Comparative Diagnosis of *Mycobacterium ulcerans* Infections by Ziehl-Neelson Microscopy, Insertion Sequence *2404* Nested Polymerase Chain Reaction and Loop-Mediated Isothermal Amplification Technique

By
Enoch Aninagyei BSc. (Hons)

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DECLARATION

I declare that this is my original work, carried out at the Noguchi Memorial Institute for Medical Research, Legon-Accra and Ga West Municipal Hospital Laboratory, Amasaman-Accra, has not been submitted for the award of degree in any other University or for any other award.

ENOCH ANINAGYEI
Student

Sign …… Date……

DR ALEXANDER YAW DEBRAH
Supervisor

Sign …… Date……

DR ANTHONY ABLORDEY
External Supervisor
Noguchi Memorial Institute for Medical Research, Legon-Accra

Sign …… Date……

DR THEOPHILUS B. KWOFIE
Head of Department

Sign …… Date……

DEDICATION

What shall I render unto the LORD for all his benefits toward me?
I will take the cup of salvation, and call upon the name of the LORD.
I will pay my vows unto the LORD now in the presence of all his people.
(Psalm 116: 12-14 KJV)

This thesis is dedicated to my dear wife, Yaa, my daughter, Nana Yaa, and son, Albert.

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God richly bless you all, Amen.

ABSTRACT

Buruli ulcer (BU) is a chronic skin disease caused by *Mycobacterium ulcerans*. The disease is often diagnosed clinically at peripheral health centres. World Health Organization has recommended that clinically suspected cases be confirmed in the laboratory. This study was designed to determine the sensitivity of loop-mediated isothermal amplification (LAMP) molecular assay, the Ziehl-Neelson (ZN) microscopy and ZN modifications as techniques for laboratory diagnosis of Buruli ulcer disease (BUD) and the field applicability of LAMP molecular assay. Clinical specimens for the study were collected from five (5) BU treatment centres in Ghana. In all, 141 clinical specimens were collected. The specimens were collected from 93 (66.0%) males and 48 (34.0%) females (p=0.0847) (aged 2-86 years; mean age 33.6 years and modal age 9 years); 103 (73.0%) swab specimens and 38 (27.0) aspirates were collected. Category I, II and III lesions were 64 (45.4%), 24 (17.0%) and 53 (37.6%) respectively. Dried smears from direct clinical specimens, specimen cellular suspensions and smears from chemically digested smears were examined with ZN technique. Suspected lesions were confirmed with dry-reagent based *IS2404* nested PCR, first run *IS2404* PCR and BULAMP molecular assay. The detection limits of the BU-LAMP, *IS2404* nested PCR and the microscopic techniques were determined. The best molecular method with the highest positivity rate was nested *IS2404* PCR 122 (86.5%) followed by BU-LAMP assay of 111 (78.7%). Kappa analysis indicated a good agreement between *IS2404* nested PCR and BU-LAMP (κ=0.72). The sensitivity of the *IS2404* nested PCR and BU-LAMP molecular assays was 30 and 3 copies of insertion sequences respectively. Direct smearing of cellular suspension had a relative higher positivity rate of 46.1%. Centrifugation and sedimentation of the cellular specimen suspension with phenol ammonium sulphate solution was the best microscopy protocol. They had positivity rate of 73 (51.7%), mean AFB yield per 100 high power field of 23.7 (95% CI, 21.1-26) and 75 (53.2%), mean AFB yield per high power field of 30.3, (95% CI, 27.0-33.8) respectively. The respective detection limits of these protocols were 16 and 4 AFBs in 1 ml of suspension. The sensitivities of the techniques were BU-LAMP molecular assay (91.5%), first run *IS2404* PCR (85.2%), sedimentation with phenol ammonium sulphate (61.5%), centrifugation with phenol ammonium sulphate (59.8%), sedimentation with phosphate buffered saline (55.7%), centrifugation with phosphate buffered saline (54.1%), direct smear microscopy using specimen cellular suspension (53.3%), sedimentation with 3.5% sodium hypochlorite (51.6%), centrifugation with 3.5% sodium hypochlorite (50.0%) and direct specimen smear (41.8%). The specificity and the positive predictive values for the techniques were 100.0%. The agreement between the *M. ulcerans* DNA detection ability of *IS2404* nested PCR and BU-LAMP from swab specimens and fine needle aspirates were perfect; κ=0.89 and κ=0.93 respectively. The best direct method for detecting AFBs in swabs and aspirates was smears from specimen cellular suspension and the best modified method for detecting AFBs in swabs and aspirates were centrifugation and sedimentation with phenol ammonium sulphate. BU-LAMP assay has a great potential in diagnosing *M. ulcerans* in clinical specimen but it is recommended that the assay methodology be improved to detect DNA from clinical specimens to enable the assay be used for field studies.

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LIST OF ABBREVIATIONS

BU	-	Buruli ulcer
PCR	-	Polymerase chain reaction
DNA	-	Deoxyribonucleic acid
Taq	-	*Thermus aquaticus*
Bst	-	*Bacillus sterothermophilus*
LAMP	-	Loop-mediated isothermal amplification
AFB	-	Acid fast bacilli
BU-LAMP	-	LAMP for Buruli ulcer detection
IS2404	-	Insertion sequence 2404
BUD	-	Buruli ulcer disease
ROS	-	reactive oxygen species
L-J	-	Lowenstein-Jenson
GWMH	-	Ga West Municipal Hospital
OHC	-	Obom Health Centre
NTGH	-	Nkawie Toase Government Hospital
TGH	-	Tepa Government Hospital
PHC	-	Paakro Health Centre
NMIMR	-	Noguchi Memorial Institute for Medical Research
PBS	-	Phosphate buffered saline
NaOCl	-	Sodium hypochlorite
PAS	-	Phenol ammonium sulphate
TBE	-	Tris-buffered-EDTA

CHAPTER 1

INTRODUCTION

1.1 Background Information

Buruli ulcer is a tropical skin disease which presents with severe necrosis and chronic cutaneous infections. The disease is caused by *Mycobacterium ulcerans* (MacCallum *et al.,* 1948) and it is the third most important mycobacteriosis globally, after tuberculosis and leprosy (Einarsdottir *et al.,* 2011). However, but in Ghana Buruli ulcer is second most prevalent mycobacterium infection after tuberculosis (Amofah *et al.,* 2002). *M. ulcerans* infection leads to extensive destruction of skin and soft tissue with the formation of large ulcers usually on the legs or arms (Marston *et al.,* 1995). The disease often starts as a painless, mobile swelling in the skin called a nodule (Asiedu *et al.,* 2000). Without treatment, massive ulcers result, with the classical, undermined borders. Sometimes, bone is affected causing gross deformities (Ellen *et al.,* 2003).

The disease leaves patients with prominent scars and lifelong disability (Agbenorku *et al.,* 2012). The distinctive features of Buruli ulcer include undermining edges, white cotton wool-like appearance and thickening and darkening of the skin surrounding the lesions. The ulcers are generally painless and progressive; about 85% of the lesions are found on the limbs with the lower limb lesions twice as common as the upper limb lesions (Amofah *et al.,* 2002).

Epidemiological studies have suggested that underreporting and improper diagnoses are some of the factors that hinder the determination of the exact disease burden in endemic areas (Coloma *et al.,* 2005). Current reporting of infections is based on clinical presentation of the symptoms. This represents a challenge due to the vast number of other skin conditions that may exhibit symptoms similar to that of Buruli ulcer (Semret *et al.,* 1999). The World Health Organization has therefore directed that all clinically diagnosed or suspected cases of Buruli ulcer be confirmed. Buruli ulcer

confirmation can only be done in reference laboratories, since the current methods are not amenable to point of care centres (Portaels *et al.,* 1997). Laboratory confirmation of Buruli ulcer is complex and has evolved over the years. *M. ulcerans* stains red in the Ziehl-Neelsen staining procedure but this method has a low sensitivity (40-58.4%). Swabs taken from lesions often do not show AFB by microscopic examination (Portaels *et al.,* 1997; Yeboah-Manu *et al.,* 2011). Culturing *M. ulcerans* from clinical samples is difficult and has a low sensitivity (35-60%) (Phillips *et al.,* 2005). *M. ulcerans* is notoriously slow-growing (6-8 weeks) and culture media are frequently contaminated with other faster growing *Mycobacterium* species (Yeboah-Manu *et al.,* 2004).

PCR methods have been developed and used for Buruli ulcer diagnosis (Ross *et al.,* 1997) and has been used as a gold standard test for several comparative studies (Portaels *et al.,* 1997). The PCR molecular assay was developed to circumvent the problems of delayed diagnosis and insensitivity of standard bacterial culture for *M. ulcerans* (Ross *et al.,* 1997). Although the sensitivity of PCR is high, about 98% (Phillips *et al.,* 2005), this method is expensive and requires technical expertise in terms of DNA extraction and equipment sophistication. The highly technical and expensive nature of the techniques confines them to reference laboratories (Ross *et al.,* 1997). The technique easily gets contaminated making it not feasible for field applicability (Ross *et al.,* 1997). The turn-around-time for PCR assay is about 14-16 hours making the search for a fast but similarly sensitive and specific molecular technique for Buruli ulcer confirmation justifiable (Kuboki *et al.,* 2003). The only enzyme used in PCR, *Taq DNA polymerase*, is often inactivated by inhibitors present in biological samples, which sometimes cause problems for sensitivity and reproducibility (Al-Soud *et al.,* 2000). Thus, there is the need for simple and rapid test that can be used for early point of care diagnosis in low-resourced laboratory settings where the disease is most prevalent. This led to the development of a simple and relatively inexpensive test for *M. ulcerans* diagnosis, which could easily be applied in basic healthcare facilities, without recourse to expensive, complex and time-

consuming methods. This new method is, loop-mediated isothermal amplification (LAMP), based on a novel DNA amplification at isothermal temperatures (Notomi *et al.*, 2000).

The loop-mediated isothermal amplification (LAMP) is a molecular method, which in comparison to the PCR is cheaper, simpler and faster, taking out three disadvantages of PCR.

The LAMP is a nucleic acid amplification method that relies on auto cycling strand-displacement DNA synthesis performed with *Bst DNA polymerase*. The principal merit of this method is that no denaturation of the DNA template is required, the *Bst DNA polymerase* can be activated at isothermal temperatures to perform DNA template strand displacement and thus the LAMP reaction can be conducted at temperatures 60-65 ^{o}C (Notomi *et al.*, 2000). The LAMP technology has been developed to detect *Trypanosoma* species (Kuboki *et al*., 2003), *Cryptosporidium* species (Karanis *et al*., 2007), *Toxoplasma gondii* (Sotiriadou *et al*., 2008), *Babesia microti* (Guan *et al*., 2008), *Microsporidia* (El-Matbouli *et al*., 2006), *Taenia* species (Nkouawa et *al*., 2009), *M. tuberculosis* (Tomotada *et al*., 2003), *Plasmodium* spp (Pöschl *et al*., 2010) and rift valley fever virus (Peyrefitte *et al*., 2008).

1.2 Problem Statement

The four major diagnostic options for Buruli ulcer disease are microscopic examination which has a sensitivity of 58.4% (Yeboah-Manu *et al*., 2011), culture which has a sensitivity between 35-60% (Phillips *et al.*, 2005), polymerase chain reaction (PCR), sensitivity is around 98% (Phillips *et al.*, 2005) and histopathology is 90% sensitive (Phillips *et al.*, 2005). Among these diagnostic methods, only microscopy is applicable to be used for point of care diagnosis but has the lowest sensitivity. The rest which are relatively sensitive are not applicable in smaller laboratories where early and prompt diagnosis of the disease is needed. This study was designed to assess the

efficiency of the modified conventional BU microscopy and the applicability of the loop-mediated isothermal amplification technology in a low resourced laboratory.

1.3 Rational of the Study

Ziehl-Neelson microscopy is considered a suitable first line diagnostic test to be applied in field settings (Herbinger *et al*., 2009). The technique is fast and less demanding but has limited sensitivity (Coloma *et al.,* 2005) due to destruction of extra-cellular acid fast bacilli (AFBs) by mycolactone (Hong *et al.,* 2008). Presently there is no simple and rapid test that is appropriate for early diagnosis and use in the low-resource settings where *M. ulcerans* is most prevalent (Ablordey *et al*., 2012). For definitive species determination, molecular techniques are needed. Most endemic communities for BU diseases are poor and their laboratories cannot afford the equipment needed for the PCR technology. The process of the PCR is time-consuming providing the results with a delay to the physician. Although PCR techniques provide sensitive and reliable diagnostic results, it is not easy to exploit in the laboratories of developing countries where these diseases are endemic. Furthermore, *Taq DNA polymerase* is often inactivated by inhibitors present in biological samples, which sometimes cause problems for sensitivity and reproducibility (Monteiro *et al.,* 1997). The loop-mediated isothermal amplification (LAMP) is a molecular method, which in comparison to the PCR is cheaper, simpler and faster (Notomi *et al.,* 2000).

The Buruli ulcer microscopy involves Ziehl-Neelsen examination of stained smears to detect acid fast bacilli (AFB). This technique is only able to detect AFBs at levels of 5,000-10,000 bacilli specimen. This method can produce false negative results due to its low sensitivity. Low sensitivity of BU microscopy can lead to misdiagnosis, delay diagnosis and eventually affect disease control. Improving the sensitivity of AFB detection by microscopy is therefore an important public health

priority (Steingart *et al*., 2006). Studies have shown that the sensitivity for the detection of AFB can be improved when diagnostic specimens are concentrated into smaller volumes by either centrifugation or overnight gravitational sedimentation (Miörner *et al.*, 1996). These procedures increase the quantum of AFB per volume per field and enhance visualization by microscopy (Ängeby *et al*., 2000; Bruchfeld *et al*., 2000). Chemical agents can also be used to facilitate the release of AFB embedded in host cell matrix. This procedure can clear the viewing field and decrease the time taken to read the slides. Chemical agents such as 3.5 % sodium hypochlorite and 5% phenol in 4% ammonium sulphate have been successfully used to improve AFB detection in *Mycobacterium tuberculosis* microscopy (Miörner *et al*., 1996; Selvakumar *et al.*, 2002). Outcomes from these studies have shown improved sensitivities especially in the area of *Mycobacterium tuberculosis* studies (Van Deun *et al.*, 2000; Ängeby *et al*., 2004).

Using PCR as the gold standard technique for identification of *M. ulcerans* (Phillips *et al.*, 2009), this study was designed to assess the sensitivity and the field applicability of LAMP for the detection of *M. ulcerans* genome in clinical specimens and the efficiency of the various modifications of the conventional ZN microscopy.

1.4 Research Questions

The purpose of the study was to answer the following research questions:

1. What are the sensitivity and specificity of ZN microscopy and BU-LAMP as a laboratory diagnostic technique for BU disease?
2. Which of the diagnostic techniques (ZN, PCR and LAMP) will be sensitive and specific for the various clinical specimens and categories of the BU disease?
3. Can the digestion of clinical specimen suspension with sodium hypochlorite and phenol ammonia sulphate improve the chances of detecting AFBs by ZN microscopy?

4. Is the diagnostic efficiency of BU-LAMP better than IS2404 nested PCR for the diagnosis of Buruli ulcer disease?
5. Is the dry-reagent-based nested *IS2404* PCR more sensitive than first run *IS2404* PCR?

1.5 Aim of the Study

The aim of this study was to determine the sensitivity of BU- loop-mediated isothermal amplification (LAMP) molecular assay, the Ziehl-Neelson (ZN) microscopy and ZN modifications as diagnostic methods for *Mycobacterium ulcerans* infections.

1.6 Specific Objectives of the Study

The following were the specific objectives of the study:

1. To confirm suspected *Mycobacterium ulcerans* infections lesions and compare the positivity rate of the dry-reagent-based first run *IS2404* PCR, dry-reagent-based *IS2404* nested PCR and loop-mediated isothermal amplification molecular assay on the purified *Mycobacterium ulcerans* DNA extract.
2. To determine the minimum detection limits of BU-LAMP assays and *IS2404* nested PCR and the various microscopy detection techniques.
3. To assess the field applicability of the loop-mediated isothermal amplification in the diagnosis of Buruli ulcer disease.
4. To determine the diagnostic sensitivity and specificity of the conventional Ziehl-Neelson microscopy, its modifications, first run *IS2404* polymerase chain reaction and the BULAMP molecular technology as a laboratory diagnostic technique for diagnosing Buruli ulcer disease.

CHAPTER 2

LITERATURE REVIEW

2.1 Characteristics and nature of Buruli ulcer

Buruli ulcer disease (BUD) is caused by *Mycobacterium ulcerans* (MacCallum *et al.*, 1948). The disease presents as a chronic ulcerative skin infection with extensive tissue damage in the absence of an acute inflammatory cells (George *et al*., 2000). The disease transmission could be linked to the environment whilst water striders are more susceptible (Wilson *et al*., 2011). A major advance in understanding transmission occurred with the detection of *M. ulcerans* DNA in predaceous aquatic insects (*Naucoridae* and *Belostomatidae*), leading to the hypothesis that insects may be involved in the transmission of the infection (Portaels *et al*., 1999). After *M. ulcerans* inoculation, cutaneous lesions appear as painless lesion and mostly ignored by patients (Asiedu *et al*., 2000). The characteristic loss of pain associated with BU lesions is attributable to the diffusion of mycolactone, which is known to directly damage sensory nerves (Junichiro *et al*., 2008). The production of mycolactone is responsible for the cytotoxic effect on skin tissues (Sarfo *et al*., 2011). Complications may occur, especially super-infections and more rarely bone involvement responsible for osteomyelitis. The prognosis is usually functional with sometimes severe sequels and skin and tendinous retraction as well as amputation are frequent (Junichiro *et al*., 2008). The disease often remains ignored and undiagnosed, leading to bad clinical presentations and consequences (Ofori-Adjei, 2011). The *M. ulcerans* disease is a progression from skin nodules with abundant extracellular acid fast bacilli (AFBs), extensive necrosis and little or absent of inflammatory response, through to ulceration with the histological hallmarks of lack of AFBs. The accumulation of mycolactone may cause lysis of macrophages and paralyze the cellular functions of infiltrating lymphocytes or macrophages. This localized immunosuppression may contribute to delaying an early systemic immune response to mycobacterial antigen (Stanford *et al*., 1975). The cytotoxicity of this mycolactone leads to progressive destruction of the inflammatory infiltrates by

post-apoptotic secondary necrosis, generating necrotic acellular areas with extracellular bacilli released by the lysis of infected phagocytes. The lack of inflammatory infiltrates in the extensive areas of necrosis seen in advanced infections results from the destruction of continuously produced inflammatory

infiltrates (Stanford *et al*., 1975).

2.2 *Mycobacterium ulcerans* –The etiological agent of Buruli ulcer disease

Mycobacterium ulcerans is the aetiological agent of Buruli ulcer disease. It is classified as a member of the phylum *Actinobacteria*, order *Actinomycetales*, in the suborder *Corynebacteriaceae* and the genus *Mycobacterium* (Connor and Lunn, 1965). Structurally, *M. ulcerans* has a typical *Mycobacterium* cell structure made up of a tightly wound nuclear structure with a high Guanine+Cytosine (G+C) DNA content of 65% (George *et al*., 1999; Siegmund *et al*., 2005). The cell cytoplasm is enclosed by the plasma membrane and the peptidoglycan layer which is bordered on the exterior by layers of complex fatty acids and lipids including mycolic acids from which the mycolactone, a toxin that mediates pathogenesis is derived (Adusumilli *et al*., 2005). The lipid rich layer of the cell wall makes it waxy. This makes it relatively impervious to hydrophilic compounds. It also makes it impermeable to most disinfectants except phenol compounds. *Mycobacterium* is generally termed 'acid fast' because, they cannot be decolorized by acids or acid/alcohol once stained. The waxy cell wall makes them clumpy in suspension and in culture. This contributes to the characteristically slow growth rate, with a generation period of 20 hours (Baron, 1994). The colonies of *M. ulcerans* range from colourless to pale yellow. The colonies have a dull texture and may be convex or flat, and not more than 3mm in diameter (Barksdale *et al*., 1977). Biochemically, *M. ulcerans* has moderate catalase activity (Collins *et al*., 1997).

2.3 Mycolactone- The virulence factor for *M. ulcerans*

Mycolactone is composed of a 12-membered macrolactone to which two polyketide-derived highly unsaturated acyl side chains are attached as shown in Figure 2.1

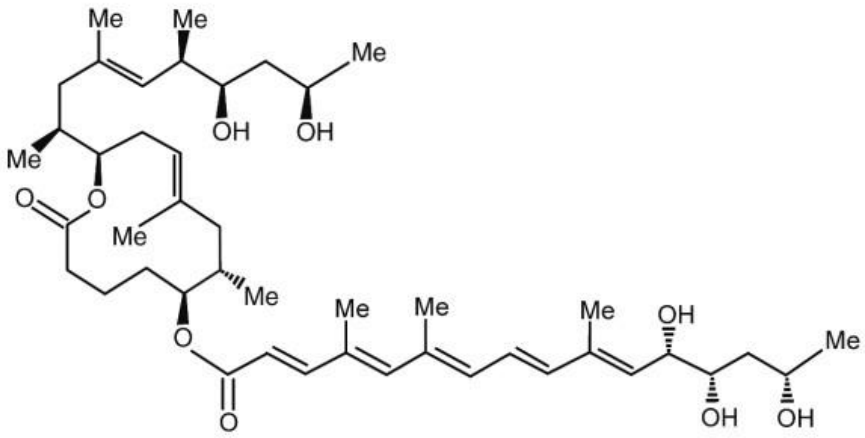


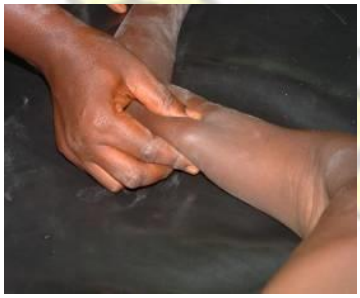
Figure 2.1: Molecular structure of mycolactone *(Source: Dr. Fred Sarfo, KATH-Kumasi)*

The initial molecule reported by George *et al* (1999) was identified on thin layer chromatography as a light yellow and ultraviolet-active lipid band (George *et al*., 1999). Mass spectrometric analysis of the toxin showed the molecular formula to be $C_{44}H_{70}0_9Na$ (molecular weight-765 g/mol). Serial dilutions of mycolactone up to 25 pg/ml when added to culture of fibroblasts in a microtitre plate led to rounding up of cells within 24 hours and detachment from the plate by 48 hours confirming the cytopathic effect of mycolactone (George *et al*., 1999). Injection of purified mycolactone intradermally into guinea pigs resulted in the appearance of a dark, necrotic and open ulcer (George *et al*., 1999). In murine study, histopathological examination of the ulcerative lesion produced by injection of mycolactone revealed it to be closely identical to that produced by inoculation of *M. ulcerans*. It was observed that at the site of mycolactone injection, there was focal necrosis extending through the dermis and adipose tissue into the muscle with few polymorphonuclear neutrophils in spite of extensive tissue destruction (George *et al*., 1999). There are variations in the type of mycolactone produced depending on the origin of strains of *M. ulcerans*. Pathogenic human

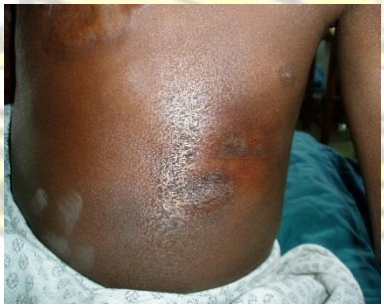
M. ulcerans strains from Africa, Australia and China predominantly produced mycolactone A/B, C and D respectively (Mve-Obiang *et al.*, 2003). *Mycobacterium liflandii*, a pathogen of frogs produces mycolactone E (Fidanze *et al*., 2001) and the fish pathogens (*Mycobacterium pseudoshottsii* and *Mycobacterium marimum*) produce mycolactone F (Song *et al*., 2002). The most cytotoxic congener of mycolactone is the mycolactone A/B produced by the African strains (Mve-Obiang *et al*., 2003).

2.4 Buruli ulcer disease clinical case definition

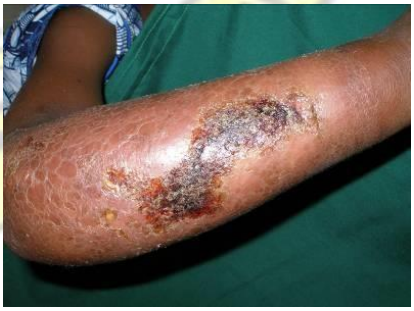
Clinically, an ulcer is said to be caused by *M. ulcerans* if the lesion has a prominent undermining edges, contains a whitish cotton wool-like appearance and thickening and darkening of the skin surrounding the lesion. The early and active form of Buruli ulcer is characterized by nonulcerative forms, which presents as papules, nodules, plaques and oedema (figure 2.2) and the late ulcerative forms (figure 2.3) present as open ulcers (Amofah *et al*., 2002). The inactive form of the disease is characterized by evidence of previous infection with a depressed stellate scar (Amofah *et al*., 2002). Diagnostically, a lesion is defined as Buruli ulcer if any one of the following techniques are positive on analysis: acid-fast bacilli (AFB) in a smear stained by either Ziehl-Neelsen (Herbinger *et al*., 2010), culture on Lowenson-Jenson (L-J) medium (Bar *et al.*, 1998), histopathology (Phillips *et al*., 2005) and *IS2404 M. ulcerans* DNA detection by PCR
(Portaels *et al*., 2001)



A nodule on the upper arm

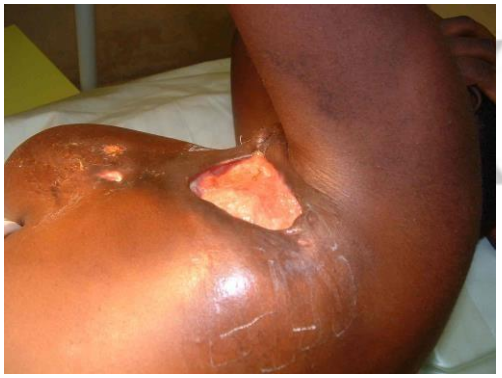


A plaque at the back

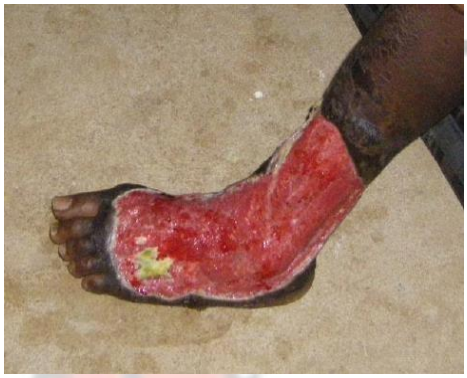


An eodema on the upper arm

Figure 2.2: Non-ulcerative forms of BU disease (*Source: Dr Albert Paintsil, Plastic Surgeon, Regenerative and Plastic Surgery, KBTH-Accra)*



Non-ulcerative lesion in the armpit

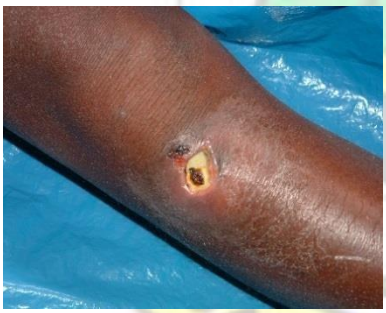


Non-ulcerative lesion on the leg & foot

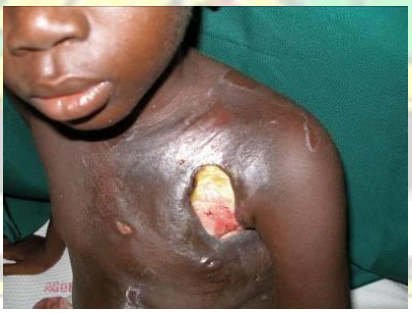
Figure 2.3: Ulcerative forms of BU disease *(Source: Dr Richard Phillips, Dept. of Medicine, KATH-Kumasi)*

2.5 Categories of BU lesions

There are three categories of BU lesions; category (CAT) 1 is a single lesion <5 cm in diameter, CAT 2 is a single lesion between 5–15 cm diameter and CAT 3 is either a single lesion more than 15 cm diameter or multiple lesions at critical sites such as the eye, genitals, breast, joints and others may be osteomyelitis (Agbenorku *et al*., 2012).



Category I (lesion < 5cm)



Category II (lesion 5-15 cm)

Category III (lesion > 15 cm)

Figure 2.4: Categories of BU Lesions *(Source: Dr Richard Phillips, Dept. of Medicine, KATHKumasi)*

2.6 Transmission and epidemiology of Buruli ulcer disease

The route of transmission of Buruli ulcer remains unknown (Johnson *et al*., 2005; Wallace *et al*., 2010). One proposition is that *M. ulcerans* enters through a break in the skin, through insect bite, cut or wound (Marsollier *et al*., 2002; Portaels *et al*., 1999). Many cases are found in poor, rural farming communities. Children under the age of 15 years are disproportionately affected. However, adults are also susceptible. Recent evidence suggests that aquatic bugs belonging to the genus *Naucoris* and *Diplonychus* may be involved in the transmission of the infection (Marsollier *et al*., 2002). Lavender *et al* (2001) reported on the possible transmission of the Buruli ulcer infection through mosquitoes (Lavender *et al*., 2011). Epidemiological evidence has not clearly supported person-to-person transmission; however, Muelder *et al* (1990) found that some patients had relatives who had also had the disease and cautioned against the dismissal of person-to-person transmission (Muelder *et al*., 1990).

2.7 Pathogenesis of *M. ulcerans* infections

The pathology of BU lesions is associated with the local production of a diffusible substance, mycolactone (Hong *et al*., 2008). Despite the extensive necrosis and tissue damage, the lesions are painless (Junichiro *et al.*, 2008). This absence of pain which is as a result of the diffusion of mycolactone into the surrounding tissues prevents patients from seeking early treatment and as a result, many patients experience severe sequelae, including limb amputation. The accumulation of toxin cause destruction of the skin tissues exposing the adipose tissues (Stanford *et al.*, 1975). Necrosis of subcutaneous tissues and dermal collagen accompanied by minimal inflammation are considered the most reliable histopathologic features for the diagnosis of Buruli ulcer disease (Connor *et al.*, 1966; Clancey *et al.*, 1961). Buruli ulcer is associated with necrosis of both the subcutis and dermal collagen and necrosis found in infected cases has been attributed to mycolactone that is produced by *M. ulcerans* and acts as an extracellular toxin (Krieg *et al.*, 1974;

George *et al*., 1999). Several authors have established that during the preulcerative stage and early in the ulcerative stage, the coagulative necrosis forms a nidus where calcifications and AFB colonies are easily visualized (Connor *et al.,* 1966; Clancey *et al.,* 1961; Krieg *et al.,* 1974). However, when the ulcer starts healing, and granulation tissue, fibrosis and granulomatous inflammation are present (Connor *et al.*, 1966).

2.8 Buruli ulcer case situation in Ghana

Ghana is the second most endemic country after Cote d'Ivoire globally (Renzaho *et al*., 2007). The first cases of Buruli ulcer in Ghana were reported in 1971 in the Greater Accra Region. The foci of the disease were along the Densu River and its tributaries. Later in 1989, additional cases were described in the Asante Akim North District of Ashanti Region (van der Werf *et al*., 1989). An active BU case search was done by Amofah *et al* (2000). Report from the exercise indicated that BU cases were identified in 90 of Ghana's then 110 districts. The most endemic districts were Ga West, Ga South, Akwapim South, Upper Denkyira East, Suhum Kraboa Coaltar, Amansie West, Amansie Central, Ahafo Ano North, Asante Akim North, Atwima Nwabiagya and Ahanta West (Amofah *et al*., 2002). Amansie West had the highest rate (prevalence 150.8 per 100,000), followed by Asante Akim North (prevalence 131.5 per 100,000) and Upper Denkyira (prevalence 114.7 per 100,000) and the Ga Districts (87.7 per 100,000). The estimated national prevalence in 2002 was 20.7 per 100,000 (Amofah *et al*., 2002).

2.9 Diagnosis of Buruli ulcer

Laboratory tests available for diagnosing BU include microscopy (ZN and fluorescent staining techniques), culture, serology and molecular assays (Phillips *et al*., 2005; Wakai *et al*., 2011; Ruf *et al*., 2011).

2.9.1 Ziehl-Neelson in Buruli ulcer microscopy

ZN microscopy is one of the techniques for diagnosing BU in the laboratory (Eddyani *et al*., 2009). The technique is done directly on swabs, aspirates, punch biopsies and surgical biopsies (Beissner *et al*., 2010). The sensitivity and specificity of ZN microscopy with concentrated specimens is 58.4% and 95.7% respectively (Yeboah-Manu *et al*., 2011). For non-ulceratve lesions, the sensitivity of ZN microscopy for FNA and punch biopsy samples is 58.3% and 55.6% respectively. For ulcerative lesions, the sensitivity of ZN microscopy for swabs, FNA, punch biopsies and surgically excised specimens is 46.4%, 22.2%, 37.5% and 20.0% respectively (Herbinger *et al*., 2010). This data indicates that the sensitivity of the ZN microscopy is higher for non-ulcerative lesions than ulcerative lesions. This is because the bacilli density reduces as the lesion progresses (Aninagyei *et al*, unpublished data). Swabs of lesions often do not show acid-fast bacilli (AFB) by microscopic (Stienstra *et al*., 2003).

2.9.1.1 Modification of the ZN technique

Microscopy for the detection of AFB can only detect bacteria load of more than 5,000-10,000 bacteria per milliliter of specimen suspension. Any bacteria load less than 5000 bacteria/ml of specimen suspension will be false negative by direct microscopy. This makes it relatively low in sensitivity in comparison to culture. Culture can detect AFB levels of 10-100 viable bacteria per millilitre of sample. Microscopy as a diagnostic method lacks specificity as it is limited in differentiating AFB from different *Mycobacterium* species (Zumla and Grange, 2002). Outcomes from studies indicate that the presence of debris and host protein material, found in diagnostic specimens, hampers the effective detection of AFB by microscopy. Lysing or digestion procedures have been adapted, by several studies to overcome this challenge (Ängeby *et al.*, 2000; Daley *et al.*, 2009).

2.9.1.2 Sodium hypochlorite lysing procedures to enhance AFB detection

Sodium hypochlorite or household bleach is a chemical compound with the formula NaOCl. It is a disinfectant with cleaning and bleaching properties. Its bleaching activity is based on its oxidizing property. This inherent property has been exploited, in the area of Mycobacteriology as digestant and disinfectant for processing of diagnostic specimens (Van Deun *et al*., 2000; Cattamanchi *et al*., 2010). Bleach improves concentration of sputum samples, especially when the sputum is processed by the overnight sedimentation method, where it is considered as a substitute to centrifugation (Yassin *et al.,* 2003). Others have attributed the improvement of sensitivity to the property of bleach to digest the debris including mucous, salivary particles and cells and help easily identify the bacteria in a much clearer field (Saxena *et al.*, 2001). Some researchers have tried the bleach method on extra-pulmonary tuberculosis. Samples such as abscess fluids, lymph node aspirates, body fluids or skin scrapes and found the method improved sensitivity (Khubnani and Munjal, 2005). The limits of the bleach method have been tested considerably over the last decade. It has proved to be an effective method to improve the sensitivity of case detection. It is a safe, cheap, applicable, easy to use and effective method applicable even in patients with low bacillary load. The sedimentation method can be used in resource poor settings to improve sputum smear sensitivity considerably (Srikanth *et al.,* 2009).

2.9.1.3 Phenol ammonium sulphate lysing procedures to enhance AFB detection

Phenol ammonium sulphate (PAS) is a chemical compound with similar properties to sodium hypochlorite. It is being used for the processing of diagnostic specimens for the detection of AFB. Phenol ammonium sulphate is a solution made from phenol and ammonium sulphate compounds (Karl-Heinz, 2012). The phenol ammonium sulphate (PAS) method of sedimentation/precipitation of sputum involves ZN staining of precipitates/floccules formed in chemically treated sputum samples. Phenol or carbolic acid is known to promptly kill the

mycobacteria. It also precipitates proteins in low concentration without decreasing the AFB counts and fixes the smear firmly on the slide (Chedore *et al.,* 2002). Ammonium sulphate prevents the formation of hydrogen bonds of proteins with water and facilitates the interaction of proteins with each other to form aggregates. This causes the mucus and other proteins in the sputum to precipitate and later sediment by the ‘‘salting out’’ phenomenon (Jakoby, 1971). The precipitate/sediment obtained may be used for smear preparation (Singhal *et al.,* 2013).

2.9.2 The fluorescence microscopy

The identification of AFB with rhodamine-auramine is due to the affinity of the mycolic acid in its cell wall for the fluorochromes. The fluorochromes bind to the cell wall and fluorescence bright yellow against a greenish background is diagnostic (Mccarter and Robinson, 1994). Auramine O and Auramine-Rhodamine fluorescent stain have been used to diagnose BU (Billie and Bereneice, 2000). Fluorescent microscopic method is more rapid (less than 2 minutes), cost effective, efficient and permits consistent AFB quantitation. The use of potassium permanganate as a quencher reduces background staining (Hendry *et al*., 2009). The sensitivity and specificity of Auramine O for the diagnosis of Mycobacteriosis is 71.8% and 99.2% respectively (Hooja *et al*., 2011). The sensitivity of Auramine O and Rhodamine B fluorescent technique is 88.5%

(Tarhan *et al*., 2003). Rhodamine-auramine stained smears are scanned under low magnification (x200 or x400) than ZN smears (x1000), thus permitting a larger area of the smear to be examined (Huebner *et al*., 1993). The fluorescent microscopy is more sensitive than the ZN technique (Githui *et al*., 1993).

2.9.3 Culture of Buruli ulcer lesions

Culture of clinical lesions are very specific for detection of viable *M. ulcerans*, however it is slow and low sensitive (King *et al*., 2001). Primary cultures from clinical specimens are usually positive within 6-12 weeks of incubation at 29-33 °C in an atmospheric oxygen tension of less than 2.5 kPa

and within a pH range of 5.4–7.4. Much longer incubation times of up to 9 months have been observed (Buntine and Crofts, 2001). In vitro culture is important for the investigation of BU drug susceptibilities, to give information on the viability of the bacilli in the lesion and for molecular epidemiology of the disease as positive cultured media always contain sufficient numbers of bacilli for fingerprinting analysis (Deun, 2004). BU samples to be cultured are decontaminated with hydrochloric acid followed by neutralization of the suspension with sodium hydroxide (Palomino and Portaels, 1998). The resultant pellets obtained after centrifugation are suspended in distilled water and is then inoculated into Lowenstein-Jensen (LJ) medium (Portaels *et al*., 1997). The culture colonies are identified to the species level on the basis of their phenotypic characteristics such as growth rate, pigmentation, photo reactivity, enzymatic characteristics, morphology and growth in the presence of some inhibitors (LevyFrebault and Portaels, 1992). LJ media that do not show growth after 12 months are considered negative (Eddyani *et al*., 2008).

2.9.5 Molecular diagnosis of Buruli ulcer

A number of molecular techniques have been developed to confirm *M. ulcerans* in clinical and environmental samples. The molecular tools for identification of *M. ulcerans* are polymerase chain reaction (Ross *et al*., 1997), loop-mediated isothermal amplification (de-Souza *et al*.,

2012), DNA sequencing (Portaels *et al*., 1996), restriction fragments length polymorphism (Stinear *et al*., 1999) and amplified fragment length polymorphism (Vos *et al*., 1995). But the commonly used technique is the *IS2404* PCR and recently the BU-LAMP technique.

2.9.5.1 Polymerase chain reaction for *M. ulcerans* identification

PCR methods that have been developed to detect *M. ulcerans* DNA in clinical and environmental samples are based on the detection of 16S rRNA gene (Portaels *et al.*, 1997), hsp65 gene (Roberts *et al.*, 1997) and the *insertion sequence 2404* (Ross *et al*., 1997). PCR based on 16S rRNA gene

and hsp65 gene target genes with low copy numbers and high sequence conservation among all mycobacteria. They utilize a genus-specific first-round PCR followed by either a second-round PCR or high-stringency probe hybridization conditions to ensure sensitivity and specificity. In these situations the potential for false positives caused by chimera formation or primer cross-reactivity is likely to be high (Zhao *et al.*, 1993). The genus-specific 65-kDa antigen demonstrates a high degree of homology between different mycobacterial species and may not be an ideal target for specific amplification (Hance *et al*., 1989). The utility of 23S rRNA sequencing has been demonstrated in a case of suspected *M. ulcerans* infection in which a single nucleotide difference was detected between *M. ulcerans* and *M. marinum* (Hofer *et al*., 1993). However, a recent report indicated variability among the 16S rRNA sequences of a number of different *M. ulcerans* strains (Portaels *et al*., 1996). The *IS2404* is specific only to *M. ulcerans* and analysis based on this insertion sequence has been shown to be effective, sensitive and reliable to identify the organism from all sources (Ross *et al*., 1997). Several PCR assays have been applied for case confirmation of BU in endemic countries and *IS2404* PCR has been shown by most mycobacteriologists to be the most sensitive and specific method for the laboratory confirmation of Buruli ulcer disease (Phillips *et al*., 2009; Herbinger *et al*., 2009). The *IS2404* PCR can be used to detect *M. ulcerans* DNA in fresh and paraffin-embedded tissues and in culture isolates (Ross *et al*., 1997). The overall sensitivity of the *IS2404* PCR is 98.0% (Phillips *et al*., 2005). However, it is 86.0% sensitive on FNA samples, 98.0% sensitive on punch biopsies (Phillips *et al*., 2009) and 90.0% for swab samples (Herbinger *et al.*, 2009).

2.10 The Loop-mediated isothermal amplification (LAMP)

2.10.1 The LAMP technique

The LAMP is a nucleic acid amplification method that relies on autocycling strand-displacement

DNA synthesis performed with *Bst DNA polymerase*. The *Bst DNA polymerase* is a thermostable DNA polymerase obtained from *Bacillus sterothermophilus* and exploits its ability to efficiently extend all of the template-primer complex, even at low substrate concentrations (Notomi *et al*., 2000). The enzyme can significantly reduce the cost associated with DNA polymerase and the amount of template and time required to perform the enzymatic sequencing reactions. *Bst DNA polymerase* has the ability to efficiently sequence nanogram amounts of DNA template (Mead *et al.,* 1991). The principal merit of this method is that no denaturation of the DNA template is required and the LAMP reaction can be conducted under isothermal conditions (Notomi *et al*., 2000). The invention of the LAMP method has given new impetus towards development of point of care diagnostic tests based on amplification of pathogen DNA (Notomi *et al.,* 2000). The LAMP technology amplifies DNA with high sensitivity relying on an enzyme with strand displacement activity under isothermal conditions (Mori *et al.,* 2001). Since LAMP is done under isothermal conditions (60 to 65°C), simple incubators, such as a water bath or a block heater, are sufficient for DNA amplification (Notomi *et al.,* 2000). The technology is highly specific because it uses four to six specially designed primers recognizing six to eight regions of the target DNA sequence. The auto-cycling reactions lead to accumulation of a large amount of the target DNA and other reaction by-products, such as magnesium pyrophosphate, that allow rapid detection using varied formats (Mori *et al.,* 2001). Hence, LAMP is a highly sensitive and specific DNA amplification tool suitable for the rapid diagnosis of infectious diseases, including parasitic diseases (Kuboki *et al.,* 2003) in a well-equipped laboratory and small-scale clinical laboratories and is expected to be highly useful and feasible in the field (Poon *et al.,* 2006).

2.10.2 General principles of LAMP technology

The LAMP methodology employs a *Bst DNA polymerase* and a set of 4-6 primer sequence that recognise a total of six distinct sequences on the DNA (Notomi *et al.,* 2000). The inner primer

containing sequences of the sense and the anti-sense strands of the target DNA initiates LAMP. Following strand displacement DNA synthesis primed by an outer primer releases a single stranded DNA (ssDNA). This serves as template for DNA synthesis primed by the second inner and outer primers that hybridize to the other end of the target, which produces a stem-loop DNA structure. In subsequent LAMP cycling one inner primer hybridize to the loop on the product and initiates displacement DNA synthesis yielding the original stem-loop DNA and a new stem-loop DNA with a stem twice long (Notomi *et al.,* 2000). The cycling reaction continues with accumulation of 10^9 copies of target in less than an hour. The final products are stem-loop DNAs with several inverted repeats of the target and cauliflower like structure with multiple loop formed by annealing between alternatively inverted repeats of the target in the same strand. Because LAMP recognizes the target by six distinct sequences initially and by four distinct sequences afterwards it is expected to amplify the target sequence with high selectivity (Notomi *et al.*, 2000).

2.10.3 Structure of target DNA and primer design

Six distinct regions are designated on the target DNA, labelled F3, F2 and F1 (5'---->3' at the beginning of the *IS2404*) and B1c, B2c and B3 (3'---->5' at the end of the IS2404). c represents a complementary sequence. The F3, F2 and F1 and their complementary sequences are inner sequences of the target and the B3, B2 and B1 and their complementary sequences are outer sequences of the target (Tomita *et al.,* 2008). The F2c and the B2 sequences are typically 23-24 nucleotides long. F2c and B2 are each 40 nucleotides inside away from F1c and B1 respectively. The B3 and the B3c are outside sequences 40 nucleotides away from F2 and F2c respectively. F3 and F3c are 17-21 nucleotides long (Notomi *et al.,* 2000). On the double stranded DNA, F3, F2 and F1 are complementary sequences to F3c, F2c and F1c respectively. Similarly, B1, B2 and B3 are complementary sequences to B1c, B2c and B3c respectively (Tomita *et al.,* 2008).

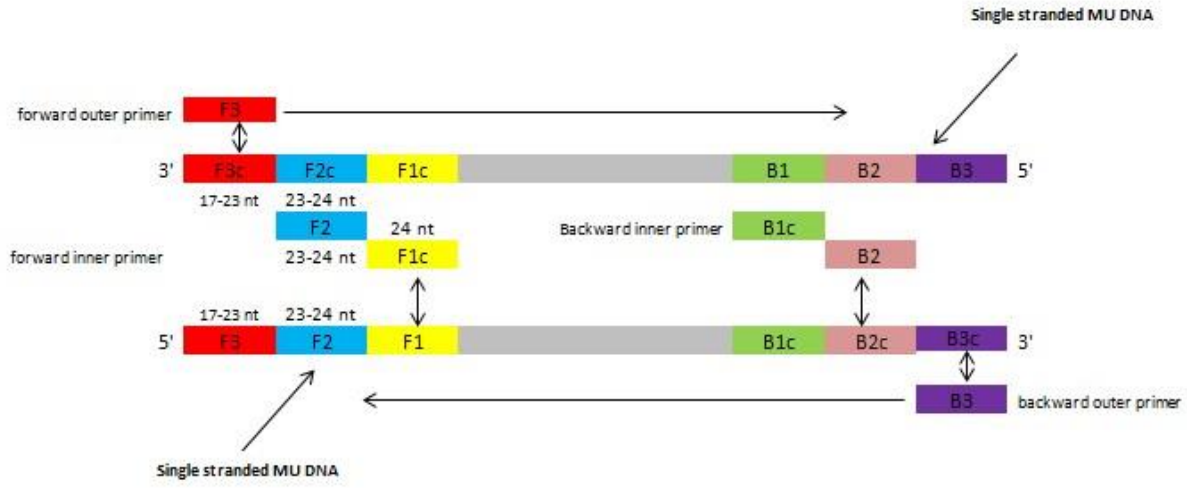


Figure 2.5: Simplfied structure of the M. ulcerans DNA and primer design. Source: Designed by student but adapted from Tomita *et al.,* (2008)

Given structure in figure 2.5, the forward inner primer (FIP) contains sequences to F1c separated by TTTT and sequences complementary to F2c (F2). The backward inner primers (BIP) contain sequences complementary to B1 (B1c), TTTT spacer and sequences to B2. The loop primers, forward loop (LF) contains primers complementary to the forward sequences on the loop structures. The backward loop (LB) also contains primers complementary to the backward sequences on the loop structures (Notomi *et al.,* 2000).

2.10.4 Molecular mechanism of the LAMP assay

LAMP methodology relies on auto-cycling strand displacement DNA synthesis that is performed by *Bst DNA polymerase* with high strand displacement activity and a set of three specially designed inner and three outer primers. In the initial steps of the LAMP reaction, four primers (two inner and two outer) are used, but later during the cycling reaction only the inner primers are used for strand displacement DNA synthesis. DNA synthesis initiated from FIP proceeds as follows: The F2 region on the FIP primer anneals to the F2c region on the target DNA and initiates the elongation. DNA amplification proceeds with BIP in a similar manner. The F3 primer anneals to the F3c region on

the target DNA and strand displacement DNA synthesis takes place. The DNA strand elongated from FIP is replaced and released. The released single strand forms a loop structure at its 5' end. DNA synthesis proceeds with the single-strand DNA as the template and BIP and B3 primer, in the same manner as described earlier which possesses the loop structure at both ends (dumbbell like structure). Self-primed DNA synthesis is initiated from the 3' end F1 region and the elongation starts from FIP annealing to the single strand of the F2c region in the loop structure (Tomita *et al*., 2008). To initiate LAMP cycling, FIP hybridizes to the loop in the stem–loop DNA and primes strand displacement DNA synthesis, generating as an intermediate one gapped stem–loop DNA with an additional inverted copy of the target sequence in the stem and a loop formed at the opposite end via the BIP sequence (Notomi *et al*., 2000). Subsequent self-primed strand displacement DNA synthesis yields one complementary structure of the original stem–loop DNA (structure 10) and one gap repaired stem–loop DNA with a stem elongated to twice as long (double copies of the target sequence) and a loop at the opposite end. Both these products then serve as template for a BIP-primed strand displacement reaction in the subsequent cycles. Thus, in LAMP the target sequence is amplified 3-fold every half cycle (Notomi *et al*., 2000).

The final products are a mixture of stem–loop DNAs with various stem lengths and cauliflowerlike structures with multiple loops formed by annealing between alternately inverted repeats of the target sequence in the same strand. The use of four primers (recognition of six distinct sequences) in the initial steps of LAMP and two primers (recognition of four distinct sequences) during the subsequent steps ensures high specificity for target amplification. Moreover, in LAMP four primers (six distinct recognition sequences) are simultaneously used to initiate DNA synthesis from the original unamplified DNA to generate a stem–loop DNA for subsequent LAMP cycling, during which the target is recognized by four sequences. Therefore, target selectivity is expected to be higher than those obtained in PCR (Notomi *et al*., 2000).

The addition of loop primers, which contain sequences complementary to the single stranded loop region on the 5' end of the hairpin structure, speeds the reaction by providing a greater number of starting points for DNA synthesis. Using loop primers, amplification by 10^9 - 10^{10} times can be achieved within 15-30 minutes (Notomi *et al*., 2000).

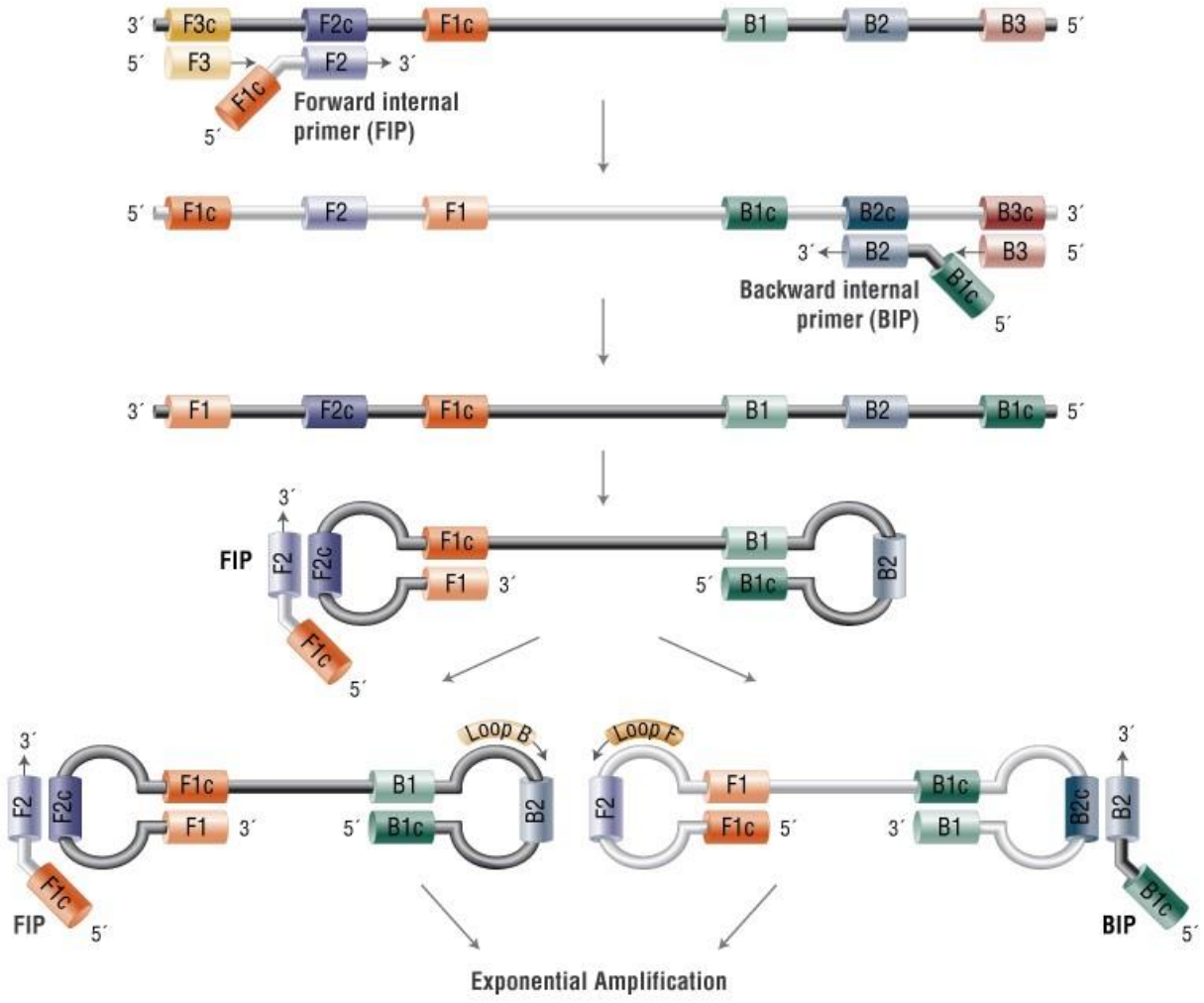


Figure 2.6 Schematic description of LAMP molecular assay (Notomi et al., 2000).

2.10.5 In Vitro LAMP reaction

The BU-LAMP reaction mixture consists of the target gene, four or six different primers, *Bst DNA polymerase* and fluorescent detection reagent. The primers are 2 outer primers (Buruli- F3 and Buruli- B3), two inner primers (Buruli-FIP and Buruli- BIP) and loop primers (Buruli-LF and Buruli- LB) (Kaneko *et al.*, 2007; de Souza *et al.*, 2012). Loop primers are not necessarily required. However, the use of loop primers shortens the amplification time by about one third (Nagamine *et al.*, 2002).

2.10.6 Detection of LAMP product

Significant progress has been made with modification of the LAMP method, but the product detection technologies have not seen similar advancement. The LAMP reaction produces large amounts of magnesium pyrophosphate (a white precipitate) and dsDNA, which allow visual inspection of results using a turbidimeter and real-time PCR machine, respectively, avoiding post DNA amplification manipulation. To achieve a white precipitate, extra Mg^{2+} is required while fluorescence dyes have a major limitation in that they can bind non-specifically to any dsDNA, such as primer-dimers, leading to erroneous result interpretation (Njiru *et al*., 2008). A higher test specificity can be achieved by targeting an internal sequence of the amplicon through incorporating fluorescent molecular beacon probe, thus minimizing non-specific signal (Dominguez *et al*., 2010) or by using a lateral flow dipstick (Njiru, 2010). The principle of the fluorescent detection of LAMP product is based on the production of large amount of pyrophosphate. Calcein included in the fluorescent kit initially binds with Mg^{2+}. When the amplification reaction proceeds, the Mg^{2+} is deprived of calcein by the generation of pyrophosphate which results in the emission of fluorescence. Free calcein binds to Mg^{2+} in the reaction mixture so that it strengthens the fluorescence emission (Tomita *et al.*, 2008).

2.10.7 BU-LAMP in BU diagnosis

De Souza *et al* (2012) developed the LAMP molecular assay as a quick and cost effective method for the diagnosis of *M. ulcerans* infections (de Souza *et al*., 2012). The LAMP technology amplifies DNA with high sensitivity under isothermal conditions by relying on the Bst DNA polymerase enzyme with strand displacement activity. The technology uses 4 to 6 specially designed primers recognizing 6 to 8 regions of the target DNA sequence and hence has a high specificity. The autocycling reactions led to accumulation of large amounts of the target

DNA and other reaction by-products such as magnesium pyrophosphate which allow rapid detection of amplicons (Mori *et al*., 2001). Amplification can be achieved using a simple incubator such as a water bath or heating block. In addition, the results can be visually inspected through a colour change or use of a chromatographic lateral flow dipstick format (Njiru, 2011). LAMP has proved a powerful tool in detection of DNA and is cheaper than other DNA-based tests (Wastling *et al*., 2010). Compared to the conventional *IS2404* PCR, LAMP is simpler and ten times more sensitive (de Souza *et al*., 2012). In order to develop a field applicable technique that offers high detection sensitivity and specificity for the diagnosis of BU, Ablordey *et al*. (2012) explored the use of the pocket warmer LAMP (pwLAMP) technique, a DNA

amplification method that uses isothermal conditions (60 ^{o}C) provided by a disposable pocket warmer (Ablordey *et al*., 2012).

2.10.8 The Versatility applications of LAMP

LAMP has the potential to be used as a simple screening assay in the field or at the point of care by clinicians. It may be a particularly useful method for infectious disease diagnosis in low and middle income countries (Poon *et al.,* 2006). Due to the specific nature of the action of LAMP primers, the amount of DNA produced is considerably higher than PCR based amplification. The corresponding

release of pyrophosphate results in visible turbidity due to precipitation, which allows easy visualization by the naked eye, especially for larger reaction volumes or via simple detection approaches for smaller volumes (Mori *et al.,* 2004). The reaction can be followed in realtime either by measuring the turbidity (Mori *et al.,* 2004) or the signals from DNA produced through fluorescent dyes that intercalate or directly label the DNA and in turn can be correlated to the number of copies initially present. Hence, LAMP can also be quantitative (Notomi *et al.,* 2000). LAMP has been observed to be less sensitive than PCR to inhibitors in complex samples such as blood, likely due to use of a different DNA polymerase (*Bst* DNA polymerase rather than *Taq* polymerase as in PCR). Several reports describe successful detection of pathogens from minimally processed samples (Curtis *et al.,* 2008; Sattabongkot *et al.,* 2014). This feature of LAMP may be useful in low-resource or field settings where a conventional DNA or RNA extraction prior to diagnostic testing may be impractical (Poon *et al.,* 2006).

2.10.9 Advantages of LAMP technology over PCR

BU-LAMP technology is less expensive in the sense that less instrumentation is required to achieve amplification at isothermal temperatures (Notomi *et al*., 2000). Results are obtained within 1 hour but in the case of *IS2404* PCR, results can take up to 6 hours (Kuboki *et al.,* 2003). The sensitivity of BU-LAMP equals or even higher than that of classical PCR targeting the same gene (Bakheit *et al.,* 2008). The technique is robust, it amplifies target DNA from partially processed or unprocessed specimen and the technique is very specific since four to six primers are used targeting six to eight DNA target regions (Kaneko *et al.,* 2007). There is ease of product detection. Large amount of dsDNA formed and magnesium pyrophosphate allow visual detection formats and amplification occurs at isothermal conditions where low heat is required, hence water bath and exothermal chemical units are sufficient (Njiru, 2012). Unlike IS 2404 PCR, BU-

LAMP is highly useful and feasible in the field (Poon *et al.*, 2006).

2.11 Alternative isothermal nucleic acid amplification methods

Nucleic acid amplification techniques are pivotal process in molecular biology and have been widely used in medical research and forensics. PCR was the first nucleic acid amplification method developed (Mullis, 1990). However, PCR has a number of limitations (Fakruddin *et al.,* 2013) and for that matter a number of alternative isothermal amplification methods have been developed (Fakruddin *et al.,* 2013). Examples of isothermal amplification techniques are LAMP, nucleic acid sequence based amplification (Guatelli *et al.,* 1990), strand displacement amplification (Walker, 1993), multiple displacement amplification (Hawkins *et al.,* 2002), rolling circle amplification (Wiltshire *et al.,* 2000), ligase chain reaction (Wu and Wallace, 1989), helicase dependent amplification (Vincent *et al.,* 2004) and ramification amplification method (Zhang *et al.,* 2001).

2.12 Treatment of Buruli ulcer disease

M. ulcerans is susceptible to several antimicrobials such as rifampin (RIF), clarithromycin (CLR), streptomycin (STR), amikacin (AMK), sparfloxacin (SPX) and clofazimine (Portaels *et al.,* 1998; Thangaraj *et al.,* 2000). Experimental studies using the mouse model demonstrated that the combination of rifampin (RIF) and an aminoglycoside was bactericidal for *M. ulcerans* (Dega *et al*., 2000). Based on these findings and subsequent studies in humans the daily administration of the streptomycin and rifampin (STR+RIF) for 2 months was recommended by the World Health Organization for the treatment of BU (WHO, 2004). Treatment with STR requires intramuscular injection which requires the use of sterile needles and syringes to avoid infection with blood borne pathogens. Therefore, the development of an entirely oral regimen is desirable (Grietens *et al.,* 2008). Almeida *et al* (2011) have suggested that an entirely oral daily regimen of rifapentine (RPT)

+ clarithromycin (CLR) may be as effective as the currently recommended combination of injected STR + oral RIF (Almeida *et al.,* 2011).

CHAPTER 3

METHODOLOGY

3.1 Profiles of the study sites

The clinical specimens were collected from five (5) Buruli ulcer treatment centres in three (3) regions in Ghana. The study sites were Ga West Municipal Hospital, Amasaman, Obom Health Centre (Greater Accra region), Nkawie-Toase Government Hospital, Tepa Government Hospital (Ashanti Region) and Paakro Health Centre (Eastern region).

3.1.1 Ga West Municipal Hospital, Amasaman

The Ga West Municipal Hospital (GWMH) is located in the capital of the Municipality. The GWMH Buruli ulcer treatment centre receives patients from Mayera, Oduman, DomeSampaman, Ofankor and Pokuase Health Centres as well as other private health facilities such as M&D Clinic (Medie), Aneeja Clinic (Tantra Hills) and St. Moses Clinic (Pokuase). Other BU cases are referred from the Eastern, Volta and Central Regions to the treatment centre (Hamzat *et al*., 2011).

3.1.2 Tepa Government Hospital

The Tepa Government Hospital is located in the Ahafo Ano North District in the Ashanti Region. It shares boundaries with three districts; Tano, Asutifi and Ahafo Ano South Districts. Ahafo Ano North District is one of the endemic Buruli ulcer districts in the country. The disease is

mostly endemic in Manfo Sub-District especially Asuhyiae zone. Tepa Government Hospital has been a Buruli ulcer research site since 2002 (Amofah *et al*., 2002).

3.1.3 Nkawie-Toase Government Hospital

Nkawie-Toase Government Hospital (NTGH) is located in the Atwima-Nwabiagya District in the Ashanti Region of Ghana. Atwima-Nwabiagya District shares boundaries with Ahafo Ano South and Atwima-Mponua Districts (to the west), Offinso District (to the North), Amansie west and Bosomtwe-Atwima–Kwanwoma Districts (to the South) and Kwabre District (to the East).

The hospital receives BU cases from Akropong, Barekese, Asuofua, Abuakwa, Toase and Maakro health centres (*Source: District Health Profile, 2013*).

3.1.4 Paakro Health Centre

Paakro health Centre is located in Paakro in the Akuapem North district in the Eastern Region in Ghana. The centre sees BU cases from mostly rural areas such as Mangoase, Dedewa-Afransu, Nsuablaso, Towoboase, Tabankro and Asuboi (Disease control unit, Paakro Health Centre).

3.1.5 Obom Health Centre

The Obom Health Centre BU treatment and research centres was set up by the STOP Buruli project of NMIMR, University of Ghana, Legon to cater for patients who find it difficult to travel to GWMH BU treatment for case management. The centres receive patients from villages and hamlets along the Densu River where the disease is more prevalent (Yeboah-Manu *et al*., 2011).

3.2 Study Design

The research work was a cross sectional study that was intended to compare the sensitivity and the specificity of direct smear microscopy, concentrated smear microscopy (conventional and

modified), polymerase chain reaction and loop-mediated isothermal amplification technology for the laboratory diagnosis of Buruli ulcer disease.

3.3.1 Inclusion criteria

Subjects included in the study were: individuals with suspected Buruli ulcer lesions that who were clinically diagnosed by experienced clinicians, individuals who consented to be enrolled on the study after obtaining detailed information on their involvement and role in the study, suspected BU cases who had agreed to provide information as required by the questionnaires administered and new patients (patients that have never being treated with antibiotics) who were suspected of having Buruli ulcer disease and whose infection status have not being confirmed by any molecular method.

3.3.2 Exclusion criteria

Subjects excluded from the study were: suspected patients with lesions but not clinically confirmed as such, clinically diagnosed BU patients who were not in agreement with the terms and conditions associated with enrolment and BU patients whose infection status has been determined and has started antibiotic treatment.

3.4 Sampling and analysis period

The specimen collection took place between November 2014 and June 2015. The night surveillance for active case search took place in December 2014 and January 2015. The microscopy aspect of the study begun after each batch of specimens have been collected. The PCR and the LAMP assays were done between April and July 2015.

3.5 Administration of questionnaire

A structured questionnaire was administered to obtain baseline demographic information from persons enrolled in the study. Information sought from persons with this questionnaire included age, sex, residential setting, occupation, etc. Data were also obtained on disease presentations including lesion type, lesion location, lesion size and others provided in the questionnaire. (See appendix 1 for sample of the questionnaire).

3.6 Night Surveillance for Buruli ulcer case search

Night surveillance for active Buruli ulcer case search was carried out in four (4) communities in the Ga West Municipality; Kojo Ashong (3rd & 4th December, 2014), Manhean (10th & 11th December, 2014), Nsakina (17th & 18th December, 2014) and Mayera and surrounding villages (14 &15th January, 2015). The team was made up of biomedical researchers from School of Public Health, University of Ghana-Legon.

3.6.1 Night surveillance

The community health officers and the disease control officers mounted a platform and showed film concerning the risk factors, pathology, clinical presentations, categories and disabilities of Buruli ulcer amidst narration of the pictures in the local languages. After the film show, an announcement was made for the community members to gather at a public place for the screening exercise the following day. The community based surveillance volunteer (CBSV) also led the team to the houses of people who exhibited the symptoms as shown in the film show but did not turn up for screening.

3.6.2 Screening of suspected Buruli ulcer patients

The screening for suspected lesions took place the following day. The clinicians examined the community members, after prior permission and consent has been sought. The screening exercise

entailed looking for nodules, plaques and edema that are suggestive of BU. Open wounds were also assessed for cardinal signs of BU.

3.6.3 Sampling of lesions

After the screening, the biomedical scientists sampled the suspected lesions. The nodules, plaques and edematous lesions were aspirated and open wounds were swabbed. A total of two

(2) swabs were taken for each ulcerative lesion and more than 15mg of cells aspirated from the pre-ulcerative stages of the disease.

3.6.4 Management of data obtained from the night surveillance

Clinical samples obtained from the night surveillance exercise were added to samples taken from the Ga West Municipal Hospital, Amasaman. Kojo Ashong, Nsakina, Manhean, Mayera and Amasaman were located in the Ga West municipality.

3.7 Clinical confirmation of Buruli ulcer disease

Suspected Buruli ulcer patients were clinically confirmed by a clinician based on defined WHO guidelines on the clinical diagnosis of Buruli ulcer. A suspected case was defined as persons manifesting with any of the following clinical signs of Buruli ulcer disease: a nodule was a firm, painless subcutaneous swelling less than 2cm in size and attached to the skin, a plaque was a firm, raised lesion irregular at the edges, with point of weakness and with a size usually greater than 10cm, an edema was painless, non-pitting swelling of an anatomical site and an ulcer as an open wound with a typical cotton-wool like basal slough with undermined edges (WHO, 2001).

3.8 Sample size estimation

Sample size for this study was determined based on achieving a 95% confidence interval of detecting the Buruli ulcer disease with significant level set at d=0.05 using the equation:

$$n = \frac{z^2\, p(1-p)}{d^2}$$

Where *n = sample size*

z = confidence interval d

= significant level

p = proportion of population with BU

1-p= proportion of population without BU

No reliable record of p was known but based on an earlier study in the study sites, p was not expected to exceed 10%. Substituting this into the equation, a minimum sample size of 138 was determined:

$$n = \frac{1.96^2\, x0.1(1-0.1)}{0.05^2} = 138$$

3.9 Materials, equipment and reagents

3.9.1 Materials and items

The major materials and items used for the study were frosted end microscope slide, staining rack, 21G-hypodermic needle, sterile swab stick, 2.0 ml sterile screw-cap tube, 0-1000 µl, 010µl, 0-20µl, 0-5µl and 0-100 µl pipette and corresponding tips, discard jar, acid washed glass beads (1.5mm diameter; Merck, Germany), eppendorf tubes (0.5ml, 1.0ml, 1.5ml, 20ml and 2.5ml) and PCR tubes.

3.9.2 Equipment

The major equipment used for the project were heating block, microscope (Zeiss, Hamburg, Germany), Applied Biosystems thermocycler (PCR machine), centrifuge (table top and eppendorf centrifuge), PCR workstation, safety cabinet, vortex mixer, mechanical shaker, electrophoresis set and UV illuminator.

3.9.3 Reagents and chemicals

The major reagents and chemicals used were carbol fuchsin stain, 20% sulphuric acid, methylene blue stain, acetone, diatomaceous earth, 70% alcohol, lysis buffer, proteinase-K, nuclease free water, phosphate-buffered saline (PBS), sodium hypochlorite, phenol ammonia sulphate, QIAamp DNA minikit, nested PCR primers (pGp1 and pGp2 for first run PCR and pGp3 and pGp4 for nested PCR), BU-LAMP DNA amplification kit, fluorescent detection kit, BU LAMP primer sequences

3.10 Sampling techniques

Fine needle aspiration (FNA) was done on the pre-ulcerative lesions and ulcerative lesions without prominent undermining edges. Samples from ulcerative lesions were taken before the wound was dressed. The un-opened skin lesions (nodules, plaques and oedematous) were disinfected with 70% alcohol before the sample was taken.

3.10.1 Swabbing from ulcerative lesions

The wound was cleaned with normal saline and the undermined edges of the wound were swabbed with a sterile swab stick. The swabbing was done in such a way that as many necrotic tissues as possible were collected. Two (2) swab samples were collected from each lesion. Two lesions that

were non-communicating were swabbed separately. The swabs were stored dried in their case at 2-8 ^{o}C.

3.10.2 Fine needle aspiration

The non-ulcerated lesion was disinfected with 70% isopropyl alcohol. The lesion was grasped and immobilized in a fixed and stable position. The needle (21G) with the syringe was inserted through the point of weakness into the center of the nodule or weaker points in case of edematous lesions. The needle was moved back and forth several times while applying and maintaining pressure. Multiple aspirations were made to aspirate enough cellular specimens. The needle was withdrawn and the content was flushed into eppendorf tube containing phosphate buffered saline. The eppendorf tubes were covered and stored at 2-8 ^{o}C.

3.11 Specimen processing and preparation

3.11.1 Preparing and processing swab specimens

A direct smear was made with one of the swab, air dried and kept in a slide rack. The slide was labeled for direct smear microscopy. The rest of the swabs were processed into specimen suspension.

3.11.2 Preparation of cellular suspension from swabs

The tip of the swabs with the necrotic cellular material were broken into a sterile BD falcon tubes with about eight (8) glass beads containing 2 ml of phosphate buffered saline (PBS). The test tube was stoppered and vortexed continuously for 1 minute or until the cotton end is free from any cellular material. The cellular suspension was poured into an eppendorf tube, covered and stored at -20^{o}C with its appropriate label. A direct smear was made with about two (2) drops of the specimen suspension, air-dried, labelled as direct specimen suspension smear and stored at room temperature in a slide rack. The specimen cellular suspensions were divided into two equal portions. One was

used for the modified ZN and the other one was used for the molecular studies; *IS2404* PCR and BU-LAMP.

3.11.3 Preparation of FNA cellular suspension

The tube containing the FNA specimen was centrifuged at 3000 rpm for a minute. The supernatant was replaced with 2 ml of PBS and vortexed. A direct smear was prepared, air-dried and labelled as direct specimen suspension smear. The rest was stored at -20^{o}C with its appropriate label.

3.11.4 Concentration of the bacilli with PBS from the specimen suspension

The frozen specimen suspension was thawed at room temperature and from which 100 µl was pipetted into an eppendorf tube already containing 100 µl of PBS. The mixture was vortexed for a minute and incubated for 15 minutes at room temperature after which 800 µl of distilled water added to the mixture. The mixture was centrifuged for 5 minutes at 3000 rpm. The supernatant was discarded and a smear prepared from the re-suspended pellets. The smear was air-dried, heat fixed and stored in a dust proof slide container (Figure 3.1).

3.11.5 The Phosphate buffered saline

The phosphate buffered saline (PBS, pH 6.8) was prepared by weighing 8.5g Sodium chloride (NaCl, BDH Chemicals Ltd, Poole, England; MW- 58.0 g/mol), 0.71g di-Sodium hydrogen phosphate (Na_2HPO_4, BDH Chemicals Ltd, Poole, England; MW- 141.96 g/mol) and 0.68g potassium di-hydrogen phosphate (KH_2PO_4, BDH Chemicals Ltd, Poole, England; MW- 174.2 g/mol). The salts were weighed and transferred into 1 litre volumetric flask containing about 500 ml of distilled water. The mixture was mixed until the chemicals were completely dissolved. The mixture was made up to the 1 litre mark with distilled water, mixed well and stored at room temperature. The pH was checked to be 6.8 by a pH meter.

Table 3.1: Mass and Molar Concentrations of the components of PBS (1X)

Buffer salt	Mass (g)	Molar concentration (mmol/L)	Mass concentration (g/L)
Sodium chloride NaCl	8.5	137	8.0
di-Sodium hydrogen phosphate (Na2HP04)	0.71	10.0	1.44
potassium di-hydrogen phosphate (KH2PO4)	0.68	1.8	0.24

3.12 Modification of the conventional PBS concentration technique

The modifications employed in this study were techniques that have been used to improve the sensitivity of the ZN TB microscopy. Ängeby *et al.* (2004) and Selvakumar *et al.* (2002) employed these methods to improve the smear preparation protocol for AFB detection in sputum samples (Ängeby *et al.*, 2004; Selvakumar *et al.*, 2002). These modifications were piloted by Dr Enid Owusu (Department of Epidemiology and Disease control, School of Public Health, College of Health Sciences, University of Ghana-Legon, in her thesis 'Evaluating and Improving Microbiological Methods for the Diagnosis of Buruli Ulcer Disease' 2013) to improve the detection of AFBs in BU clinical specimens.

The modifications techniques were:

1. centrifugation of 3.5% sodium hypochlorite (NaOCl) digested cellular specimen suspension
2. centrifugation of phenol-ammonium sulphate (PAS) digested cellular specimen suspension
3. sedimentation of 3.5% sodium hypochlorite digested cellular specimen suspension
4. sedimentation of phenol-ammonium sulphate digested cellular specimen suspension
5. sedimentation of phosphate buffered saline (PBS) cellular specimen suspension

3.12.1 Preparation of sodium hypochlorite and phenol ammonium sulphate

3.12.1.1 3.5% Hypochlorite solution

The 3.5% sodium hypochlorite was prepared by weighing and dissolving 3.5g sodium hypochlorite salt (Fisher Scientific UK; NaOCl; MW- 56.5 g/mol) in 100ml of distilled water. The components were mixed by shaking to completely dissolve the salt. The reagent was stored at room temperature until ready to be used.

3.12.1.2 5% Phenol 4% Ammonium sulphate solution

The phenol ammonium sulphate (PAS) solution was prepared by weighing 5g phenol crystals (Fisher Scientific UK, C_6H_5OH; MW-94.11g/mol) into a conical flask; 4g ammonium sulphate (Adarsh Scientific, Haryana, India; $(NH_4)_2SO_4$; MW-132.14 g/mol) was also weighed and added to the phenol crystals followed by addition of 95 ml of distilled water, the components were mix by vigorous shaking till all the salts were dissolved. The reagent was stored at room temperature until ready to be used.

3.12.2 Digesting cellular suspension with 3.5% hypochlorite

The BU cellular suspension was allowed to thaw at room temperature. Equal volumes (100 µl) of 3.5% sodium hypochlorite and well mixed BU specimen suspension (swab or FNA cellular suspension) were vortexed for 1 minute, followed by room temperature incubation for 15 minutes with intermittent mixing after which 800 µl of sterile distilled water was added and mixed to neutralize effect 3.5% NaOCl used (Ängeby *et al.*, 2004). This set up was made in duplicate.

3.12.3 Digestion cellular suspension with phenol-ammonia sulphate

The BU cellular suspension was allowed to thaw at room temperature. Equal volumes (100 µl) of phenol-ammonia sulphate and well mixed BU cellular suspension (swab or FNA cellular suspension) were vortexed for 1 minute, followed by room temperature incubation for 15 minutes

with intermittent mixing after which 800 µl of sterile distilled water was added and mixed to neutralize effect of PAS used (Selvakumar *et al.*, 2002). This set up was made in duplicate.

3.12.4 Concentration of Digested Cellular Suspension by Centrifugation

The neutralized digested cellular suspension (PBS, NaOCl and PAS) was vortexed for a minute and centrifuged at 3,000 rpm for 5 minutes using a cyto-centrifuge. The supernatant was carefully aspirated; a smear was prepared from the deposit, air-dried and heat fixed. The slide was labelled appropriately and stored in a dust proof slide rack.

3.12.5 Concentration of digested cellular suspension by sedimentation

The neutralized digested cellular suspension (PBS, NaOCl and PAS) was vortexed for a minute and incubated undisturbed at room temperature overnight. The supernatant was aspirated and a smear was prepared from the deposit, air-dried and heat fixed. The slide was labelled appropriately and stored in a dust proof slide rack.

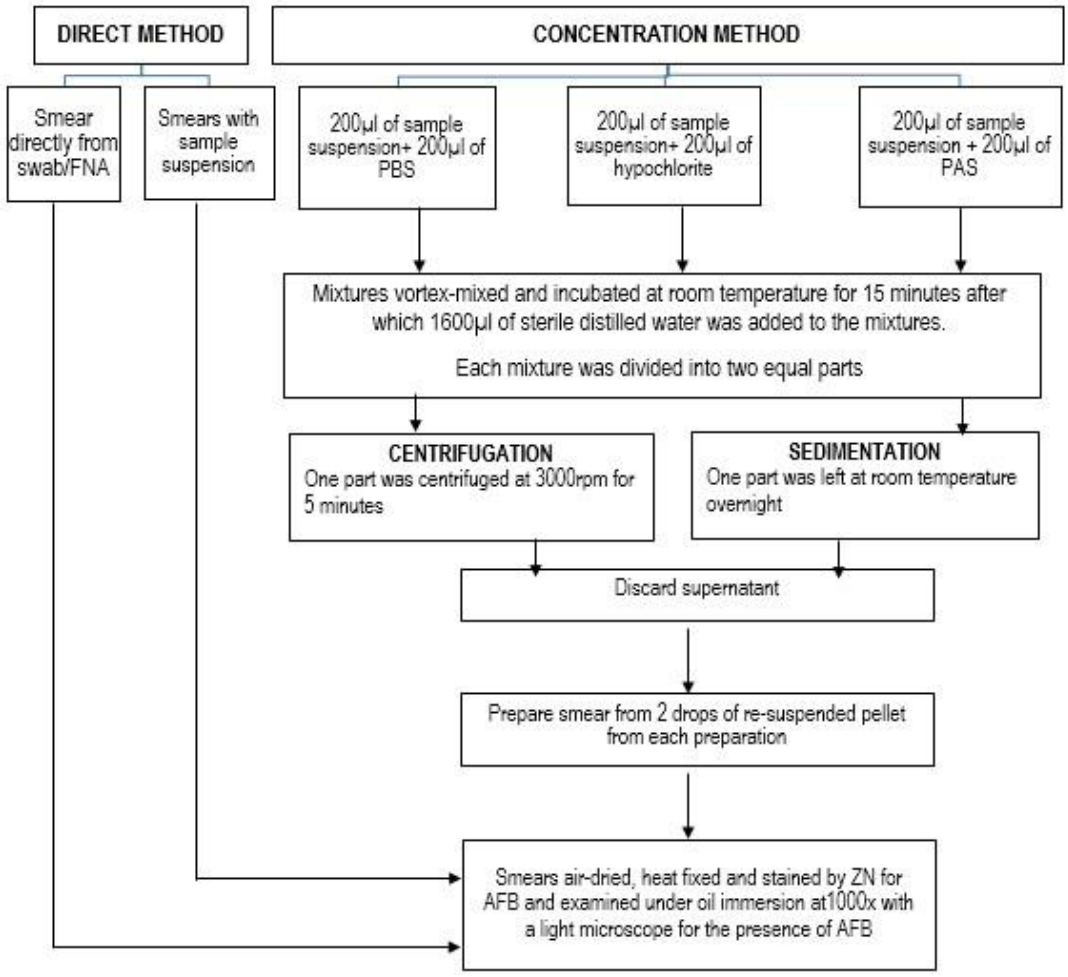


Figure 3.1 Summarized schematic diagram of modified laboratory procedures

3.13 The ZN microscopy technique

The air dried smears were flooded with the carbol fuchsin stain. The slides were flamed till steam just begun to appear. The stain was left on the smear for 5 minutes and the smear was washed with clean water and decolorized with 20% sulphuric acid (H_2SO_4) till the smear did not produce any more pinkish colour. They were washed with clean water and counterstained with methylene blue stain for 1 minute. The slide was washed with clean water, drained in a slide rack till they were air dried. The slides were examined with oil immersion using the X100 objective of the microscope. The staining procedure and the efficacy of the staining solutions were controlled with a positive *M. tuberculosis* sputum smear.

3.13.1 Evaluation criteria for AFB detection

The smears were graded based on evaluation standards of the American Thoracic Society in accordance with published standards (American Thoracic Society, 1981).

More than 10 AFB/field … … … …	report +++
1–10 AFB/field … … … … …	report ++
10–100 AFB/100 fields … … … …	report +
1–9 AFB/100 fields … … … … …	report the exact number
No AFB/100 fields … … … … …	No AFB seen

3.13.2 Quantification of AFB

The numbers of AFBs in the ZN stained smear were quantified by counting all the AFBs in one hundred fields of high power oil immersion fields.

3.13.3 Detection limits of the microscopy techniques

The cellular specimen suspensions that gave concordance results with all the eight protocols were pooled together till it produced an average ZN AFB count of 128 bacilli per 100 fields. A total cellular suspension volume of 2.0 ml was made with phosphate buffered saline (PBS). Serial doubling dilutions of the suspensions were made with PBS. Table 3.2 indicates how the doubling dilutions were made.

Table 3.2: Doubling dilutions of cellular suspensions

Tube	Volume of PBS in tube (ml)	Doubling dilutions	Dilution factor of content
1	2 ml of undiluted suspension	The cell suspension was vortexed for 10s. 1.0 ml was transferred into tube 2.	1
2	1.0	The 2.0ml content was vortexed for 10s and 1.0ml transferred into tube 3.	2
3	1.0	The 2.0ml content was vortexed for 10s and 1.0ml transferred into tube 4.	4
4	1.0	The 2.0ml content was vortexed for 10s and 1.0ml transferred into tube 5.	8

5	1.0	The 2.0ml content was vortexed for 10s and 1.0ml transferred into tube 6.	16
6	1.0	The 2.0ml content was vortexed for 10s and 1.0ml transferred into tube 7.	32
7	1.0	The 2.0ml content was vortexed for 10s and 1.0ml transferred into tube 8.	64
8	1.0	The 2.0ml content was vortexed for 10s and 1.0ml discarded.	128

The concentration of the bacilli in each tube was done as described in figure 3.1. Replicate smears were prepared for each dilution, air dried and stained with ZN stain. Average AFBs of the duplicate smears were determined.

3.14 Buruli Ulcer *IS2404* nested PCR methodology

3.14.1 Sample processing for *IS2404* nested PCR

The swab tip was cut into a sterile tube containing about eight glass beads to facilitate the removal of the bacterial cells from the cotton wool and 2.0 ml of phosphate buffered saline (PBS) was transferred into the tube. The tube was capped and vortexed. The mixture was transferred into a 2.0 ml screw capped sterile microtube.

3.14.2 DNA extraction using the modified Boom method

The extraction and purification of DNA was done by using the Boom DNA extraction method (Boom *et al.*, 1990) based on the protocol modified by Ablordey *et al.* (2012). The clinical specimen suspension was transferred into a tube containing 2 ml nuclease free distilled water and gently vortexed for 5 seconds. Portions (250 µl) of the sample suspensions were transferred to separate new sterile eppendorf tubes containing 250 µl of lysis buffer (1.6 M GuHCl, 60 mM Tris pH 7.4, 1% Triton X-100, 60 mM EDTA, Tween-20 10%), 50 µl proteinase-K and 250 µl glass beads (acid washed). The mixtures were incubated horizontally in a shaker (200 rpm) at 65 ^{o}C overnight. To

capture the DNA, 250 µl of diatomaceous earth solution (10 g diatomaceous earth obtained from Sigma Aldrich Chemi GmbH in 50 µl of H_2O containing 500 µl of 37%

(wt/vol) HCl) was added to the suspensions and incubated at 37 ^{o}C with shaking (200 rpm) for 60 min. The mixtures were centrifuged at 14,000 rpm for 10 sec and the resulting pellets were washed twice with 900 µl of 70% ethanol (2–8 ^{0}C) followed by 900 µl of acetone. The pellets were dried at 50 ^{o}C for 20 min and resuspended in 100 µl nuclease free distilled water and centrifuged at 14,000 rpm for 10 sec. The purified DNA was used as templates for both the *IS2404* nested PCR and the BU-LAMP assays to detect *M. ulcerans.* Positive and negative extraction controls were extracted along with the clinical specimens.

Figure 3.2: Extraction of DNA in a bacteriological hood

3.14.3 Dry-reagent-based *IS2404* nested PCR amplification

In order to avoid PCR contamination, a three room approach was followed. In this approach, preparation of the PCR master mix, addition of template DNA to the PCR master mix and PCR amplification were carried out in three separate compartments.

3.14.4 Primer set for dry-reagent based *IS2404* nested PCR

The primer (invitrogen) set used for the conventional and the nested PCR was the primers designed and used by Ablordey *et al.* (2012). The characteristics of the primers are indicated in Table 3.3.

Table 3.3: Oligonucleotide sequences used for the IS2404 nested PCR

Name of Primer	DNA Oligonucleotide sequences (5'-3')	Number of bases
Primers for first run *IS2404* PCR (conventional)		
pGp1 (Forward)	AGGGCACCGCGCTGATACGG	20
pGp2 (Reverse)	CAGTGGATTGGTGCCGATCGAG	22
Primers for nested *IS2404* PCR (Second run)		
pGp3 (Forward)	GGCGCAGATCAACTTCGCGGT	21
pGp4 (Reverse)	CTGCGTGGTGCTTTACGCGC	20

3.14.5 Preparation of stock PCR Primer solution

The lyophilized primer set came in various concentrations. An equal primer stock concentration of 100µM was prepared. The initial molarity of the lyophilized primers and the volume of nuclease free water to that was added to obtain a stock concentration of 100µM are presented in Table 3.4.

Table 3.4: Reconstitution of lyophilized IS2404 PCR primers

IS2404 PCR Primers	Initial concentration (nM) of the lyophilized primers	Volume (µl) of nuclease free distilled water	Final concentration (µM) of stock primers
pGp1 (Forward)	24.2	242.0	100.0
pGp2 (Reverse)	28.8	288.0	100.0
pGp3 (Forward)	25.3	253.0	100.0
pGp4 (Reverse)	28.3	283.0	100.0

3.14.6 Preparation of *IS2404* primer working solution

An equal 25.0pmol primer working solution was prepared. The volume of the stock solution and the volume of nuclease free water needed to prepare the desired molarity of the primer working solution are indicated in the table below.

Table 3.5: Preparation of PCR primer working solutions

IS2404 PCR Primer	Concentration of required working solution (pM)	Final volume of the working solution (µl)	Volume of stock solution required (µl)	Volume of nuclease free water to add (µl)
pGp1 (Forward)	25.0	100.0	25.0	75.0
pGp2 (Reverse)	25.0	100.0	25.0	75.0
pGp3 (Forward)	25.0	100.0	25.0	75.0
pGp4 (Reverse)	25.0	100.0	25.0	75.0

3.14.7 Preparation of PCR master mix

The *IS2404* sequence was amplified in a 30 µl reaction volume using Qiagen Hotstar PCR amplification kit. Each reaction was made in 27.0µl of the master mix.

Table 3.6: The PCR master Mix

Component	Vol (µl) for 1 Reaction	Vol (µl) for 141 reactions
Nuclease free water	15.5	2,185.5
PCR buffer	3	423.0
Q-solution	6	846.0
Deoxynucleotides (dNTPs)	2.0	282.0
HotStar Taq DNA pol	0.1	14.1
pGp1 primers	0.3	42.3
pGp2 primers	0.3	42.3
Total Volume	**27.0**	**3,927.0**

3.14.8 The *IS2404* first run PCR amplification reaction

The first run (conventional) PCR reaction was done with 27.0µl of the master mix and 3µl of the DNA template. The reaction mixture was vortexed for 10 seconds before amplification. The reaction was carried out in a thermocycler (Applied Biosystems) set with the amplification conditions as indicated in the table below. After 15 minutes initial denaturation at 95 ^{o}C, the reactions underwent 40 cycles of denaturation, annealing and extension at 94^{o}C, 64^{o}C and 75^{o}C respectively. The time taken for amplification of the DNA template to be completed was 145 minutes (3 hours 25 minutes).

Table 3.7: Amplification conditions for conventional (first run) PCR

Stage	Temperature (^{o}C)	Time	No. of cycles
Initial denaturation	95	15 min	1 cycle
Denaturation	94	30 sec	40 cycles
Primer annealing	64	60 sec	
Oligonucleotide extension	75	90 sec	

Final extension	72	10 min
Holding	4	until amplicons are removed

3.14.9: The *IS2404* nested PCR reaction (Second run)

The second run of the *IS2404* (nested) PCR was performed by using 1µl of the first run PCR amplicons as the template DNA and 24µl of master mix. The master mix for the nested reaction was the same as that for the first run PCR except that primer set pGp1 and pGp2 were replaced with the primer set pGp3 and pGp4 with the same volume. The same PCR amplification condition was used for the nested PCR except 30 cycles of denaturation, annealing and extension. The time taken for the completion of the nested PCR was 115 minutes (2 hours 25 minutes). The amplicons were kept at 4^{o}C until gel electrophoresis was done.

3.14.10 Agarose gel electrophoresis

3.14.10.1 Preparation of 10X TBE (Tris/Borate/EDTA) buffer

Stock TBE buffer (10X) was prepared according to the formula shown in Table 3.8. The salts were weighed in a precision chemical balance. The salt mixture was poured into a measuring cylinder containing 400ml of distilled water. The mixture was shook vigorously until all the salts were dissolved. The mixture was topped with distilled water to 1 litre mark. ***Table 3.8: Components of 10X stock TBE buffer***

Component	Amount	10X Stock Concentration
Tris Base	108 g	890 mM
Boric Acid	55 g	890 mM
EDTA (pH 8.0)	40 ml	20 mM

3.14.10.2 Preparation of 1X TBE (Tris/Borate/EDTA) buffer

About 100ml of stock 10X TBE was measured with a measuring cylinder and transferred into a 1 litre reagent bottle after which 900 ml of distilled water was measured with a measuring cylinder

and transferred into the bottle. The bottle was covered with a lid and shaken to mix the TBE solution thoroughly.

3.14.10.3 Agarose gel preparation

Preparation of 2% agarose gel was carried out by weighing 2 g of agarose into 100 ml of 1X TBE. The mixture was gently heated to boil in a microwave until all the agarose particles were completely dissolved in the TBE buffer. The bottle was removed from the microwave, allowed to stand for some time to cool down to about 70 ^{o}C and 1 µl of ethidium bromide was carefully pipetted into the agarose solution and swirled to mix well. A gel tray was set up with the gel comb in its proper place and the gel solution after cooling to about 65^{o}C was carefully poured into the prepared gel tray to avoid air bubbles been trapped in the gel. After the gel is poured, the gel tray was allowed to stand at room temperature for the gel to set, after which the gel comb was carefully removed from the gel.

3.14.10.4 Agarose gel electrophoresis

The electrophoretic chamber was filled with 1X TBE buffer and the agarose gel was placed into it. Additional 1X TBE buffer was added to completely cover the gel. The first and the last two wells of the gel were loaded with 2 µl of 100 bp DNA ladder and BU positive and negative controls respectively. Five microlitre (5 µl) amplicon was pipetted, mixed with 1µl of a loading dye and carefully loaded into to each well. The electrophoretic chamber was covered with a lid and electrophoresis was carried out at 135 V for 20 minutes. After the electrophoresis, the gel was removed from the electrophoretic chamber and placed in a UV illuminator for viewing.

3.14.10.5 Ultra-violet visualization of electrophoretic pattern

The gel was viewed under UV light and the electrophoretic patterns on the gel was captured and saved using a gel documenting system (Gel logic, Kodak, Japan). Samples were considered positive

if they yielded a 150-200 bp product that line up exactly with positive control. All negative controls were negative and the PCR control as well as the extraction control was negative. The gel picture and the wells were labelled with their corresponding sample identities.

3.15 The BU-LAMP methodology

The step-by-stem LAMP methodology used was the technique developed by Ablordey *et al.* (2012).

3.15.1 BU-LAMP primer arrangement and sequences

The specific primers used for the specific identification of *M. ulcerans* were the primer set designed and used by de Souza *et al.* (2012). A set of six (6) primers were used to amplify the *M. ulcerans* genome; they are two (2) inner primers, two (2) outer primers and two (2) loop primers. The specific primers were BU-LAMP forward inner primer (BU-LAMP FIP), BU-LAMP backward inner primer (BU-LAMP BIP), BU-LAMP forward outer primer (BU-LAMP F3), BULAMP backward outer primer (BU-LAMP B3), BU-LAMP forward loop primer (BU-LAMP FLP) and BU-LAMP backward loop primer (BU-LAMP BLP).

Table 3.9: The DNA oligonucleotide sequences primers used for LAMP

Name of Primer	DNA Oligonucleotide sequences (5'-3')	Number of bases
BU-LAMP FIP	GCATCTCCGGCCACCCCAACGCCCAACGACCGCTA	35
BU-LAMP BIP	GTGGTGGGCCCCTGGGAAACCGCTGTCGAACTGTGC	36
BU-LAMP F3	ACGGATCGTCGAGGATGG	18
BU-LAMP B3	GCGCCAGGTCCCTTGA	16
BU-LAMP FLP	GAGCCTGCTGGGCGGTC	17
BU-LAMP BLP	CATATCCCACCCTGGTG	17

3.15.2 DNA extraction and purification from clinical specimens

Refer to session 3.13.2

3.15.3 Preparation of BU-LAMP stock primer solution

The six (6) BU-LAMP lyophilized primers (*meta*Bio, Japan) came in various concentrations and they needed to be reconstituted with nuclease free distilled water to equal concentration of 100µM as presented in Table 3.6. The stock primers were stored in an eppendorf tubes at -20 ºC.

Table 3.10: Reconstitution of lyophilized BU-LAMP primers

BU-LAMP Primer	Initial concentration (nM) of the lyophilized primers	Volume (µl) of nuclease free distilled water	Final concentration (µM) of stock primers
Buruli-FIP	52.0	520	100
Buruli-BIP	68.9	689	100
Buruli-LF	59.6	596	100
Buruli-LB	61.5	615	100
Buruli-F3	74.7	747	100
Buruli-B3	58.6	586	100

3.15.4 Preparation of primer working solution

Various concentrations of the primer working solutions were prepared. The working solution of the inner primers (forward and backward) was 40µM, loop primers (forward and backward) were 20µM and outer primers (forward and backward) were 5µM (Table 3.11). The stock primers were stored in an eppendorf tubes at -20 ºC.

Table 3.11: Preparation of primer working solutions

BU-LAMP PRIMER	**Concentration of required working solution (µM)**	**Final volume of the working solution (µl)**	**Volume of stock solution required (µl)**	**Volume of nuclease free water to add (µl)**

Buruli-FIP	40.0	30.0	12.0	18.0
Buruli-BIP	40.0	30.0	12.0	18.0
Buruli-LF	20.0	30.0	6.0	24.0
Buruli-LB	20.0	30.0	6.0	24.0
Buruli-F3	5.0	30.0	1.5	28.5
Buruli-B3	5.0	30.0	1.5	28.5

3.15.5 Preparation of BU-LAMP primer mix

Calculated portions of the BU-LAMP primer working solution was pipetted and add together into a master mix. The final volume of the primer mix per sample was 2.6µl. Table 3.12 indicates the proportions of each primer working solution available in the primer mix. A total of 366.6 µl of primer mix was prepared for the 141 clinical specimens.

Table 3.12: Volumes of each primer working solution in the primer mix

BU-LAMP Primer	Primer concentration (nmol/µl)	Volume of working solution for 1 BU-LAMP reaction	Volume of working solution for 141 BU-LAMP reactions
Buruli-FIP	40.0	0.8 µl	112.8 µl
Buruli-BIP	40.0	0.8 µl	112.8 µl
Buruli-LF	20.0	0.4 µl	56.4 µl
Buruli-LB	20.0	0.4 µl	56.4 µl
Buruli-F3	5.0	0.1 µl	14.1 µl
Buruli-B3	5.0	0.1 µl	14.1 µl

3.15.6 The LAMP master mix

The BU-LAMP master mix was prepared with LoopAmp amplification kit (Eiken Chemical, Japan). The component was made up of 12.5µl of reaction mix, 1 µl of fluorescent detector (Eiken Chemical, Japan), 2.6 µl of primer mix (Eiken Chemical, Japan), 1 µl of Bst DNA polymerase (Eiken Chemical, Japan) and 2.9 µl of nuclease free distilled water (Promega, USA). The total reaction volume per sample was 20 µl; therefore 2,820 µl of master mix was prepared

(Table 3.13). The composition of the reaction mix is shown in Table 3.10

Table 3.13: The BU-LAMP Master Mix

Component	Vol (µl) per sample	Vol (µl) per 141 sample
Bst DNA polymerase	1 µl	141 µl
2x Reaction Mix	12.5 µl	1,762.5 µl
Primer mix	2.6 µl	366.6 µl
Nuclease free water	2.9 µl	408.9 µl
Fluorescent detector	1 µl	141 µl

The reaction mix was composed of tri-buffer (pH=8.8), KCl, $MgSO_4$, $(NH_4)_2SO_4$, tween-20, betaine, dNTPs, $MnCl_2$ and calcein.

Table 3.14: Composition of 2x LAMP reaction mix

Component	Concentration
Tri-buffer (pH=8.8)	40.0mM
KCl	20.0mM
$MgSO_4$	16.0mM
$(NH_4)SO_4$	20.0mM
Tween-20	0.2%
Betaine	1.6mM
DNTPs	2.8mM each
$MnCl_2$	1mM
Calcein	50µM

3.15.7 The LAMP reaction mixture

The BU-LAMP reaction mixture was prepared by mixing 20 µl of the master mix and 5 µl of the DNA extract in 0.2 ml microAmp™ tubes (Applied Biosystems, Singapore). The mixture was vortexed using a vortex mixture fitted with microAmp™ tubes vortex adaptor.

3.15.8 Amplification of DNA

The DNA amplification was done by incubating the reaction mixture on a heating block at a temperature of 65 ^{o}C for 1 hour and the Bst DNA polymerase was inactivated at 80^{o}C for 5 minutes.

3.15.9 Visualization and determination of results

The final reactions were determined by visual examination of the final colour production. A final greenish yellow colour development signifies a positive reaction and a light brown final colour signified a negative reaction (Figure 3.3).

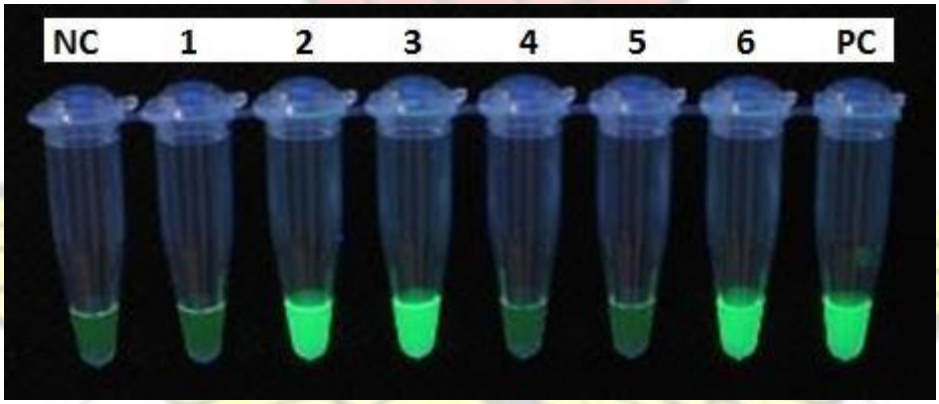


Figure 3.3: Final LAMP reaction Products.
Positive reactions: samples 2, 3 and 6. Negative reactions: sample. Samples 1, 4 and 5. PC and NC are positive and negative controls respectively

3.16 Detection limits of BU-LAMP and PCR

The detection limits of the BU-LAMP and the *IS2404* nested PCR was determined by isolating *M. ulcerans* DNA from colonies growing on L-J medium. Approximately 10µl loopful of pure *M. ulcerans* colonies was scraped from the L-J agar slopes. DNA was isolated from the colonies using the modified Boom methods described by Ablordey *et al.* (2012) and stated in section 3.14.2. The whole isolated DNA from 10µl of colonies contains $3.0x10^{5}$ copies of *IS2404* (Stinear *et al.,* 2000). Serial dilution of genomic DNA was done with nuclease free water in 10 folds. The

10 fold dilutions were achieved by mixing 10µl of DNA in the previous tube with 90µl of nuclease free water in the next tube. This generated 6 serially diluted DNA with titres $3.0x10^4$, $3.0x10^3$, $3.0x10^2$, $3.0x10^1$ and 3.0. The BU-LAMP assay was done with the same methodology as described in 3.15.1-3.15.11. The PCR amplification and visualization of the amplicons of the serially diluted DNA extract was done as described in 3.14.4-3.14.10.

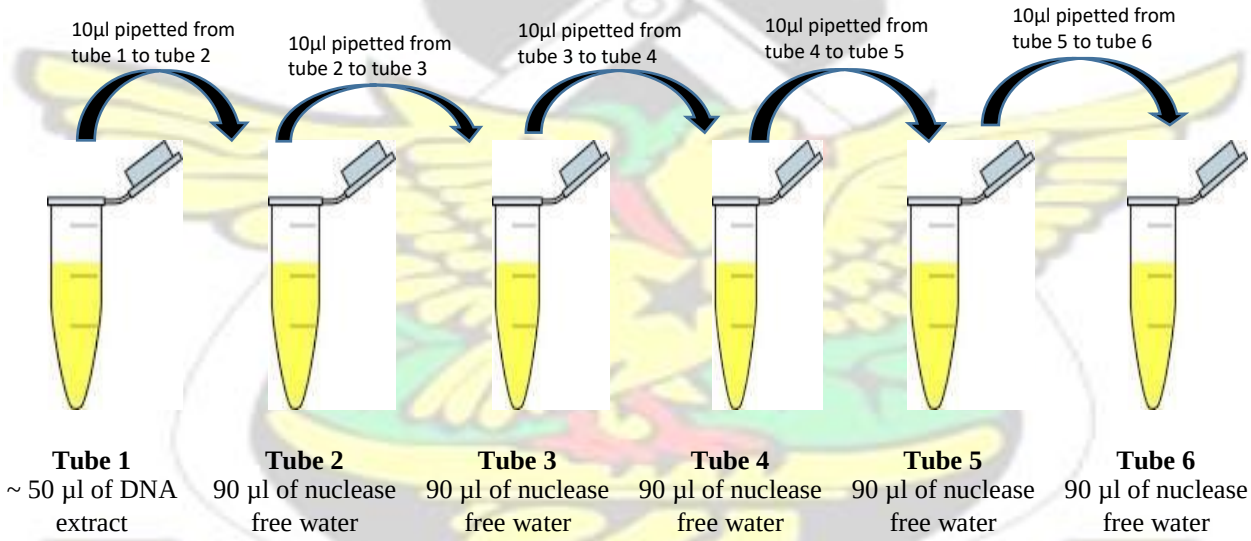


Figure 3.4: Schematic description of diluting the colonial DNA extract

3.17 Data management and statistical analysis

The data were managed by Microsoft Office (Excel 2010). The same package was used to determine percentages and proportions. The statistical analyses were done using SPSS (version 16.0). Most of p-values were determined using Pearson Chi square. Statistical significant differences were defined as p-values <0.05. Dependent variables were diagnostic laboratory results

and independent variables were type of lesions, categories of lesions, residential settings, age, gender, occupation, education level reached and type of diagnostic specimen.

3.18 Determination of the diagnostic indices

The sensitivity, specificity, efficiency, positive predictive value (PPV) and the negative predictive value (NPV) of the various techniques were calculated. The PCR results were taken as the gold standard test.

3.18.1 Diagnostic sensitivity of a technique Sensitivity of diagnostic test is calculated as:

$$\text{Sensitivity} = \frac{TP}{TP+FN} \times 100\%$$

3.18.2 Diagnostic specificity of a technique

$$\text{Specificity} = \frac{TN}{TN+FP} \times 100\%$$

3.18.3 Positive Predictive Value (PPV) of a technique

$$\text{Positive Predictive Value (PPV)} = \frac{TP}{TP+FP} \times 100\%$$

3.18.4 Negative Predictive Value (NPV) of a technique

$$\text{Negative Predictive Value (PPV)} = \frac{TN}{TN+FN} \times 100\%$$

3.18.5 Positivity rate (PR) Positivity rate (PR) =

$$\frac{\text{Number of patients with positive by a particular protocol}}{\text{Number of patients tested}} \times 100\%$$

3.18.6 Negativity rate (PR)

Negativity rate (PR) =

$$\frac{\text{Number of patients withnegative by a particular protocol}}{\text{Number of patients tested}} \text{ X } 100\%$$

3.18.7 Determination of inter-diagnostic agreement (kappa statistic)

The inter-diagnostic agreement (kappa statistic or kappa coefficient) between a reference methodology and comparative methodology was determined manually and confirmed electronically with Crosstab tool in SPSS (version 6.0). The manual method was adopted from Vieira and Garrett (2005).

Kappa coefficient (κ) was calculated as follows:

$$\kappa = \frac{Po - Pe}{1 - Pe}$$

Where κ - kappa statistic or coefficient

Po - observer agreement

Pe - expected agreement

Considering Table 3.15, Po and Pe were calculated as follows:

Table 3.15: Diagnostic indices for reference and observed methods

		Reference diagnostic method		
		Positive	Negative	Total
Diagnostic method to be compared	Positive	A	b	a+b
	Negative	c	d	c+d
	Total	a+c	b+d	a+b+c+d

Indices 'a' and'd' are agreed values for the methods and indices 'c' and 'b' are disagreed values for the methods.

3.18.7.1 Determination of observer agreement (Po) Observer agreement was calculated as follows:

$$Po = \frac{a + b}{a + b + c + d}$$

3.18.7.2 Determination of expected agreement (Pe) Expected agreement was calculated as follows:

$$Pe = \left[\frac{a+c}{a+b+c+d}\right]\left[\frac{a+b}{a+b+c+d}\right] + \left[\frac{b+d}{a+b+c+d}\right]\left[\frac{c+d}{a+b+c+d}\right]$$

Table 3.16: Interpretations of kappa (statistic) coefficients (Source: Vieira and Garrett, 2005).

Cohen's kappa statistic	Interpretation
0.01-0.20	Slight agreement
0.21-0.40	fair agreement
0.41-0.60	Moderate agreement
0.61-0.80	Substantial agreement
0.81-0.99	Almost perfect agreement
1.0	Perfect agreement

3.19 Ethical clearance and informed patient consent

This study was reviewed and approved by the Committee on Human Research Publication and Ethics, Kwame Nkrumah University of Science and Technology, Kumasi (Reference Number: CHRPE/AP/036/15). Informed patients consent was sought from participating adults and from the parents or guardians of children. Participation was voluntary and enrolment was subject to individual's approval through signature or thumb-printing.

CHAPTER 4

RESULTS

4.1 Age, sex and residential setting distribution of the participants

Out of the one hundred and forty-one (141) clinically diagnosed Buruli ulcer patients recruited for the study, 93 (66.0%) were males and 48 (34.0%) were females (Table 4.1). More cases were recruited from the Ga West Municipal Hospital; 52 cases in all; 13 (25.0%) were clinical cases and 39 (75.0%) were patients recruited from the active case search in Kojo Ashong (14 suspected cases), Manhean (10 suspected cases), Nsakina (6 suspected cases) and Mayera (9 suspected cases).

Table 4.1: Gender distribution among the study sites

Study site	Gender		Total
	Female n (%)	**Male n (%)**	
Ga West Municipal Hospital	18 (34.6%)	34 (65.4%)	52
Nkawie-Toase Government Hospital	10 (47.6%)	11 (52.4%)	21
Obom Health Centre	6 (35.3%)	11 (64.7%)	17
Paakro Health Centre	9 (29.0%)	22 (71.0%)	31
Tepa Government Hospital	5 (25.0%)	15 (75.0%)	20
Total	**48 (34.0%)**	**93 (66.0%)**	**141**

The rest of the cases that is 21 were seen in Nkawie-Toase Government Hospital (14.9%), 17 from Obom Health Centre (12.0%), 31 from Paakro Health Centre (21.9%) and 20 from Tepa

Government Hospital (14.2%). From Table 4.1, the statistical differences between gender of the patients recruited for the study from each study site were not significant ($p>0.05$).

4.1.1: Age distribution of the participants

The patients were aged between 2-86 years. The mean age was 33.6 years and the modal age was 9 years, the modal age range was 0-19 years and the least encountered age range was 80+ years (Table 4.2). The differences between the distribution of the patients within the age ranges among each study site were not statistically significant ($p>0.05$, Pearson Chi Square). The overall age (yrs.), gender and residential settings distribution of the patients are represented in Table 4.2, Table 4.3 and Table 4.4 respectively. Fisher exact test indicated very high statistical differences between the distribution of the age ranges in the various residential settings ($p=0.004$). From Table 4.3, there was disproportional distribution of the patients in the study sites from which they were seen. There was a very high statistical difference between the distributions of the various age ranges seen in the study sites among the three residential settings (Table 4.4).

Table 4.2: Age (yrs.) distributions among the Study Sites

	Study Sites					**Total**
Age group	**GWMH**	**NTGH**	**OHC**	**PHC**	**TGH**	
0-19	19 (36.5%)	6 (11.5%)	9 (17.3%)	11 (21.2%)	7 (13.5%)	52
20-39	13 (41.9%)	6 (19.4%)	2 (6.5%)	7 (22.6%)	3 (9.7%)	31
40-59	15 (34.9%)	5 (11.6%)	5 (11.6%)	8 (18.6%)	10 (23.3%)	43
60-79	4 (40.0%)	2 (20.0%)	1 (10.0%)	3 (30.0%)	0 (0.0%)	10
80+	1 (20.0%)	2 (40.0%)	0 (0.0%)	2 (40.0%)	0 (0.0%)	5
Total	**52 (36.9%)**	**21 (14.9%)**	**17 (12.1%)**	**31 (22.0%)**	**20 (14.2%)**	**141**

GWMH-Ga West Municipal Hospital, NTGH-Nkawie-Toase Government Hospital, OHC-Obom Health Centre, PHC-Paakro Health Centre, TGH-Tepa Government Hospital

Table 4.3: Age and gender distribution of the Buruli ulcer patients

Age group (yrs)	Gender			p-value
	Female n (%)	Male n (%)	Total	
0-19	12 (23.1%)	40 (76.9%)	52	0.036
20-39	13 (41.9%)	18 (58.1%)	31	0.294
40-59	16 (37.2%)	27 (62.8%)	43	0.599
60-79	4 (40.0%)	6 (60.0%)	10	0.680
80+	3 (60.0%)	2 (40.0%)	5	0.212
Total	**48 (34.0%)**	**93 (66.0%)**	**141**	**0.0847**

p-value determined by Pearson Chi square

4.1.2: Residential settings of the participants

The residential settings distributions of the patients were rural 109 (77.3%), peri-urban 22 (15.6%) and urban 10 (7.1%). there was unequal distribution of the cases among residential settings with respect to age range. In all the age groups, more than 75% were rural residents.

Table 4.4: Residential settings distribution of the BU patients

	Residential Setti ıg			Total	p-value
Age group (yrs)	Peri-urban n (%)	Rural n (%)	Urban n (%)		
0-19	7 (13.5%)	41 (78.8%)	4 (7.7%)	52	0.001

20-39	8 (25.8%)	21 (67.7%)	2 (6.5%)	31	0.007
40-59	6 (14.0%)	34 (79.1%)	3 (7.0%)	43	0.009
60-79	1 (10.0%)	8 (80.0%)	1 (10.0%)	10	0.043
80+	0 (0.0%)	5 (100.0%)	0 (0.0%)	5	0.7522
Total	**22 (15.6%)**	**109 (77.3%)**	**10 (7.1%)**	**141**	**0.004**

4.2: Education level

Most of the patients recruited have had some form of formal education except 54 (38.3%) of the patients. Out of the rest of the patients who had had some formal education, 43 (49.4%) did not progress above primary education, 30 (34.5%) could not progressed above middle or junior high school, 8 (9.2%) completed secondary or senior high school and 6 (7.0%) have had education up to tertiary (Figure 4.1).

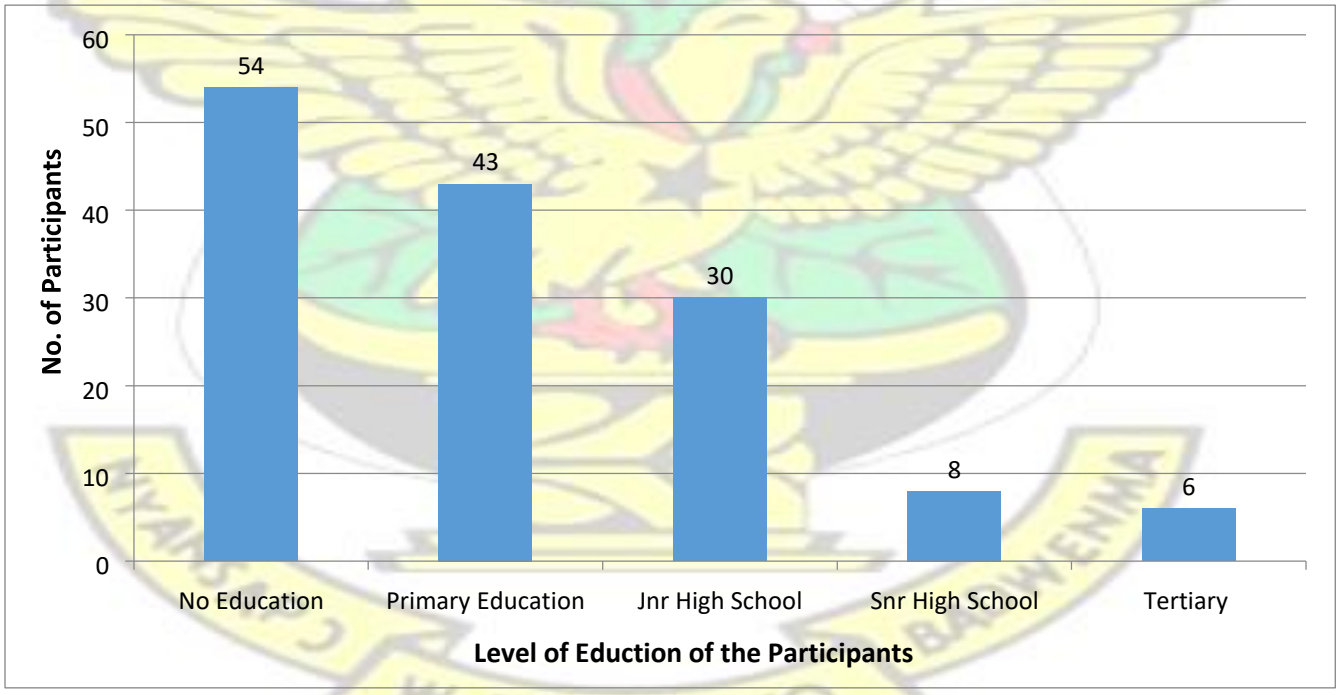


Figure 4.1: Educational Level

4.3: Occupation of the participants

Most of the patients below 22 years were school children in various stages of their education. Thirty (30) of such patients were in primary school (11), junior high school (16) and senior high schools (3). The rest were engaged in various forms of employment; trading (16), hairdressing (3), various forms of farming (62), mechanic (3), masonry (5), steel bending (3), driving (3) and 18 were unemployed.

4.4 Endemic villages within the study sites

Out of the 46 positive cases, 10 (21.7 %), 8 (17.4 %) and 7 (15.2 %) of the cases seen in the Ga West Municipal Hospital resided in Kojo Ashong, Manhean and Nsakina respectively. Out of the 19 positive cases seen in Nkawie Hospital, most of them resided in Achiase 4 (20.1%), Nyinahin 4 (20.1 %) and Nkawie Panin 3 (15.8 %). Majority of the 25 positive cases seen in Paakro Health Centre resided in Mangoase 7 (28.0 %) and Nsuablaso 5 (20.0 %), in Tepa Government Hospital most of the 16 positive cases seen resided in Subonpan 3 (18.7 %), Adrobaa 3 (18.7%) and Nfanibu 2 (12.5 0%) and majority of the 16 positive cases that were seen in Obom Health Centre resided in Dankyira 4 (25.0 %), Amanfrom 3 (18.7 %) and Hobor 3 (18.7 %). The rest of the villages had only one case each.

4.5 Clinical data of the BU patients

4.5.1 Location of the lesions

The Buruli ulcer clinical specimens were taken from eight (8) different anatomical sites from the patients who were recruited for the study. The sites were hands, legs, thigh, calf, foot, shoulder, buttock and stomach. From Table 4.5, it can be seen that majority of the cases sampled 121 (85.8%) were located at the lower limb whilst 17 (12.1 %%) were located at the upper limb and the rest 3

(2.1%) were located at shoulder and the buttock. The most encountered site was the leg and the least encountered site was the stomach. Males were significantly associated with lesions on the hand (p=0.03) whilst females were significantly associated with lesions located on the shoulder (p=0.047) and the calf (p=0.012).

Table 4.5: Location of lesions (upper and lower limbs)

Regional location of lesions	Female n (%)	Male n (%)	Total	p-value
Lower limb	44 (37.0%)	75 (63.0%)	119 (84.8%)	0.199
Upper limb	4 (21.1%)	15 (78.9%)	19 (13.5%)	0.087
Others	0 (0.0%)	3 (100.0%)	3 (2.1%)	0.208
Total	**48 (34.0%)**	**93 (66.0%)**	**141 (100.0%)**	

Table 4.6: Anatomical locations of the lesions

Anatomical location of lesion	Gender		Total	p-value
	Female	Male		
Thigh	4 (44.4%)	5 (55.6%)	9	0.496
Calf	6 (75.0%)	2 (25.0%)	8	0.012
Foot	4 (22.2%)	14 (77.8%)	18	0.257
Shoulder	2 (100.0%)	0 (0.0%)	2	0.047
Buttock	1 (50.0%)	1 (50.0%)	2	0.631
Stomach	0 (0.0%)	1 (100.0%)	1	0.471
Hand	3 (15.8%)	16 (84.2%)	19	0.03
Leg	28 (34.1%)	54 (65.9)	82	0.976

The p-value was determined by Pearson Chi square

4.5.2 Clinical form of the lesions encountered

Out of the five possible clinical forms of the BU disease, three (3) were encountered during the study. Majority of the lesions 103 (73.0 %) presented as ulcers. The other clinical forms seen were 23 nodules (16.3%) and 15 oedematous lesions (10.6%). There were significant differences between the distribution of the clinical forms of the disease and gender; number of ulcerative lesion

were higher than the non-ulcerative lesion (p=0.011). From Table 4.7, ulcerative, nodular and oedematous lesions were significantly seen among males than females.

Table 4.7: Clinical Forms of the lesions with respect to gender

	Gender			
Clinical forms of the lesions	**Female n (%)**	**Male n (%)**	**Total**	**p-value**
Oedema	3 (20.0%)	12 (80.0%)	15	0.005
Nodule	3 (13.0%)	20 (87.0%)	23	0.020
Ulcer	42 (40.8%)	61 (59.2%)	103	0.225
Total	**48 (34.0%)**	**93 (66.0%)**	**141**	**0.011**

The p-value was determined by Pearson chi square

The frequency of the ulcerative forms of the disease was high among the 40-59 years age group (36.9%) and low among 80+ year group (3.5%). The 0-19 year's age group had the highest number of the nodular forms (60.8%) however no nodules were seen in patients older than 79 years. The oedematous lesions were very high among the 0-19 years group (73.3%) but no oedematous lesion was seen in patients older than 59 years (Table 4.8). There was unequal distributions of the clinical forms among the age groups. In all the age groups, the ulcerative forms were significantly high follow by the nodular forms and the oedematous forms. In patients more than 80 years old, no nodules or oedematous forms were seen (Table 4.8). From Table 4.8, there was significant difference between the distribution of the clinical forms of the disease and age (p=0.0086).

Table 4.8: Clinical Forms of the lesions with respect to age in years

Clinical forms of lesions	**Age groups**					**Total**	**p-value**
	0-19	**20-39**	**40-59**	**60-79**	**80+**		
Oedema	12 (80.0%	2 (13.3%)	1 (6.7%)	0 (0.0%)	0 (0.0%)	15	<0.0001
Nodule	13 (56.5%)	4 (17.4%)	3 (13.0%)	3 (13.0%)	0 (0.0%)	23	0.083
Ulcer	27 (26.2%)	25 (24.3%)	39 (37.9%)	7 (6.8%)	5 (4.9%)	103	0.007

Total	**52 (37.0%)**	**31 (22.0%)**	**43 (30.5%)**	**10 (7.1%)**	**5 (3.5%)**	**141 (100.0%)**	**0.0086**

The p-value was determined by Pearson chi square

4.5.3 Category of lesion encountered

All the three categories of BU lesions were encountered; 64 (45.4 %) of the lesion were category I, 24 (17.0%) were category II and 53 (37.6 %) were category III. The males have relatively high incidences of the three categories than the females (Table 4.9) but there was no statistical differences between them (p=0.1027). Out of the 64 category I cases seen, 38 (59.4%) of them were pre-ulcerative cases and 26 (40.6%) were ulcerative cases less than 5 cm. Most of the preulcerative cases were seen among the males 32 (84.2%) as against 6 (15.8%) in females.

Table 4.9: Categories of the Lesions

Category of lesions	Gender		Total	p-value
	Female	**Male**		
Category 1	16 (25.0%)	48 (75.0%)	64	0.039
Category II	12 (50.0%)	12 (50.0%)	24	0.07
Category III	20 (37.7%)	33 (62.3%)	53	0.473
Total	**48 (34.0%)**	**93 (66.0%)**	**141**	**0.103**

The p-value was determined by Pearson chi square

4.6 Types of clinical specimen sampled

Out of the 141 samples collected, 103 (73.0%) were swabbed and 38 (27.0%) were aspirated (Table 4.10). Only 12 (8.5%) of the patients had multiple lesions; 4 were two noncommunication ulcers, 6 were two (2) non-ulcerative lesions (both nodule and oedema) and 2 were both ulcers and nodules.

Table 4.10: Types of clinical specimens

Specimen taken	Number of patients	%
Swab	103	73.0
FNA	38	27.0

Total	**141**	**100.0**

4.7 Patient's treatment history

Almost all the patients who were sampled for the study were not on streptomycin-rifampicin treatment at the time the samples were taken. Only 1 patient (0.71%) was on treatment not exceeding 14 days at the start of treatment. Aside that, 140 (99.29%) of the patients were new cases (Table 4.11).

Table 4.11: Patients' treatment history

Number of complete days of SR treatment	Number of patients	%
Yet to start	140	99.29%
1-14 days	1	0.71%
15-28 days	0	0.00%
29-42 days	0	0.00%
43-56 days	0	0.00%
Treatment completed	0	0.00%

SR- streptomycin-rifampicin

4.8 Microscopic and molecular analysis of the clinical samples

Table 4.12 shows the analysis of the test positivity and negativity rate of the microscopy and the molecular analysis of the Buruli ulcer lesions. Analysis of the three molecular methods indicated that the nested *IS2404* PCR had the highest positivity rate of 122 (86.5%) followed by BULAMP assay of 111 (78.7%) and first run *IS2404* PCR 104 (73.8%). Kappa analysis indicated moderate

agreement between the *IS2404* nested PCR and the first run PCR (κ=0.60) however, there was substantial inter-protocol agreement between *IS2404* nested PCR and BU-LAMP (κ=0.72). None of the negative *IS2404* nested PCR results was positive in the LAMP assays.

Direct smearing of cellular suspension had a relative higher positivity rate of 65 (46.1%) compared to that of the direct smear of clinical specimen which had 51 (31.7%) positivity rate, however, there was almost a perfect inter-technique agreement between them (κ=0.83). The inter-protocol agreement between the gold standard and the direct smear microscopy was poor (κ=0.15) and there was fair inter-protocol agreement between the gold standard and the direct smears prepared from the cellular suspension (κ=0.23).

Centrifugation of the cellular specimen suspension following prior digestion with phenol ammonium sulphate had a better positivity rate 73 (51.7%), followed by digestion with phosphate buffered saline 66 (46.8%) and sodium hypochlorite 61 (43.3%). Even though the phenol ammonium sulphate method yielded more positivity rate in detection AFBs, there was perfect inter-technique agreement between the phosphate buffered saline technique and the sodium hypochlorite technique (κ=0.93) and that of phosphate buffered saline and phenol ammonium sulphate technique (κ=0.90). However, there was fair inter-protocol agreement between IS2404 nested PCR gold standard and the centrifugation techniques (nested PCR vs. PBS, κ=0.24; nested PCR vs. NaOCl, κ=0.21; nested PCR vs. PAS, κ=0.28).

On analysis of the overnight sedimentation protocol prior chemical digestion, phenol ammonium sulphate had the highest positivity rate of 75 (53.2%) followed by phosphate buffered saline 68 (48.2%) and sodium hypochlorite 63 (44.7%). There was a near perfect inter-technique agreement between the diagnostic performance of phosphate buffered saline and sodium hypochlorite (κ=0.92) and also between phosphate buffered saline and phenol ammonium sulphate (κ=0.90).

However, the inter-protocol agreement between the gold standard and the sedimentation technique was fair (nested PCR vs. PBS, κ=0.25; nested PCR vs. NaOCl, κ=0.22; nested PCR vs. PAS, κ=0.30).

Comparing the mean acid fast bacilli yield per 100 high power fields of the ZN microscopy smears (Table 4.13), the best three diagnostic techniques that yielded the highest mean AFBs count per 100 high power fields were sedimentation with phenol ammonium sulphate (χ=30.3, 95% CI, 27.0-33.8), centrifugation with phenol ammonium sulphate (χ=23.7 [95% CI, 21.1-26.6) and sedimentation with phosphate buffered saline (χ=19.8, 95% CI, 17.7-21.8).

Table 4.12: Positivity rates and Intra-protocol kappa analysis

			Inter-rater agreement (kappa statistic)						
			Molecular		Direct	Centrifugation		Sedimentation	
Protocol	**Diagnostic Technique**	**Positivity Rate n (%)**	**Nested Vs. First run**	**Nested Vs. BULAMP**	**Clinical specimen vs. suspension**	**PBS Vs. NaOCl**	**PBS Vs. PAS**	**PBS Vs. NaOCl**	**PBS Vs. PAS**
Molecular Methods	Nested *IS2404* PCR	122 (86.5)	0.60	0.72	0.83	0.92	0.90	0.92	0.90
	First run *IS2404* PCR	104 (73.8)							
	BU-LAMP assay	111(78.7)							
Direct Smears	Clinical Specimen	51 (31.7)							
	Cellular Suspension	65 (46.1)							
Centrifugation	Conventional with PBS	66 (46.8)							
	Modified with NaOCl	61 (43.3)							
	Modified with PAS	73 (51.7)							
Sedimentation	Modified with PBS	68 (48.2)							
	Modified with NaOCl	63 (44.7)							
	Modified with PAS	75 (53.2)							

PBS-phosphate buffered saline, NaOCl-Sodium hypochlorite, PAS-phenol ammonium sulphate

Table 4.13: AFB yield of the conventional and the modified ZN Protocols

Protocol	Diagnostic Technique	Mean AFB count per 100 high power fields	95% CI
Direct Smears	Clinical Specimen	10.5	9.3-11.6
	Cellular Suspension	12.4	10.8-13.9
Centrifugation	Conventional with PBS	17.05	15.5-18.9
	Modified with NaOCl	11.5	10.4-12.6
	Modified with PAS	23.7	21.1-26.5
Sedimentation	Modified with PBS	19.8	17.7-21.8
	Modified with NaOCl	11.1	10.0-12.3
	Modified with PAS	30.3	27.0-33.8

PBS-phosphate buffered saline, NaOCl-Sodium hypochlorite, PAS-phenol ammonium sulphate

4.8.1 Detection limits of the microscopy protocols

From Table 4.14, all the protocols were able detect AFBs in the tube with bacilli density 128 bacilli per ml, 64 bacilli per ml and 32 bacilli per ml. All the protocols detected AFBs in tube with density 16 bacilli/ml except centrifugation with 3.5% sodium hypochlorite. However, only one of two smears was positive with centrifugation with phosphate buffered saline. Only two sedimentation protocols (phosphate buffered saline and phenol ammonium sulphate) were able to detect AFBs in tube with bacilli density 8 bacilli/ml. Only sedimentation with phenol ammonium sulphate protocol out of the six protocols was able to detect AFBs in tube with bacilli density 4 bacilli/ml. No protocol was able to detect AFBs in tubes with bacilli density less than 4 bacilli/ml in 100 high power fields. The detection limits of the centrifugation protocol modified with phosphate buffered saline, sodium hypochlorite and phenol ammonium sulphate was 16 bacilli/ml, 62 bacilli/ml and 16 bacilli/ml respectively. The detection limit of the sedimentation protocol modified with phosphate buffered saline, sodium hypochlorite and phenol ammonium sulphate was 8 bacilli/ml, 16 bacilli/ml and 4 bacilli/ml respectively.

Table 4.14: Detection limits of the microscopy protocols

		Concentration by centrifugation			Concentration by sedimentation		
Tube (dilution factor)	**AFB density in 1 ml of diluted solution**	**PBS**	**NaOCl**	**PAS**	**PBS**	**NaOCl**	**PAS**
1 (1)	128	+	+	+	+	+	+
2 (2)	64	+	+	+	+	+	+
3 (4)	32	+	+	+	+	+	+
4 (8)	16	+[1]	-	+	+	+	+
5 (16)	8	-	-	-	+[1]	-	+
6 (32)	4	-	-	-	-	-	+
7 (64)	2	-	-	-	-	-	-
8 (128)	1	-	-	-	-	-	-

[1]*One of two slides was positive*

4.8.2 Background deposits of the protocols

The phenol ammonium sulphate digested suspension produced a smear with few background deposits. The smears were easy to examine and the bacilli stood out visibly on a clear background. Smears from phosphate buffered saline had moderate deposits on the background which easily mask the bacilli. Smears from the sodium hypochlorite treated specimen had heavy deposits of background particles. The bacilli are almost hidden by the deposits which makes microscopy very difficult. The time taken to examine slides from sodium hypochlorite were twice or thrice the time taken to examine the phenol ammonium sulphate and the phosphate buffered saline smears.

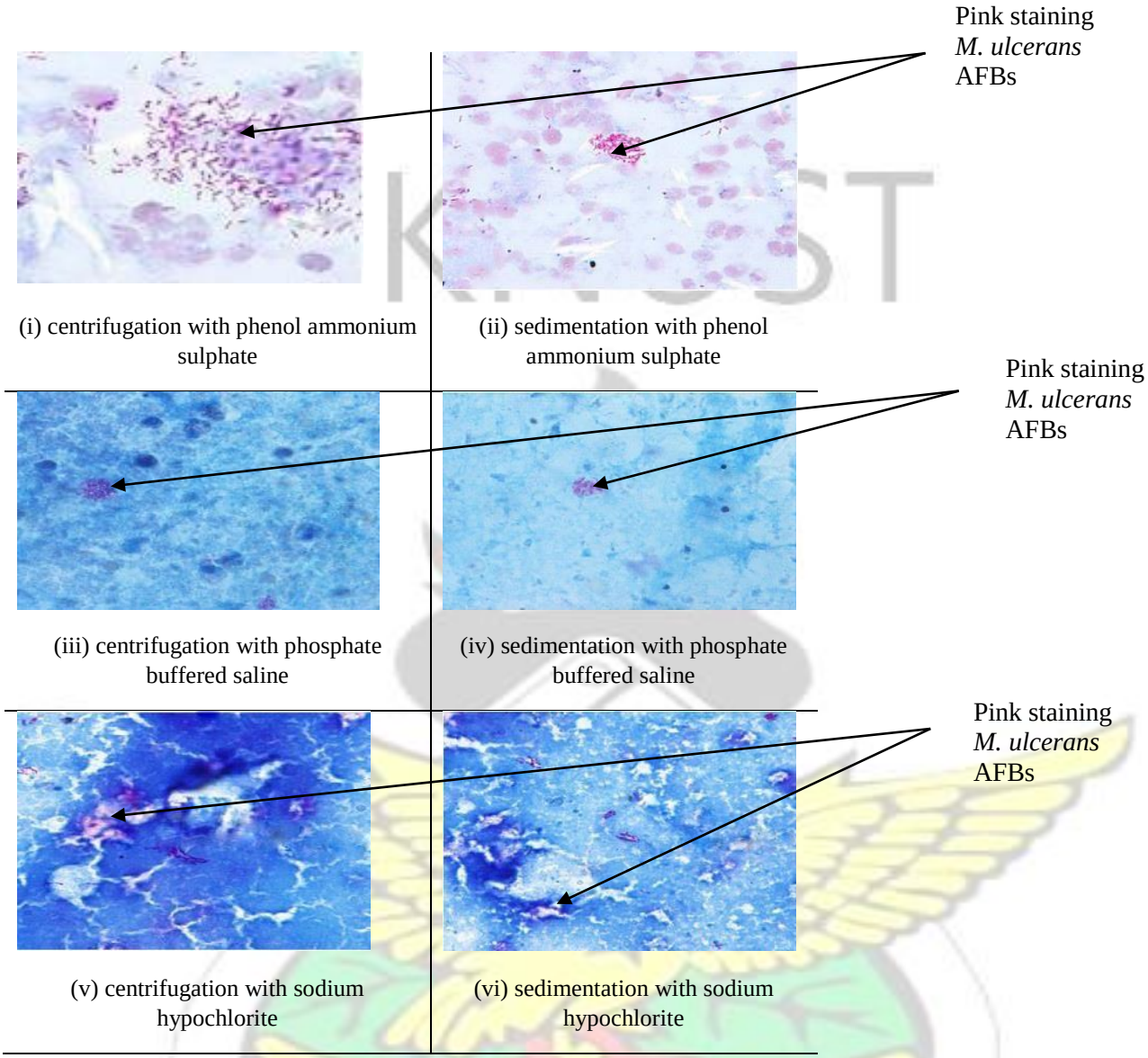


Figure 4.2: Micrographs of the microscopy protocols

4.8.1 Study site, gender and age distribution of laboratory results

Table 4.15 indicates the distribution of laboratory results by the gold standard among the study sites, gender and age group. Sixteen out of 17 specimens collected from Obom Health Centre were positive (94.1%), 19 (90.5%) of the specimens collected from Nkawie-Toase Hospital was positive, 46 (88.5%) of the specimens collected from Ga West Municipal Hospital were positive,

25 (80.6%) of specimens collected from Paakro Health Centre were positive and 16 (80.0%) of the specimens from Tepa Government Hospital were positive. However, there was no significant differences in the positivity rate among the study sites (p=0.473) (Table 4.15A).

The difference between the distributions of positive cases among gender was not statistically significant even though 83 (89.2%) of the specimen collected from males were positive as compared to females 39 (81.3%) (Table 4.15B). Analysis of positive results by the gold standard stratified by age indicated that 49/52 (94.2%) of patients aged 0-19 years were positive, followed by 20-39 years 27/31 (87.1%), 40-59 years 37/43 (86.0%), 60-79 years 8/10 (80.0%) and above 80 years 1/5 (20.0%). The statistical differences between the age distribution was significant (<0.0001) (Table 4.15C).

Table 4.15: Confirmed cases stratified by study site gender and age group (yrs.)

Stratum	**Number confirmed positive by the gold standard n (%)**	**p-value**
A. Study site		
Ga West Hospital (n=52)	46 (88.5)	0.473
Nkawie-Toase Hosp (n=21)	19 (90.5)	
Obom Health Centre (n=17)	16 (94.1)	
Paakro Health Centre (n=31)	25 (80.6)	
Tepa Govt Hospital (n=20)	16 (80.0)	
B. Gender		
Male (n=93)	83 (89.2)	0.188
Female (n=48)	39 (81.3)	
C. Age group (yrs.)		
0-19 (n=52)	49 (94.2)	

20-39 (n=31)	27 (87.1)	<0.0001
40-59 (n=43)	37 (86.0)	
60-79 (n=10)	8 (80.0)	
80+ (n=5)	1 (20.0)	

4.8.2 Sensitivity and specificity of the diagnostic techniques

Table 4.16 indicates the various diagnostic indices of the techniques being compared. Using *IS2404* nested PCR as the gold, the sensitivities of the techniques were BU-LAMP molecular assay (91.5%), first run *IS2404* PCR (85.2%), sedimentation with phenol ammonium sulphate (61.5%), centrifugation with phenol ammonium sulphate (59.8%), sedimentation with phosphate buffered saline (55.7%), centrifugation with phosphate buffered saline(54.1%), direct smear microscopy using specimen cellular suspension (53.3%), sedimentation with sodium hypochlorite (51.6%), centrifugation with sodium hypochlorite (50.0%) and direct specimen smear (41.8%). The techniques had specificity and positive predictive value of 100.0%.

Among the two molecular assays, BU-LAMP agreed more with the *IS2404* nested PCR (κ=0.72) than first run PCR (κ=0.60). However, there was poor to fair agreement between the *IS2404* nested PCR gold standard and the microscopy techniques (κ=0.15-0.30) even though the intermicroscopy agreement between the microscopy technique was almost perfect (κ=0.83-0.90).

There was varied % for negative predictive value (NPV). The %NPV for the techniques were as follows: BU-LAMP molecular assay (63.3%), first run *IS2404* PCR (51.3%), sedimentation with phenol ammonium sulphate (28.8%), centrifugation with phenol ammonium sulphate (27.9%), sedimentation with phosphate buffered saline (26.0%), centrifugation with phosphate buffered saline(25.3%), direct smear microscopy using specimen cellular suspension (25.0%), sedimentation with sodium hypochlorite (24.4%), centrifugation with sodium hypochlorite

(23.7%) and direct specimen smear (21.1%). The positive predictive value and the specificity of the diagnostic techniques were 100.0%.

Table 4.16: Diagnostic indices of the molecular and the microscopy techniques

Protocol	Technique	TP	TN	FP	FN	Sens (95% CI)	Spec (95% CI)	PPV	NPV
Direct Smear Microscopy	Clinical Specimen	51	19	0	71	41.8 (32.9 – 51.0)	100.0 (82.4-100.0)	100.0	21.1
	Specimen suspension	65	19	0	57	53.3 (44.0-62.4)	100.0 (82.4-100.0)	100.0	25.0
Centrifugal concentration Smear Microcopy	Conventional with PBS	66	19	0	56	54.1 (44.8-63.2)	100.0 (82.4-100.0)	100.0	25.3
	Modified with NaOCl	61	19	0	61	50.0 (40.8-59.2)	100.0 (82.4-100.0)	100.0	23.7
	Modified with PAS	73	19	0	49	59.8 (50.6-68.6)	100.0 (82.4-100.0)	100.0	27.9
Concentration by sedimentation Smear Microcopy	Modified with PBS	68	19	0	54	55.7 (46.5-64.7)	100.0 (82.4-100.0)	100.0	26.0
	Modified with NaOCl	63	19	0	59	51.6 (42.4-60.7))	100.0 (82.4-100.0)	100.0	24.4
	Modified with PAS	75	19	0	47	61.5 (50.6-68.6)	100.0 (82.4-100.0)	100.0	28.8
Molecular Technique	First run IS2404 PCR	104	19	0	18	85.2 (77.6-91.2)	100.0 (82.4-100.0)	100.0	51.3
	BU-LAMP Assay	111	19	0	11	91.5 (84.4-95.4)	100.0 (82.4-100.0)	100.0	63.3

TP-True Positive, TN-True Negative, FP-False Positive, FN-False Negative, PPV-Positive Predictive Value, NPV-Negative Predictive Value, PBS-Phosphate buffered saline, NaOCl - Sodium hypochlorite, PAS – phenol ammonium sulphate, sensitivity, specificity and 95% CI was determined by Fisher's exact test.

Table 4.17: Inter-technique kappa analysis: Gold standard vs. other techniques

			Inter-rater agreement (kappa statistic)							
			Direct protocols		Centrifugation			Sedimentation		
Protocol	**Diagnostic Technique**	**Positivity Rate n (%)**	**Nested Vs. Direct smear**	**Nested Vs. Cell suspension**	**Nested vs. PBS**	**Nested vs. NaOCl**	**Nested vs. PAS**	**Nested vs. PBS**	**Nested vs. NaOCl**	**Nested vs. PAS**
Molecular Methods	Nested *IS2404* PCR	122 (86.5)	0.15	0.23	0.24	0.21	0.28	0.25	0.22	0.30
	First run *IS2404* PCR	104 (73.8)								
	BU-LAMP assay	111 (78.7)								
Direct Smears	Clinical Specimen	51 (31.7)								
	Cellular Suspension	65 (46.1)								
Centrifugation	Conventional with PBS	66 (46.8)								
	Modified with NaOCl	61 (43.3)								
	Modified with PAS	73 (51.7)								
Sedimentation	Modified with PBS	68 (48.2)								
	Modified with NaOCl	63 (44.7)								
	Modified with PAS	75 (53.2)								

4.8.3 Gel electrophoretic products of First run and nested *IS2404* PCR

Figure 4.3A indicates the electrophoretic pattern of the first run *IS2404* PCR. Out of the first twenty (20) samples run only one was positive (sample 8). The positivity rate was increased by 900% when nine out of the twenty samples (as shown in figure 4.3B) were positive with the nested PCR. The first run yielded about 500 bp and the nested *IS2404* PCR yielded DNA of 200 bp molecular weight.

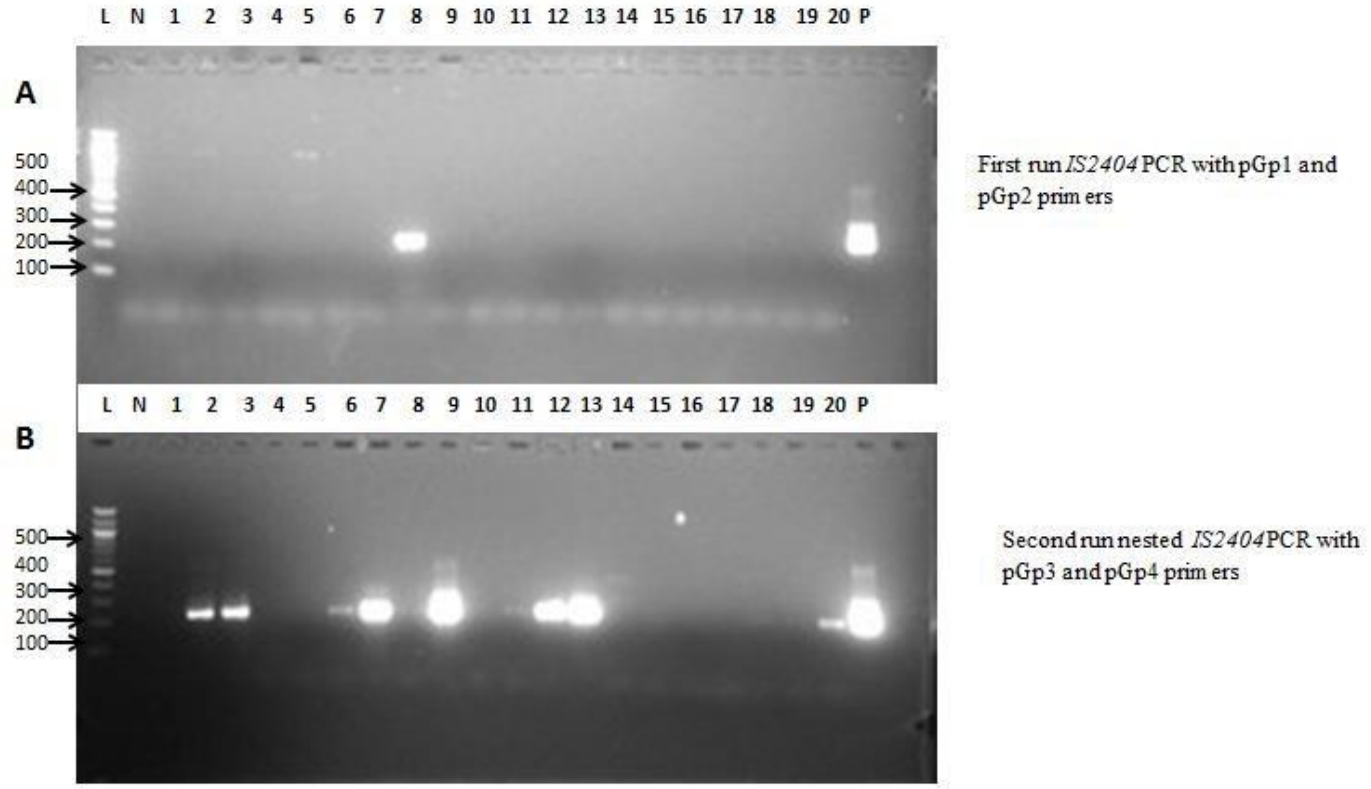


Figure 4.3: Electrophoretic pattern of the first run and the second run nested PCR A-first run *IS2404* PCR, B-second run (nested) *IS2404* PCR, L-100 bp molecular ladder size marker, N-negative control, P-positive control, positive samples for first run-sample 8, positive samples by nested PCR-samples 1, 2, 5, 6, 8, 11, 12, 19 and 20.

4.8.4 Analysis of results stratified by education level and occupation

Out of the 54 patients who had had no formal education, 44/54 (81.5%) were positive, 39/43 (90.7%) of the patients who had just had primary education were positive, 25/30 (83.3%) of patients who only completed middle school or junior high were positive. However, all the

patients who had completed secondary education or its equivalent and tertiary education were positive (Table 4.18).

Table 4.18: Analysis of results with respect to highest education attained

Highest education attained	No. of Patients	*No. of Patients tested Positive	No. of Patients tested Negative
No Education	54	44 (81.5)	10 (18.5)
Primary Education	43	39 (90.7)	4 (9.3)
Middle/JSS/JHS	30	25 (83.3)	5 (16.7)
Secondary Education	8	8 (100.0)	0 (0.0)
Tertiary	6	6 (100.0)	0 (0.0)

* *Positive by gold standard*

There were unequal frequencies of the results with respect to the occupation of the patients. From Table 4.19, all the lesions that were taken from mechanics, hairdressers and other professionals were positive on analysis. The respective percentages of pupils, traders, farmers and those not working whose lesions were positive were 35/39 (89.7%) and 14/16 (87.5%), 51/62 (82.3%) and 16/18 (88.9%).

Table 4.19: Analysis of results with respect to Occupation

Occupation	Number of Patients n (%)	No. of Patients tested Positive (%)	No. of Patients tested Negative (%)	p-value
Pupil	39	35 (89.7)	4 (10.3)	0.214
Trading	16	14 (87.5)	2 (12.5)	0.903
Farming	62	51 (82.3)	11 (17.7)	0.189
Mechanic	3	3 (100.0)	0 (0.0)	0.490
Hairdresser	3	3 (100.0)	0 (0.0)	0.490
Not occupied	18	16 (88.9)	2 (11.1)	0.671
Others	6	6 (100.0)	0 (0.0)	0.490

[1] *Positive by gold standard test*

4.9: Analysis of the efficiency of the protocols on clinical forms of the disease

4.9.1 The molecular methods

With reference to Table 4.20, the best molecular assay for detecting DNA of the *M. ulcerans* in ulcerative lesion was nested *IS2404* PCR (positivity rate (PR) =86.4%) followed by BULAMP assay (PR=79.6%). For nodular lesions, *IS2404* PCR detected *M. ulcerans DNA* in 87.0% of the lesions and BU-LAMP assay detected the DNA of the bacteria 82.6% of the nodular lesions. Nested *IS2404* PCR and first run PCR had equal efficiency of detecting *M. ulcerans* DNA in oedematous lesions (86.7%). There were no statistical differences between the efficiencies of the molecular assays in detecting *M. ulcerans* DNA among the three clinical forms of the disease ($p>0.05$, chi square).

4.9.2 The direct methods

Direct smear of cellular suspensions detected AFBs in 40.7% of ulcerative lesions compared to 27.2% when direct smear of clinical specimens were examined. Similarly, direct smears of cellular suspensions detected AFBs in 53.3% of oedematous lesions compared to 46.7% when direct smears from clinical specimens were examined. However, direct smears from clinical specimens detected AFBs in 69.5% of nodular lesions compared to 65.2% when cellular suspension smears were examined. The differences between the efficiency of the direct smear microscopy in detecting AFBs among the clinical forms were statistically significant ($p<0.0001$, chi square). Similarly, there was statistical difference in the efficiency of the direct smears from cellular suspension in detecting AFBs among the three clinical forms ($p=0.029$, chi square) (Table 4.20).

4.9.3 Centrifugation methods

Centrifugation of cellular suspension after prior digestion with phenol ammonium sulphate demonstrated AFBs in 46.6% of ulcerative lesions whilst phosphate buffered saline detected

AFBs in 43.6% of ulcerative lesions. Phenol ammonium sulphate demonstrated AFBs in 73.9% of nodular lesions and sodium hypochlorite also demonstrated AFBs in 69.5% of nodular lesions. In oedematous lesions, all the three methods detected AFBs with equal ability (53.3%). With the exception of phenol ammonium sulphate protocol, there was statistical difference in the efficiencies of the other protocols in detecting AFBs among the clinical forms of the disease ($p<0.05$, chi square) (Table 4.20).

4.9.4 Sedimentations methods

Overnight sedimentation of cellular suspension with phenol ammonium sulphate detected AFBs in 48.5% and 74.0% of ulcerative and nodular lesions. However, phosphate buffered saline detected AFBs in oedematous lesions better than phenol ammonium sulphate (60.0% against 53.3%). In all the sedimentation techniques, there was statistical differences in their ability to detect AFBs in the three clinical forms of the disease ($p\leq0.05$) (Table 4.20).

Table 4.20: Performance of the protocols on clinical forms of the disease

Protocol	Technique	Ulcer (n=103)	Nodule (n=23)	Oedema (n=15)
Molecular Assay	Nested *IS2404* PCR	89 (86.4%)	20 (87.0%)	13 (86.7%)
	First Run *IS2404* PCR	74 (71.8%)	17 (74.0%)	13 (86.7%)
	BU-LAMP assay	82 (79.6%)	19 (82.6%)	10 (66.7%)
Direct smears	Clinical specimen	28 (27.2%)	16 (69.5%)	7 (46.7%)
	Cellular suspension	42 (40.7%)	15 (65.2%)	8 (53.3%)
Centrifugation	Phosphate buffered saline	45 (43.6%)	13 (56.5%)	8 (53.3%)
	Sodium hypochlorite	37 (36.0%)	16 (69.5%)	8 (53.3%)
	Phenol ammonium sulphate	48 (46.6%)	17 (74.0%)	8 (53.3%)
Sedimentation	Phosphate buffered saline	43 (41.7%)	16 (69.5%)	9 (60.0%)
	Sodium hypochlorite	39 (37.8%)	16 (69.5%)	5 (33.3%)

	Phenol ammonium sulphate	50 (48.5)	17 (74.0%)	8 (53.3%)

4.10 Analysis of the efficiency of the protocols on category of lesions

4.10.1 Molecular methods

Nested *IS2404* PCR, first run *IS2404* PCR and BU-LAMP assay detected DNA in 90.6%, 86.05 and 82.8% of category I lesions respectively. Nested *IS2404* PCR, BU-LAMP assay and first run *IS2404* PCR detected DNA in 87.5%, 83.3% and 54.2% category II lesions respectively. Similarly, Nested *IS2404* PCR, BU-LAMP assay and first run *IS2404* PCR detected DNA in 81.1%, 71.7% and 67.9% category III lesions respectively. There was no statistical differences in the ability of *IS2404* nested PCR and BU-LAMP in detecting *M. ulcerans* DNA in all the categories of the lesions ($p>0.05$), however, the DNA detecting ability of first run IS2404 PCR among the three categories was significant ($p=0.025$).

4.10.2 Direct methods

Direct smears from cellular suspensions detected AFBs in more lesions than direct smears from clinical specimens in all the categories of lesions: category I (56.3% vs. 53.1%), category II (58.3% vs. 16.7%) and category III (28.3% vs. 24.5%). There was statistical difference between the ability of the two direct methods to detect AFBs from the three categories of lesions ($p<0.05$) with the direct smearing of cellular suspension of specimen detecting comparatively more AFBs that the direct smearing of the clinical specimen among the categories of lesions.

4.10.3 Centrifugation methods

Centrifugal concentration of cellular suspensions detected AFBs in 59.4% of category I lesions when digested in phenol ammonium sulphate whilst sodium hypochlorite and phosphate buffered saline had equal efficiency of 56.3%. For category II lesions, there was equal efficiency of phenol ammonium sulphate and phosphate buffered saline (58.3%). For category III lesions, phenol ammonium sulphate, phosphate buffered saline and sodium hypochlorite detected AFBs in 39.6%, 30.2% and 22.6% of lesions respectively.

4.10.4 Sedimentation methods

Phenol ammonium sulphate was better at detecting AFBs in category I and III lesions with detecting rate of 60.1% and 41.5% respectively but in category II lesions, phenol ammonium sulphate shared honours with phosphate buffered saline (58.3%). There was a highly significant differences (p=0.009), high significant differences (p=0.030) and no statistical differences (p=0.058) in the respective ability of sodium hypochlorite, phosphate buffered saline and phenol ammonium sulphate in detecting AFBs from the three categories of lesions.

Table 4.21: Performance of the protocols on the category of the lesions

Protocol	Technique	Category I (n=64)	Category II (n=24)	Category III (n=53)	p-value
Molecular Assay	Nested *IS 2404* PCR	58 (90.6)	21 (87.5)	43 (81.1)	0.946
	First run *IS2404* PCR	55 (86.0)	13 (54.2)	36 (67.9)	0.025
	BU-LAMP Assay	53 (82.8)	20 (83.3)	38 (71.7)	0.245
Direct smears	Clinical specimen	34 (53.1)	4 (16.7)	13 (24.5)	0.001
	Cell suspension	36 (56.3)	14 (58.3)	15 (28.3)	0.013
Centrifugation	PBS	36 (56.3)	14 (58.3)	16 (30.2)	0.024
	Sodium hypochlorite	36 (56.3)	13 (54.2)	12 (22.6)	0.002
	PAS	38 (59.4)	14 (58.3)	21 (39.6)	0.167
Sedimentation	PBS	37 (57.8)	14 (58.3)	17 (32.1)	0.030
	Sodium hypochlorite	36 (56.3)	13 (54.2)	14 (26.4)	0.009
	PAS	39 (60.1)	14 (58.3)	22 (41.5)	0.058

% in parenthesis

4.11 Cohen's Kappa analysis of the molecular assays on sampling methods

Out of the 103 swabs specimens collected, *IS2404* nested PCR detected *M. ulcerans* DNA in 89 of them. First run PCR as well as BU-LAMP detected DNA in 74 and 82 respectively.

There was perfect inter-molecular assay agreement between the DNA detecting ability from swab specimens of *IS2404* nested PCR and BU-LAMP (κ=0.89) and the agreement between

IS2404 nested PCR and first run PCR was substantial (κ=0.63). Among the 38 fine needle aspirates, *IS2404* nested PCR detected DNA in 33 specimens, first run PCR detected DNA in 30 specimens and BU-LAMP detected DNA in 29 of the 38 FNA specimens. Despite these differences, the inter-molecular diagnostic agreement of the three molecular assays were perfect (κ=0.93-0.94).

Table 4.22: Cohen's kappa analysis of the molecular methods

Sampling method	**Positive by Nested *IS2404* PCR**	**Positive by First run *IS2404* PCR**	**Positive by BU-LAMP assay**	**Nested PCR vs. First run PCR**	**Nested PCR vs. BU-LAMP**
Swabs (n=103)	89 (86.4)	74 (71.8%)	82 (79.6%)	**0.63**	**0.89**
Fine Needle Aspirates (n=38)	33 (86.8)	30 (79.0)	29 (76.3)	**0.94**	**0.93**

Out of the 103 swabs specimen collected, direct smears detected AFBs in 28 of them and direct smearing of cellular suspension detected AFBS in 42 specimens. The inter-microscopy agreement between the techniques was substantial (κ=0.74). The two direct methods had equal ability of detecting AFBs in fine needle aspirates. Each technique detected AFBs in 23 specimens. The inter-rater agreement in the ability to detect AFBs form FNA was equal (κ=1.0).

Table 4.23: Cohen's Kappa analysis of the direct smear methods

Sampling method	**Positive by direct smearing of clinical specimen**	**Positive by direct smearing from cellular suspension**	**Kappa value**
Swabs (n=103)	28 (27.2)	42 (40.8)	**0.74**
Fine Needle Aspirates (n=38)	23 (60.5)	23 (60.5)	**1.0**

Out of the 103 swabs specimen collected, the PAS concentration methods detected the highest number of AFBs in the specimen (sedimentation-50 specimens and centrifugation-48

specimens), followed by PBS concentration methods (sedimentation-43 specimens and centrifugation-45 specimens) and NaOCl concentration methods (sedimentation-39 specimens and centrifugation-37 specimens). In spite of all these differences in AFB detection rate in swab specimens among the techniques, these differences were not statistically significant (p=0.953). Among the fine needle aspirates, sedimentation by PAS, PBS and centrifugation by PAS detected AFBs in 25 (65.8%) of the aspirates. The next technique that performed better was centrifugation by NaOCl which detected AFBs in 24 aspirates and finally sedimentation by NaOCl and centrifugation by PBS detected AFBs in 21 specimens each, however the differences between these figures were not statistically significant (p=0.961).

Table 4.24: Comparison of the modifications of the ZN microscopy on specimen type

	Positive results from concentration by centrifugation			**Positive results from concentration by sedimentation**		
Clinical Specimen	PBS	NaOCl	PAS	PBS	NaOCl	PAS
Swabs (n=103)	45 (43.7)	37 (35.9)	48 (46.6)	43 (41.7)	39 (37.9)	50 (48.5)
Fine Needle Aspirates (n=38)	21 (55.3)	24 (63.2)	25 (65.8)	25 (65.8)	21 (55.3)	25 (65.8)

PBS-phosphate buffered saline; NaOCl-sodium hypochlorite; PAS-phenol ammonium sulphate

4.12 Molecular sensitivity of BU-LAMP and *IS2404* nested PCR

Figure 4.4 indicates the detection limits of the molecular methods. BU-LAMP and IS2404 nested PCR were able to detect DNA densities as low as 30 copies of IS2404 (sample number 1-5). All the agarose gel electrophoresis of the PCR amplicons of the serially diluted DNA extract gave a band with molecular weight approximately 200 bp. The LAMP reaction of all the same serially diluted DNA extract that was positive gave the same intensity of fluorescence. The PCR could further detected DNA as few as only 3 copies of *IS2404* but BU-LAMP could not detect this level of DNA copies.

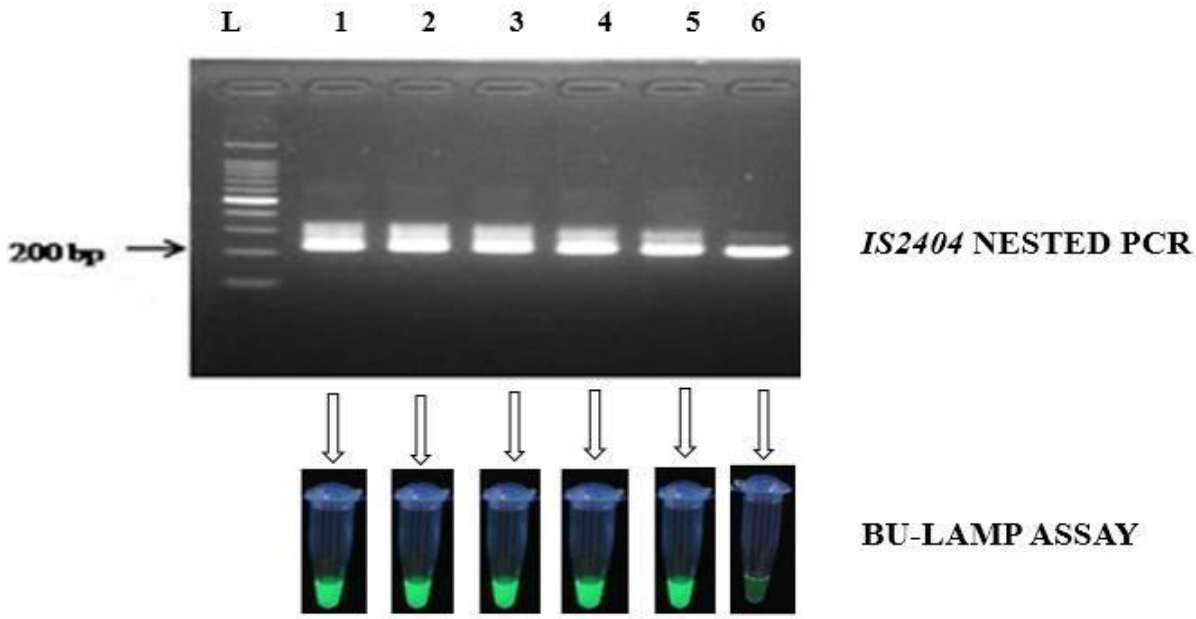


Figure 4.4: The detection limits of the BU-LAMP and *IS2404* nested PCR

L-100 bp molecular ladder size marker; **1** - $3.0x10^5$ copies of *IS2404*; **2** - $3.0x10^4$; **3** - $3.0x10^3$; **4** - $3.0x10^2$; **5** - $3.0x10^1$; **6** - 3.0. Samples 1-5 were detectable by both BU-LAMP and PCR. Sample 6 detected by PCR but not LAMP

CHAPTER 5

DISCUSSION

5.1 Gender, age and Residential Distribution of the Participants

Most epidemiological studies designed to understand the dynamics of Buruli ulcer disease (BUD) have associated the disease to be more prevalent among females than males. Herbinger *et al.* (2008) reported 59.1% females and 40.9% males in a study involving 384 suspected cases. Phillips *et al.* (2009) also reported 60.0% and 40.0% prevalence of Buruli ulcer in females and males respectively among 45 suspected Buruli ulcer patients. This study found a contrasting gender association of BU when out of the 141 suspected patients that were studied, 68.0% were males and 32.0% were females. The study found no statistical differences in gender distributions among the study sites ($p>0.05$) (Table 4.1). The differences in gender association of Buruli ulcer obtained by this study may be due to the fact the adults males are now considering hospital-based ulcer care as alternative to crude herbal treatment.

Buruli ulcer disease has been reported to be disproportionately distributed among age groups with patients less than 15 years significantly affected (Herbinger *et al.,* 2008). In this study, 52 (36.9%) of the patients were children below 20 years. Out of this 48 (92.3%) of the children were below 16 years and all of them were infected with *M. ulcerans*. This data confirm the premise that children up to 15 years are commonly infected by *M. ulcerans* (Asiedu *et al.,* 2000; Amofah *et al.,* 2002; Herbinger *et al.,* 2008). The observed high prevalence of the disease among children in this and other studies could be due to the fact that children and young adults usually get into contact with the soil and water bodies unprotected. Soil and water bodies are known perceived sources of infections of the *M. ulcerans* in endemic communities (Portaels, *et al*, 2008; O'Brien *et al*., 2011).

Buruli ulcer disease is still associated with rural life. Residents in rural and economically deprived areas are mostly farmers who cultivate the land. *M. ulcerans* is an environmental mycobacterium (Hayman, 1991) and they predispose rural residents to the infections more than

urban dwellers. Due to lack of potable water, rural dwellers depend on streams, dams and ponds as their source of water. *M. ulcerans* infections are associated with slow flowing or stagnant water (Marston *et al.*, 1995). Even though the mode of transmission of *M. ulcerans* is still unknown (Portaels *et al.*, 1996), virtually all the risk factors and conditions that are perceived to aid the transmission of the disease are all associated with rural life (Phillips *et al.*, 2009).

5.2 Clinical data of the BU patients

Data obtained from this study confirmed the anatomic association of Buruli ulcer lesions to the lower limbs. There was no statistical difference in the distribution of the locations of the lesions among gender ($p>0.05$) (Table 4.5) There is consistency with this and data obtained from other studies. In 2002, Amofah *et al.* (2002) reported 65.6% lesions located on the lower limbs and Agbenorku *et al.* (2012) reported 57.5% association of Buruli ulcer lesions to the lower limbs. This study found out that 84.4% of the lesions were on the lower limb. The lower limbs are always in constant touch with the soil and water bodies during normal daily activities. The *M. ulcerans* might have been transmitted due to the persistent of the Mycobacterium in the environment of endemic areas. This site specific association of Buruli ulcer can be due to not wearing lower body clothing while farming (Pouillot *et al.*, 2007).

In spite of extensive education and clinical interventions to reduce the incidence and disease severity of Buruli ulcer (Adu *et al.*, 2011), most of the encountered lesions in this study were in the ulcerative forms. A hundred and three (73.0%) of the lesions were ulcers out of which 53 (37.5%) were category III ulcers. Category III ulcers are large surface area ulcers more than 15 cm in diameter (Sarfo *et al.*, 2010) and they require both antibiotic and surgical interventions for management unlike category I and II lesions that may completely heal with antibiotics treatment alone (WHO, 2008). Late reporting of cases delay healing time and demands more resources for management.

5.3 Positivity rate of the diagnostic techniques

Current reporting of cases of BU infections in non-specialized centres is based on the presentation of clinical symptoms. This represents a challenge due to the vast number of other skin infections or conditions that may exhibit symptoms similar to that of Buruli ulcer (Coloma *et al.,* 2005). WHO has therefore directed that all suspected cases of Buruli ulcer are confirmed in the laboratory (WHO, 2008). The ideal diagnostic technique that will be able to achieve this purpose is a method with high positivity rate. Among the three molecular methods, the *IS2404* nested PCR had the highest positivity rate of 86.5% followed by BULAMP assay with positivity rate of 78.7% and then first run *IS2404* PCR (the first amplification stage of nested PCR using *M. ulcerans* specific primers) with positivity rate of 73.8%. With respect to the *IS2404* nested PCR gold standard, the positivity rate of the BU-

LAMP substantially agreed with the gold standard (κ=0.72, Cohen's kappa) than first run

PCR which agreed with the gold standard moderately (κ=0.60). *IS2404* nested PCR has been used in several study as the gold standard for detecting *M. ulcerans* due to its high sensitivity, specificity and relative high positivity rate (Phillips *et al.,* 2009; Herbinger *et al.,* 2010; Yeboah-Manu *et al.,* 2011; Ablordey *et al.,* 2012;). There were 19 discordant results between the gold standard and the BU-LAMP molecular assay. This discordant results could be due to possible reason that the DNA concentration in the eleven samples was below the LAMP detection level (less than 3 copies of the insertion sequences) or DNA might have been degraded in the samples since the samples from outside Accra were stored for a long period (November 2014 to April 2015). Analysing specimens immediately they are taken will probably help prevent these false negatives. All the eight (8) microscopy techniques were able to detect some AFBs in the ZN smears. The average positivity rate of the direct methods was 41.1% and that of all the sedimentation methods was 48.7% and 47.3% for the centrifugation methods. Among the direct smear protocol, specimen cellular suspension increased the positivity rate over the direct smear by 14.4% (31.7% vs. 46.1%). Among the centrifugation

protocol, centrifugation with phenol ammonium sulphate had the highest positivity rate of 51.7%, 4.9% higher than centrifugation with phosphate buffered saline and 8.4% higher than centrifugation with sodium hypochlorite. In all, the sedimentation protocol gave relatively the best positivity rate. However, sedimentation with phenol ammonium sulphate gave the highest positivity rate of 53.2%; 8.5% better than sodium hypochlorite and 5.0% efficient than phosphate buffered saline. The overall chemical modification that gave the best results was the phenol ammonium sulphate treated specimen (average AFB positivity detection rate of 52.4%), followed by phosphate buffered saline (47.5%) and sodium hypochlorite (43.9%) (Table 4.12). The phenol ammonium sulphate gave the best positivity rate probably due to its ability to precipitate proteins in low concentrations without decreasing the AFB counts and also it fixes the smear firmly on the slide to prevent being washed away (Selvakumar *et al.,* 2002). Phenol ammonium-sulphate also prevents the formation of hydrogen bonds of proteins with water and facilitates the interaction of proteins with each other to form aggregates. This causes the mucus and other proteins in the smears to precipitate. This precipitates are washed away leaving a smear with clear background with reduced obstructive deposits (Chedore *et al.,* 2002).

5.4 Diagnostic Sensitivity and specificities of the techniques

For a diagnostic technique to be highly sensitive, when compared to its appropriate gold standard, the technique should be able to produce very few or no false negative. In the same way a highly specific diagnostic technique should yield few or no false positives when its diagnostic products are compared to an appropriate gold standard (Ablordey *et al.,* 2012). The *IS2404* nested gold standard used for this study confirmed 122 lesions as true positive and 19 true negatives. When results of first run *IS2404* PCR and BU-LAMP were analyzed, BU-LAMP proved to be the most sensitive molecular assay for the confirmation of *M. ulcerans* infections. The sensitivity of BU-LAMP assay was 91.5% (95% CI, 84.4-95.4) and the sensitivity of first run *IS2404* PCR was 85.2% (95% CI, 77.6-91.2). The sensitivity of the BU-

LAMP assay was comparative higher than that of first run *IS2404* PCR due to the utilization of three specific primers pairs involved in the technique (Ablordey *et al.,* 2012; deSouza *et al.,* 2012) as against only one specific primer pairs used for the first run *IS2404* PCR (Stienstra *et al.,* 2003). Secondly, this study found the detection limit of BU-LAMP to be 30 copies of *IS2404*. The faint electrophoretic bands that corresponded to 30 copies of insertion sequences with the *IS2404* nested PCR molecular assay were all negative with the first run PCR confirming that the detecting limits for the first run *IS2404* PCR was greater than 30 copies of insertion sequence. This accounted for the relative lower sensitivity of the first run PCR compared to the BU-LAMP.

When the chemically digested cellular suspension was concentrated by sedimentation, the sedimentation with phenol ammonium sulphate gave the best sensitivity of 61.5% (95% CI, 50.6-68.6) followed by 55.7% (95% CI, 46.5-64.7) sensitivity with sedimentation by phosphate buffered saline and then a sensitivity of 51.6% (95% CI, 42.4-60.7) with by sedimentation sodium hypochlorite. The high speed centrifugation post-chemical digestion yielded similar results trend with the sedimentation technique. The sensitivity of the techniques were PAS 59.8% (95% CI, 506-68.6), PBS 54.1% (95% CI, 44.8-63.2) and NaOCl 50.0% (95% CI, 40.8-59.2). With respect to the *IS2404* nested PCR gold standard, there was poor to fair agreement with the eight microscopy techniques (κ=0.15-0.28) even though sedimentation and centrifugation with PAS were the most efficient.

The use of sodium hypochlorite (bleach) can be traced back to 1909, where it was used as a mucolytic agent before centrifuging sputum samples. These sample processing methods were able to able to increase the sensitivity of the ZN technique in the diagnosis of pulmonary tuberculosis by 26.0% (Gebre-Selassie, 2003). The bleach method is proven to be efficient on extra-pulmonary tuberculosis. Samples such as abscess fluids, lymph node aspirates, body fluids or skin scrapes and found the method and documented that bleach did improve sensitivity (Khubnani and Munjal, 2005). Even though the average sensitivity of

the bleach concentration method (50.5%) performed better than the direct method (47.5%), the former performed poorly when compared with centrifugation with PBS and PAS. This study found the mean AFB count using sodium hypochlorite to concentrate AFBs to be 11.1-11.5 AFBs/100 high power fields. This is possibly because sodium hypochlorite as a disinfectant is proven to be effective in reducing the population of microbes and therefore it is being used to reduce the infection risk to health personnel (Best *et al.,* 2005). Sodium hypochlorite is also reported to destroy Mycobacteria and reduce their density in solution and in smears (Githui *et al.,* 2007). Phenol has some bactericidal property but its effect is not as strong as sodium hypochlorite. Phenol ammonium sulphate yielded a mean AFB count of 23.7-30.3 AFBs/100 high power fields which is significantly higher than that of sodium hypochlorite.

The sensitivity of the PBS treatment yielded better results than the direct and the NaOCl treated specimen but poorer than the PAS. This is because PBS is an isotonic solution and for that matter, it can only give an isotonic environment to the bacilli and their surrounding debris without positively influencing the detection of the AFBs in smears.

Each of the techniques yielded diagnostic specificity of 100.0%. This result is consistent with study conducted by Phillips *et al.* (2005) which also yielded a specificity of 100.0% although they had varying degree of sensitivities (Phillips *et al.,* 2005). With this high specificity of these techniques, the study techniques will be able to detect only *M. ulcerans* in the lesion and other lesions containing AFB, such as those of tuberculosis, leprosy or non-tuberculous mycobacterial infections are unlikely to be confused clinically with *M. ulcerans* disease. Similarly, the techniques being studied and those reported by Phillips *et al.* (2005) had positive predictive value of 100.0% each. This further confirms the specificity of the techniques in diagnosing *M. ulcerans* infections.

5.5 Efficiency of study protocols on clinical forms and categories of lesions

IS2404 nested PCR detected 86.4% of the ulcerative lesions compared to 79.6% and 71.8% detection rate of BU-LAMP and first run *IS2404* respectively of *M. ulcerans* DNA in ulcers.

In nodular lesions, the detection rate of *IS2404* nested PCR was 87.0% as against 82.6% and 74.0% with BU-LAMP and first run PCR respectively. In oedematous lesions, the two PCR assays had equal detection rate of 86.7% and BU-LAMP detected DNA in 66.7% of lesions. The reasons for these differences were not explored in this study.

Table 4.20 shows that the performance of all the microscopy techniques was better among the non-ulcerative lesions than the ulcerative forms. Similarly, both the molecular and the microscopy techniques detected more *M. ulcerans* DNA and AFBs among the category I lesions (small lesions less than 5 cm in diameter) than category II (moderate lesion 5-10 cm in diameter) and category III lesions (large lesions more than 15 cm in diameter). These observations stems from the fact that as the disease progresses, the number of bacilli present in the lesions reduces. *M. ulcerans* is known to produce a cytotoxic lipid called mycolactone that induces apoptosis in human patients (George *et al.,* 1999). Mycolactone-positive, cytotoxic strains are virulent and multiply progressively. The cytotoxicity of these strains leads to progressive destruction of the inflammatory infiltrates by post-apoptotic secondary necrosis, generating necrotic acellular areas with extracellular bacilli released by the lysis of infected phagocytes (Martinha *et al.,* 2005). The extracellular bacilli are also destroyed by the presence of mycolactone released by the bacilli in a concentration-dependent rate (Sarfo *et al.,* 2010). This confirms that the non-ulcerated and small lesions less than 5 cm in diameter (category I) contains high number of the bacilli and for that matter easily detected by molecular and microscopy methods. Data presented on Table 4.21 indicates that the chances of detecting the bacilli in non-ulcerative lesions, on the average, are twice the chance of detecting the bacilli in category II and III ulcers. The bacilli in ulcers are unevenly distributed (Guimaraes-Peres *et al*., 1999) and this further reduces the sensitivity of the microscopy techniques of detecting the AFBs especially if exudates sampled were less than 40.9mg (Aninagyei *et al.,* unpublished data).

5.6 Ziehl-Neelson BU Microscopy, modifications and detection limits

This study presents an evaluation of laboratory procedures aimed at improving the detection of *M. ulcerans* from Buruli ulcer samples for detection of the disease. The conventional procedures (direct smear microscopy) were evaluated by comparing their performance with that of modified specimen processing procedures. The most efficient technique was a method which involved the treatment of the samples with phenol ammonium sulphate (PAS) solution concentrated by sedimentation. This is a protocol that has been investigated and found to be useful in *M. tuberculosis* studies (Selvakumar *et al*., 2002). Equally favourable results have been reported on the performance of PAS where it was used in conjunction with basic fuchsin and also in overnight sedimentation for the detection of AFB for *M. tuberculosis* diagnosis (Selvakumar *et al*., 2002). Smears from this protocol appeared relatively clearer with good contrast. The bacilli were clearly visible and with few background deposits. The microscopy detection limits for the sedimentation and the concentration with PAS was 4 and 16 bacilli/1 ml of PBS solution. This detection limit makes the PAS technique very sensitive in detecting *M. ulcerans* bacilli as low as 4 bacilli in 1 ml of suspension. Micrographs of smears prepared from PAS showed clearly visible fields (figure 4.2 (i) and (ii). In a tuberculosis study similar to this, the author reported sputum samples treated with PAS method had a relatively shorter slide reading period, the smears made from PAS processed samples remained intact on the slide after periods of washing but this was not the case with smears from sodium hypochlorite (NaOCl) processed samples (Sighal *et al.*, 2013).

The direct applications of swabbed or aspirated materials from lesion on a slide, recorded the lowest mean AFBs per 100 high power fields. As far as this study is concerned, the direct smear microscopy is routinely done but it has a poor performance for detecting AFBs in clinical samples. Poor performance of the direct smear microscopy could be as a result of the poor release of the exudates from the tip of the swab during smear preparation and also due the uneven distribution of the bacilli in the lesions. Outcomes from other studies on *M. tuberculosis* diagnosis showed that concentration by centrifugation recorded the highest average number of

AFBs per microscopic field (Gebre-Selassie, 2003) but this study yielded a better results with concentrated by sedimentation. The PAS-treated samples performed well but NaOCl did not do so well. The detection limits of the sodium hypochlorite technique, centrifugation and sedimentation were 32 and 16 bacilli in 1ml of cellular suspension respectively. This suggests that bacilli density less than these limits will not be detected by this method but PAS-treated specimen will detect. In the case of smears processed with sodium hypochlorite, the field appeared relatively darker with moderate background deposits
(figure 4.2 v and vi). This observation was consistent with findings made by Van Deun *et al.* (2000) and agreed by Ängeby *et al*. (2004) (Van Deun et al,, 2000; Ängeby et al,, 2004). In this study samples treated with hypochlorite before various concentration methods did not perform as well as the other protocols. Although other studies had reported on the method improvement after bleach treatment (Chew *et al*., 2011). The observed differences in smear reading in terms of clarity, may be due to procedural differences; whilst this study used a concentration of 3.5% hypochlorite, Frimpong *et al.* (2005) used 1% hypochlorite whilst working on MTB (Frimpong *et al*., 2005). This may probably explain the findings by Chew *et al* (2011) which reported that hypochlorite sedimentation seemed to have a ten-fold decrease in AFB present (Chew *et al*., 2011).

The smears from the phosphate buffered saline (PBS) treated samples produced smears with mild background deposits (figure 4.2 iii and iv). These deposits could probably be crystalized sodium chloride used in preparing PBS. The detection limit of the PBS technique was 16 and 8 bacilli per 1 ml of cellular suspension for centrifugation and sedimentation smears respectively, making PBS-treated specimen unsuitable to detect AFBs less than these limits. From these findings, sedimentation of BU lesions with phenol ammonium phosphate is the ideal protocol to determine treatment successes with streptomycin-rifampicin treatment.

5.7 Efficiency of FNA and swabs for laboratory diagnosis of BU disease

The sensitivity of swab sampling technique in the diagnosis of *M. ulcerans* infections was 86.4%, 79.6% and 71.8% respectively with *IS2404* nested PCR, BU-LAMP and first run PCR. The BU-LAMP assay agreed more perfectly with the gold standard (κ=0.89, Cohen's kappa) than first run PCR which agreed substantially (κ=0.63, Cohen's kappa). For fine needle aspirates, the respective sensitivities of the *IS2404* nested PCR, first run PCR and BULAMP in detecting *M. ulcerans* DNA was 86.8%, 79.0% and 76.3%. The agreement of the FNA sampling technique with the gold standard was perfect (κ=0.93-0.94). These findings were consistent with the findings obtained by Phillips *et al.* (2009) where the sensitivity of the FNA in detecting *M. ulcerans* DNA was 86.0% and Herbinger *et al.* (2008) also had a sensitivity of 90.0% with swab specimens when a molecular method was used (Phillips *et al.*, 2009; Herbinger *et al.*, 2008). In previous studies in Ghana, PCR of swab samples confirmed 60%–70% of suspected cases of BU disease with ulcerative lesions (Siegmund *et al.*, 2007; Bretzel *et al.*, 2007). The data on the comparative sensitivity of PCR and LAMP on swab samples obtained in this study suggests considering LAMP assays using swab samples to be the method of choice for cases that involve ulcerative lesions due to the advantages of the LAMP assay over PCR. A similar recommendation can also be made for the FNA specimen since the inter-technique agreement of *IS2404* nested PCR and LAMP was perfect (κ=0.93). LAMP assays can effectively replace PCR assays due to its relatively affordability, sensitivity, specificity, user-friendliness, robustness and rapidity with which results are obtained, equipment free and deliverable to the end user in a very short time.

There was substantial agreement of the direct smear microscopy with the direct cellular suspension to detect AFBs in swab specimen when the Cohen's kappa coefficient of 0.74 was obtained upon analysis of the data obtained by the two direct methods. Compared to the *IS2404* nested PCR gold standard, the sensitivity of direct smear microscopy to detected AFBs in clinical swab specimen was 27.2% and that of the cellular suspension of clinical specimen

was 40.8%. The sensitivity of FNA sampling technique to detect AFBs in both direct smear and direct cellular suspension was 60.5% each. The sensitivity of the modified microscopy techniques to detect AFBs in swab specimen was 35.9%-48.5% and that of the FNA specimen was 55.3%-65.8%. The statistical differences between the efficiencies of the modified techniques among the clinical specimens were not significant ($p>0.05$). The low sensitivity of the microscopic detection of *M. ulcerans* bacilli is due to the destruction of the bacilli by mycolactone (Read *et al.,* 1974) and consequently reduce the number of the bacilli or clear the bacilli from the infected site as the lesion progresses (Kathleen *et al.,* 2000).

5.8 Molecular detection limits of BU-LAMP and *IS2404* nested PCR

An experimental design was set up to determine the minimum molecular sensitivity of the *IS2404* nested PCR and the BU-LAMP assays. The set ups had 10-fold dilutions of purified DNA from $3.0x10^5$ to 3 copies of insertion sequences. Both assays could detect DNA up to 30 copies of *IS2404*. *IS2404* Nested PCR could further detect DNA in tube containing just 3 copies of the insertion sequence. Based on the premise that one *M. ulcerans* bacilli contains approximately 250 copies of insertion sequence *2404* (Stinear, 2005), these methods can amplify just 12.0% of a segment of the *M. ulcerans* genome but nested PCR can amplify up to 1.2% of a segment of *M. ulcerans*. This makes *IS2404* nested PCR ten times more sensitive than BU-LAMP. In a similar study, the detection limit of BU-LAMP was compared with conventional *IS2404* primers; the results contradicted the findings in this study. Desouza *et al.* (2012) found the molecular sensitivity of the BU-LAMP to be ten times that of conventional *IS2404* PCR (de-Souza *et al.*, 2012). These differences on the detection limit were possibly because this study used nested PCR primers which are more sensitive than conventional PCR. Nested PCR uses two pairs of primers (pGp1, pGp2, pGp3 and pGp4) and makes it more sensitive than conventional PCR which uses one pair of primer (MU1 and MU2) set (Stienstra *et al.,* 2003). This findings make it unsuitable to use *IS2404* nested PCR and BU-LAMP to

determine antibiotic treatment successes since these assays can detect DNA as few as 30 copies of *IS2404* (0.1 M. ulcerans). Cultivation of *M. ulcerans* remains the best technique to determine treatment outcome (Herbinger *et al.,* 2008).

5.9 Field applicability of the BU-LAMP molecular technology

A lot of studies have tried to assess the field applications of LAMP technology (Ablordey *et al.,* 2012; de-Souza *et al.,* 2012). Considering the methodology and handling of reagents associated with BU-LAMP assay, some aspects of this protocol must be improved to make the technique amenable for field studies. Data obtained from this study with BU-LAMP gave insignificant differences in positivity rate and sensitivity results that agreed substantially with the gold standard, indicating the suitability of the *IS2404* nested PCR to be replaced with BULAMP assay in confirming *M. ulcerans* infections. However, the 19 discordant results produced by the BU-LAMP, in this study, when pure DNA was used indicates that the techniques cannot perform well with unprocessed or crude DNA as was confirmed by Ablordey *et al.* (2012). BU-LAMP stock and working primers must be stored at temperatures below -20^{o}C. This temperature condition is difficult to attain in low resourced and field laboratories. To make BU-LAMP assay amenable for field use, the technology must be improved to make the assay sensitive with clinical samples and the primers should be manufactured and aliqouted in lyophilized forms that can be stored at 2-8^{o}C.

CHAPTER 6

CONCLUSION, RECOMMENDATIONS AND LIMITATIONS

6.1 Conclusion

This study compared the diagnostic sensitivity of BU-microscopy and its modifications, *IS2404* nested and first run polymerase chain reaction and *IS2404* loop-mediated isothermal amplification assay for detection of *M. ulcerans*. Among the three molecular methods, nested

PCR was the most sensitive followed by BU-LAMP assay. Similarly, the molecular sensitivity of nested PCR was higher than BU-LAMP since nested PCR had the capacity to detect up to 3 copies of *M. ulcerans insertion sequence 2404.*

Among the conventional and the modified microscopy methods, the most sensitive technique was overnight sedimentation of specimen suspension with phenol ammonium sulphate followed by centrifugation of the specimen suspension using the same lysing agent. The microscopic detection limit of the phenol ammonium sulphate concentration by sedimentation was however better than its centrifugal replicate since the former detected up to 4 AFB/ml of specimen suspension.

The loop-mediated isothermal amplification molecular method can be used for field studies or as point of care molecular technique provided *M. ulcerans* DNA can be isolated with good yield using alternative simple and hassle free method that will be amenable to low resource laboratory.

6.2 Recommendations

On the basis of the search for rapid, sensitive and affordable diagnostic test for point of care early detection of *M. ulcerans* in clinical specimens, the following recommendations are suggested.

1. Concentration of cellular suspension with phenol ammonium sulphate should be adopted to detect *M. ulcerans* AFBs in peripheral laboratories. However, it is further recommended that the sedimentation time should be experimented hourly to determine the shortest incubation time which will give a comparable results with the overnight sedimentation.

2. To make loop-mediated isothermal amplification molecular techniques suitable to be used at low resource laboratory for field studies, the DNA isolation protocol should be simplified by using a single chemical compound that will have the capacity to isolate DNA with a good yield since LAMP does not perform better with unprocessed specimen.
3. Finally, the LAMP primer and the master mix components should be manufactured in one thermostable lyophilized form that will only need reconstitution prior to use.

6.3 Limitations

The following limitations were encountered during the study:

1. The sample size was calculated based on estimated prevalence of Buruli ulcer disease due to lack of availability of information on the prevalence of the disease in Ghana.
2. Due to the numerous study sites involved in this study, the molecular assays have to delay till the samples arrive in Accra.
3. Procurement, freight and delivery issues associated with the LAMP technology delayed the analysis of the clinical specimens.

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APPENDIX 1

KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY
SCHOOL OF GRADUATE STUDIES
DEPARTMENT OF CLINICAL MICROBIOLOGY
INFORMED PATIENT CONSENT

Patients ID number: _____

I, _____, Age _____ years, Sex [ ] M/ [ ] F have been informed about the study **"Comparative Diagnosis of Buruli ulcer disease by Ziehl-Neelson Microscopy, *Insertion Sequence 2404* Polymerase Chain Reaction and Loop-Mediated Isothermal Amplification Technique"** that is being carried out in Kwame Nkrumah University of Science and Technology, Kumasi, I have been explained about this study in my local language in detailed. I have no objections in providing the necessary requirement for the study and I will

give my full co-operation for the study. I have been informed that I will not receive any remuneration for this study and my personal details have been assured to be kept confidential.

Signature/Left thumb impression: _____ Date: _____/_____/ 20____

Basic Demographic and Clinic Information [Please tick √ the applicable response]

1. Name of Study Site: ……
2. Residential town/village:……..

   Residential setting: [ ] Rural [ ] Peri-urban [ ] Urban [ ] Others…… 3.

Highest educational: [ ]No education [ ]Primary [ ]JHS [ ]SHS [ ]Tertiary

4. Occupation:……..
5. Clinical form of the lesion: [ ]Ulcer [ ]Nodule [ ]Plaque [ ]Healed [ ]Other_____
6. Location of lesion:……
7. Category of lesion: [ ]Cat I [ ]Cat II [ ]Cat III
8. How long has the patient been on streptomycin & rifampicin treatment?

   [ ] Yet to start [ ] 1-14 days [ ] 15-28 days [ ] 29-42 days

   [ ] 43-56 days [ ] Treatment Completed
