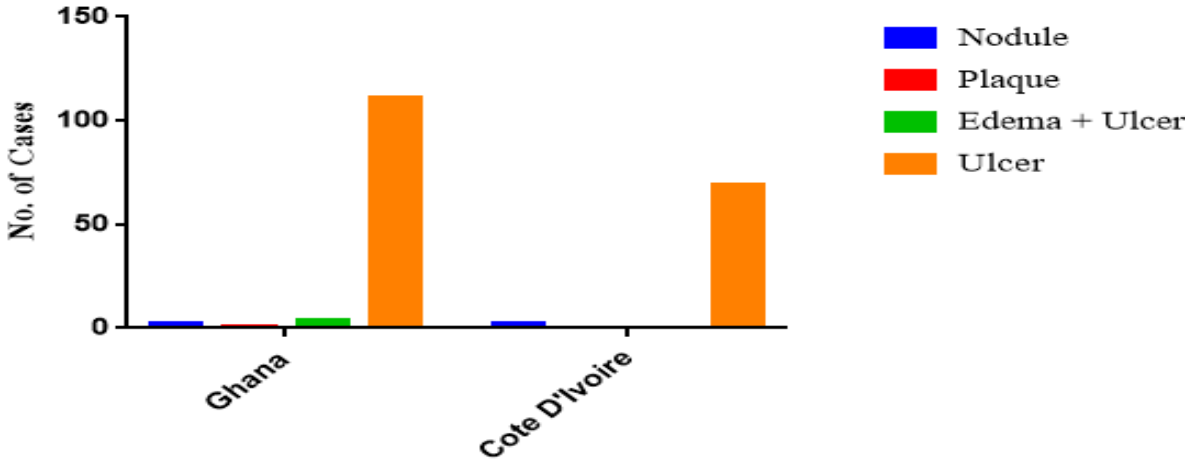


Figure 9: *Stages of Buruli ulcer disease in suspected participants. Most of the cases recruited were in the ulcerative stage*

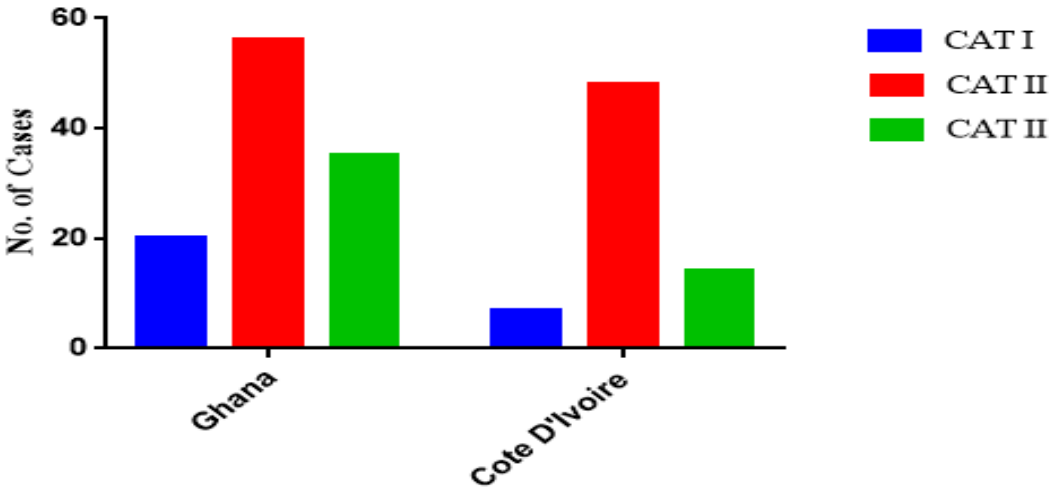


Figure 10: *Categories (CAT) of lesions of suspected Buruli ulcer patients in Ghana and Côte d'Ivoire*

Most of these infections were observed on the lower and the upper limbs in both countries. Rare infections on the trunk/breast area were also observed in both Ghana and Côte d'Ivoire with infections on the genitalia

and head/neck observed in only Côte d'Ivoire. One hundred and fifty-six (156) and twenty-seven (27) cases reported were on the lower (82.5%) and upper (14.1%) limbs respectively, with one (1), two (2) and three (3) infections observed on the neck/head area (0.5%), genitalia (1.1%) and trunk/breast area (1.6%) (Figure 11)

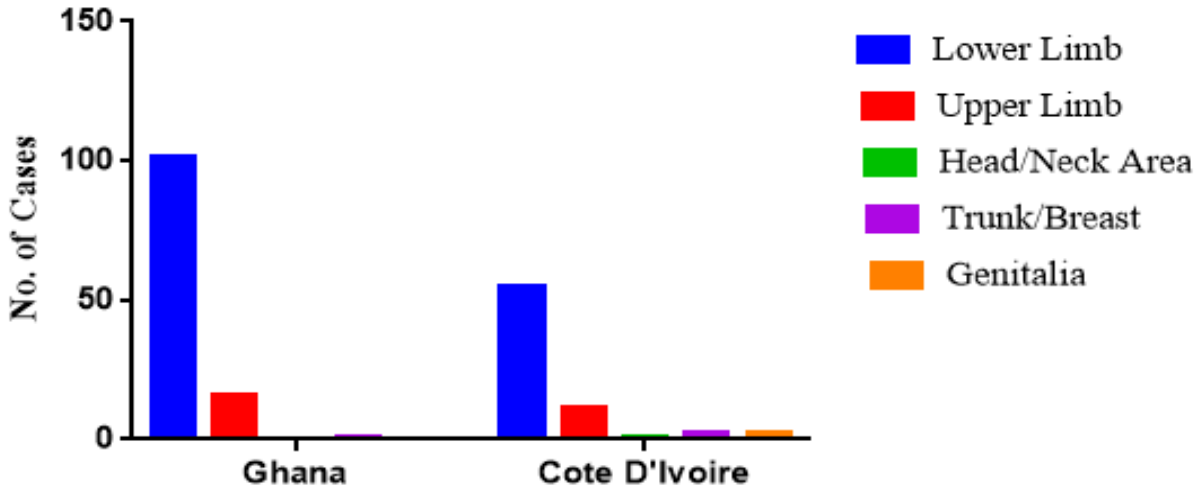


Figure 11: *Different body parts affected. In Ghana, infections were only on the lower and upper limbs and the trunk and breast area with Côte d'Ivoire recording infections at the same parts as well as the genitalia, head and neck area.*

4.2. Case Confirmation

4.2.1. Microscopy

All one hundred and eighty-nine (189) samples were tested for the presence of Acid-fast bacilli using Ziehl neelsen's staining. Only five samples (2.7%) were positive by microscopy and acid fast coccobacilli clustered together were observed in Figure 12.

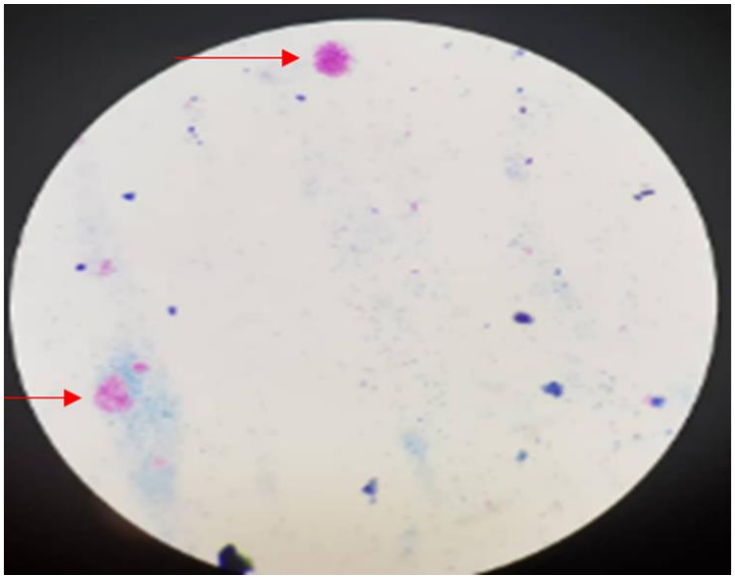


Figure 12: *Acid fast stained slide from a sample collected in Côte d'Ivoire. Red arrows shows a clump of bacilli*

4.2.2. PCR targeting IS2404 and Enoyl Reductase gene

Conventional and nested PCR targeting the insertion sequence IS*2404* were performed and validated by qPCR targeting the same insertion sequence for the presence of Buruli ulcer among patients. Out of all the one hundred and eighty-nine(189) samples tested, only twenty-four (24) samples were positive for IS*2404* representing 12.7% positivity. Fifty-eight (58) samples tested positive by nested IS*2404* PCR representing 30.7% positivity. Positivity of samples were further validated by qPCR which produced one hundred and fifty-nine (159) positive samples for Buruli ulcder representing 84% positivity.

PCR targeting the Enoyl reductase gene was perform to confirm the presence of MPM infection. Out of the one hundred and eighty-nine (189) samples tested, thirty-four samples tested positve re presenting 18% positivity.

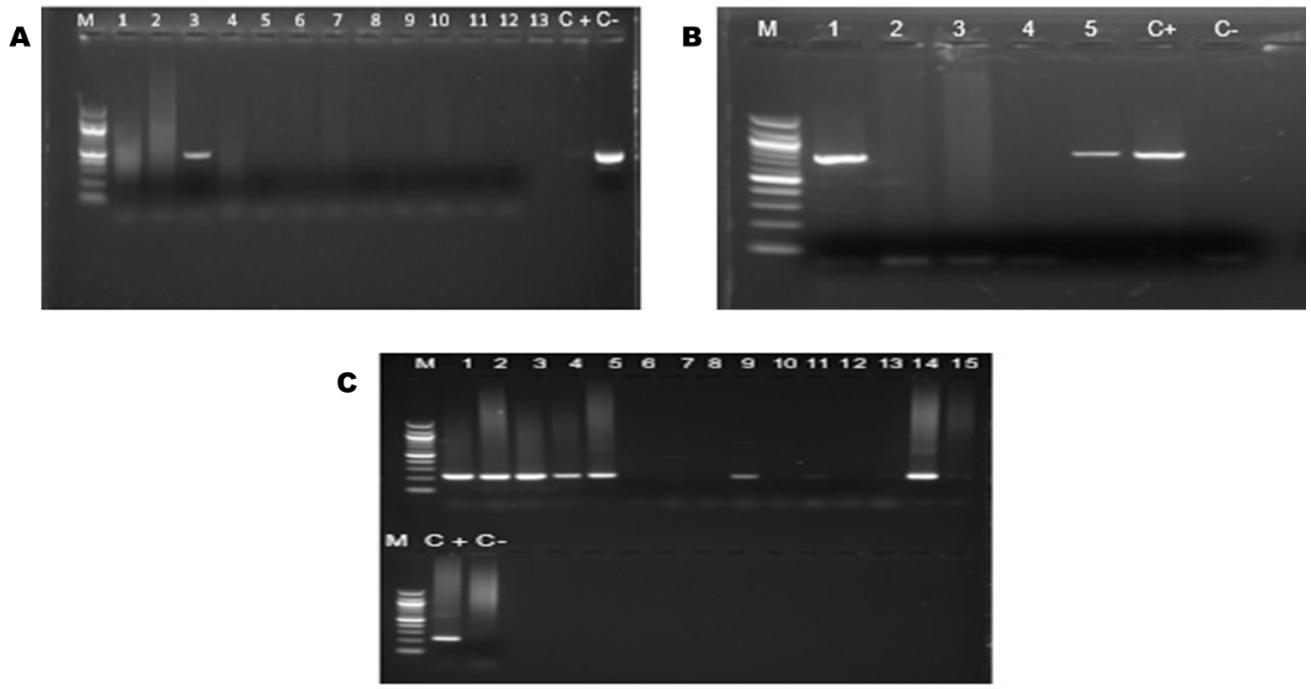


Figure 13: *Representitive 1.5% Gels of ethidium bromide stained PCR amplicons. A - amplicons of IS2404 PCR with band size of 400bp. B - Amplicons for PCR targeting the Enoyl reductase gene with band size of 720bp. C - amplicons of nested IS2404 PCR with band size of 210bp. M :100bp Molecular Ladder, C+ : Positive Control, C- : Negative Control, 1- 15 : Samples*

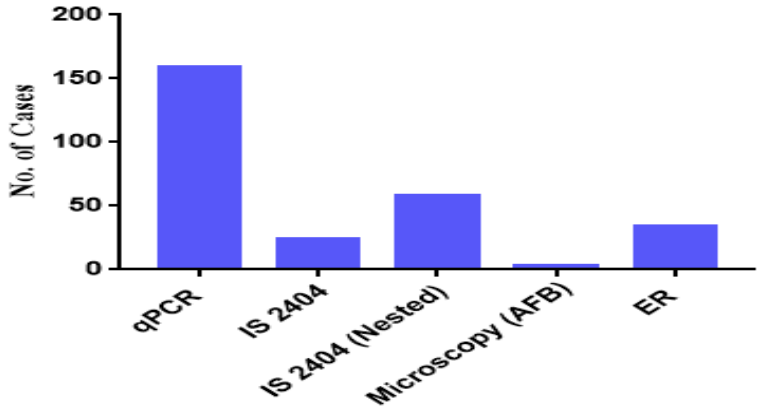


Figure 14: *A summary of molecular confirmatory tools used for confirming the presence of Buruli ulcer in patients. All samples positive for qPCR were further typed at sixteen (16) VNTR loci.*

4.2.3. VNTR Typing of Isolates

PCR targeting different VNTR loci in the *M. ulcerans* strain Agy *99* genome was performed for qPCR IS*2404* positive (159/189) samples. Each reaction was run independently for all sixteen (16) VNTR loci as described in section 3.9. Repeat sizes were confirmed by Sanger sequencing as described for controls in section 3.12. Table 3 below shows the VNTR profiles and designated genotypes of isolates based on published data from Williamson et al., 2014; Narh et al., 2015. Amplification was achieved at all loci for all controls and most isolates (11% - 69% success rate) (Table 5) with band sizes ranging from 200bp to 900bp (Figure 15).

Figure 15 is a representation of an ethidium bromide stained gel of VNTR profile at nine loci of six controls. Most of the samples amplified at polymorphic loci Miru 1 (65%), locus 6 (65%), locus 19 (53%), locus 16 (52%), locus 15 (53%) and ST1 (69%) as well as monomorphic loci such as locus 4 (68%) and locus 10 (50%). Most samples could not be successfully amplified (>50% amplification) at loci such as locus 33 (18%), locus 13 (15%), locus 1 (11%), locus 8 (33%), locus 9 (8%), locus 18 (39%), locus 14 (30%) and Miru 9 (13%) (Table 5). All controls and samples (165) were monomorphic at six (6) loci; locus 1, locus 4, locus 8, locus 9, locus 10 and locus 14 and hence were not used for typing isolates.

Genotyping was achieved by three stages of assignment based on the ten (10) polymorphic loci out of the sixteen (16) loci. The first stage involved typing at the four (4) standard and most used loci; Miru 1, locus 6, locus 19 and ST1. Isolates with an indefinite or unassigned genotype at this first stage were further typed at two loci, locus 15 and locus 33. These two loci distinguished *M. ulcerans* strain 1615 from the other MPM controls used in this study and thus was used to distinguish isolates as either a strain of *M. ulcerans* or MPM. Isolates without a definite, or

unassigned genotype at this stage, were further typed at the remaining four loci, locus 13, locus 16, locus 18 and Miru 9. These four loci generally differentiated all the controls. Samples with less than five positively amplified loci out of the sixteen were not assigned a genotype.

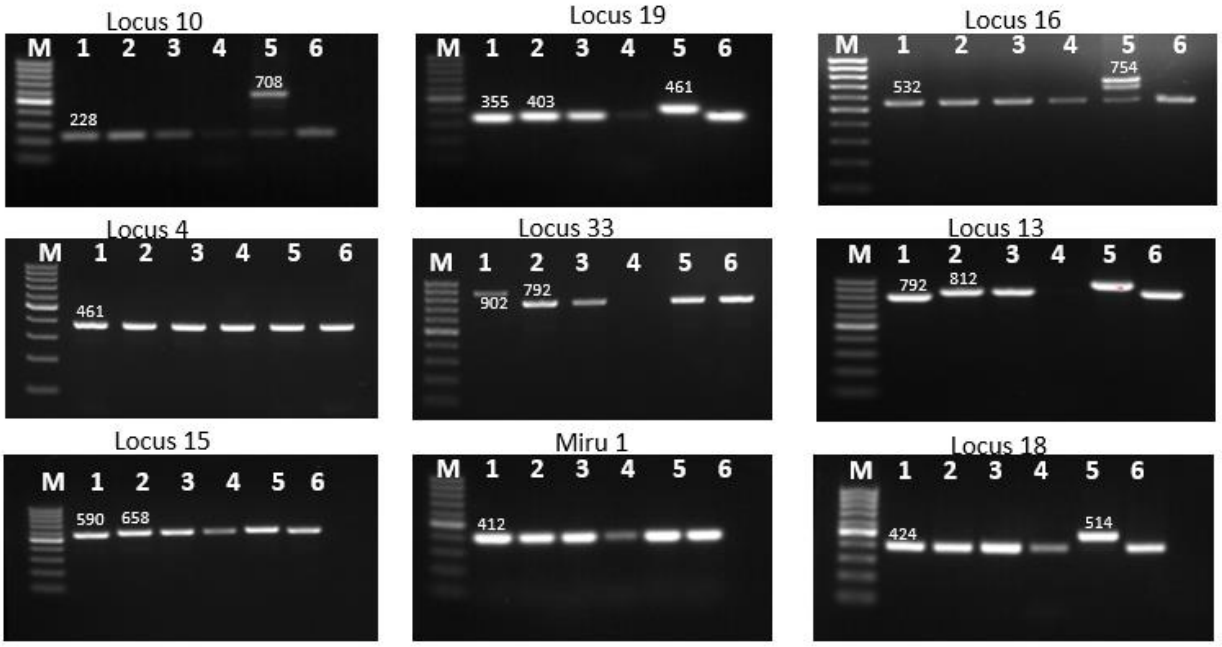


Figure 15: *Ethidium bromide stained gels of controls at nine different loci. M- Molecular weight ladder, 1- M. ulcerans 1615, 2- M. marinum hybrid 270995, 3- M. marinum DL 180892, 4- M. pseudoshottsii, 5- M. marinum CL, 6- M. marinum SA 200695*

4.2.4. Genotype Assignment

Seven (7) *M. ulcerans* genotypes designated A, B, C, D, E, F and G as well as five (5) MPM genotypes designated MHB, MDL, MCL, MSA and MLF were generated for assigning genotypes in this study as shown and seen in tables 4 and 5. Most isolates could not be predominantly assigned genotypes due to unsuccessful amplification at the particular loci required to differentiate

them (Figure 16). Predominant definitely assigned genotypes that were identified in Ghana were Genotype D present in all three (3) communities, Genotype E present only in Amansie Central District, Genotype C and F present only in Pakro and MLF present only in Amasaman.

Genotypes prominently observed in Côte d'Ivoire were A, C, D, F and G across all communities with Genotype F found only in Yamoussoukro and Genotype A and G found only in Djekanou. Genotype D was observed in Djekanou, Kongouanou and Zoukougbeu while Genotype C was observed in Kongouanou, Bouake and Zoukougbeu. Genotypes present in both Ghana and Côte d'Ivoire include Genotypes C, D and F. Genotypes E and MLF were only found in Ghanaian samples while that of Genotypes A and G were found in Ivorian samples.

In Ghana, Genotype C was associated with Category II lesions while Genotype F was associated with Category I lesions. Genotype E was also associated with Category I and II lesions while Genotype D was associated with all categories. Conversely as observed in samples from Côte d'Ivoire, Genotype A was associated with Category II lesions, Genotype F associated with Category II and III lesions, Genotype G associated with Category II and III lesions as well as an edematous ulcer, while Genotype C and D were associated with all categories (Figure 17).

Data from Narh et al., 2015 and Williamson et al., 2014 was included in this study and compared with genotypes present from 2008 to 2019 and the changes that have occurred over time. These two studies as well as this current study all observed Genotype D (1, 1, 2, 2) among samples (Table 3). Narh et al., 2015 and Williamson et al., 2014 both observed MDL (1, 3, 2, 1). This current study observed Genotypes A (1, 1, 1, 2) and C (3, 1,2,2) with Narh et al., 2015 and Genotypes G (1, 1, 2, 1) and MLF (1, 2, 2, 1) among isolates. However, there were genotypes peculiar to each study. Narh et al., 2015 observed Genotype Z (1, 2, 2, 2), Williamson et al., 2014 observed

Genotype B (3, 1, 1, 2) and MPS (1, 4, 2, 2) whiles Genotypes E (1, 1, 2, 3) and F (2, 1, 2, 2) was observed in this study only.

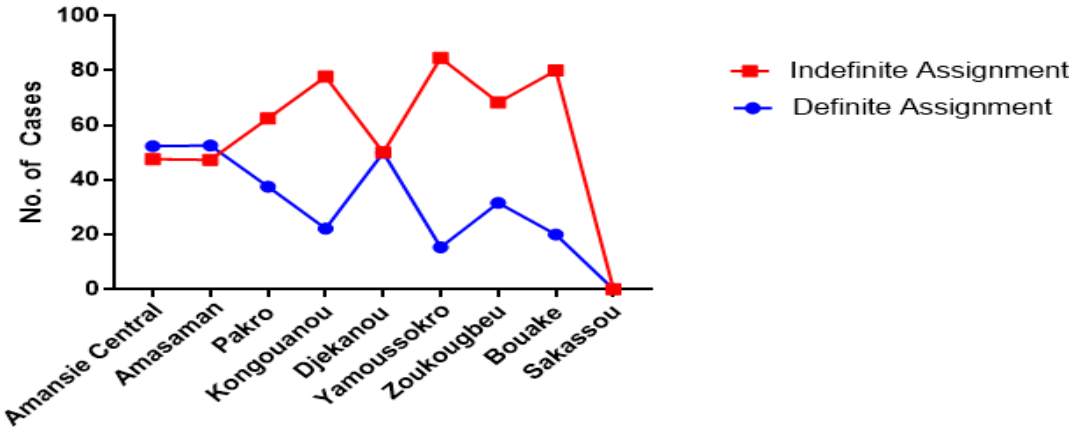


***Figure 16**: Percentage genotype assignment of samples from various communities in Ghana and Côte d'Ivoire.*

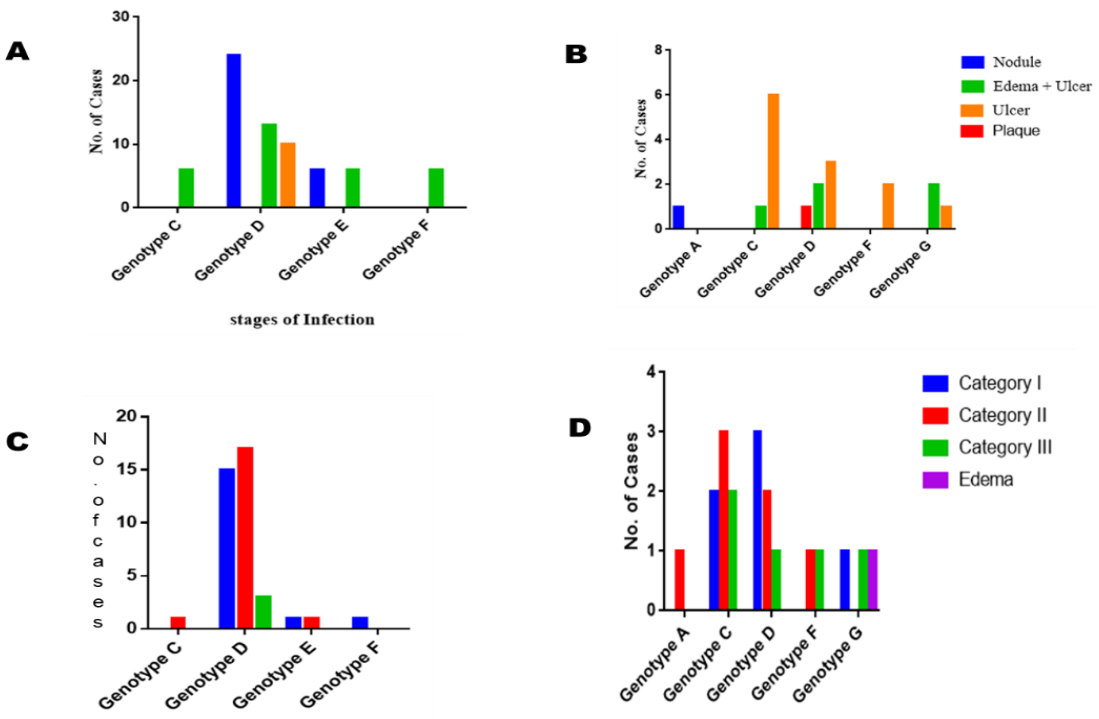


***Figure 17:** Genotypes associated with different disease stages and categories in Ghana (A &C) and Côte d'Ivoire (B&D).*

Table 3*:* Genotyping coding for samples based on four standard loci, Miru 1, Locus 6, ST1 and Locus 19

Designated genotype	VNTR Profiles				Reference (Sample size)
	MIRU1	Locus 6	ST1	Locus 19	
A (MU)	1	1	1	2	Current study (159)
B (MU)	3	1	1	2	
C (MU)	3	1	2	2	
D (MU)	1	1	2	2	
E (MU)	1	1	2	3	
F (MU)	2	1	2	2	
G (MU)	1	1	2	1	
MHB	1	3	2	1	
MDL	1	3	2	1	
MCL	1	2	2	3	
MSA	1	3	3	1	
MLF	1	2	2	1	
W	1	1	2	1	Narh et al., 2015 (15)
X	1	1	2	2	
Y	1	2	2	1	
Z	1	2	2	2	
A	1	1	1		Williamson et al., 2008 (50)
B	3	1	1		
C	3	1	2		
D (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	

Table 4a: Mycobacteria species identified using Miru-VNTR Profiles of isolates (Indefinitely assigned)

Country	**Community/District**	***M. ulcerans***							**Other Mycolactone Producing Mycobacteria**					**UNASSIGNED**
		A	**B**	**C**	**D**	**E**	**F**	**G**	**[1]MHB**	**[2]MDL**	**[3]MCL**	**[4]MSA**	**[5]MLF**	
Ghana	Amansie Central	11	2	3	40	21	9	0	0	0	0	0	1	4
	Amasaman	1	1	2	17	5	2	2	1	0	1	0	2	1
	Pakro	1	1	4	11	6	3	3	3	3	1	0	3	4
Côte d'Ivoire	Kongouanou	1	1	5	7	3	5	0	1	1	1	0	1	0
	Djekanou	1	0	5	8	4	3	5	3	3	2	1	2	0
	Yamoussoukro	2	1	3	8	8	6	4	5	5	5	1	2	0
	Zoukougbeu	3	2	7	12	1	3	9	6	6	1	3	6	1
	Bouake	3	2	3	3	2	3	2	1	1	0	1	2	5
TOTAL		**23**	**10**	**32**	**106**	**50**	**34**	**25**	**20**	**19**	**11**	**6**	**19**	**15**

[1] *M. marinum* hybrid *270995*; [2]*M. marinum DL 180892;* [3] *M. marinum CL;* [4]*M. marinum SA 200695;* [5]*M. liflandii*

Table 5b: Mycobacteria species identified using Miru-VNTR Profiles of isolates (Definitely assigned)

Country	Community/ District	*M. ulcerans*							MLF	MU	MPM	MU/ MPM	UNASSIGNED
		A	B	C	D	E	F	G					
Ghana	Amansie Central	0	0	0	20	2	0	0	0	17	0	4	8
	Amasaman	0	0	0	10	0	0	0	0	6	1	1	0
	Pakro	0	0	1	4	0	1	0	1	7	2	0	7
Cote D'Ivoire	Kongouanou	0	0	2	1	0	0	0	0	5	1	1	0
	Djekanou	0	0	1	0	0	0	0	0	2	0	2	5
	Yamoussoukro	0	0	0	0	0	2	0	0	4	0	5	0
	Zoukougbeu	0	0	3	2	0	0	0	0	8	2	4	0
	Bouake	1	0	2	3	0	0	3	0	4	1	2	0
	Sakassou	0	0	0	0	0	0	0	0	1	0	0	0
TOTAL		**1**	**0**	**9**	**40**	**2**	**3**	**3**	**1**	**54**	**7**	**19**	**20**

Table 6: VNTR typing success at sixteen (16) loci

LOCI	Ghana (%) N=88	Côte d'Ivoire (%) N=70	Total (%) N=158
Locus 6	51(58)	51(73)	102(65)
Locus 13	14(16)	9(13)	23(15)
Locus1	9(10.2)	8(11)	17(11)
Locus 4	59(67)	49(70)	108(68)
ST1	61(69)	48(69)	109(69)
Locus 8	31(35)	21(30)	52(33)
Locus 9	8(9)	5(7)	13(8)
Locus 15	48(55)	35(50)	83(53)
Locus 16	50(57)	32(46)	82(52)
Locus 18	48(55)	14(20)	62(39)
Locus 19	51(58)	32(46)	83(53)
Locus 33	23(26)	6(9)	29(18)
Miru 1	68(77)	34(49)	102(65)
Miru 9	14(16)	6(9)	20(13)
Locus 10	30(34)	49(70)	79(50)
Locus 14	29(33)	18(26)	47(30)

4.3. Allelic Frequencies of Loci

Most of the isolates and controls were polymorphic at eight loci, Miru 1, ST1, locus 19,locus 8, locus 13,locus 15, locus 18 and locus 33. The highest number of observed alleles for any loci was 3 with the least being 1.

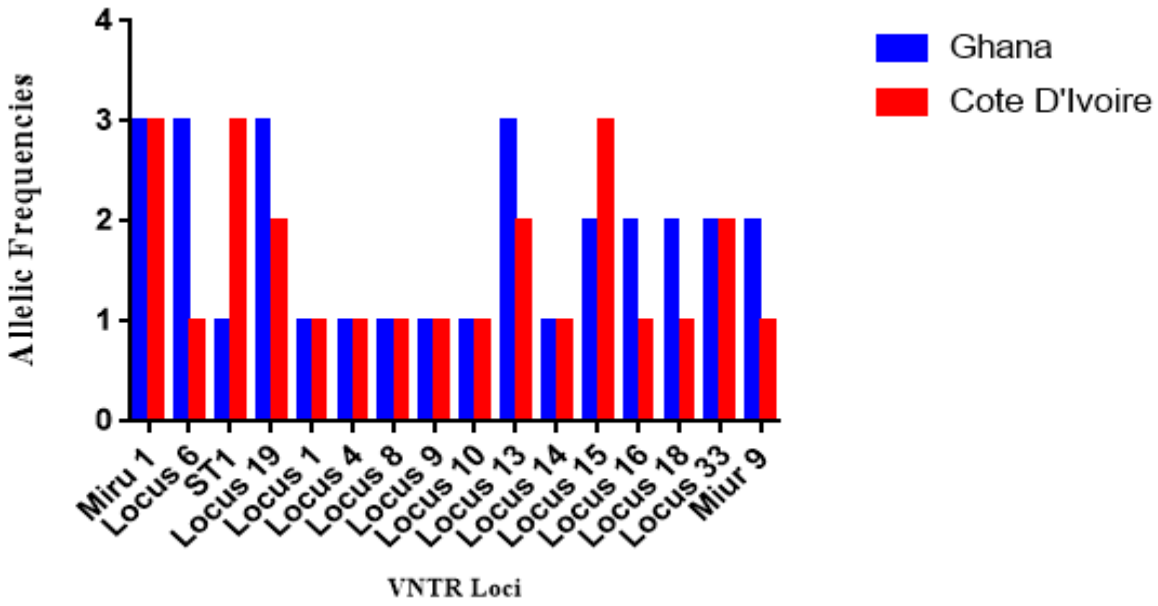
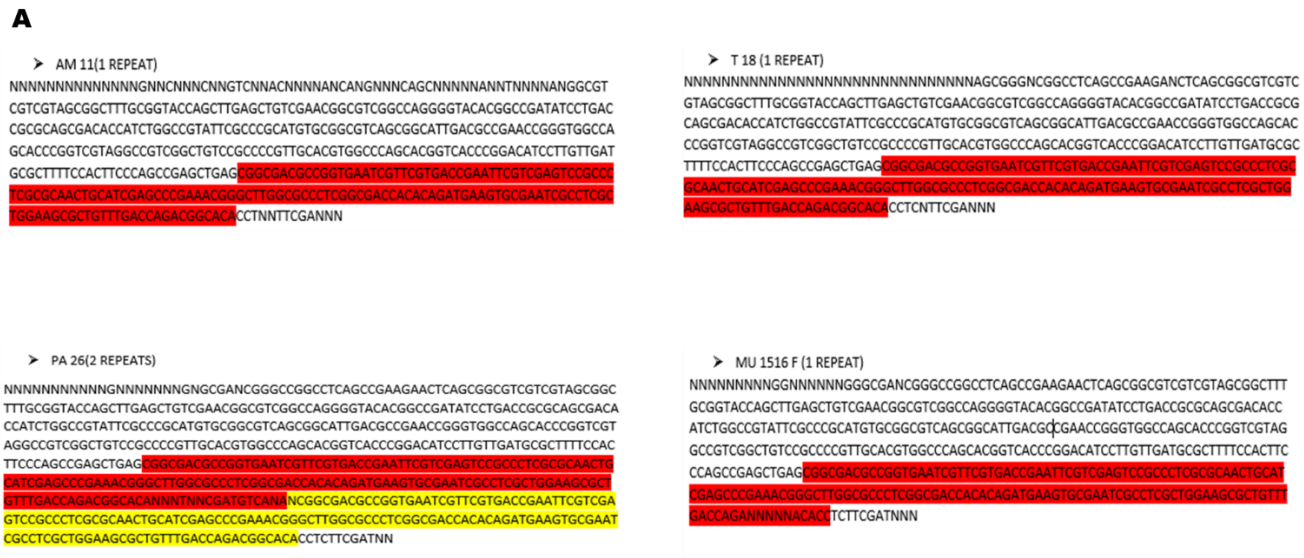


***Figure 18**: Allelic frequencies of VNTR loci typed for samples collected in Ghana and Côte d'Ivoire*

4.4. Sequence Confirmation

All controls and randomly selected isolates (10% of 159 isolates) at the sixteen (16) different loci were Sanger sequenced to confirm tandem repeats. Figure 19 shows sequences of some selected isolates and controls at locus 6, a polymorphic locus. Repeats are color coded with each color representing a repeat. All repeats for corresponding isolates confirmed repeat typing using band sizes. During sequencing, some sequences were trimmed at both ends and hence sequence lengths do not always correspond exactly with band sizes on agarose gel. Figure 19 (B) shows the phylogenetic tree of representative isolates plotted using their locus 6 sequences. As shown, there was clustering of sample T 18 with controls *M. Pseudoshottsii*, *M. marinum DL*, *M. marinum SA and M. marinum* hybrid. Samples T01, PA26, T07, PA 22 and PA 25 clustered with control MU 1615.



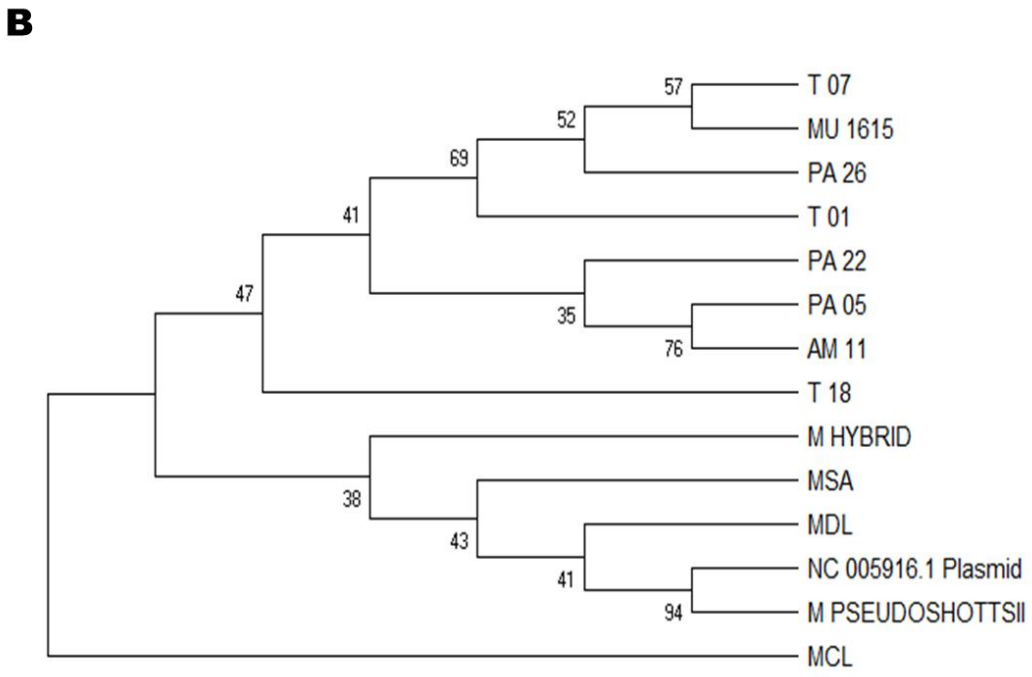


Figure 19: *Sequence confirmation of VNTR repeats and phylogeny of isolates. M. ulcerans 1615 (MU1615), M. marinum DL 180892 (MDL), M. pseudoshottsii, M. marinum hybrid 270995 (MHBD), M. marinum CL (MCL), M. marinum SA 200695(MSA) were controls included in the study. NC005916.1 Plasmid is AGY 99 reference strain. PA22, PA05, AM11, T07, PA26, T01 and T18 are cases from Ghana and Côte d'Ivoire. M. ulcerans 1615 (MU1615) clustered closely with T07 as well as PA26 and T01. Samples, PA22, PA05 and AM11 clustered together with T18. All controls besides MU1615 clustered closely with the reference strain.*

CHAPTER FIVE

5.0. DISCUSSION, CONCLUSION, AND RECOMMENDATION

5.1. DISCUSSION

Buruli ulcer has remained a neglected tropical disease over decades. The mode of transmission remains a mystery. Pure cultures of MPMs are difficult to obtain from the environment, hence most studies fall on genomic material of *M. ulcerans* and other MPMs in several aquatic environments to infer transmission with proximity and activities performed in slow moving water bodies and also as potential reservoirs of these MPMs (Johnson et al., 2005)(Marsollier et al., 2004; Mosi et al., 2008). MPMs are therefore essentially of immense importance because of their ability to cause ulcers in both animals and man (Fyfe et al., 2010).

Not until recently, other Mycolactone Producing Mycobacteria other than *M. ulcerans* causing infections in fish and frogs have been identified to be somewhat associated with Buruli ulcer in these animals (Dassi et al., 2017; Tano et al., 2017). Other researchers (Dassi et al., 2017; Tano et al., 2017) have reported unsuccessful VNTR typing at the four most typed loci, Miru 1, locus 6, locus 19 and ST1 hence the need to increase the loci used for typing to increase the discriminatory power of this tool. *M. ulcerans* as well as other MPMs have similar sequence of various house-keeping genes as well as other very similar genetic elements especially among isolates from the same geographic region hence hindering substantial genomic studies (Portaels et al., 1996; Stinear et al., 2000; Ablordey et al., 2005). VNTR Typing has proven to be extremely beneficial over the years to categorically differentiate isolates even found within the same geographical locations, hence fine tuning would greatly benefit genomic studies on such isolates. This section discusses data from the study to support the assertion that other MPMs besides *M.*

ulcerans may be implicated in Buruli ulcer and are genotypically diverse. In this study, isolates were typed at 16 VNTR loci to access the genetic diversities at selected loci.

Samples for the study were collected from Ghana and Côte d'Ivoire. These two countries were explored for this particular studies due to the endemicity of the disease in both countries and ties to other Buruli ulcer resource centers. Côte d'Ivoire remains the most endemic country with disease burden closely tailed by Ghana which is the second most endemic country (Zingue et al., 2018). In Ghana, Amasaman is a small community located in the Ga West Municipal district of the Greater Accra Region and happens to be the districts' capital. Resident in that community is the Amasaman Hospital which has a Buruli ulcer treatment center. This treatment center is one of the only two Buruli ulcer treatment centers present in the Greater Accra region and hence is utilized by a large number of Buruli ulcer and other tropical ulcer patients within the region and from surrounding regions.

This deduction was made from responses obtained from patients on questionnaires administered during interviews. Pakro is also a community situated in Akuapim South Municipal district in the Eastern Region. Unlike Amasaman Hospital, the hospital located in Pakro lacked a Buruli ulcer treatment centre and Buruli ulcer like cases were referred to general Out Patients Department OPD) and were treated as usual tropical ulcers. Practices on Buruli ulcer patients and other tropical ulcers in both hospitals were somewhat similar following routine practices such as periodic wound cleaning and management and drug (mostly antibiotics and NSAIDs) administration. Patients with severe ulcers that usually affects easy movement to and from the health center were admitted. The only difference between this two health centers besides geographical location was that patients in Amasaman hospitals resided in Buruli ulcer treatment

wards while patients admitted in Pakro resided in general wards with patients suffering from other ailments other that Buruli ulcer or other tropical ulcers. Patients from these two centers were recruited as and when they visited the hospital and consented to the study and were sampled passively. This reflects in the low number of samples obtained in both health centers.

Amansie Central District (then Amansie West) is a district located in Ashanti region of Ghana. Its district capital, Jacobu is home to one of the districts biggest hospitals. Ashanti region alone has been reported to have more than sixty percent (60%) of all total infections in Ghana (Amofah et al., 2002) In this particular district and the region at large, high cases of Buruli ulcer disease have been reported and is a hub for most researchers (Asiedu and Etuaful, 1998; Duker et al., 2004; Hilty et al., 2006; Owusu-Sekyere et al., 2013; Ablordey et al., 2015). A oneday workshop was organized in this district which brought about fifty (50) nurses and community health workers from different parts of the district to sensitize them on disease identification, patient recruitment and sample collection. This exercise led to an active case search in this particular area and with active sampling. It is therefore not surprising to have obtained fifty-six (56) samples from Amansie Central representing 47.5% of the entire samples obtained as supposed to twenty-six (26) and thirty-six (36) samples representing 22% and 30.5% of the entire samples obtained from Amasaman and Pakro respectively over a six (6) months period.

In Côte d'Ivoire, seven communities were selected for sampling based on the endemicity of the disease in such areas. The seven communities include Yamoussoukro, Kongouanou, Zoukougbeu, Divo, Bouaké, Sakassou and Djenedoufla. Being the most endemic country, all the health facilities visited had well-furnished Buruli ulcer treatment centers were patients are treated and observed. The facilities had admission wards for Buruli ulcer as well as other tropical ulcers, wound dressing

area, common play grounds, a small resource laboratory, a kitchen and a common eating area which is not surprising. Nurses and Community health workers were likewise trained in a oneday workshop on disease identification, patient recruitment and sample collection. Samples from patients in Côte d'Ivoire were either collected by trained nurses and community health workers at the various resource centers and shipped or collected by researchers during periodic visits.

A lot of cases (189) were observed in this short study period (3-6 months) due to the incorporation of active sampling where patients are identified from endemic communities due to failure to visit health facilities. Aguiar et al., 1997 identified eight hundred and sixty-seven (867) Buruli ulcer cases passively by waiting for patients to visit health facilities and recruit as well as sample them over a five (5) year period (1992-1996). The number of cases identified also in another five (5) year period study (1997-2001) by Debacker et al., 2004 doubled to one thousand and seven hundred (1700) cases which could be attributed to either robust passive case search system or increased number of Buruli ulcer cases. The latter however which is most likely to be cause is downplayed by underreporting of cases. Active case search is highly recommended for early case detection and treatment due to the bad roads among other poor infrastructure in remote areas where the disease usually occurs to manage underreporting.

There were more female than male cases observed in this study but this was not statistically significant ($p= 0.202$). This was however in contrast with a study by Debacker et al., 2004 and case control studies by Raghunathan et al., 2005 and Sopoh et al., 2010 that reported more males than female cases and Debacker et al., 2004 who reported a 1:1.01 male to female ratio.

Ages of patients with cases ranged from one (1) year to ninety (90) years with most of the cases reported in the Adult category (18-50). This finding contradicts what is perceived and has been

reported as children especially boys below the ages of fifteen (15) are considered to be the most at risk of contracting Buruli ulcer. The adult category aged between fifteen (15) and fifty (50) years however are frequently exposed to wetlands mostly due to their occupation (most being farmers) and daily activity which validates the preponderance observed among this age group. Incidence in aged category in this study (50 – 90years) was also high and this could possibly be in relation to a waning and down regulated immune state allowing the reactivation of latent disease (Debacker et al., 2004) usually caused by trauma (Meyers et al. 1974). The number of cases in children aged between zero (0) and fifteen (15) years were lesser and may be attributed to more effective immune protection of children within this age group.

Lesions in all body areas were observed in this study as observed in other studies (Pluschke et al., 2005) with more cases reported on the extremities of the body (hundred and fifty-six (156) and twenty-seven (27) cases reported on the lower (82.5%) and upper (14.1%) limbs respectively).This preponderance observed was expected and is consistent with reports by Portaels et al., 2001, Debacker et al., 2004, Merritt et al., 2010 and Adu and Ampadu, 2015. Infections on the trunk and breast area and the head and neck area were observed but in a few cases as also observed by Adu and Ampadu, 2015. These observation support reports by Amofah et al., 2002; Portaels et al., 1999 and Wilson et al., 2011 that *M. ulcerans* is an environmental pathogen found in aquatic habitats and may possibly be transmitted by close proximity to such environment or surroundings. Interestingly, two (2) cases on the genitalia which rarely occurs was observed in Côte d'Ivoire in two (2) children (1 male and 1 female) between the ages of one to ten (1-10). Cases as such are not often seen in the hospital, as Debacker et al., 2004 reported only one (1) female case (age range 30-44) and six (6) male cases (age range 0-14) on the genitalia in a total of one thousand and seven hundred (1700) Buruli ulcer cases collected over a five (5) year period (1997-2001).

Gender and age does not significantly affect the presentation of the lesion (Zingue et al., 2018). The variation in lesion topology by gender and age may be attributed to occupation, daily and recreational activities. For example, adult males may be more likely to weed and farm with cutlasses and may sustain injury or trauma on the limbs more as supposed to adult females who are likely to carry gathered farm produce or even fetch water and carry on their head thus making that area more prone to infection.

Mycolactone's analgesic effects make it highly ignored by patients at the earlier stages with most seeking treatment at the very late stages. This was evident in the cases identified in this study as the earlier disease presentations such as the nodule (3.8%) and the edema (2.1%) were very few. These earlier disease stages may also be misdiagnosed as insect bites or allergic reactions by only relying on clinical diagnosis. The few observed cases were treatable with antibiotics. Most of the cases were already in the late disease stages possibly requiring surgical excision, skin grafting and corrective treatment post antibiotic administration. The late forms of the disease were mostly ulcerative. In Ghana, more of these cases were in Category III (lesions greater than 15cm in diameter) while more Category II (lesions within 5-15cm in diameter) cases were observed in Côte d'Ivoire. This may be due to the treatment seeking behavior of the patients as Côte d'Ivoire had well established Buruli ulcer treatment centers where patients often visited and were treated and observed whereas most patients in Ghana usually seek traditional healers or treat themselves at home with just a handful visiting the hospitals.

Cases were confirmed by non-culture based laboratory assays specifically microscopy and PCR. Culture was not included in the confirmation of cases due to the long period required by the bacterium for growth (over 4 weeks) and short study period. Only five (5/189) samples (2.7%)

were positive for microscopy with acid fast bacilli and coccobacilli observed in singletons and clusters. Being the simplest and widely used diagnostic tool, it's the least sensitive and requires residence in an endemic community coupled with strong clinical presentations hence may produce a lot of false negative results (Wansbrough-Jones and Phillips, 2006). WHO recommends PCR targeting the insertion sequence, IS*2404* as the gold standard for disease confirmation. However, other mycolactone producing mycobacteria also possess this same insertion sequence which may impede proper diagnosis. IS*2404* has been reported in only MPMs and not all NTMs (Yip et al., 2007). Only twenty-four (24/189) samples (12.7%) were positive for IS*2404* PCR using conventional PCR methodology. The copy number of this insertion sequence is higher in *M. ulcerans* (213 copies) than other MPMs (91 copies) thus other MPMs or *M. ulcerans* strains with low copy numbers may be missed out. This low amplification outcome was thus validated by nested PCR targeting the same insertion sequence. PCR amplifications were achieved after several optimization conditions. This correlates with reports by Williamson et al., (2014) who achieved successful amplification in a single reaction. This produced fifty-eight (58/189) positive samples increasing the positivity to 30.7%. Enoyl Reductase PCR was also performed to increase the chance of detecting other MPMs other than *M. ulcerans*. Only thirty-four (34/189) representing 18% of the samples were positive. To further increase the validity of these findings, qPCR targeting IS*2404* was used as the last line of sample confirmation as shown and used by Sarpong-Duah et al., 2017. The low copy numbers of *1S 2404* of MPMs (91 copies) make qPCR more effective in detecting MPMs which would have been missed by convention PCR by having a higher cycle threshold (CT) value compared to *M. ulcerans* which has 213 copies of the same insertion sequence and hence a lower CT value. Positivity among samples increased from 30.7% to 84% as one hundred and fifty-nine (159/189) samples were positive using qPCR. The one

hundred and fifty-nine (159) cases were confirmed as positive for Buruli ulcer with laboratory evidence and corroborates results from other study cases that relied solely on clinical diagnosis (Kanga and Kacou, 2001). The high rate of laboratory confirmed cases in this study, irrespective of the stage of the disease indicates that clinical diagnosis is enough to commence treatment in endemic areas. Diagnosis in most rural areas have however moved from using mere clinical symptoms only for diagnosis (Bratschi et al., 2013) to the use of laboratory based diagnosis which is highly recommended by WHO.

SNP typing has been shown to be successful for the typing of MPMs (Yip et al., 2007), however VNTR typing was used in this particular study to increase its efficiency and discriminatory power as it is a cheaper and faster tool to use and extensively employed for MPM Typing. VNTR typing in this study was achieved at sixteen (16) loci; locus 1, locus 4, locus 6, locus 8, locus 9, locus 10, locus 13, locus 14, locus 15, locus 16, locus 18, locus 19, locus 33, ST1, Miru 1 and Miru 9. VNTR analysis of two polymorphic loci, MIRU1 and ST1 on seventy-two (72) African isolates (fifty-seven (57) Ghanaian isolates) revealed three different genotypes with clonal clustering, suggesting genetic diversity of *M. ulcerans* in Ghana (Hilty et al., 2006) which could have been more if other polymorphic loci were included. Using sixteen (16) loci, this study increased the discrimination power of VNTR typing as against two (2), three (3) and four (4) loci separately reported by Hilty et al., 2006; Williamson et al., 2014 and Narh et al., 2015 respectively. Controls used (*M. ulcerans* 1615, *M. marinum* DL 180892, *M. pseudoshottsii*, *M. marinum* hybrid 270995, *M. marinum* CL, *M. marinum* SA 200695) and samples (159) were monomorphic at six (6) loci; locus 1, locus 4, locus 8, locus 9, locus 10 and locus 14. Data from these loci were not used for genotyping of samples because it did not distinguish samples.

Samples were classified into seven (7) *M. ulcerans* genotypes and five (5) MPMs genotypes at ten (10) loci based on controls and published literature (Williamson et al., 2014; Narh et al., 2015).The four (4) standard loci, Miru 1, locus 6, ST1, locus 19 were first used to genotype and the other six (6) used to supplement data for samples that poorly performed at the former. Interestingly, locus 15 and locus 33 were able to distinguish *M. ulcerans* from the other MPMs. As a results, samples that could not be categorized as either *M. ulcerans* or an MPM due to poor amplification at the standard four loci were identified at this loci. This would serve as another simple PCR based method of distinguishing *M. ulcerans* from the other MPMs which only relies on qPCR targeting IS*2404* due to varying copy numbers of this insertion sequences hence reflecting in their CT value. The remaining four loci, locus 13, locus 15, locus 16 and locus 18 generally distinguished all MPMs indiscriminatorily and was used when samples could not be genotyped at the first six loci mentioned above. Loci, Miru 1 and ST1 with repeats 1 and 2 respectively were observed in this study which is consistent with reports by Hilty et al., (2006) for *M. ulcerans* strains from Amansie Central which happens to be one of the sites under this study. Williamson et al., (2014) also obtained repeats 1, 1 and 1; 3, 1 and 2; 3, 1 and 1 as well as 1, 1 and 2 at loci, Miru 1, locus 6 and ST1 respectively which were observed in this study as well; however, Repeats 1, 4 and 2 observed by Williamson et al., (2014) at the above mention loci were absent in this study with samples monomorphic at locus 19 but polymorphic in this study.

Both Ghana and Côte d'Ivoire had five (5) genotypes in circulation. Genotype C (3, 1, 2, 2), D (1, 1, 2, 2) and F (2, 1, 2, 2) was identified in both Ghana and Côte d'Ivoire. Genotypes A (1, 1, 1, 2) and G (1, 1, 2, 1) were only found in Côte d'Ivoire while Genotype E (1, 1, 2, 3) and the MPM, MLF (1, 2, 2, 1) were only identified in Ghana. In Ghana, Genotype D was found in all three communities. Genotypes E was found only in Amansie Central, Genotype MLF in Amasaman and

Genotype C and F only in Pakro. The presence of two genotypes in Amasaman suggests that MPMs in circulation in that area is low. This may be attributed to the small of samples (26) obtained in this area. Three genotypes observed in Pakro in relation to the sample size (36) is suggests that different species of *M. ulcerans* exist in Pakro. Amansie central was the community in Ghana with the most cases (56) due to active case search but only two genotypes were identified. The high sample size in Amansie central coupled with few genotypes in circulation may be attributed to the treatment regime amongst patient. In Amansie central, active case searches led to the early identification of cases and follow up to clinic and treatment commencement. Hence fewer genotypes observed in this community is inferable.

In Côte d'Ivoire, all the communities sampled had Buruli ulcer treatment centers that are highly utilized by individuals. Unlike Ghana, no single genotype was found across all communities. Djekanou had the most genotypes (A, C, D and G) with Yamoussoukro and Bouake having the least number of genotypes, F and C respectively. Common genotypes (C and D) were found in Kongouanou and Zoukougbeu. Fewer genotypes were expected in Côte d'Ivoire than in Ghana due to the approximately 1:2 sample size ratio which was however not observed. A higher number in the former observed may be linked to the acquisition of infections in rare positions in the body such as the genitalia as observed in samples from only Côte d'Ivoire. Data from similar studies conducted by Narh et al., 2015 and Williamson et al., 2014 were included in this study. Both studies were conducted in Ghana. Genotype D was observed among all studies which makes it the most prevalent genotype from 2008 to 2019. Apart from Genotype D, Genotype A, B and C were observed in this current study and Williamson et al., 2014. However, Genotype C was the prominent genotype in the later study whereas Genotype D was the prominent in the former. There could possibly be a change from one genotype to another to aid disease establishment and

adaptation to a particular niche over time. Also, Ivorian samples included in this present study may have influenced this pattern observed as the former study did not have any Ivorian samples. Genotype G and the MPM genotype MLF represented as W and Y by Narh et al., 2015 were common to both studies. The absence of these strains in Williamson et al., 2014 study indicates that there may be possible emerging strains as both studies were conducted between 2015-2019. Distinctively, Genotype E and F were only found in this present study further validating the emergence of possible new strains. Genotypes Z was only found in Narh et al., 2015 study whereas Genotype B and MPM, MPS (*M. Pseudoshottsii*) suggesting that strains of *M. ulcerans* of may be adapting to favor infection and MPMs may be implicated also in Buruli ulcer causation.

In both Ghana and Côte d'Ivoire, Genotype D was associate with all stages of infection. This observation is consistent with the high number of D genotypes identified in both countries. Genotypes C and F were associated with the earlier stages of the infection in Ghana and conversely the late BU stages in Côte d'Ivoire. Similarly, Genotype E was associated with the earlier disease stage in Ghana whiles Genotype F was associated with late stages of the disease in Côte d'Ivoire. Genotype A was only involved in nodular infections in Côte d'Ivoire, however inferring that it's actually causes nodular infections may be wrong as only a few samples were Genotype A.

Most of the cases were however either in the late ulcerative stage or had advanced and were categorized as based on lesion size and presentation. In Ghana, Genotype C was associated with Category II lesions whiles Genotype F was associated with Category I lesions. Genotype E was also associated with Category I and II lesions while Genotype D was associated with all categories. Conversely as observed in Côte d'Ivoire isolates, Genotype A was associated with

Category II lesions, Genotype F associated with Category II and III lesions, Genotype G associated with Category II and III lesions as well as an edematous ulcer while Genotype C and D was associated with all categories (Figure 17).

This study addressed three key objectives: Identification of MPMs, MPM genotypes causing infections in Ghana and Côte d'Ivoire and the dynamics of genotype differences over the years as well as between two study sites. Genotypes responsible for Buruli ulcer in Ghana and Côte d'Ivoire were duly accessed.

5.2. CONCLUSION

A higher number of Buruli ulcer patients than expected were identified. More adults were infected than children and aged. Out of the sixteen (16) VNTR loci used for typing cases, six (locus 1, locus 4, locus 8, locus 10, locus 14 and Miru 9) were monomorphic whiles polymorphic at ten (Miru 1, locus 6, locus 19, locus 15, locus 13, locus 16, locus 18, locus 33, locus 9 and ST1). Locus 15 and locus 33 distinguished *M. ulcerans* from the other MPMs. Five genotypes were observed in Ghana and Côte d'Ivoire. Genotypes C, D and F were found in both Ghana and Côte d'Ivoire. Genotype E and MLF found only in Ghana whiles Genotypes A and G were only found in Côte d'Ivoire. Genotype D was prominent from 2008 to 2019.VNTR typing results with published literature gave indications for the possible emergence and spreading of new genetic variants of *M. ulcerans* and other MPMs within Ghana and Côte d'Ivoire. 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.

5.3. RECOMMENDATION

Whole genome metagenomics sequencing of both IS*2404* positive and negative will be essential to see what microbial community may be responsible for varying genotypes observed in the positives and similar lesion and clinical presentations of the negatives. It would be quite interesting as more information will be available to definitely assign genotypes. Similar studies can be done in other endemic communities other than the ones in this study to see if findings will corroborate that observed in this study. A similar study should also be done in the same communities but this time around with environmental samples in show if similar genotypes would be observed to infer local transmission.

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APPENDICES

Appendix 1: Demographic characteristics of participants

Country	Ghana	Côte d'Ivoire	Total
		Frequency (%)	
Education			
No Formal Education	33 (21.7)	33 (50)	66 (37.2)
Primary	47 (42.3)	18 (27.3)	65 (36.7)
Secondary	27 (24.3)	13 (19.7)	40 (22.6)
Tertiary	4 (3.6)	0 (0.0)	4 (2.3)
Koranic School	0 (0.0)	2 (3.0)	2 (1.1)
Total	**111**	**66**	**177**
Occupation			
None	14 (12.5)	17 (26.1)	31 (17.5)
Driver	5 (4.5)	0 (0.0)	5 (2.8)
Farmer	42 (37.5)	13(20)	55 (31.1)
Student	15 (13.4)	14 (21.5)	29 (16.4)
Artisan	9 (8.0)	5 (7.7)	14 (7.9)
Trader	14 (12.5)	9 (13.8)	23 (13.0)
Other	13 (11.6)	7 (10.8)	20 (11.3)
Total	**112**	**65**	**177**
Gender			
Male	52 (46.4)	33 (50.0)	85 (47.8)
Female	60 (53.6)	33 (50.0)	93 (52.2)
Total	**112**	**66**	**178**
Age Categories			
0-10	5 (5.7)	12 (21.4)	17 (11.9)
11-20	10 (11.5)	9 (16.1)	19 (13.3)
21-30	11 (12.6)	5 (8.9)	16 (11.2)
31-40	14 (16.1)	9 (16.1)	23 (16.1)
41-50	15 (17.2)	7 (12.5)	22 (15.4)
51-60	10 (11.5)	9 (16.1)	19 (13.3)
61-70	11 (12.6)	4 (7.1)	15 (10.5)
71-80	9 (10.3)	1 (1.8)	10 (7.0)
81-90	2 (2.3)	0 (0.0)	2 (1.4)
Total	**87**	**56**	**143**

Appendix 2: Correlation table; Infection Status by the other Variables. Negative sign means negative correlation and Positive sign means positive correlation.

	Infection status	Lesion category	sex	Marital status	age	Level of education	profession
Infection status	1.000						
Lesion category	-0.170	1.000					
	(-0.103)						
sex	-0.123	0.217	1.000				
	(0.202)	(0.036)					
Marital status	0.074	-0.096	-0.076	1.000			
	(0.449)	(0.359)	(0.428)				
age	-0.101	0.077	-0.061	-0.023	1.000		
	(0.294)	(0.461)	(0.522)	(0.809)			
Leve of education	0.104	-0.184	0.180	0.096	-0.229	1.000	
	(0.288)	(0.083)	(0.062)	(0.329)	(0.017)		
profession	0.028	0.006	-0.020	0.016	-0.450	0.029	1.000
	(0.773)	(0.952)	(0.838)	(0.872)	(0.000)	(0.768)	

Infections status by profession

	Infections Status		
Profession	First Time	reinfection	Total
Artisan	7	2	9
Driver	5	0	5
Farmer	39	3	42
None	13	0	13
Other	11	1	12
Student	13	2	15
Trader	12	2	14
Total	100	10	110
	Pearson chi2(6) = 4.6628 Pr = 0.588		

Infections status by lesion category

	Infection status		
Lesion category	First time	reinfection	Total
I	11	3	14
II	25	2	27
III	49	3	52
Total	85	8	93

Pearson chi2(2) = 3.5093 Pr = 0.173

Infections status by age

	Infection status		
Age	First time	reinfection	Total
children	9	1	10
adult	50	7	57
aged	41	2	43
Total	100	10	110

Pearson chi2(2) = 1.7373 Pr = 0.420

Appendix 3: Buruli ulcer project Questionnaire

DATE:……. /……../……..

INTERVIEWER……

Has consent been sought Yes/ No

PART ONE: SOCIOLOGY

SECTION A - DETAILS OF SUSPECTED CASE

A1. Name (Surname, First Name(s))

…….

A2. Age ……..

A3. Sex: □ M □ F

A4. Profession ……

A5. Marital Status ……

A6. Ethnic group (Optional)…….....

A7. Level of education. □Primary □ Secondary □ Tertiary

A8. Where do you stay? ……..

A9. Duration of stay in the community.……

(If duration is less than 6 months, ask question A8)

A10. Where were you previously staying? ……

A11. Do you know of other skin diseases prevalent in the community? Mention them.

…….……

A12. Is this your first time of contracting the disease? Yes / No

A13. If yes, how do you think you contracted it? ……

A14. How long did it take before it progressed to an ulcer (sore) ……

SECTION B - HISTORY OF LESION/ ULCER PRESENTATION (Both Bu and Non BU)

B1. When did you first notice the lesion/ ulcer?

Establish approximate date through a seasonal calendar. (__/__/____) or describe date ……

B2. Where is the lesion / ulcer located? Circle the portion.

B3. Describe your behavioral activities when the lesion/ulcer was first noticed (Be as specific as possible.) ……

B4. How did the lesion present itself during its development? Provide a brief narrative of the order of development stages. Note whether the ulcer first appeared from trauma.

A. Nodule B. Plaque C. Ulcer D. Nodule>Ulcer E. Plaque>Ulcer

F. Oedema>Ulcer G. Nodule>Oedema>Ulcer

B5. Are you undergoing any treatment? □ Yes □ No

B6. If Yes, which form of treatment?

A. Hospital/Clinic B. Traditional healer C. Self-treatment

B7. What medication do you take? ……....

B8. How long have you been on treatment? …….

SECTION C

SOCIAL FACTORS RELATED TO REPORTING AND TREATMENT OF BURULI ULCER

C1. Do you know the disease Buruli ulcer (BU)? Yes □ No □

If participant answers no, move to section D C2. What is BU called in your local language? .....

C3. Does BU exist in your village? Yes □ No □

C4. If yes, do you know people suffering from it? Yes □ No □

C4.1. Is the person a family member, friend, neighbor? …….

C4.2. If it's a family member, identify their relationship to you.

□ Mother □ Father □ Child□ Sibling (same mother) □ Sibling (same father)

□ Other genetic relationship □ Non-genetic relationship

C4.2. If it's a friend or neighbor, identify his/her relationship to you……..

C5. What are some symptoms of this disease that you know?

Fever □ Headache □ Cough □ Itching □ Wound (open wounds) □, Other (be precise) □

C6. Which part of the body is particularly affected?

Arm □ Body □ Hands □ Legs □ Foot □ Eye □ Genitals □ Others (be Precise)

C7. Do you know what can happen to you if you have the disease? Yes □ No □

C8. If yes, cite at least one (1) of them ……...

C9. Do you know if it is treatable? Yes □ No □

C10. If yes, How? ……

SECTION D - TYPE OF SAMPLE TAKEN (TICK THE BOX)

D1. Nodule : FNA □ 2 PBS □ 1 M7H9 □ 1 Methanol □ 1 Glass slide

Skin Excision : Ulcerated skin □ 2 PBS □ 1 M7H9 □ 1 Methanol □ 1 Glass slide

Ulcer: Swab □ 2 PBS □ 1 M7H9 □ 1 Methanol □ 1 Glass slide

D2. Has patient's incentive been given? Yes □ No □

……. (Signature of Patient)

Thank you

Appendix 4: Consent form for patients

CONSENT FORM

Title: Metabolites in MPM and BU infection: Diagnostic Biomarkers through Metabolomics.

Principal Investigator: Dr. Lydia Mosi

Address: Department of Biochemistry, Cell and Molecular Biology, University of Ghana. P. O. Box LG 54, Legon.

General Information about Research

This study involves research aimed at determining the 'complete' set of metabolites (small molecules, such as sugars, amino acids or hormones) of *Mycobacterium ulcerans*, which will be used to find metabolites of Buruli ulcer patients. This will lead to the identification of markers which could serve as a means of diagnosing this disease. This study will run for 2years. If you agree to be in this study, you will be asked to provide two types of samples; a small portion of the skin around your wound and also a small amount of fluid that will be drawn near your wound. Your engagement in the study for the taking your samples and answering of questionnaire will last about 20 to 30 minutes

Possible Risks and Discomforts

There is no major risk associated with this study apart from the slight discomfort you may get from parts of your wounds when the sample is being taken. When you are emotionally disturbed in the course of the study, you will need to inform the researcher. In such instance, you will be required to take a break and resume when you feels better. However if you wants to discontinue due to the slight discomfort, you can freely do so.

Possible Benefits

The study will be of no direct benefit to you. However, the results of the sample to be taken and the information you provide will contribute significantly in the identification of differences that exist among the bacteria causing Buruli ulcer as well as the best way of treating them. In addition, you will be offered the opportunity to test for Buruli ulcer which will help you know your status.

Confidentiality

We will protect information about you to the best of our ability. Your records will be kept in a secure location at the Department of Biochemistry Cell and Molecular Biology for 2 years after

which it will be destroyed. 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 your name. Only the research team will have access to your name associated with the study number and for special reasons such as treatments. It is likely that data obtained from tests done on you may be published in medical journals. However, your identity will not be disclosed. The samples taken will not be used for any future work.

Compensation

You will be given an incentive in a form of provisions which will amount to Gh₵25.00 as appreciation for partaking in the study.

Voluntary Participation and Right to Leave the Research

Your participation in this research is voluntary and you are free to withdraw at any point without penalty.

Notification of Significant New Findings

Any significant new findings that will be obtained in the course of the research that concerns you and requires further participation will be communicated to you.

Contacts for Additional Information

Lydia Mosi PhD,
Department of Biochemistry, Cell and Molecular Biology,
University of Ghana.
P. O. Box LG 54, Legon
054 089 0352, lmosi@ug.edu.gh

Your rights as a Participant

This research has been reviewed and approved by the Institutional Review Board of Noguchi Memorial Institute for Medical Research (NMIMR-IRB). If you have any questions about your rights as a research participant you can contact the IRB Office between the hours of 8am-5pm through the landline 0302916438 or email addresses:

nirb@noguchi.ug.edu.gh

VOLUNTEER AGREEMENT

The above document describing the benefits, risks and procedures for the research titled **Metabolites in MPM and BU infection: Diagnostic Biomarkers through Metabolomics** has been read and explained to me. I have been given an opportunity to have any questions about the research answered to my satisfaction. I agree to participate as a volunteer.

_____ _____

Date Name and signature or mark of volunteer

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.

_____ _____

Name and signature of witness Date

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.

_____ _____

Name and signature of Person Who Obtained Consent Date

Appendix 5: Parental consent form for parents with infant patients

PARENTAL CONSENT FORM

Title: Metabolites in MPM and BU infection: Diagnostic Biomarkers through Metabolomics.

Principal Investigator: Dr. Lydia Mosi

Address: Department of Biochemistry, Cell and Molecular Biology,
University of Ghana.
P. O. Box LG 54, Legon
054 089 0352, lmosi@ug.edu.gh

General Information about Research

This study involves research aimed at determining the 'complete' set of metabolites (small molecules, such as sugars, amino acids or hormones) of *Mycobacterium ulcerans,* which will be used to find metabolites of Buruli ulcer patients. This will lead to the identification of markers which could serve as a means of diagnosing this disease. This study will run for 2years. If you agree for your child to be in this study, you will be asked to provide two types of samples; a small portion of the skin around your wound and also a small amount of fluid that will be drawn near your wound. Your engagement in the study for the taking your samples and answering of questionnaire will last about 20 to 30 minutes.

Possible Risks and Discomforts

There is no major risk associated with this study apart from the slight discomfort your child may get from parts of their wounds when the sample is being taken. When your child is emotionally disturbed in the course of the study, you will need to inform the researcher. In such instance, he/ she will be required to take a break and resume when he/she feels better.

Possible Benefits

The study will be of no direct benefit to your child. However, the results of the sample to be taken and the information your child provides will contribute significantly in the identification of biomarkers (markers relating to living organism) associated with the study. In addition, your child will be offered the opportunity to test for Buruli Ulcer which will help you know his/her status.

Confidentiality

We will protect information about your child to the best of our ability. Your child's records will be kept in a secure location at the Department of Biochemistry Cell and Molecular Biology for a year after which it will be destroyed. All information collected during the study will be stored in a file which will not have your child's names on it, but a study number assigned to each of your child's names. Only the research team will have access to your child's names associated with the study numbers and for special reasons such as treatments. It is likely that data obtained from tests done on you may be published in medical journals. However, your identity will not be disclosed. The samples taken will not be used for any future work.

Compensation

You will be given an incentive in a form of provisions which will amount to Gh₵25.00 as appreciation for partaking in the study.

Voluntary Participation and Right to Leave the Research

Your child's participation in this research is voluntary and he/she is free to withdraw at any point without penalty.

Notification of Significant New Findings

Any significant new findings that will be obtained in the course of the research that concerns your child and requires further participation will be communicated to you.

Contacts for Additional Information

Magdalene Dogbe, Department of Biochemistry, Cell and Molecular Biology, University of Ghana. P. O. Box LG 54, Legon 0209337398, magden2916@gmail.com

Your Child's Rights as a Participant

This research has been reviewed and approved by the Noguchi Memorial Institute for Medical Research Institutional Review Board (NMIMR-IRB). If you have any questions about your child's rights as a research participant you can contact the IRB Office between the hours of 8am-5pm through the landline 0302916438 or email addresses:

nirb@noguchi.ug.edu.gh

VOLUNTEER AGREEMENT

The above document describing the benefits, risks and procedures for the research title: "Metabolites in

MPM and BU infection: Diagnostic Biomarkers through Metabolomics" has been read and explained to me. I have been given an opportunity to have any questions about the research answered to my satisfaction. I agree that my child should participate as a volunteer.

_____ _____

Name and signature or mark of parent or guardian Date

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 child's parent or guardian. All questions were answered and the child's parent has agreed that his or her child should take part in the research.

_____ _____

Name and signature or mark of parent or guardian Date

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.

_____ _____

Name and signature of Person Who Obtained Consent Date

Appendix 6: Child consent form for juveniles

CHILD ASSENT FORM

Introduction

My name is Dr. Lydia Mosi and I am from the Department of Biochemistry, Cell and Molecular Biology, at University of Ghana. I am conducting a research study entitled **Metabolites in MPM and BU infection: Diagnostic Biomarkers through Metabolomics**. I am asking you to take part in this research study because I am trying to learn more about the 'complete' set of metabolites (small molecules, such as sugars, amino acids or hormones) of *Mycobacterium ulcerans,* which will be used to find metabolites of Buruli ulcer patients. This will lead to the identification of markers which could serve as a means of diagnosing this disease. This study will run for a 2years. If you agree to be in this study, you will be asked to provide two types of samples; a small portion of the skin around your wound and a small amount of fluid that will be drawn near your wound. Your engagement in the study for the taking your samples and answering of questionnaire will last about 20 to 30 minutes.

Possible Benefits

The study will be of no direct benefit to you. However, the results of the sample to be taken and the information you provide will contribute significantly in the identification of biomarkers (markers relating to living organism) associated with the study. In addition, you will be offered the opportunity to test for Buruli Ulcer which will help you know your status.

Possible Risks and Discomforts

There is no major risk associated with this study however, there may be slight discomfort you may get from parts of the wounds when the sample is being taken. When you are emotionally disturbed in the course of the study, you will need to inform the researcher. In such instance, you will be required to take a break and resume when you feel better.

Voluntary Participation and Right to Leave the Research

You can stop participating at any time if you feel uncomfortable without any persuasion.

Confidentiality

We will protect information about you to the best of our ability. Your records will be kept in a secure location at the Department of Biochemistry Cell and Molecular Biology for a year after which it will be destroyed. All information collected during the study will be stored in a file which will not have your names on it, but a study number assigned to your sample and data. Only the research team will have access to your name associated with the study number and for special reasons such as treatments. It is likely that data obtained from tests done on you may be published in medical journals. However, your identity will not be disclosed. The samples taken will not be used for any future work.

Contacts for Additional Information

You may ask me any questions about this study. You can call me at any time on 0262211948/0540890352 or talk to me the next time you see me.

Please talk about this study with your parents before you decide whether or not to participate. I will also ask permission from your parents before you are enrolled into the study. Even if your parents say "yes" you can still decide not to participate.

Your rights as a Participant

This research has been reviewed and approved by the Institutional Review Board of Noguchi Memorial Institute for Medical Research (NMIMR-IRB). If you have any questions about your rights as a research participant, you can contact the IRB Office between the hours of 8am-5pm through the landline 0302916438 or email addresses: nirb@noguchi.ug.edu.gh.

VOLUNTARY AGREEMENT

By making a mark or thumb printing below, it means that you understand and know the issues concerning this research study. If you do not want to participate in this study, please do not sign this assent form. You and your parents will be given a copy of this form after you have signed it.

This assent form which describes the benefits, risks and procedures for the research titled '**Metabolites in MPM and BU infection: Diagnostic Biomarkers through Metabolomics** has been read and or explained to me. I have been given an opportunity to have any questions about the research answered to my satisfaction. I agree to participate.

Child's Name:……

Researcher's Name:……

Date:……

Child's Mark/Thumbprint……..

Researcher's Signature:……

Date:……