UNIVERSITY OF GHANA

COLLEGE OF BASIC AND APPLIED SCIENCES

EXAMINATION OF HUMAN SKIN SURFACES FOR THE DETECTION OF *MYCOBACTERIUM ULCERANS*

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DECLARATION

I, Isaac Prah, do hereby declare that with the exception of the references cited to other people's work which has been duly acknowledged, this work is the result of my own research work, done under supervision and has neither in part or whole been presented elsewhere for another degree.

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ABSTRACT

Mycobacterium ulcerans, causes Buruli ulcer (BU), a necrotizing skin disease endemic in 33 countries globally. Its environmental reservoir and mode of transmission are unknown. Portaels hypothesized that prior skin contamination followed by penetration through existing skin abrasion or trauma may be a possible route through which *M. ulcerans* is transmitted to humans. Comparative epidemiological and case control studies have outlined some risk activities associated with BU that could result in contamination of human skin with *M. ulcerans*. In this study, skin surfaces of inhabitants involved in specific risk activities in eight selected communities either BU endemic or non-endemic were examined for the presence of *M. ulcerans*. Skin swabs were taken from exposed limbs of 50 individuals from each community for the detection and isolation of *M. ulcerans*. Two DNA extraction methods were compared in extracting *M. ulcerans* DNA from pooled skin suspensions. The presence of *M. ulcerans* DNA from the extracts was first detected using Taqman real time IS*2404* PCR multiplex with internal positive control and then IS*2606* multiplex with KR for all IS*2404* positives. None of the DNA extracts using modified Boom DNA extraction method was positive for IS*2404* target sequence while three of the extracts using power soil DNA isolation kit were IS*2404* positive. Only one of the IS*2404* pooled positives was positive for both IS*2606* and KR. Five individual samples within these three pools accounted for the pools positivity for IS*2404*, IS*2606* and KR results. The difference in Ct value between IS*2606* and IS*2404* for one out of the five samples that was positive for all the targets was 2.34, an indication of *M. ulcerans* DNA. This was detected on an individual who was returning from the farm without protective clothing in a non-endemic community. No *M. ulcerans* was isolated from the LJ culture after 15 weeks of cultivation. Detection

of *M. ulcerans* DNA on the skin surface of a farmer shows that the skin can be contaminated with *M. ulcerans* and supports Portaels' hypothesis about skin surface contamination with *M. ulcerans.* The findings also support the case control studies establishing association between BU with absence of protective clothing during farming.

DEDICATION

I dedicate this work to the Almighty God for accomplishing what He began in my life

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LIST OF ABBREVIATIONS

AFB	Acid-fast bacilli
BU	Buruli ulcer
Ct	Cycle threshold
FNA	Fine needle aspirate
IS*2404*	Insertion sequence *2404*
IS2606	Insertion sequence *2606*
KR	Ketoreductase
LJ	LÖwenstein Jensen
MPM	Mycolactone Producing Mycobacteria
WHO	World Health Organization
ZN	Zeihl-Neelson

CHAPTER ONE

INTRODUCTION

1.0 Introduction

Buruli ulcer (BU) is an indolent, necrotizing disease of the skin, soft tissue and bone caused by a slow-growing environmental bacterium, *Mycobacterium ulcerans*. Incidence of BU is gradually increasing (WHO, 2015). Fifteen out of the 33 BU endemic countries report an estimate of 6000 cases annually, with higher frequencies occurring within rural populations of sub-Saharan African countries (WHO, 2014). Early clinical manifestations include papules, nodules, edema, and plaques, these pre-ulcerative lesions when left untreated eventually form characteristic ulcers with undermined edges.

Despite remarkable achievement attained in treatment of the disease, efforts toward BU prevention have severely been undermined by limited information on how *M. ulcerans* is transmitted from its ecological niche to humans. Although the exact mechanism by which humans become infected with *M. ulcerans* still remains unknown, case control studies have outlined some risk factors associated with contracting BU. These include residing or working near a slow flowing water body, wading and swimming in contaminated water bodies (Merritt *et al.*, 2010) as well as bites from mosquitoes probably infected with *M. ulcerans* (Quek *et al*., 2007).

Critically reviewing these potential risk factors have led to the suggestion that transmission of the etiological agent of BU might occur through direct inoculation of the bacteria into the skin by way of either contact with contaminated environmental

source (Portaels *et al*., 2001) where trauma may facilitate the introduction of the microbe into the skin or through insect bites (Portaels *et al*., 1999).

The potential role of biting insects in transmission of BU at the moment seems limited to some foci where infection occurs. This was made evident when after an outbreak of BU at Point Lonsdale in Australia, *M. ulcerans* DNA was detected from some species of mosquitoes within that region (Johnson *et al*., 2007). Contrary to some studies in West Africa, where DNA was detected in non hematophagous insects (Williamson *et al*., 2014) making the role of insect as vector in transmission of BU uncertain (Marion *et al*., 2015; Merritt *et al*., 2010).

Portaels in 2001, hypothesized that *M. ulcerans* could enter the human host through trauma after introduction from an overlying contaminated skin surface (Debacker *et al*., 2003). Human activities that increase direct skin contact with environments where traces of *M .ulcerans* DNA have been documented (Merritt *et al*., 2010) could result in contamination of the human skin. This appears to be a contributory factor for the transmission of *M. ulcerans* in some endemic foci especially in Africa where there is constant and direct interaction with such environments.

1.1 Statement of Problem

Buruli ulcer disease (BUD) is a rapidly re-emerging neglected tropical disease (WHO, 2007) which is poorly understood but associated with rapid environmental changes (Ravensway *et al*., 2012)**.** Though mortality is low, morbidity is extremely high and has associated economic implications not only on the individual but on the family as well (Peeters *et al*., 2008; Asiedu and Etuaful, 1998). Epidemiological evidence reveals the spatial clustering of BU cases often around a particular water

body and its focal distribution where endemic and non-endemic communities are usually separated by only a few kilometres (WHO, 2008). Transmission route for BU still remains unknown, although it is known to be linked to contaminated water (Merritt *et al*, 2010; Veitch *et al*, 1997). In Africa, the major risk factor for BU is proximity to stagnant or slow flowing water (Merritt *et al*, 2010). Other risk factors for contracting BU as revealed through case control studies include the use of unprotected water from swamps (Debacker *et al*., 2006) and agricultural lands (Wagner *et al*., 2008).

Despite the identification of these risk factors, precisely how *M. ulcerans* gets into its human host still remains unclear. It has been hypothesized that *M. ulcerans* is transmitted through skin abrasions or skin injuries after contact with contaminated water, vegetation or soil. This implies that, the individuals' skin surfaces must first be contaminated with *M. ulcerans*, before invading the human host. A key factor in the development of wound infections is the human – microbe interface and as *M. ulcerans* is not part of the normal skin flora, detailed studies need to be conducted to examine the skin surfaces of individuals in endemic areas for the presence of *M. ulcerans*, in an effort to understand how *M. ulcerans* is transmitted.

1.2 Justification

Poor understanding of BU transmission processes despite description of the mycobacterium about 60 years ago has severely hindered the prevention and control of the disease. Absence of a proven strategy for preventing the infection has serious public health implications for the health system in endemic countries like Ghana.

Ghana' national BU prevalence in 1998 was 20.7/100,000 and currently over 426 communities mainly in the Ashanti, Brong -Ahafo, Eastern, Greater Accra and

Western region have reported cases of BU (Kenu *et al*, 2014). Akwapim South municipality of the Eastern region of Ghana is endemic with BU with prevalence rate of 151.4 per 100,000 populations and currently is the hottest BU district spot in Ghana (Kenu *et al*, 2014). How the indigenous contract the disease still remains enigmatic. Case control studies conducted by Kenu and his colleagues recently in the district highlighted washing in the Densu river, use of adhesive when injured, the presence of wetlands, insect bites in water and not covering limbs during farming as some risk factors for BU in the locality (Kenu *et al*, 2014).

Prevention and control activities are based on existing knowledge of the risk factors. Establishing the presence of *M. ulcerans* on the skin surface of these risk groups would enormously support the hypothesis that transmission of *M. ulcerans* from the environment to humans could occur through direct contact with a contaminated environmental source. This would ultimately help in tailoring targeted preventive measures, as well as the provision of information to be used in public health education.

1.3 Aim

To examine the skin surfaces of inhabitants of BU endemic communities for the presence of *M. ulcerans*.

1.4 Specific Objectives

The following objectives were taken to help address the question posed above

1. To detect the presence of *M. ulcerans* on the skin surface of individuals involved in specific risk activities.

2. To compare the detection rate of *M. ulcerans* on skin surfaces of inhabitants from endemic and non-endemic communities.
3. To compare two DNA extraction protocols for the detection of *M. ulcerans* from skin swabs.
4. To isolate *M. ulcerans* from skin surfaces of inhabitants of the study communities.

CHAPTER TWO

LITERATURE REVIEW

2.1 The genus *Mycobacterium*

Mycobacterium belongs to the order Actinomycetales, family Mycobacteriaceae and genus *Mycobacterium*. They are aerobic, non-motile, non-capsulated and asporogenous that replicate by binary fission (Grange, 1996). Mycobacteria are significantly smaller in size than other type of bacteria and the cell shape varies from cocco –bacilli to elongated rods but pleomorphic morphology is common. The genome of mycobacteria is estimated to be in equivalent range of 4.6 - 8.4 Mb (Baess and Mansa, 1978) and like other prokaryotes is arranged as closed circle. The nucleotide composition of mycobacterial DNA is extraordinarily rich in guanine and cytosine with a G-C ratio of 61-71% (Levy –Frebault and Portaels, 1992).

Another unique characteristic of mycobacterium is their cell wall. Mycobacterial cell wall is highly complex and has a lipid content that approximates 60% of the structure (Brennan and Nikaido, 1995). This high mycolic acid content confers alcohol and acid-fast staining properties on mycobacteria distinguishing them from most bacteria. This cell wall characteristic allows the mycobacterial species to survive in different environments such as in biofilms, in water habitats or particulate matter in soils.

Mycobacteria are classified as either slowly or rapidly growing. This distinction is based on whether isolated colonies are observed after more than 7 days or less than 7 days on solid medium (Levy–Frebault and Portaels, 1992). Solid media commonly used to culture mycobacterium is the Lowenstein-Jensen (L-J) medium though other media such as Ogawa egg yolk medium and Middlebrook 7H10 or 7Hll agar could as

well be used. Mycobacteria may synthesize carotenoid pigments which confer a yellow to red pigmentation to colonies. Carotenogenesis can be achieved in the absence of light by scotochromogenic mycobacteria. Photochromogenic mycobacteria require exposure to light and oxygen for carotenogenesis to occur (Levy –Frebault and Portaels, 1992).

The genus *Mycobacterium* comprises over 150 parasitic and free living acid-fast species of bacteria (Dai *et al*., 2011). Majority of the species are non- pathogenic environmental bacteria. However, only a few species are highly successful pathogens, and include *Mycobacterium tuberculosis* complex, *Mycobacterium leprae*, and *Mycobacterium ulcerans*, the causative agents of tuberculosis, leprosy, and Buruli ulcers, respectively.

2.1.1 Environmental Mycobacteria

Environmental mycobacteria also referred to as atypical mycobacteria or non tuberculous mycobacteria (NTM) are fascinating group of human, animal, and bird pathogens (Dawson, 2000). They have significant impacts on the morbidity and mortality of humans and have important economic impacts on agriculture. Environmental opportunistic mycobacteria are distinguished from the members of the *Mycobacterium tuberculosis* complex and *Mycobacterium leprae* by the fact that they are not obligate pathogens but are true inhabitants of the environment. They are normal inhabitants of a wide variety of environmental reservoirs, including natural and municipal water, soil, aerosols, protozoans, animals, and humans. They can be found as saprophytes, commensals and symbionts (Primm *et al*., 2004).

Environmental mycobacteria have extraordinary starvation survival (Smeulders *et al*., 1999), persisting despite low nutrient levels (Primm *et al*., 2004). Some are also tolerant to temperature extremes (Schulze-Robbecke and Buchholtz, 1992).

The most common environmental mycobacterial pathogens can be divided into two groups based on growth rate; the slowly growing species include *M. avium*, *M. intracellulare*, *M. kansasii*, *M. marinum*, *M. xenopi*, *M. malmoense* and *M. ulcerans* (Falkinham, 2009). Rapidly growing species include *M. abscessus*, *M. chelonae* and *M. fortuitum* (Falkinham, 2009). Pathogenic environmental mycobacteria are more likely transmitted from environmental sources to their host by ingestion, inhalation or through inoculation of mycobacterium bacilli. These environmental sources may include aerosols, water, soil, dust, food products and contaminated medical equipment (Gangadharam and Jenkins, 1998).

2.1.2 *Mycobacterium ulcerans*

Mycobacterium ulcerans is a slow-growing environmental mycobacterium and the causative agent of BU, a skin infection characterized by extensive ulceration with scarring. It is a strong acid fast rod, non-motile and non-sporing. It has limited range of distribution and is found in communities associated with rivers, swamps, wetlands, and human-linked changes in the aquatic environment, particularly those created as a result of environmental disturbance such as deforestation, dam construction, and agriculture (Merritt *et al*. 2010). Environmental reservoir of *M. ulcerans* is yet to be identified despite its linkage to aquatic habitats. Molecular epidemiological studies reveal clustering of *M. ulcerans* according to the geographical origin of isolates (Chemlal *et. al*., 2001).

Successful cultivation of *M. ulcerans* from primary cultures depends on several parameters, including cultivation conditions and decontamination methods (Palomino and Portaels, 1998). *M. ulcerans* grows best at temperatures of 30 to 33^{o}C, reduced oxygen tension (pO2< 2.5Kpa) and a pH range of 5.4 - 7.4 (Palomino *et al.*, 1998). Visible colonies are seen in culture within 6-8 weeks after inoculation producing yellow or cream colonies. Although cultivation of *M. ulcerans* from clinical specimens have successfully been documented, attempts to cultivate the mycobacterium from the environment have been greatly unsuccessful except for work by Portaels and colleagues in 2008 where they isolated *M. ulcerans* from aquatic Hemiptera on Lowenstein-Jensen medium (LJ) after several passages in mouse footpads (Portaels *et al*., 2008). Failure to cultivate this organism from nature may be attributable to conditions of transport, decontamination method and its scarcity in the environment.

2.1.2.1 Genome of *Mycobacterium ulcerans*

Whole genome sequence of *M. ulcerans* was determined by Stinear and colleagues from a clinical isolate called Agy99, isolated in 1999 from a Ghanaian patient (Stinear *et al*., 2007). The Agy99 genome comprises two circular replicons (Fig. 2.1), a chromosome of 5,631,606 bp with a 174,155 bp virulence plasmid referred to as pMUM001. The chromosome harbors 4,160 protein-coding genes, 771 pseudogenes, two prophages phiMU01 (18 kb, 18CDS) and phiMU02 (24 kb, 17CDS) as well as several copies of insertion sequences (IS) elements, IS*2404* (209 copies) and IS*2606* (83 copies) (Stinear *et al*., 2007). The virulence plasmid also harbors 81 protein coding DNA sequences, 4 copies of IS*2404* and 8 copies of IS*2606*. The G+C content of the chromosome (65%) is slightly higher than that of the plasmid which is 62.5%

(Stinear *et al*., 2007). IS*2404* sequences account for 6% of the genome of *M. ulcerans*. Insertion sequence elements are mobile genetic entities encoding transposases which catalyse DNA copying or movement. IS*2404* is 1,368 bp long, containing 41 bp perfect inverted repeats and producing 10 bp target-site duplications whereas IS*2606* is 1,438 bp long, with 31 bp imperfect inverted repeats and producing target site duplications of 7 bp (Stinear *et al*., 2005).

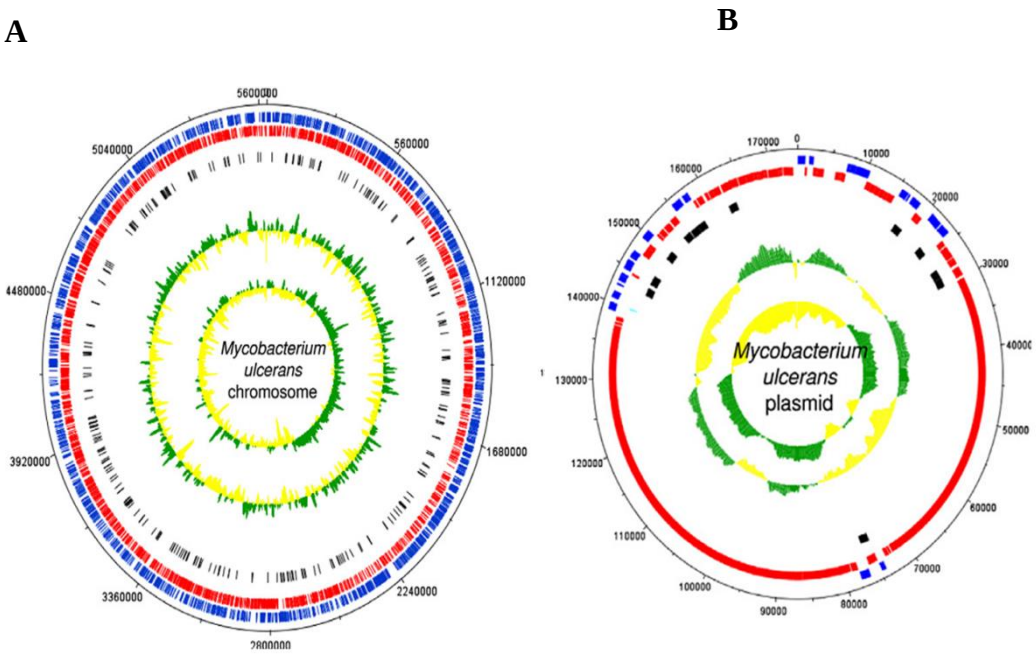


Figure 2.1 Circular representation of the M. ulcerans Agy99 replicons.

The outer black circle shows the scale in bases. The next two inner circles illustrate the forward and reverse strand CDS in blue and red, respectively. IS element regions are displayed in the next inner circle in black. The innermost circles show GC plot and GC skew. (A) 5632 kb chromosome (B) 174 kb virulence plasmid (Picture adapted from Röltgen *et al.*, 2012).

2.1.2.1.1 Plasmid pMUM001

Primary function of pMUM001 of *M. ulcerans* is seemingly for toxin production. It carries a cluster of genes encoding giant polyketide synthases (PKSs), and polyketide-modifying enzymes that are necessary for synthesis of the lipid toxin, mycolactone (Fig. 2.2). Polyketide synthase responsible for producing the mycolactone core structure are encoded by the genes *mlsAl* and *mlsA2* with respective sizes of 50,973

bp and 7,233 bp. Enzyme responsible for synthesizing side chain is encoded by *mlsB* gene having size of 42,393 bp. (Stinear *et al*., 2004). All three PKS genes are highly related, with nucleotide sequence similarity of 99.7%. The polyketide-modifying enzymes including a P450 monooxygenase, type III ketosynthases (KS) and type II thioesterase (TE) are encoded by the respective genes *mup053*, *mup045* and *mup037*. P450 monooxygenase is responsible for hydroxylation at carbon 12 of the side chain, KS catalyzes ester bond formation between the mycolactone core and side chain whereas TE is required for removal of short acyl chains from the PKS loading modules, arising by aberrant decarboxylation (Heathcote *et al*., 2001).

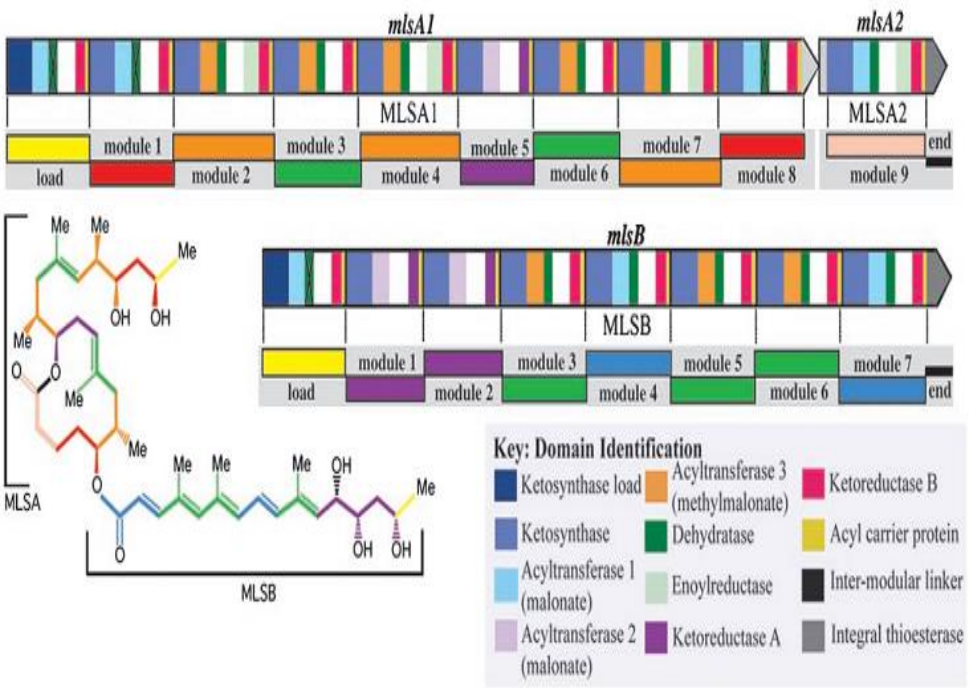


Figure 2.2 Domain and module organization of the mycolactone PKS genes.

Within each of the three genes (*mlsA1*, *mlsA2*, and *mlsB*), different domains are represented by a colored block. The domain designation is described in the key. White blocks represent interdomain regions of 100% identity. Module arrangements are depicted below each gene, and the modules are color-coded to indicate identity both in function and sequence (98%). The structure of mycolactone has also been color-coded to match the module responsible for a particular chain extension. (Picture adapted from Stinear *et al*., 2004).

2.1.2.2 Evolution of *Mycobacterium ulcerans*

Availability of the genome sequence of *M. ulcerans* strain Agy99 has afforded comparative mycobacterial genomics analysis which has provided insight to the evolutionary scenes of *M. ulcerans*. *M. ulcerans* and *M. marinum* are genetically related species that cause quite different human skin diseases.

M. ulcerans causes BU whereas *M. marinum* causes relatively minor granulomatous skin lesions, often referred to as "fish tank granulomas". *M. ulcerans* is not photochromogenic contrary to that of *M. marinum* which produces bright yellow pigments when exposed to light. They both seem to occupy the aquatic environments (Stinear *et al*., 2000b). In spite of apparent phenotypic difference, phylogenetic analysis has revealed a clear delineation between strains of *M. ulcerans* and *M. marinum* indicating that all *M. ulcerans* strains have evolved from a common *M. marinum* progenitor.

This evolutionary process is also reinforced by genomic comparison between the two species revealing sharing of more than 4,000 orthologous and syntenic protein-coding DNA sequences and having an average sequence identity of 98.3%. This genomic analysis has also revealed the loss of over 1.1Mb of DNA owing to deletions by that of *M. ulcerans* and acquisition of 168kb by *M. marinum* mainly in the form of prophages and chromosome rearrangements resulting in disruption of some genes facilitated at least in part by the high number IS*2404* and IS*2606*.

The fact that there is high mutual sequence identity between *M. ulcerans* and *M. marinum* suggests that *M. ulcerans* is probably derived from a *M. marinum* progenitor after acquisition of the plasmid through horizontal gene transfer and has evolved through reductive evolution, massive expansion of IS*2404* and IS*2606*, extensive

pseudogene formation, genome rearrangements and gene deletion. The divergence event has been estimated to have occurred between 470,000 and 1,200,000 years ago, as evidenced by the acquisition of the 174 kb virulence plasmid pMUM001 by *M. ulcerans* (Stinear *et al*., 2000b).

2.2 Buruli ulcer

The human necrotizing skin disease that results from infection with *M. ulcerans* is commonly known as BU. It takes its name from a series of cases described earlier in the Ugandan region of Buruli, near the river Nile. BU is one of the 17 neglected tropical diseases identified by the WHO and is the second most prevalent mycobacteriosis in Ghana after tuberculosis and third globally, after tuberculosis and leprosy (Asiedu *et al*., 2000). This atypical mycobacteriosis has been reported in at least 33 countries worldwide and remains an emerging infection primarily in riverine rural regions of West and Central Africa where it is recognized as a serious health problem (Reynaud *et al*., 2015).

Non-endemic areas such as North America and Europe have reported cases of BU as a result of international travel (Semret *et al*., 1999; Ezzedine *et al*., 2009). BU can affect all age group although in West Africa, children are disproportionately affected with a peak age group of 5 to 15 years (Debacker *et al*., 2004). There is no important difference among sex group susceptible for BU infection (WHO, 2015) although Barker (1973) reported prevalence to be higher among women than men and among boys than girls. Fatality associated with the disease is low compared to its high morbidity.

In 1998, the World Health Organization (WHO) established the Global Buruli Ulcer Initiative to focus on prevention, awareness, and improving treatment options for

those suffering from this disease (WHO, 1998). To date, public health effort toward BU control has been undermined by poor understanding of how humans acquire *M. ulcerans* from the environment which still remains an enigma in BU research (Merritt *et al*., 2010).

2.2.1 Diagnosis of Buruli ulcer

BU is diagnosed on the basis of clinical examination of lesions on suspected patients as well as laboratory testing of clinical samples. Specimens obtained by swabs are taken from the undermined edges of ulcers. Fine-needle aspiration (FNA) is mainly used to obtain samples from clinically-diagnosed non-ulcerative lesions (nodule, plaque and oedema). Punch biopsy is preferable when larger diagnostic specimens are required for histopthological analyses. Four laboratory tests are recommended for the diagnosis of BU. These include microscopic examination, culture, histopathology and PCR (Fig. 2.3), all having varied levels of specificity and sensitivity (Herbinger *et al*., 2009). Microscopic examination is the most rapid and least expensive method of diagnostic that detects acid-fast bacilli (AFB) in smears after employing staining techniques such as the Zeihl Neelson (ZN). The method has low sensitivity (29-78%) (Ablordey *et al*., 2012). Histopathologic features for the diagnosis of BU include necrosis of subcutaneous tissues and dermal collagen accompanied by minimal inflammation and presence of AFB (Guarner *et al.*, 2003). Cultivation takes approximately 6-8 weeks to see visible yellow to cream colonies of *M. ulcerans* in pure cultures. Owing to the long-time taken to obtain culture results, this method is not considered as an alternative to rapidly confirm BU. The most commonly used target sequence in PCR assays for molecular detection of *M. ulcerans* is IS*2404*

(Stinear *et al*., 1999). Both real time PCR and conventional PCR targeting the IS*2404* sequence have been used for prompt and accurate diagnosis of *M. ulcerans* infection.

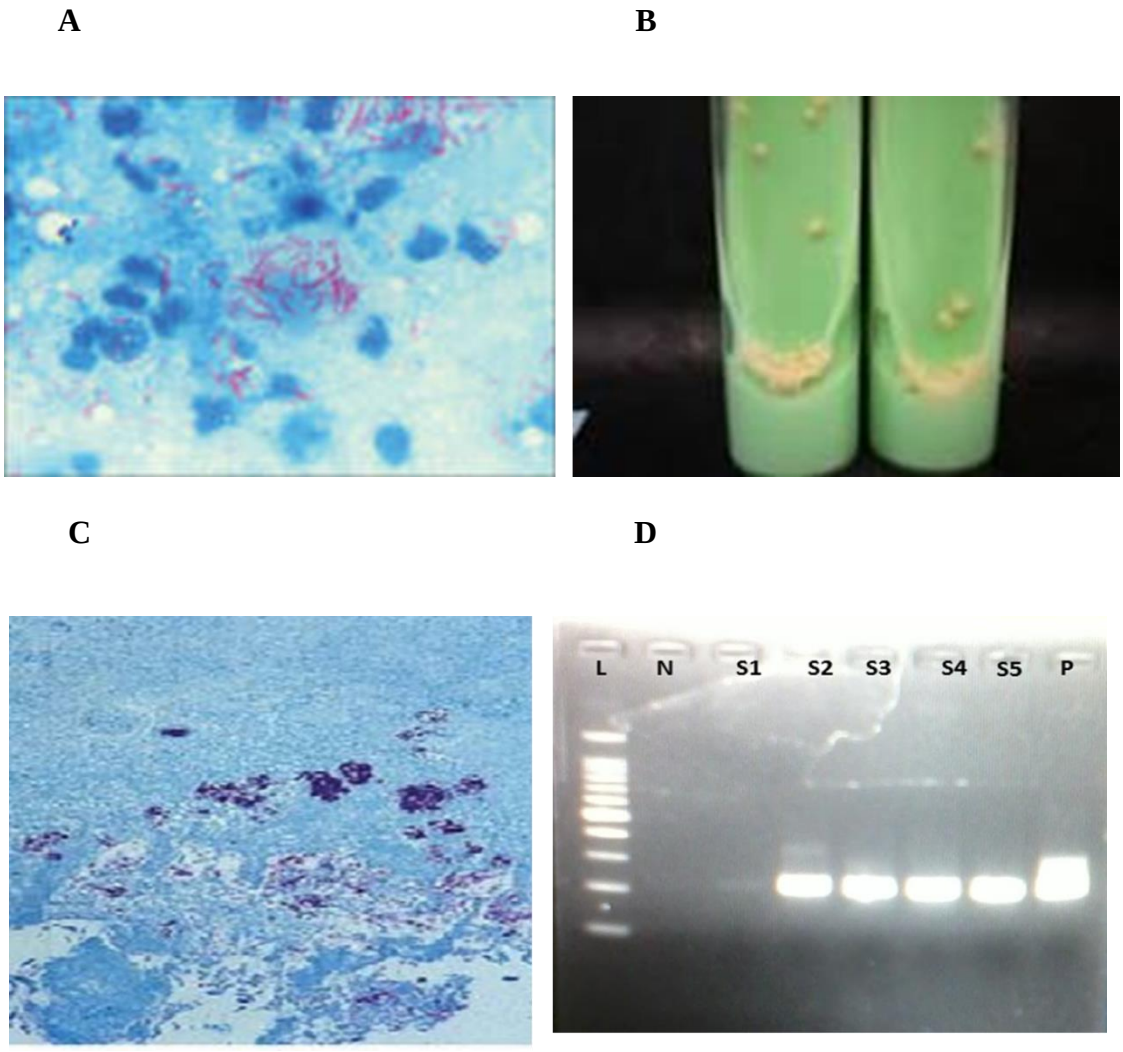


Figure 2.3 Laboratory tests for Buruli ulcer diagnosis.

(A) Ziehl -Neelsen staining technique showing acid fast bacilli (AFB) of *M .ulcerans* (B) Yellow colonies of *M. ulcerans* cultivated on Lowenstein-Jensen (LJ) media (C) clumps of AFB in necrotic tissues in histological analysis (D) Gel picture showing the amplified products of IS*2404* PCR, L = molecular weight marker, N= negative PCR control, S1-S5 = are clinical samples ; S1 was negative for *M . ulcerans* DNA, S2-S5 were positive for *M . ulcerans* as depicted by the band sizes (approximately 200bp) , P = PCR positive control. (Picture (C) adapted from Guarner *et al*., 2003).

2.2.2 Treatment of Buruli ulcer

Before 2004, antibiotics were believed to be ineffective for the treatment of BU as surgical excision and repair was the main regimen. Challenges associated with the use of this regimen such as the high recurrence rates (Kibadi *et al.*, 2009), high cost as

well as difficulties in accessing treatment especially in endemic rural areas and also the significant cosmetic morbidity, paved way for the use of antibiotics for treatment of BU, after empirical evidence from animal and human studies.

The latest WHO recommendations are eight weeks combination antibiotic therapy of intra-muscular streptomycin 15 mg/kg and oral rifampicin 10 mg/kg. Surgical intervention is recommended only to hasten healing of more extensive ulcers if antibiotics are contraindicated or not tolerated, or at a patient's request. In addition, if surgery is required, an initial four weeks of antibiotics prior to surgery is recommended (Cowan *et al*., 2015).

The efficacy of rifampicin combined with streptomycin in humans was first established by a small case series of patients with early lesions in Ghana. Since its introduction, there have been little reports on its side effects though attempts are geared toward the use of all-oral regimens to avoid the toxicity associated with the use of aminoglycoside such as streptomycin. Published and observational data have confirmed the effectiveness of all oral regimens of rifampicin-based drug combinations with a second oral agent such as clarithromycin, moxifloxacin or ciprofloxacin (O'Brien *et al*., 2007). Report indicates that only few patients after treatment with antibiotic develop worsening appearance of their lesion referred to as paradoxical reaction or an immune reconstitution inflammatory reaction. This presents as deterioration in the clinical appearance of the lesion after initial improvement, with increasing induration, pain, wound discharge and occasionally new ulceration (O'Brien *et al*., 2013).

Aside the use of antibiotics for the treatment of BU, Bertolotti and colleagues reported for the first time use of ozone therapy for the treatment of BU. This could provide a cheaper and simple alternative for the management of BU (Bertolotti *et al*., 2013).

2.2.3 Pathogenesis of Buruli ulcer

M. ulcerans is considered an intracellular parasite despite the predominance of free bacilli in necrotic acellular areas of tissues from BU lesions (Torrado *et al*., 2007). This postulate is based on the fact that *M. ulcerans* infections are associated with cell mediated immunity (CMI) and delayed-type hypersensitivity (DTH) responses characteristic of an intracellular parasite. Its cycle within host cell encompasses phases of intra-macrophage and extracellular multiplication (Silva *et al*., 2009).

After transmission of the bacterium into the subcutaneous fat of exposed parts of the body, there is approximately 4 to 10 weeks of dormant phase followed by progressive coagulative necrosis of the dermis and subcutis resulting in the varied forms of BU lesions (Sizaire *et al*., 2006). Extensive necrosis is the hallmark of BU histopathology and has been associated with the high toxigenicity of the exotoxin, mycolactone, secreted by *M. ulcerans.* Other features relevant for *M. ulcerans* pathogenicity are the low optimum growth temperature that makes the skin its almost exclusive territory and its slow growth rate that translates into slowly progressing lesions (Silva *et al*., 2009).

2.2.3.1 Mycolactone

Mycolactone is a polyketide macrolide lipid-like secondary metabolite synthesized by *M. ulcerans* (Hall and Simmond, 2014) as well as other mycolactone producing mycobacteria (MPM). It is a highly diffusible heat-stable exotoxin that causes extensive, chronic, and necrotizing damage to the papillary dermis, subcutaneous fat,

bones and muscles (Boleira *et al*., 2010). Mycolactone is the only virulence factor identified for *M. ulcerans* to date. It consists of a 12-membered lactone core and two polyketide side chains produced by enzymes encoded by the giant 174 kb plasmid, pMUM (Stinear *et al*., 2004) present in all MPM.

Strains of *M. ulcerans* produce a range of mycolactones, each containing the lactone ring and varied length of the side chain, location of the hydroxy groups and double bonds within the side chain. Variation in the structure of polyketide side chains is responsible for differences in virulence among the family of mycolactones. At least five structurally distinct mycolactones are produced by the different strains of *M. ulcerans* and that of the close relatives from fish and frogs (Fig. 2.4). These are mycolactone A/B, C, D, E and F. Strains of *M. ulcerans* from Africa, Malaysia and Japan produce mycolactone A/B, Australian strains produce mycolactone C, Chinese strains produce mycolactone D and *Mycobacterium liflandii* and *Mycobacterium Pseudoshottsii* produce the unique mycolactones E and F (Hong *et al*., 2008). The alphabetical grading denotes the potency of the mycolactone thus mycolactone A/B is the most potent and mycolactone F is the least potent (Hong *et al*., 2007).

Mycolactone is believed to play a central role in determining the extracellular localization of the bacteria and modulation of immunological responses to *M. ulcerans* (Adusumilli *et al.,* 2005). Purified mycolactone has cytotoxic, apoptotic, and immunosuppressive properties. These unique properties of mycolactone appear to be of crucial importance to the pathogenesis of the disease (Mve-Obiang *et al*., 2003).

Studies using L929 murine fibroblasts revealed that mycolactone induced cell-cycle arrest at the G1/G0-phase, cytoskeletal rearrangements and the cells became apoptotic (George *et al*., 1999). Susceptibilities of cells to mycolactone appear to differ.

Whereas immature DCs (dendritic cells) are relatively more sensitive to mycolactone after 48 h, (Coutanceau *et al*., 2007) purified human monocytes show no significant reduction in viability after 24 h (Simmonds *et al*., 2009).

The mechanism underlining mycolactone induced cell death has recently been elucidated by Guenin-Mace et al. (2013). Their studies revealed how low doses of mycolactone cause cells to rapidly form filopodia, clusters of actin bundles associated with wound healing and cell–cell interactions. These resulted from mycolactone-dependent enhancement of actin polymerization following its binding to the GTPase domain of the WASP (Wiskott–Aldrich syndrome protein) family of proteins. Activation of WASP and relocalization of the actin-nucleating complex Arp2/3 (actin-related protein 2/3) are believed to explain the cytoskeletal rearrangement in mycolactone-treated cells. These were associated with a loss of cell adhesion and E-cadherin (epithelial cadherin) thus *in vitro*, cell death is related to anoikis, an apoptosis owing to detachment.

Mycolactone can suppress both innate and adaptive immune responses in patients and in experimentally infected mice. Mycolactone suppresses the innate responses by restricting the functions of resident tissue macrophages. At the early stage of *M. ulcerans* infections, *M. ulcerans* are phagocytosed by macrophages (Coutanceau *et al*., 2005) and presumably escaping digestion by preventing phagolysosome formation. Over short time periods it proliferates inside macrophages until mycolactone causes the apoptosis/necrosis of the macrophages and the release of the bacilli into the intercellular space (Torrado *et al*., 2007).

A key event that normally occurs upon phagocytosis of a microbe is the activation by PAMPs (pathogen-associated molecular patterns) resulting in intracellular signalling

cascades that end in a rapid, profound and controlled inflammatory response, including the production of cytokines and chemokines to recruit other inflammatory cells to the site of infection. These have been shown to be interfered by mycolactone. Failure to produce cytokines and chemokines at the site of infection is considered a probable explanation for the lack of an inflammatory infiltrate close to bacilli, with the peripheral infiltrate indicating the limit of biologically active doses of mycolactone (Silva *et al*., 2009).

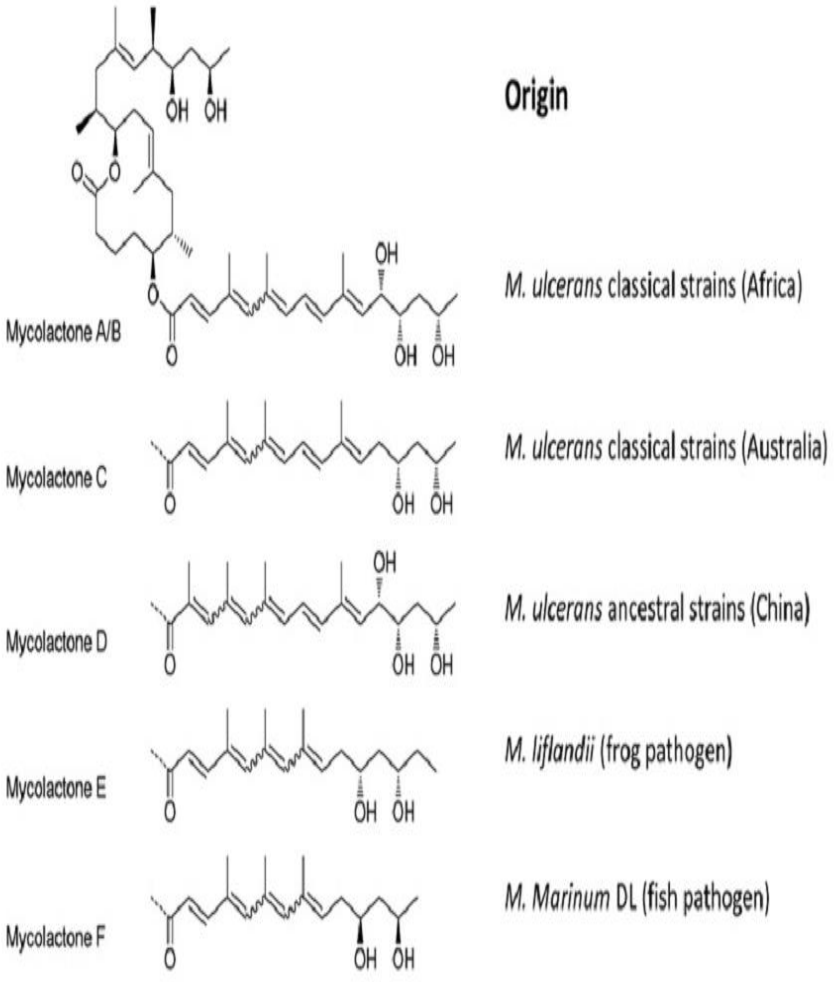


Figure 2.4 Structures of naturally occurring mycolactones and their geographical origin.

The complete structure of mycolactone A/B is shown, which exists as 3:2 ratio of Z-/E- isomers of the C-4 – C-5 bond in the long polyketide side chains. Variation in the structure of polyketide side chains responsible for structural differences in the family of mycolactone is shown for mycolactone C- F. The species of mycobacterium secreting the mycolactone as well as it origin indicated. (Figure adapted from Hall and Simmonds, 2014)

2.2.4 History of Buruli ulcer

First description of cutaneous ulcers probably caused by *M. ulcerans* was in 1897 by Sir Albert Cook, in Uganda but it was in 1948 when MacCallum and his colleagues isolated *M. ulcerans* from some patients in Australia. The most common name for the *M. ulcerans* infection originated from Buruli district near Lake Kyoga in Uganda (now called Nakasongola District) after the district recorded high cases in 1950. Depending on the geographical origin, the name of the infection could vary. For instance in southern Australia, it is still called Bairnsdale ulcer following the detection of the disease from Bairnsdale area near Melbourne by a team lead by Professor Peter MacCallum.

BU has emerged rapidly to other parts of the world and in the last decade, there have been consistent and increased cases of *M. ulcreans* infection particularly in West Africa (Amofah *et al.*, 2002). The disease has been recorded in at least 33 countries spanning across the globe from tropical and subtropical regions of Asia, Latin America, Western Pacific region, Africa as well as temperate region in southern Australia **(**Merritt *et al*., 2010; Walsh *et al*., 2008; Duker *et al*., 2006; Horsburgh and Meyers, 1997).

2.2.5 Epidemiology of Buruli ulcer

Epidemiological pattern for establishment of *M. ulcerans* infection is determined by the presence or absence of Buruli ulcer foci (Pouillot *et al.*, 2007**).** BU occurs in discrete foci in endemic areas and unevenly distributed within an endemic country. BU endemic areas are usually near human disturbed aquatic habitats (Johnson *et al*., 2005) and rare in drier and savanna regions (Merritt *et al*., 2010). Increased incidences have been reported to be linked to deforestation practices, construction of

agricultural irrigation systems, resettlement and migration closer to water bodies, unprecedented flooding of lakes and rivers during heavy rainfall (Merritt *et al*., 2010). Globally, the burden of BU is increasing and WHO estimates an annual case confirmation of 6000 from at least 15 of the 33 countries affected with BU (WHO, 2015). Actively reporting countries for BU are Benin, Cameroon, Côte d'Ivoire, Congo, Democratic Republic of the Congo, Gabon, Ghana, Guinea, Liberia, Nigeria, Sierra Leone, Togo (all in Africa) , French Guiana (Americas), Australia , Japan, and Papua New Guinea (in Western Pacific) (WHO, 2015).

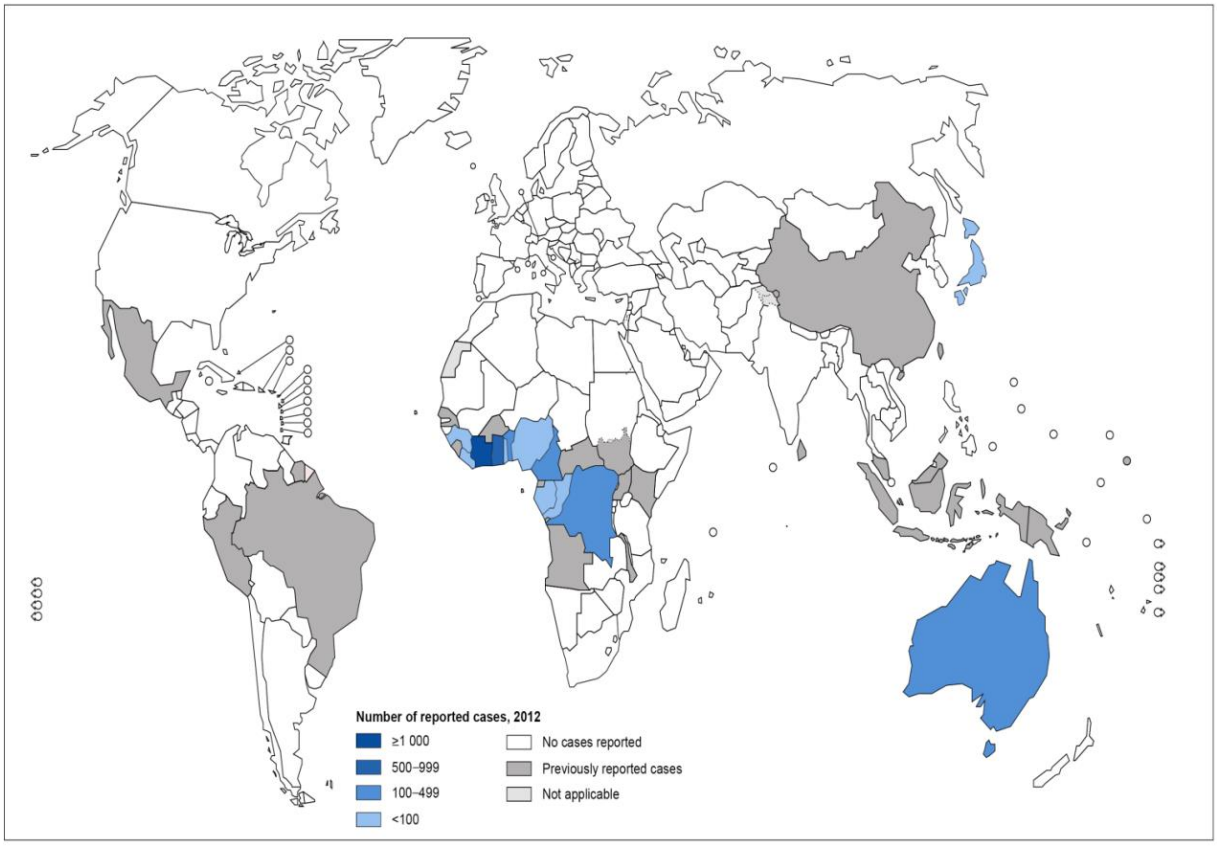


Figure 2.5 Global distribution of Buruli ulcer cases reported in 2012.
Picture adapted from WHO, 2013 control of neglected tropical disease.

2.2.5.1 Buruli ulcer Prevalence in Ghana

The first probable case of Buruli ulcer in Ghana was in 1971 recorded in the Greater Accra region in a child from Nsawam in the Eastern region. Within the same year some probable cases were also identified along the tributaries of the Densu River (Bayley, 1971). van der Werf and colleagues in 1989, also described the disease in some patients in the Asante Akim North district in the Ashanti region (van der Werf *et al*., 1989) and later on in the same region, a major endemic foci was described in Amansie West district by Amofah *et al*. (1993). Since then isolated cases have been found in scattered communities in many parts of the country warranting the need for a national surveillance study.

National case search conducted in 1999 revealed that there were about 5,600 people with suspected BU lesions at various stages of development (Amofah *et al*., 2002) and cases were distributed across all the ten regions with Central region having the highest prevalence followed by the Ashanti region. The overall national prevalence then was estimated to be 20.7/100,000 and Amansie West district had the highest prevalence with 150.8/100,000. This data made BU the second most common mycobacteriosis in Ghana after tuberculosis (Amofah *et al*., 2002). Kenu and colleagues recently reported that over 426 communities mainly in the Ashanti, Brong -Ahafo, Eastern, Greater Accra and Western region are endemic for BU (Kenu *et al*., 2014). Ghana currently reports an average of 1000 cases annually (Ackumey *et al*., 2011). Akwapim South district of the Eastern region of Ghana at the moment is the hottest BU spot in Ghana with a prevalence rate of 151.4 per 100,000 populations (Kenu *et al*., 2014).

2.2.6 Transmission of Buruli ulcer

In contrast to tuberculosis where definite mode of transmission is ascertained same cannot be said for BU. The former is characterized by person-to-person transmission while the latter hypothesized to be acquired through environmental exposure. Exposure to *M. ulcerans* is thought to occur from a yet unknown, but persistent, environmental niche. Molecular studies have detected *M. ulcerans* DNA in water, detritus (Stinear *et al*., 2000a; Ross *et al*., 1997), aquatic insects (Portaels *et al*., 1999), plants (Marsollier *et al*., 2004a), snails (Marsollier *et al*., 2004b) and small fish (Eddyani *et al*., 2004). Recently Portaels and colleagues described isolation of *M. ulcerans* in pure culture from an insect which further sheds more light on *M. ulcerans* as an environmental pathogen (Portaels *et al*., 2008). However, regardless of identifying *M. ulcerans* DNA from these environmental factors, exactly how *M. ulcerans* gets into human host still remains elusive. This has led to generation of hypotheses explaining how this could occur.

2.2.6.1 Role of insects in transmission of *M. ulcerans*

Portaels and her colleagues were first to propose the role of insecst as vectors in transmission of *M. ulcerans*. They used PCR detection of the insertion sequence IS*2404* to document *M. ulcerans* association with predaceous insects (Naucoridae and Belostomatidae) and led to the hypothesis that insects may be involved in the transmission of *M. ulcerans* (Portaels *et al*., 1999).

Naucorids and belostomatids are aquatic hemiptera that exploit a wide range of prey, including snails, fish, anuran larvae, and other terrestrial and aquatic insects (Swart *et al*., 2006). They do not feed on humans but can bite if they are disturbed. This hypothesis links the occurrence of *M. ulcernas* infection to an aquatic niche as it

maintains that *M. ulcerans* found in biofilms of aquatic habitats are first concentrated by grazing or filter-feeding invertebrates which are then consumed by predators known to bite humans. Marsollier and colleagues later reinforced this hypothesis on the basis that in an experimental setting, the salivary glands of *Naucoris cimicoides* were colonised with *M. ulcerans* and transmitted it to laboratory mice (Marsollier *et al*., 2005). Thus through biting the infected *Naucoris cimicoides* inoculated the bacilli that had accumulated in their salivary glands to the mice (Marsollier *et al*., 2005).

The quest to isolate *M. ulcerans* from the environment after nearly six decades had a major breakthrough when Portaels and colleagues isolated *M. ulcerans* from water strider an aquatic Hemiptera collected in Benin (Portaels *et al*., 2008). This further strengthens the range of hypothetical hemipteran transmitters of *M. ulcerans*. None of the predaceous hempitera implicated in transmission of *M. ulcerans* in Africa are hematophagous and as suggested by Portaels and colleagues they may be only passive, incidental and transient reservoirs of *M. ulcerans* without an obvious role in the transmission of BU to humans (Portaels *et al*., 2008).

Mosquitoes are also suspected to participate in transmission of BU to humans in Australia after an outbreak of the disease coincided with the detection of *M. ulcerans* DNA in mosquitoes (Johnson *et al*., 2007). Detection of PCR positive *M. ulcerans* DNA in mosquitoes does not link mosquitoes to transmission but probably an indicator of cohabitation of both organisms resulting in surface contamination of mosquitoes with *M. ulcerans*.

2.2.6.2 Transmission of *M. ulcerans* upon exposure to contaminated environment

There are two basic hypotheses about the portal of *M. ulcerans* into human body (Duker *et al*., 2006). First when the skin is traumatized it provides a site for introduction of *M. ulcerans* into the body. Second is through existing abrasion or

laceration (Jacobsen and Padgett, 2010). When susceptible body parts come into contact with contaminated water, soil, aquatic plants or biofilm on aquatic plants where traces of *M. uclerans* DNA have been documented, direct transmission of *M. ulcerans* into the skin from the overlying *M. ulcerans* contaminated surface could occur following entry of the pathogen through the described portals above (Portaels *et al*., 2001).

The likelihood of *M. ulcerans* transmission through direct inoculation in abrasion was recently shown to be a less favourable route of entry. Studies by Williamson and colleagues revealed how BU failed to develop after inoculation of *M. ulcerans* in abrasions on skin surfaces of guinea pigs. Rather ulcers were present at intra- dermal injection sites in all infected mice and lent support to the hypothesis that *M. ulcerans* infection occurs through injection of bacteria rather than through entrance of pre-existing, superficial skin abrasion (Williamson *et al*., 2014). Their work further strengthened what Debacker and colleagues described on how through slight trauma as in the case of hypodermic injection or severe trauma for example, mine wound, snake or human bites, *M. ulcerans* could be introduced from an overlying contaminated skin surface into the human host (Debacker *et al.*, 2003).

2.2.7 Risk factors associated with Buruli ulcer

Most case control and cross sectional studies have determined an association between age and the occurrence of BU. Children and adolescents have higher infection rate than adult. Specifically children less than 15 years are severely affected which have drawn the attention of some researchers to investigate the behaviour of this age group to possibly ascertain the transmission mode (Jacobsen and Padgett, 2010; Sopoh *et al*., 2007; Debacker *et al*., 2006 ; Phanzu *et al*., 2006).

Comparative studies that evaluated sex as a risk factor in all cases reported no association between sex and Buruli ulcer (Pouillot *et al*., 2007; Quek *et al*., 2007; Debacker *et al*. 2006; Raghunathan *et al*., 2005; Stienstra *et al*., 2004).

Epidemiological evidence mostly links the occurrence of BU to swampy and riverine environment. Infection with *M. ulcerans* has not been found to be associated with the source of water used for cooking or bathing in endemic communities (Aiga *et al*., 2004). Some studies have found an increased risk of infection associated with wading and washing in water (Raghunathan *et al*., 2005; Kenu *et al*., 2014). While other studies found no association between BU and swimming, Aiga and colleagues reported such an association (Aiga *et al*., 2004). Wearing of certain types of clothing has been evaluated extensively. Wearing long legged trousers (long pants) has been found to reduce the risk of Buruli ulcer (Pouillot *et al*., 2007; Raghunathan *et al*., 2005) but not wearing protective clothing increases the risk of infection (Pouillot *et al*., 2007; Quek *et al*., 2007; Raghunathan *et al*., 2005; Marston *et al*., 1995). Critical evaluation and examination of these potential risk factors for BU as spelt out in comparative epidemiological studies for the presence of *M. ulcerans* could likely lead to a better understanding of *M. ulcerans* transmission.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

Difco™ Middlebrook 7H9 broth (Becton, Dickinson and Company, USA), 4mm diameter glass beads (Fisher Scientific UK limited), Heat treated Diatomaceous Earth (Supelco Park, Belleforte, PA, USA), Power soil DNA isolation kit (MO BIO Laboratories, Inc), TaqMan Universal PCR Master Mix (Applied Biosystem), Primers for real time PCR (Metabion), Probes (Applied Biosystem)

3.1.1Instrument

Rotor Gene Q real time PCR machine (Qiagen), MP Fast Prep- 24™ bench top homogenizer (MP Biomedicals), Microfuge 22R Centrifuge (Beckman Coulter), Vortex – Genie® 2 (Scientific Industries, Inc.), Incubator (Ikemoto Rikakogyo Companny Limited, Tokyo, Japan)

3.2 Methods

3.2.1 Location and demography of study areas

The study was conducted in eight selected communities within the districts of Akwapim South, Akwapim North and Nsawam-Adoagyiri Municipalities of Eastern region of Ghana. It comprised of four BU endemic communities which were Pakro, Oparekrom, Mangoase and Dago. The remaining four non- endemic communities were Boahenakrom, Obosono, Obotweri and Okomfo. Definition for endemic or non-endemic community used in the study was a community with or without reported case of BU within the community for the last three years. These communities were

selected based on their prevalence data obtained from the BU register available at the BU clinic of the Pakro Health Centre.

3.2.2 Nsawam-Adoagyiri Municipality

Nsawam-Adoagyiri municipality is a peri-urban area approximately 23km from Accra the national capital and situated in the south eastern part of the Eastern region between latitude 5.45^{o} N and 5.58^{o} N and longitude 0.07^{o} W and 0.27^{o} W. It is bordered to the south by the Ga and Tema municipalities of the Greater Accra region, to the north by Akwapim North municipality, to the west by Suhum municipality and Upper West Akim District. Nsawam is the municipality capital and administratively the municipality is divided into four zonal councils namely Nsawam, Adogyiri, Nkyenenkyene and Fotobi zonal councils. The municipality has twenty-six electoral areas and one constituency. From the 2010 census, the municipality has a population of 86,000 comprising of 49.7% males and 50.3% females. The major ethnic group found in Naswam Adogyiri municipality is the Akan and the municipality is considered as an endemic area for BU disease.

3.2.2.1 Oparekrom

Oparekrom is a town located within Nsawam-Adoagyiri municipality. It falls under Gyankrom sub-municipal and have an estimated population of 5,482. The main occupations of the people are farming and trading. It lies in the Densu river basin. Water from this source is used for domestic chores such as washing and bathing. The community is endemic with BU. The Pakro health centre has recorded four confirmed BU cases from this town between 2012 to 2014.

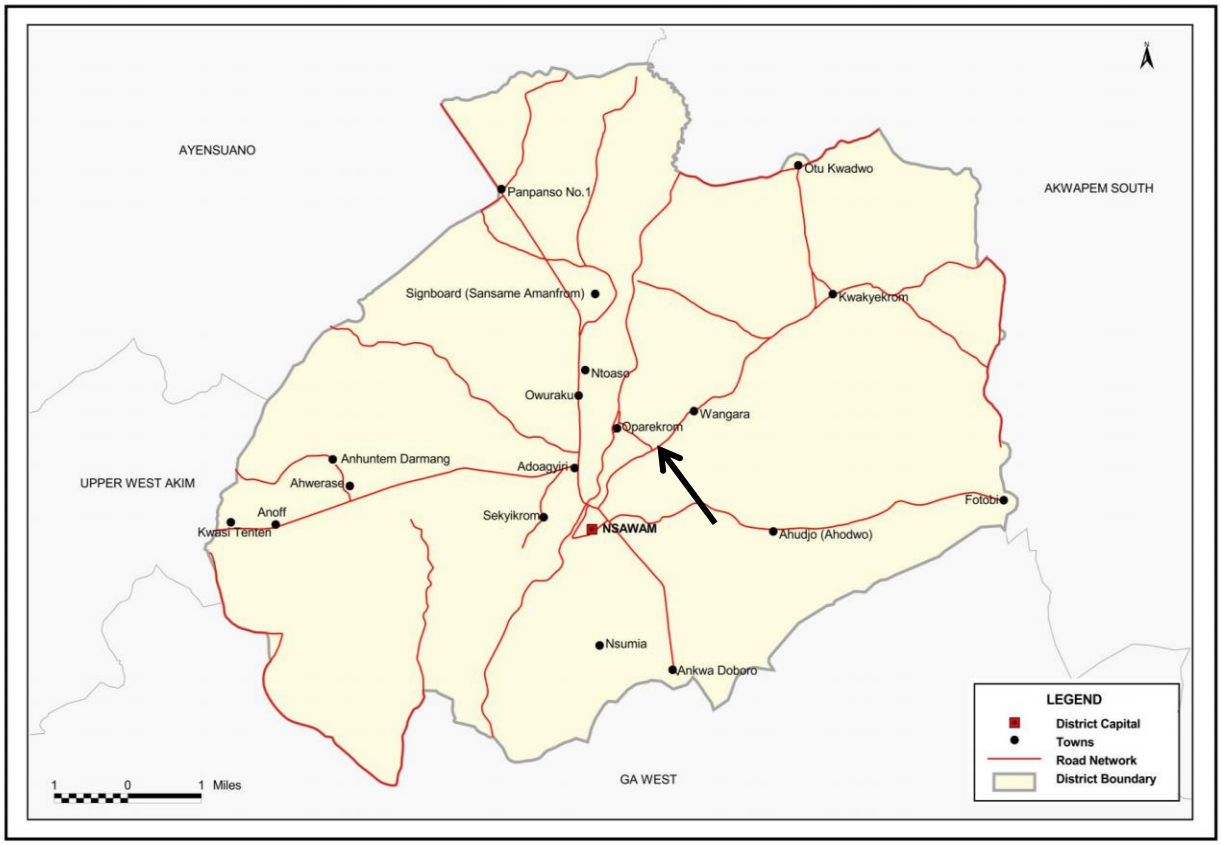


Figure 3.1 District map of Nsawam-Adoagyiri Municipality.

Black arrow indicating the position of Oparekrom. Picture adapted from 2010 population and house census for Nsawam-Adoagyiri Municipality.

3.2.3 Akwapim South Municipality

The Akwapim South District is one of the recently created districts in the Eastern region of Ghana. It was carved out from the old Akwapim South municipality. The district is located at the south eastern part of the Eastern region of Ghana between latitudes 5.45^{o} N and 5.58^{o} N, and Longitudes 0.0^{o} W and covers a land area of about 224.13 kilometres square. It lies in the Densu River basin. It is bordered to the west by the Nsawam-Adoagyiri municipality, to the south-east by the Kpone-Katamanso District, to the south by the Ga East District and to the North-East by the Akwapim North municipality. The district has Aburi as the capital. The population of Akwapim South District, according to the 2010 Population and Housing Census, is 37,501

representing 1.4% of the region's total population. Males constitute 48.5% and females represent 51.5%. Almost 73.4% of the District's population lives in the rural areas and more than one third of the labour force are employed in the agricultural sector. The district is predominantly made up of Akwapims, who are part of the Akan ethnic group. There are other ethnic groups who have migrated to settle in the district and these include Ewes, Gas and Hausas. The district is considered most endemic for BU in the region and currently the district is the hottest Buruli ulcer spot in Ghana with prevalence rate of 151.4 per 100,000 populations (Kenu *et al.*, 2014).

3.2.3.1 Pakro

Pakro is a town within Akwapim South municipal district with an estimated population of 4,376. The main occupation is farming. It lies in the Densu river basin where residents depend on the river for some domestic activities like washing and bathing. Pakro is known to be endemic for BU within the Akwapim South municipal district. The community has recorded 15 confirmed cases of BU out of 25 suspected cases recorded at the Pakro health centre from 2013 – May 2015.

3.2.3.2 Dago

Dago is a community within the Akwapim South municipal district having an estimated population of 1,421. The inhabitants are mainly engaged in farming. As revealed by the records from Pakro Health centre, the community has started recording isolated case of BU. From January to May 2015, 3 cases of BU have been confirmed from the community out of 4 suspected cases reported at the centre.

3.2.3.3 Obotweri

Obotweri is within Akwapim South municipal district having an estimated population of 1,241. Farming is the main occupation of the residents. The community has not

recorded any BU case from 2012 to May 2015 as revealed by the BU records from Pakro health centre.

3.2.3.4 Obosono

Obosono falls under Pakro sub- district within the Akwapim South municipal. Estimated population of the community is 1,527. Residents are mostly engaged in farming as the main occupation. The community has also not recorded any BU case from 2012 to May 2015 as revealed by the BU records from Pakro health centre.

3.2.3.5 Okomfo

Okomfo is also under Pakro sub –district having an estimated population of 983. The main occupation of the natives is farming. The community is known to be non-endemic for BU

3.2.3.5 Boahenakrom

Boahenkrom is a community within Akwapim South municipal and has an estimated population of 587. Residents within this community are mainly engaged in farming. The community is also known to be non- endemic for BU.

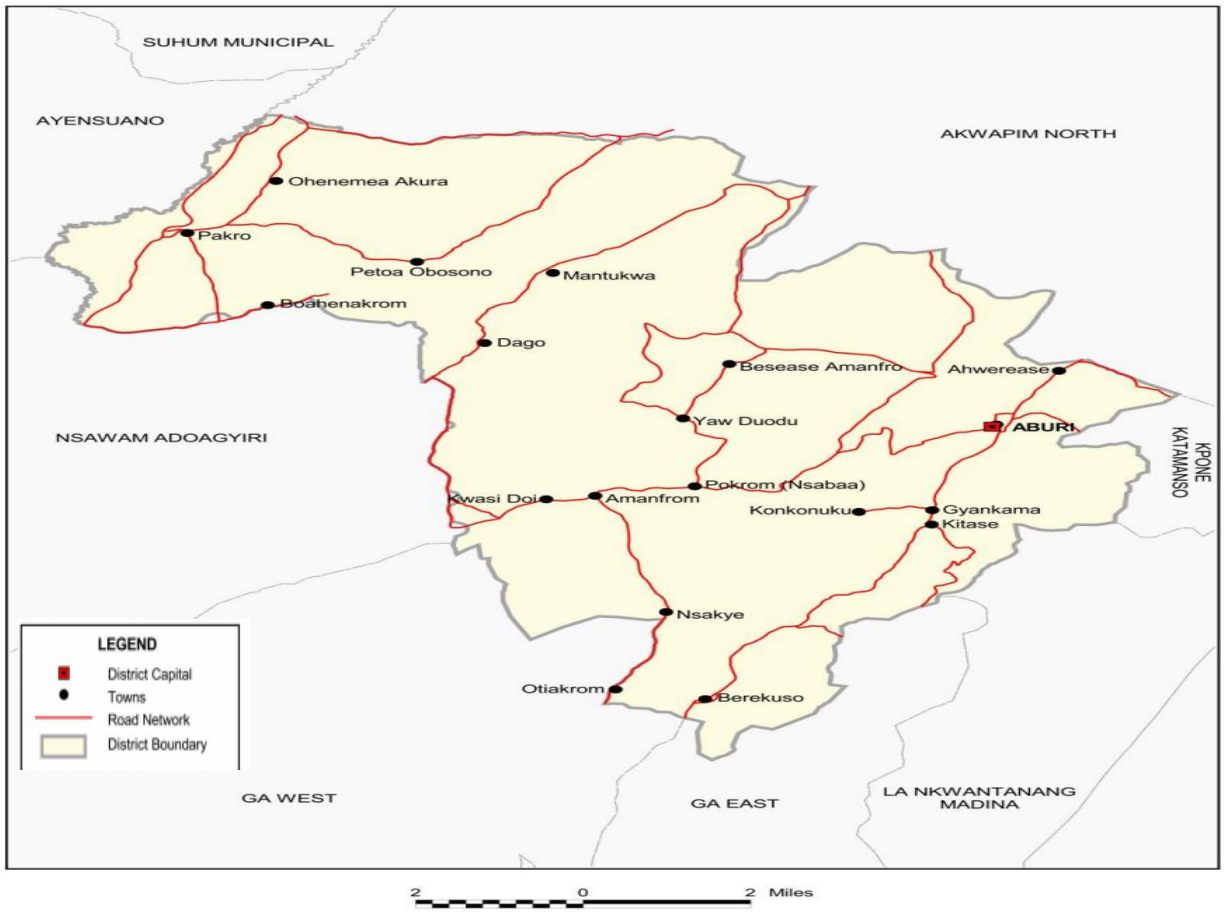


Figure 3.2 District map of Akwapim South Municipality.

Picture adapted from 2010 population and house census for Akwapim South district

3.2.4 Akwapim North Municipality

Akwapim North District is located in the south - eastern part of the Eastern region and is about 58km from Accra. The district covers a land area of about 450km^2, representing 2.3% of the total area of Eastern region. It has an estimated population of 122,063 and 3.1% growth rate. The district comprises nineteen towns, with Akropong as the district capital. According to the Eastern regional Health Directorate report in 2011, the district recorded isolated cases of BU in that year under review.

3.2.4.1 Mangoase

Mangoase is a town within Akwapim North municipality with an estimated population of 8,465. Farming and trading are the main occupations engaged by the residents. It lies within the Densu river basin. In 2014, the town recorded 7 cases of BU as disclosed by the records at the Pakro health centre.

3.3 Study Design

The study's design was a cross sectional study conducted from December 2014 – July 2015.

3.3.1 Study population

The targeted population of residents within the eight communities for the study were people engaged in specific risk activities such as farming without protective clothing, children playing with the soil, wading, swimming, or fetching water from a slow flowing water body or stagnant water in pursuing their normal activities that might result in contamination of their skin surfaces with *M. ulcerans*. Other activities like the usage of mud by individuals for construction, toddlers crawling in soil or individuals with visible soil contamination on their skin were also considered. All individuals who met the study' eligibility criteria were recruited after they consented.

3.3.2 Exclusion Criteria

Individuals diagnosed of BU by WHO guidelines as well as individuals who at the time of sampling did not appear to be engaged in any of the above mentioned specific risk activities.

3.3.3 Sample size

A total of 50 participants were recruited from each of the eight communities making a total of 400 participants.

3.4 Organization of Field work

3.4.1 Community Entry

The first point of call at the study communities were the premises of community elders, assembly men or the opinion leaders where meetings were held to explain the purpose, the scope, eligibility and exclusion criteria of the study to them.

3.4.2 Field Operations

A team comprising the Principal investigator (PI), a disease control officer from Pakro Health Centre and a community volunteer visited the eight communities to recruit, and sample participants.

3.4.3 Data and Sample Collections

A structured questionnaire (Appendix 2) was designed by the PI containing questions to solicit answers about the participant's demographics and the type of risk activity engaged with. Types of risk activities were classified into two groups based on the outlined risk factors for BU revealed by some case control studies. These groups were classified as either contact with slow flowing water body or having direct contact with the soil. Participants who were swimming, fetching or wading through slow flowing water bodies in the respective communities were captured under the first group and the activity engaged with indicated. In the other group, participants who were playing or crawling in the soil or farming without protective clothes and had visible soil

contamination on the skin surface were captured in this group as having direct contact with the soil and the respective activity engaged recorded.

Sterile cotton swabs were used to swab the exposed limbs (upper and lower) of participants after engagement with their respective activities and stored in sterile universal bottles containing Middle Brook 7H9 medium (5ml) to preserve the viability of any mycobacterium. Individual bottles were labelled with participant ID and the date of sample collection. The samples were transported on the same day on ice to the laboratories of Noguchi Memorial Institute for Medical Research (NMIMR) for analyses.

A

B

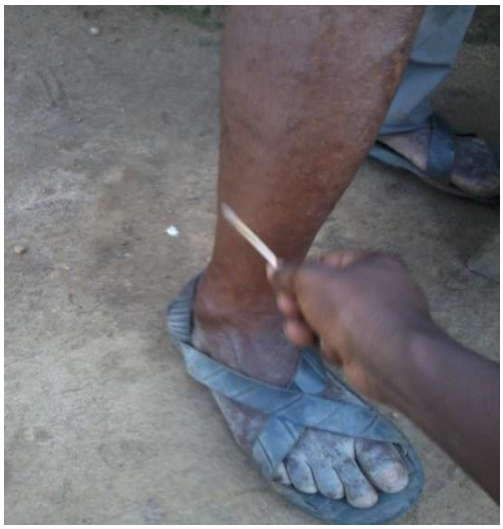
C

Figure 3.3 Specific activities engaged by recruits ;

(A.) Children swimming in the Densu River at Pakro. (B.) children fishing in a stagnant water at Pakro (C) children playing with the soil at Pakro

A

B



C

D

Figure 3.4 Sampling, sample storage and transportation.

(A) Skin swabs taken from exposed upper limb of a farmer (B) Skin swab taken from exposed lower limb of a farmer (C) Swabs in universal bottle containing 7H9 media (D) Cold box containing individual universal bottles for easy transportation to the laboratory.

3.5 Laboratory analyses

3.5.1 Sample processing and pooling strategy

Ten sterile glass beads were added to the content of each universal bottle containing the swabs and vortexed vigorously for two minutes to obtain suspensions. A portion (2 ml) from five samples was pooled into a 50 ml falcon tube. Samples were mostly pooled based on similarity of their risk activities and location (Appendix 3). A total of 80 pools were obtained from the 400 samples. The pooled samples were used for the DNA extractions as well as culture. PCR positive pooled samples were traced to the individual level to identify the type of activity engaged by the subject.

3.5.2 DNA extraction procedures

DNA was extracted from all the pooled samples as well as some individual samples using the modified Boom method (Durnez *et al*., 2009) and the power soil DNA isolation kit, a commercial DNA isolation kit.

3.5.2.1 Genomic DNA extraction using modified Boom protocol

Skin suspensions (500 µl) were added to 250 µl of lysis buffer (1.6 M GuHCl, 60mM Tris at pH of 7.4, 1% Triton X-100, 60 mM EDTA, 10% Tween-20), 50 µl of Proteinase K at concentration of 20 mg/ml and 500 µl glass beads in sterile 1.5 ml cryovial tubes. The tubes were secured in styrofoam box and incubated horizontally in an orbital shaker (200 rpm) at 60^0C for 12 hours. A combination of chemical and mechanical lysing steps employed in this protocol was to facilitate effective lysis of the mycobacterium cell.

Diatomaceous earth solution (40 µl) was added to the lysates to capture the released DNA and incubated at 37^{0}C for 1 hour. The mixtures were centrifuged at 14,000 rpm for 1 min, supernatants discarded and pellets washed twice with 900 µl of ice cold 70% alcohol and 900 µl of absolute acetone, pulsed vortexed and centrifuged at 14,000 rpm for 1min. The supernatants were discarded and the pellets containing DNA bound to diatomaceous earth were dried at 50^{0}C for 20 min using a heat block to evaporate any acetone residues.

The DNA was resuspended in 100 µl of nuclease free water after incubating at 68 ^{0}C for 20 min on a heat block with intermittent vortexing. The mixtures were centrifuged at 14,000 rpm for 1 min and 50 µl of the supernatants containing the extracted DNA were pipetted into sterile tubes and stored at -20^{0}C until used. Controls (negative and positive controls) were also run alongside the samples. The negative extraction control included all the reagents for DNA extraction with exception of the sample. Instead of the skin suspension, suspension containing *M. ulcerans* isolate was used in the positive control test.

3.5.2.2 Genomic DNA extraction using power soil DNA isolation kit

DNA extraction was performed with the use of the power soil DNA isolation kit with slight modification to the manufacturer instructions. Briefly, skin suspensions (500 µl) were added to the power bead tubes provided and followed by 60 µl of solution C1 and the tubes vortexed for 5 seconds. C1contains sodium dodecyl sulphate (SDS) and other disruption agents required for cell lysis. The power beads tubes were secured vertically on MP Fast Prep- 24^{TM} bench top homogenizer and run at maximum speed for five minutes. The MP Fast Prep- 24^{TM} uses unique optimized motion to disrupt cells through multidirectional, simultaneous beating of specialized

Lysing Matrix beads in the power beads tube on the sample material. A combination of this mechanical step as well as the chemical lysing step helps to effectively lyse the mycobacterium cell.

The power beads tubes were then centrifuged at 10,000 g for 30 seconds at room temperature. The supernatants were transferred into clean 2 ml collection tubes provided and 250 µl of solution C2 (Solution C2 is a patented inhibitor removal containing reagent to precipitate non-DNA organic and inorganic material including humic acid substances, cell debris and proteins) were added to the respective tubes. The mixtures were vortexed for 5 seconds, incubated for 5 minutes at 4^{o}C and centrifuged at10,000 g for 60 seconds at room temperature.

Avoiding the pellet and any precipitation, 800 µl of the supernatants were transferred into another clean 2 ml collection tubes provided and 200 µl of solution C3 added (Solution C3 is another patented inhibitor removal containing reagent to precipitate additional non-DNA organic and inorganic material including humic acid substances, cell debris and proteins). The mixtures were vortexed for another 5 seconds and incubated at same condition of 4^{o}C for 5 minutes and centrifuged at room temperature for 1 minute at 10,000 g. Into another clean 2 ml collection tubes, 750 µl the supernatants were transferred and 1.2 ml of solution C4 (Solution C4 is a high salt concentration that helps DNA to bind tightly to the silica spin filter) added to the individual tubes.

Tubes were vortexed for 2 seconds and 675 µl of the mixtures transferred to another collecting tubes containing spin filters. The tubes were centrifuged at room temperature for 1 munite at 10,000 g and the flow beneath the spin filter discarded. These steps were repeated three times till the whole volume in the 2ml collection

tubes were finished. Solution C5 which was an ethanol based washing solution required to further clean the DNA bound to the silica filter membrane in the spin filter were added (500 µl) to tubes containing the spin filters and centrifuged at 10,000 g for 1 minute at room temperature. The flow beneath the spin filters were discarded and the tubes centrifuged again to remove all traces of the washing solution as ethanol in Solution C5 can interfere with downstream application like PCR.

The spin filters were carefully transferred into another clean 2 ml collection tubes and 100 µl of solution C6 containing 10mM Tris solution were added at the centre of filter membrane to elute the bound DNA. The tubes were centrifuged at room temperature at 10,000 g for 30 seconds and the spin filter discarded. The tubes containing the DNA extract were kept at -20^{0}C until used. Controls (negative and positive control) were also run alongside the samples. The negative extraction control included all the reagents for DNA extraction with exception of the sample. Instead of the skin suspension, suspension containing *M. ulcerans* isolate was used in the positive control test.

3.5.3 Detection of *M. ulcerans* DNA by qPCR targeting IS*2404* multiplexed with IPC

Quantitative real time PCR targeting IS*2404* multiplexed with an internal positive control (IPC) to monitor inhibition was performed on all the DNA extracts in duplicate. The qPCR mixture contained 1.25 µl of each primer (18 µM), 1.25 µl of the probe (5 µM), 0.5 µl of Exo IPC DNA (50X), 2.5 µl Exo IPC Mix, 12.5 µl TaqMan® Universal PCR Master Mix (2X), 4.75 µl Nuclease Free Water and 1 µl of template DNA (samples) or 1 µl no template control (NTC) or 2.5 µl Exo IPC Block (10X) in a total volume of 25 µl.

Amplification and detection was performed using Rotor Gene Q thermal cycler using the following program: 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. The results were analysed with Rotor Gene Q series software version 1.7. Negative results are indicated as negative, positive results by the respective Ct (cycle threshold) value and inhibition indicated as blank.

3.5.4 Detection of *M. ulcerans* DNA by qPCR targeting IS*2606* and KR

Another multiplex TaqMan® assay targeting IS*2606* and ketoreductase-B domain (KR) designed to augment the specificity of the first IS*2404*/IPC PCR was performed on all IS*2404* PCR positive samples in duplicate. The qPCR mixture contained 1.25 µl of each primer (18 µM), 1.25 µl of the probes (2 µM), 12.5 µl TaqMan® Universal PCR Master Mix (2X), 4 µl Nuclease Free Water and 1 µl of template DNA (samples) or 1µl no template control (NTC).

Amplification and detection was performed with the Rotor Gene Q thermal cycler using the following program: 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. The results were analysed with Rotor Gene Q series software version 1.7. Negative results are indicated as negative, positive results by the respective Ct (cycle threshold) value and inhibition indicated as blank.

3.5.5 Cultivation of skin suspensions on Löwenstein Jensen (LJ) media

Pooled skin suspensions (2 ml) were transferred into 2 ml cryovials and centrifuged at 14,000 rpm for 5 min. The supernatants were discarded and the pellets decontaminated using the method by Portael *et al*. (1988) with sight modification.

Briefly to the pellets, 500 µl NaOH (1M), 100 µl of 0.8% cycloheximide and 500 µl of 0.2% malachite green were added. The content of the tubes were mixed and incubated at room temperature for 30min.

The mixtures were centrifuged at 14,000 rpm for 5 min and neutralized with 1 ml of oxalic acid and incubated for 20 min at room temperature. And again, centrifuged at 14,000 rpm for 5 min and washed twice with 1ml sterile distilled water followed by another round of centrifugation at 14,000 rpm for 5 min. Pellets were resuspended in 100 µl phosphate buffered-saline (PBS) and 100 µl volume of the decontaminated sample was inoculated onto Löwenstein Jensen (LJ) slopes and incubated at 31°C. Cultures were observed weekly for growth of mycobacterial colonies.

CHAPTER FOUR

RESULTS

4.1 Demographic Characteristics

A total of 400 participants were enrolled in the study and out of these 224 (56%) were males and 176 (44%) females. The sex ratio of participants was balanced between endemic and non-endemic communities, p = 0.009. Median age of the participants was 18 years (range of 1 to 85 years). Participants from endemic and non-endemic communities had median ages of 14 years (range of 1 to 84 years) and 25 years (1 to 85 years) respectively. Age was disproportionately represented, p= 0.075, with participants less than 16 years being predominant from both endemic and non-endemic communities. Their corresponding percentages were 52% and 41.5% respectively. Percentages of participants with age ranges of 16-45 and 45-85 were 31.5 and 16.5 for endemic communities and 35 and 23.5 for non-endemic communities (Table 4.1).

Table 4.1 Comparison of age and sex characteristics of participants in endemic and non- endemic communities.

Characteristcs		Endemic communities	Non- endemic communities	p - value
Sex				
Male	n(%)	125 (62.5%)	99 (49.5%)	0.009
Female	n(%)	75 (37.5%)	101 (50.5%)	
Age Median (range) year		14 (1-84)	25 (1-85)	
< 16	n(%)	104 (52%)	83 (41.5%)	0.075
16-45	n(%)	63 (31.5%)	70 (35%)	
> 45	n (%)	33 (16.5%)	47 (23.5%)	

n = number of participants (%) = percentage of participants

4.1.1 Age characteristic of recruits within the selected communities

Participants from Oparekrom had the lowest median age of 13 years (range of 1 to 48 years) whereas the highest median age of 32.5 years (range of 2 to 48 years) was recorded by participants from Dago. The age characteristics from the other two selected endemic communities Pakro and Mangoase had respective median ages of 14 years (range of 3 to 80 years) and 18 years (4 to 80 years). Among the non- endemic communities, recruits from Boahenekrom had the lowest median age of 14.5 years (range of 1 to 70 years) and that of Obosono had the highest of 32 years (1 to 80 years). Recruits from Okomfo and Obotweri had respective median ages of 20 years (range of 1 to 85 years) and 25 years (range of 1 to 75 years).

The highest percentage of participants with ages less than 16 years was 68% recorded by recruits from Oparekrom whereas the least of 28% was recorded by recruits form Obosono. Recruits from Obosono had the highest percentage of 44% for the age category 16 – 45 years and the least of 18% recorded by recruits from Okomfo. Dago had majority of the participants with ages above 45 years with a percentage of 34% whereas Oparekrom recorded the least percentage of 0.2% for the same age category (Fig. 4.1).

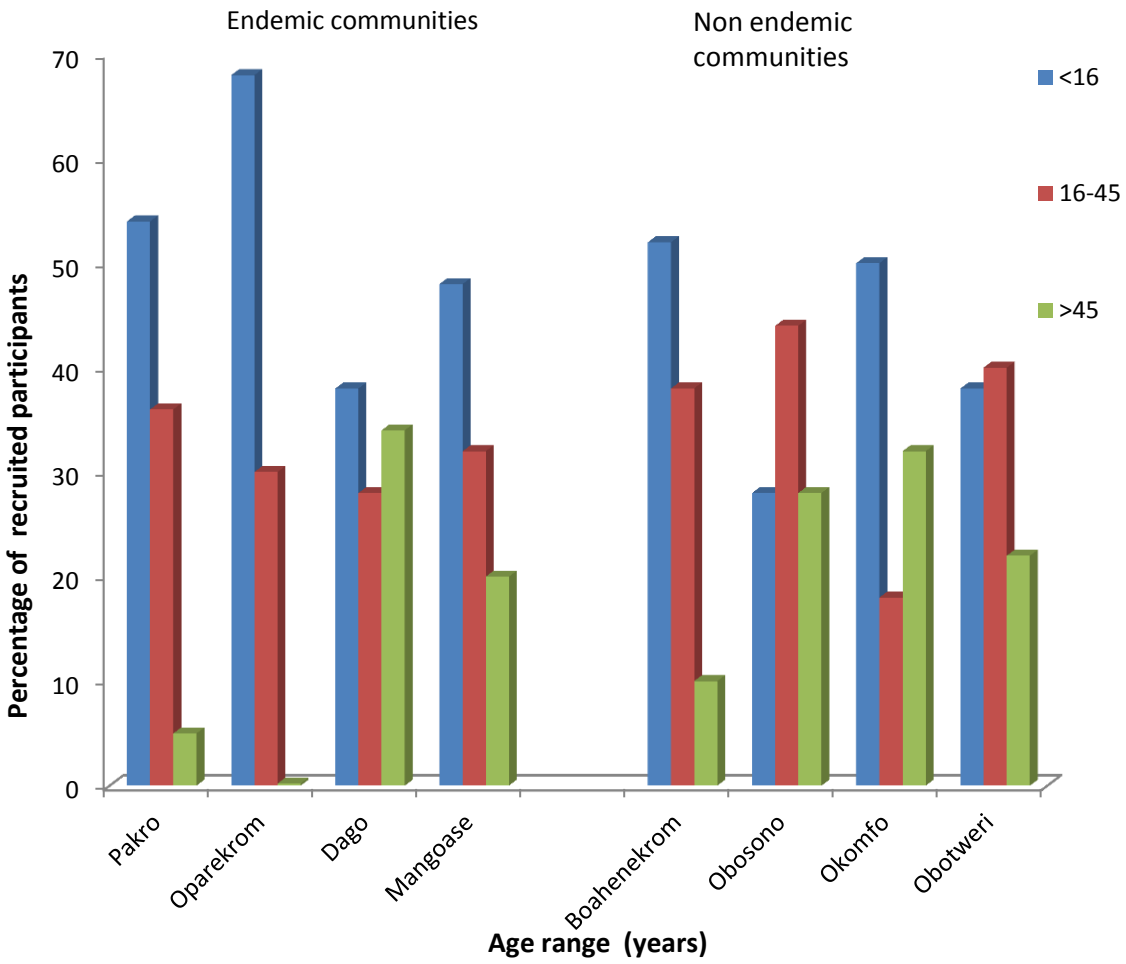


Figure 4.1 Age characteristics of participants within the eight selected communities.

Blue bars represent recruits with ages less than 16years. Red and green bars represents ages of recruits with ages 16 – 45 years and greater than 45 years respectively.

4.2 Statistics on the type of risk activities engaged by participants

The highest percentage of recruits (36%) was engaged in activities involving playing, closely followed by farming without protective clothing (29%). Participants that were crawling and those using mud for construction together formed 2%. In total, participants whose activities involved direct contact with soil were found to be 67%. Participants who had direct contact with slow flowing water or stagnant water in pursuing their normal activity formed 14 %, with those swimming having the highest

percentage of 7%. Participants who had visible soil contamination on the skin surface formed 19% and were captured as others (Fig. 4.2).

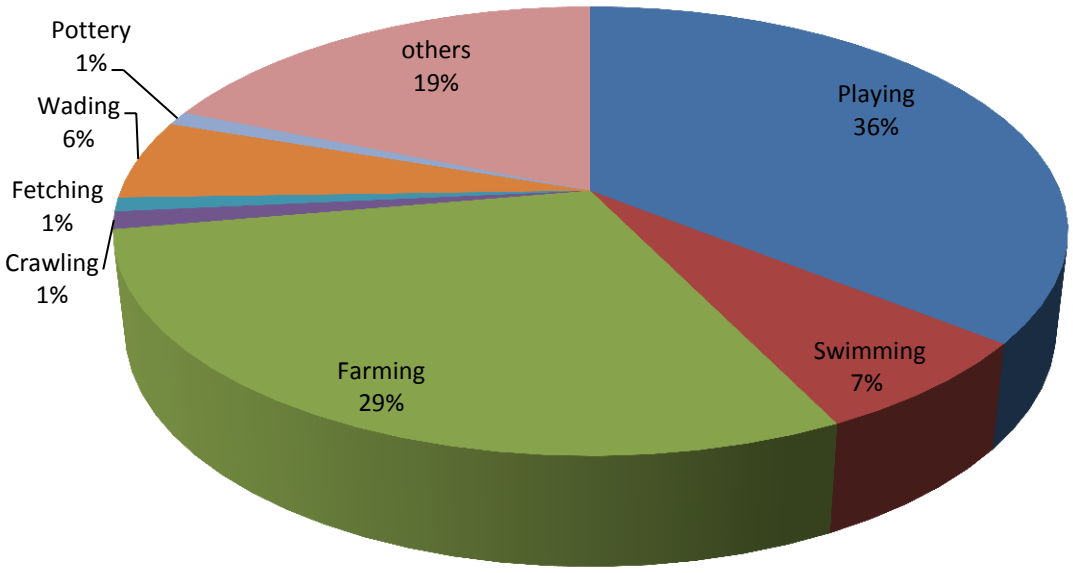


Figure 4.2 Percentages of participants engaged in specific risk activities.

4. 3 Pools composition

A total of ten pools (five different skin suspensions were pooled into a group) were generated from each of the sampled communities. Five out of the total pools from Dago (A1, A2, A3, A6 and A10) were those involved in the same activities. Pool A1 was made of samples from participants who were playing in the soil. Pools (A2, A3 and A6) were from participants who farmed without protective clothing, while A10 consisted of those classified as others as explained in Fig 4.2. The rest of the pools (A4, A5, A7, A8 and A9) comprised a mix of samples from participants engaged in different risk activities (playing, farming and others). Only three of the pools (B1, B2 and B8) from Mangoase were engaged in similar activities. Pools B1 and B2 were involved in playing while pool B8 encompassed those engaged in farming without

protective clothing. Pools B6 and B7 predominantly constituted samples from participants wading through portions of the Densu river within the town. The rest of the pools (B3, B4, B5, B9 and B10) were composed of samples from participants involved in different activities (playing, farming and others).

The first four pools (C1- C4) generated from the samples collected from Pakro consisted of individuals swimming, fetching or wading through the portion of the Densu river within that town. Pool C5 was from participants wading through a stagnant water body while pool C8 was from participants who farmed without protective clothing. The rest of the pools (C6, C7, C9 and C10) comprised samples from participants involved in different risk activities (playing and farming).

Pools D1 to D4 from Oparekrom were samples from participants swimming in the Densu river streaming through that town. D6 pool was mostly from participants wading through a stagnant water body believed to be the source of BU by section of the inhabitants whereas D5 pool was involved in playing. Pools D7 to D10 composed of samples from participants engaged in different activities (playing, farming and others).

The pools pattern (1A to 1J) generated from Boahenekrom' samples were composed of samples from participants engaged in different activities (playing, farming and others) except for pools IF and 1J. Pool 1F was composed of samples from participants who were involved in playing whereas 1J pool was also composed of samples from participants that were using mud in constructions (pottery). Pools (2A-2J), (3A- 3J) and (4A -4J) generated from samples from Okomfo, Obosono and Obotweri respectively mainly consisted of samples from participants engaged in different risk activities (playing, farming and others) except for pool 2H composed of

samples from participants who were involved in playing. Pool 3G also composed of samples from participants who were classified as others as explained from Fig. 4.2 above.

4.4 Detection of *M. ulcerans*

Detection of *M. ulcerans* DNA was assessed by detecting the presence of the multiple insertion sequence elements IS*2404* and IS*2606* as well as the gene that codes for the enzyme involved in synthesis of mycolactone, ketoreductase.

4.4.1 Detection of IS*2404* target from pooled DNA extracts

IS*2404* RT PCR revealed a higher positivity with DNA extracts obtained from the power soil DNA isolation kits than those from the modified Boom method. Out of the 80 pooled samples, 3 pools were positive for IS*2404* target sequence using extract from power soil DNA isolation kits while DNA extracted using the modified Boom methods were all negative.

The positive pooled samples were 2E (Okomfo), 3C and 3D (Obosono) Their respective mean Ct (cycle threshold) values were 31.44, 38.85, and 37.3 (Appendix 5; Table 1).

4.4.1.1 Analysis of individual component samples within IS*2404* pools positive

Individual DNA extracts from all three pooled IS*2404* positives were also tested for the presence of IS*2404*. The mean IS*2404* Ct value of the individual sample within 3C pool was 36.27. This value was slightly lower than the group mean Ct value of 38.85. Mean IS*2404* Ct value of the individual sample within pool 3D was 37.77. Three out of five samples within 2E pool were all positive for the target sequence and had mean Ct values of 38.63, 38.11 and 38.42 (Appendix 5; Table 1).

4.4.2 Detection of IS*2606* and KR targets from pooled DNA extracts

Pooled samples positive for the IS*2404* were tested for the additional targets IS*2606* and ketoreductase-B domain sequence (KR) in another multiplex TaqMan real time assay. All three IS*2404* positive pooled samples were also positive for KR target sequence but only one (pool) 2E was positive for IS*2606*. The mean KR Ct value recorded by Pool 3C and 3D were 35.85 and 39.35 and both had IS*2606* assay inhibited. Mean KR Ct and IS*2606* Ct value for pool 2E were 31.1 and 34.42 respectively (Appendix 5; Table 2).

4.4.2.1 Analysis of individual component samples within IS*2606* and KR pools positive

Individual DNA extracts constituting the IS*2606* and KR positive pools (2E, 3C and 3D) were also tested for the presence of these targets. Corresponding mean Ct values for KR and IS*2606* recorded by the only IS*2404* positive within 3C pool were 35.52 and 38.61 while that from pool 3D were 39.31 and 39.35. Only one out of the three IS*2404* positives within pool 2E was positive for the both KR and IS*2606* target with mean Ct of 38.26 and 39.63 respectively. The other two IS*2404* positive samples within pool 2E had their IS*2606* inhibited assays while their corresponding mean Ct values for KR were 93.38 and 37.18 (Appendix 5; Table 3).

4.5 Specific activities resulting in *M. ulcerans* DNA skin contamination

Five samples from participants were positive for IS*2404* target sequence. Two out of the five (40%) positive samples were from participants involved in farming without protective clothing while the other two (40%) were from toddlers crawling in the soil. The fifth IS*2404* positive sample was from a child playing with the soil. All IS*2404* positive samples obtained from participants involved in farming without protective

clothing also tested positive for IS*2606* and KR. Only one of the toddlers' positive for IS*2404* target sequence was positive for both targets while the other was positive for only KR. The only participant involved in playing and positive for IS*2404* target sequence also had IS*2606* assay inhibited. None of the DNA extracts from participants engaged in activities like wading, fetching, swimming and pottery were positive for IS*2404* target sequence or the other targets sequence.

4.6 Mycobacteria species cultivated from pooled skin suspensions

Out of 80 cultures from the pooled samples, 27 (34%) were heavily contaminated with fungi growth and were discarded after the 11th week of cultivations. A total of 32 (40%) cultures had no visible growth on Löwenstein Jensen (LJ) whereas 15 (19%) of the cultures were slightly contaminated after 15 weeks of cultivations. Only six (7%) cultures exhibited buff to yellow or red colonies after 11 weeks of cultivation. These cultures were observed from pooled suspensions from two endemic (Pakro and Dago) and non-endemic communities (Obosono and Boahenekrom).

One out of the ten pooled samples (C2) had the observed growth from Pakro whereas from Dago, two out of the ten pooled samples (A1 and A4) observed the recorded growth. Obosono also had two of its pooled samples (3B and 3G) recording yellow colonies after 11 weeks of cultivations. A pooled sample (1B) from Boahenekrom recorded the observed red colonies and was seen in culture as early as the third week of cultivation. Pooled sample (2E) from Okomfo also recorded a small yellow colony at the fourth week of cultivation but was lost to contamination.

All the six cultures exhibiting colonies were acid fast bacilli but negative for TaqMan real time PCR targeting IS*2404* multiplex with internal positive control and the other TaqMan assays targeting IS2*606* and KR.

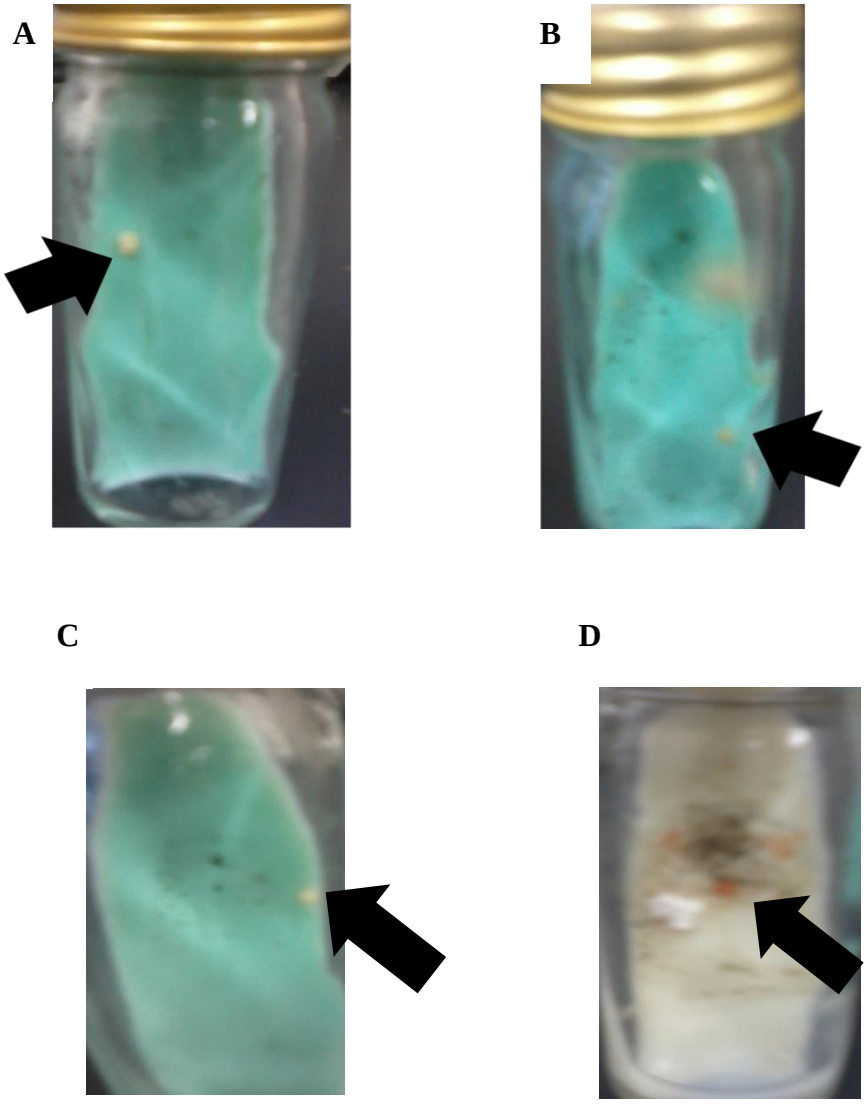


Figure 4.3 Mycobacteria colonies harvested after 11 weeks of cultivation**.**

The Black arrow indicates the position of the mycobacteria colonies on Löwenstein Jensen media. (A) Yellow colony of mycobacteria from pool C2 (B) Yellow colony of mycobacteria from pool 3G. (C) Yellow colony of mycobacteria from pool 3B (D) red colonies of mycobacteria from pool 1B.

CHAPTER FIVE

DISCUSSION AND CONCLUSION

5.0 Discussion

Despite the outlined risk factors for contracting BU as revealed in some case control studies, precisely how *M. ulcerans* is transmitted from the environment to human hosts still remains enigmatic. Portaels and colleagues hypothesized direct transmission of *M. ulcerans* into human skin from overlying *M. ulcerans* contaminated skin surface following entry of the pathogen through an abrasion or trauma (Portaels *et al*., 2001). Possible sources of *M. ulcerans* skin contamination could occur when there is contact with contaminated water, soil, aquatic plants or biofilm on aquatic plants where traces of *M. uclerans* DNA have been documented**.** The use of contaminated water such as those from swamps and slow flowing water (Debacker *et al*., 2006) or contaminated soil for agricultural purposes have been considered as risk factors for BU. Examining the skin surfaces of individuals who are considered at risk for BU for the presence of *M. ulcerans* would help shed more light on the hypothesis by Portaels and colleagues and could ultimately contribute towards efforts to understand the transmission route for *M. ulcerans*.

Skin swabs were taken from participants from four BU endemic and non- endemic communities within Akwapim South, Akwapim North and Nsawam/Adoagyiri municipalities. DNA was extracted from skin suspensions from participants using two different DNA extraction methods. While none of the DNA extracted from the pooled skin suspensions using Modified Boom method was positive for the TaqMan IS*2404*/IPC multiplex real time PCR assay targeting IS*2404*, three pools (3/80) were

positive for that target sequence using DNA extracts from power soil DNA isolation kit. Incorporation of the internal positive control (IPC) in the multiplex assay was to monitor the presence of inhibitors that could be present in the sample and inhibits the activity of the DNA polymerase. None of the pooled DNA extracts from both methods were inhibited in TaqMan IS*2404*/IPC multiplex real time PCR assay thus the low number of IS*2404* target sequence detected from the pooled samples was a true reflection from the samples and not as a result of presence of inhibitors in the samples. Though the discrepancy from the two extraction methods in detecting the target DNA was not exploited in this study, it could be attributed to the elaborate cell lysis as well as stringent purification steps incorporated in the kit' protocol. All the three pooled IS*2404* positives were from Okomfo and Obosono which were classified as BU non- endemic communities.

Unlike clinical samples where detection of IS*2404* positive in PCR assays is confirmatory for the presence of *M. ulcerans* DNA (Phillips *et al*., 2005), detecting the multi copy IS*2404* of *M. ulcerans* DNA from environmental samples is not confirmatory as this target is not specific to *M. ulceran*s DNA alone (Yip *et al*., 2007). The use of additional targets and internal probes are steps taken to increase the confidence in the interpretation of PCR positive tests for environmental samples (Fyfe *et al*., 2007). Pools positive for IS*2404* target sequence were further tested with the TaqMan real time multiplex PCR assay targeting IS*2606* and KR.

All pools negative for IS*2404* target were not tested for the presence of these targets. The three pools were positive for KR but only one (2E) was positive for IS*2606* target sequence as well. There were inhibitions in the assays targeting IS*2606* sequence for the other two pools. The same DNA extracts from pool 3C and 3D used for the first

TaqMan IS*2404*/IPC multiplex real time PCR assay were as well used for the second TaqMan multiplex assay. None of these pools were initially inhibited in TaqMan IS*2404*/IPC assay indicating that the inhibition observed for the assay targeting IS*2606* could not be the presence of inhibitors but could explain the less sensitive nature of that assay (Fyfe *et al*., 2007). The presence of these targets in the pooled DNA extracts is only indicative of genetic material from mycolactone producing mycobacteria (MPM) which also includes *M. ulcerans* (Fyfe *et al*., 2007). These targets have different copy numbers in both genomes of *M. ulcerans* and other MPM. This difference has been exploited in real time PCR assays to distinguish the occurrence of *M. ulcerans* genetic element from the other MPM which is as a result of the inherent ability of real time PCR assay to detect the relative differences in the copy numbers of *IS2404*, IS*2606*, and KR in any given sample. Estimated copy number ratio of IS*2404*-to-IS*2606* from the *M. ulcerans* reference strain Agy99 sequence is 2.3:1 (Stinear *et al*., 2007). A comparable value of 2.37 (2.17 to 2.79) for the median change in Ct values between IS*2606* and IS*2404* (ΔCt, IS*2606*- IS*2404*) was achieved by Fyfe and colleagues after detecting these targets from DNA extracts using clinical and environmental samples spiked with *M. ulcerans* isolate (Fyfe *et al*., 2007). Similar work using DNA extracts spiked with other MPM isolates revealed higher value of 7.60 (6.94 to 8.07) for the median Ct values between IS*2606* and IS*2404* (Fyfe *et al*., 2007). This difference in ΔCt for IS*2606* and IS*2404* has served as a discriminatory tool in real time PCR assays to differentiate the occurrence of genetic elements of *M. ulcerans* from other MPM. The difference in median ΔCt values for the presence of *M. ulcerans* genetic element and other MPM is as result of fewer copies of IS*2606* relative to IS*2404* for the other members of MPM (Fyfe *et al*., 2007). The change in mean Ct values between IS*2606* and IS*2404* (ΔCt, IS*2606*-

IS*2404*) for 2E pool was 2.98 **(**34. 42-31.44**)** which could indicate the presence of *M. ulcerans* DNA rather than from other MPM. The mean Ct values for the other two pools (3C and 3D) were not calculated as results of the inhibition observed in their IS*2606* assay.

Corresponding culture results for the pools that were positive for any of the three targets sequence exhibited no mycobacteria colonies after 15 weeks of cultivation. The culture for pool 2E which had tiny yellow colonies growing after the fourth week of incubation was lost to contamination. Only six cultures (6/80) from pools from Dago (A1 and A4), Pakro (C2), Boahenekrom (1B) and Obosono (3B and 3G) exhibited buff to yellow or red colonies after 11 weeks of cultivation. None of the DNA extracts from these isolates were positive for any of the targets tested. This implies that these isolates do not contain the genetic elements IS*2404*, IS*2606* and ketoreductase-B domain (KR) which are present in the genome of all MPM. Therefore, these isolates could not be from *M. ulcerans* or other MPM. The results further strengthen the specificity of the TaqMan real time multiplex PCR assays used.

Pools (2E, 3C and 3D) that were positive for any of the three targets were traced to the individual level. Individual sample within pool 3C positive for IS*2404* target sequence had a mean Ct value of 36.27 which was slightly lower than the group Ct of 38.85. This could indicate that the pooling had little effect in the detection of the target sequence, thus pools that were negative for the targets sequence were not as a result of the pooling, rather due to the absence of the target sequence. The difference in mean Ct value between IS*2606* and IS*2404* for that sample was 2.34 (38.61-36.27) (Appendix 5; Table 4) which is an indication of the presence of *M. ulcerans* DNA.

Individual samples within pool 2E and 3D positive for all three targets had difference in mean Ct values between IS*2606* and IS*2404* to be 1.52 (39.63-38.11) and 1.58 (39.35- 37.77) respectively. These values are less than the range values (2.17 to 2.79) to indicate the presence of *M. ulceans* DNA (Fyfe *et al*., 2007). Therefore, only one out of the total skin suspensions (n= 400) from individuals considered at risk for BU was positive for the presence of *M. ulcerans* DNA. This was from a participant from Obosono, a non- endemic BU community.

Kenu and colleagues recently used Geographic Information System (GIS) to map BU cases along entire stretch of the Densu river and found an association between BU cases and wading in the Densu river (Kenu *et al*., 2014). This finding could imply that the Densu river, especially where it is heavily contaminated could play a role in the transmission of BU. About 50% of the recruits from Pakro and Oparekrom had direct contact with some section of the Densu river in the form of swimming, wading or fetching from it. Analyses on skin suspensions from these people for the targets sequences were all negative. Results on skin suspensions from some recruits that were using some stagnant waters in the communities of Pakro and Oparekrom, were as well negative for the target sequences. This study did not detect *M. ulcerans* from the skin suspensions from all individuals engaged with the Densu river within the selected three communities of Pakro, Oparekrom and Mangoase.

Majority of the participants in this study were captured under two risk activity headings: farming and playing. These were recruits returning from farm not wearing protective cloths (long sleeve and trouser) and shoe or were playing in the soil in their respective community and had visible soil contamination on their skin surface. Analyses on the skin suspensions from these groups of people within the selected

endemic communities and non-endemic communities except for Okomfo and Obosono were all negative for the targeted sequences. Two individuals from Okomfo and Obosono who were farming without protective clothing were positive for all three targets but only one had difference in Ct value between IS*2606* and IS*2404* to be indicative of the presence of *M. ulerans* DNA. This links the occurrence of the only *M. ulcerans* DNA detected to the risk factor "farming without protective clothes and shoe**"**. This could emphasize the protective effect of wearing long sleeves to farm against contraction of BU (Kenu *et al*., 2014)**.**

Skin suspension from a toddler at Obosono captured under crawling was positive for all the targets but had difference in Ct value between IS2*606* and IS*2404* not indicative of presence of *M. ulcerans* DNA. Another toddler and a child from Okomfo captured under crawling and playing respectively were only positive for IS*2404* and KR target sequences.

Several case control studies have established an association between *M. ulcerans* infection and age. Children less than 15 years are disproportionately affected (Jacobsen and Padgett, 2010; Sopoh *et al*., 2007; Debacker *et al*., 2006; Phanzu *et al*., 2006). Majority of the participants were less than 16 years of age (46.8%, Table 4.1). However the participant whose sample turned positive for *M. ulcerans* DNA was 32 years.

5.1 Conclusion

M. ulcerans DNA was detected on the skin surface of a farmer supporting the hypothesis that skin contamination may be important in BU transmission. It further supports the case control studies establishing association between BU with absence of protective clothing during farming. Detection of *M. ulcerans* in a non-endemic area also supports the findings of Williamson *et al*. (2008), that *M. ulcerans* is present in the environment of endemic and non -endemic areas.

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APPENDICES

Appendix 1

Preparation of Transport Medium

Difco Middlebrook 7H9 broth (4.7g) and 2 ml glycerol was dissolved in 900 ml of distilled water and autoclaved for 10 minutes at 121°C. Volumes of 5ml were then dispensed into sterile universal bottles.

Appendix 2

Questionnaire used for sampling

Questionnaire

Questionnaire Number: ☐☐☐☐☐☐

Name of community: ☐

Date interviewed: ☐☐☐☐☐☐☐☐

Informed consent sought: Yes ☐ No ☐

Participant Information

Name of participant: ☐

Residential Address: ☐

Phone number: ☐

Age ☐ Gender ☐

Educational Status: ☐

Environmental exposure

A. Contact with slow flowing water body

Activities performed

Swimming ☐ Fetching ☐ wading ☐ Others ☐

B. Direct contact with the soil

Activities performed

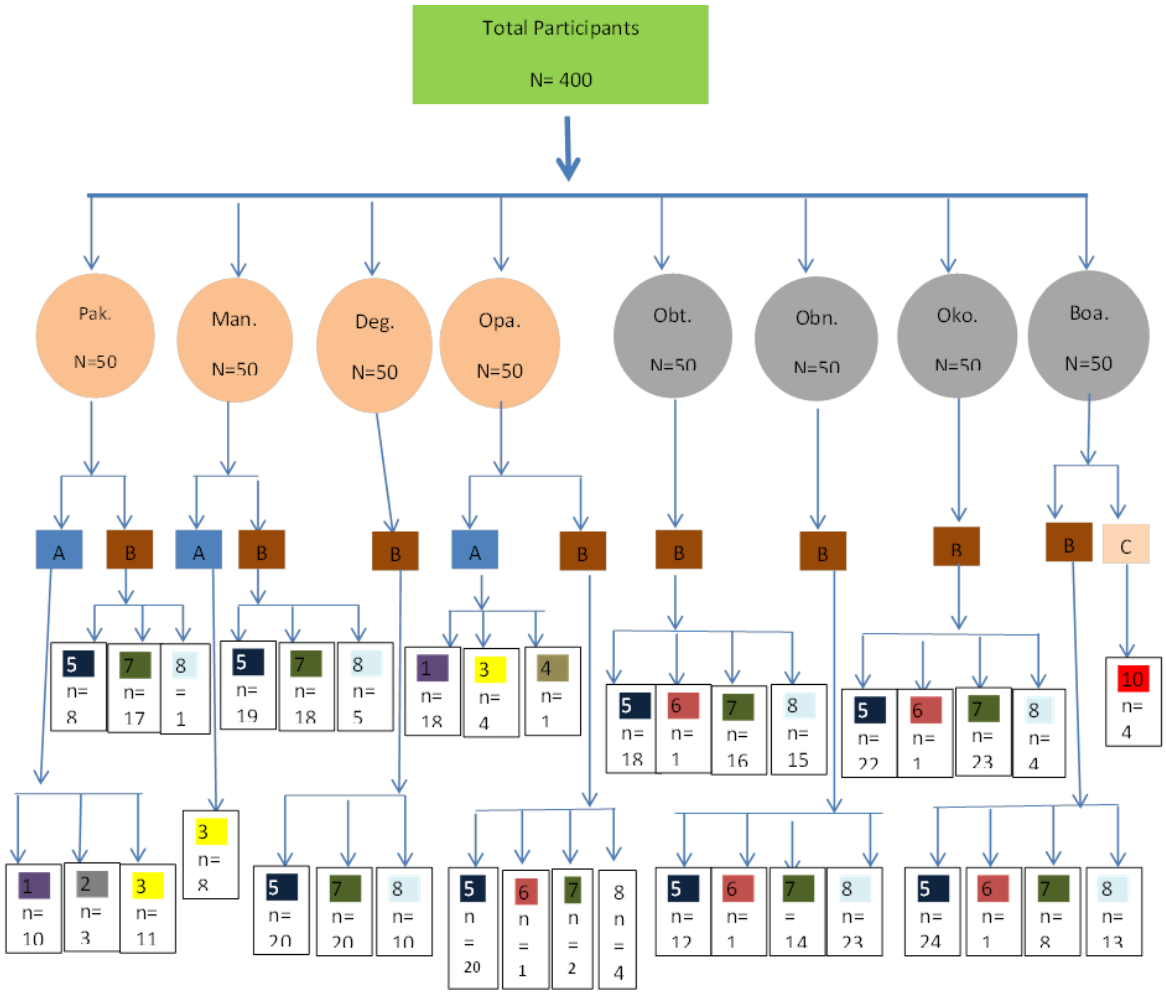
Playing ☐ Crawling ☐ Farming ☐ others ☐

Occupational exposure

Galamsey ☐ Pottery ☐ Fishing ☐ Others ☐

Appendix 3

Sampling scheme



KEY

	Risk factors considered	**Communities selected**
Endemic communities	1 **Swimming**	**Boa - Boahenekrom**
Non – endemic communities	2 **Fetching**	**Deg - Dego**
A **Contact with slow flowing water**	3 **Wading**	**Man - Mangoase**
B **Direct contact with the soil**	4 **Others**	**Obn - Obosono**
C **Occupational Exposure**	5 **Playing**	**Obt - Obotwere**
	6 **Crawling**	**Oko - Okomfo**
	7 **Farming**	**Opa - Oparekrom**
	8 **Others**	**Pak - Pakro**
	10 **Pottery**	

Appendix 4

Primers and sequences for IS*2404*, IS*2606* and KR Taqman real time PCR

Primer	Sequence (5'-3')
IS2404 TF	AAAGCACCACGCAGCATCT
IS2404 TR	AGCGACCCCAGTGGATTG
IS2606 TF	CCGTCACAGACCAGGAAGAAG
IS2606 TR	TGCTGACGGAGTTGAAAAACC
KR TF	TCACGGCCTGCGATATCA
KR TR	TTGTGTGGGCACTGAATTGAC

Probes and sequences for IS2404, IS2606 and KR Taqman real time PCR

Probe	Sequence (5'-3')
IS2404 TP	6 FAM-CGTCCAACGCGATC-MGBNFQ
IS2606 TP	VIC-TGTCGGCCACGCCG-MGBNFQ
KR TP	6 FAM-ACCCCGAAGCACTG-MGBNFQ

Appendix 5

Table 1: Mean Ct values for pooled and individual samples positive for IS*2404* target

Pool ID	Individual sample	*IS2404* Ct value
Pool 2E		31.44
	OK 21	38.63
	Ok 24	38.11
	Ok 25	38.42
Pool 3C		38.85
	Ob 13	36.27
Pool 3D		37.3
	Ob 17	37.77

Table 2: Mean Ct values for pooled samples positive for KR and IS*2606*

Pool ID	KR Ct value	IS*2606* Ct value
Pool 2E	31.1	34.42
Pool 3C	35.85	inhibited
Pool 3D	39.35	inhibited

Table 3: Mean Ct values for KR and IS*2606* for individual samples

Pool ID	Individual sample	KR Ct value	IS*2606* Ct value
2E	OK 21	39.38	inhibited
	Ok24	38.26	39.63
	OK 25	37.18	inhibited
3C	Ob 13	35.52	38. 61
3D	Ob 17	39.31	39.35

Table 4: Difference in Ct values between IS*2606* and IS*2404*, Δ*Ct(IS2606-IS2404*) for the individual samples positive for any of three targets

Pool ID	Individual ID	IS*2404* Ct value	IS*2606* Ct value	KR Ct value	ΔCt(IS*2606*-IS*2404)*
2E	OK 21	38.63	inhibited	39.38	
	Ok24	38.11	39.63	38.26	1.52
	OK 25	38.42	inhibited	37.18	
3C	Ob 13	36.27	38.61	35.52	2.34
3D	Ob 17	37.77	39.35	39.31	1.58

Appendix 6

SPSS (version 16.0) read out for the variables endemicity and gender

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
Gender * Endemicity	400	99.0%	4	1.0%	404	100.0%

Gender * Endemicity Crosstabulation

			Endemicity		
			Bu endemic comm.	Non -endemic comm.	Total
Gender	Males	Count	125	99	224
		Expected Count	112.0	112.0	224.0
		% within Gender	55.8%	44.2%	100.0%
		% within Endemicity	62.5%	49.5%	56.0%
		% of Total	31.2%	24.8%	56.0%
		Residual	13.0	-13.0	
		Std. Residual	1.2	-1.2	
	Females	Count	75	101	176
		Expected Count	88.0	88.0	176.0
		% within Gender	42.6%	57.4%	100.0%
		% within Endemicity	37.5%	50.5%	44.0%
		% of Total	18.8%	25.2%	44.0%
		Residual	-13.0	13.0	
		Std. Residual	-1.4	1.4	
Total		Count	200	200	400
		Expected Count	200.0	200.0	400.0
		% within Gender	50.0%	50.0%	100.0%
		% within Endemicity	100.0%	100.0%	100.0%
		% of Total	50.0%	50.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	6.859[a]	1	.009		
Continuity Correction[b]	6.341	1	.012		
Likelihood Ratio	6.880	1	.009		
Fisher's Exact Test				.012	.006
Linear-by-Linear Association	6.842	1	.009		
N of Valid Cases[b]	400				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 88.00.

b. Computed only for a 2x2 table

Symmetric Measures

		Value	Approx. Sig.
Nominal by Nominal	Phi	.131	.009
	Cramer's V	.131	.009
N of Valid Cases		400	

Appendix 7

SPSS (version 16.0) read out for the variables endemicity and age

Age * Endemicity Crosstabulation

			Endemicity		
			BU endemic communities	Non endemic communities	Total
Age	<16 years	Count	104	83	187
		Expected Count	93.5	93.5	187.0
		% within Endemicity	52.0%	41.5%	46.8%
		Residual	10.5	-10.5	
		Std. Residual	1.1	-1.1	
	16-45 years	Count	63	70	133
		Expected Count	66.5	66.5	133.0
		% within Endemicity	31.5%	35.0%	33.2%
		Residual	-3.5	3.5	
		Std. Residual	-.4	.4	
	> 45 years	Count	33	47	80
		Expected Count	40.0	40.0	80.0
		% within Endemicity	16.5%	23.5%	20.0%
		Residual	-7.0	7.0	
		Std. Residual	-1.1	1.1	
Total		Count	200	200	400
		Expected Count	200.0	200.0	400.0
		% within Endemicity	100.0%	100.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	5.177[a]	2	.075
Likelihood Ratio	5.195	2	.074
Linear-by-Linear Association	5.126	1	.024
N of Valid Cases	400		

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 40.00.

Symmetric Measures

		Value	Approx. Sig.
Nominal by Nominal	Phi	.114	.075
	Cramer's V	.114	.075
N of Valid Cases		400	