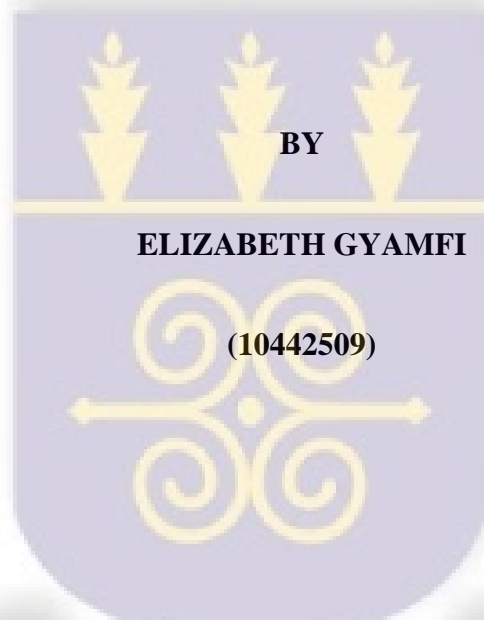


**GENOTYPING AND TREATMENT OF SECONDARY BACTERIAL
INFECTIONS AMONG BURULI ULCER PATIENTS IN THE
AMANSIE CENTRAL DISTRICT OF GHANA**



**THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON IN
PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF A
MASTER OF PHILOSOPHY DEGREE IN MEDICAL BIOCHEMISTRY**

JULY, 2015

DECLARATION

I ELIZABETH GYAMFI, do hereby declare that with the exception of references to other people's work, which have been duly acknowledged, this thesis is the outcome of my own research conducted at the Department of Medical Biochemistry, University of Ghana Medical School, College of Health Sciences and the Department of Cell, Molecular Biology and Biochemistry, University of Ghana, College of Basic and Applied Science under the supervision of Dr. Lydia Mosi and Dr. Bartholomew Dzudzor. Neither all nor parts of this project have been presented for another degree elsewhere.

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ABSTRACT

Background

Buruli ulcer (BU) is a skin disease caused by *Mycobacterium ulcerans*. BU is the third most common mycobacterial disease after tuberculosis and leprosy, but in Ghana and Cote d' Ivoire, it is the second. *M. ulcerans* produces mycolactone, an immunosuppressant macrolide toxin which makes the infection painless. However, some patients have complained of painful lesions and delay healing. Painful ulcers and delay healing experienced by some patients may be due to secondary bacterial infections.

Main Objective: To identify secondary microbial infections of BU patients, their genetic diversity as well as determine the levels of antibiotics resistance of these microorganisms.

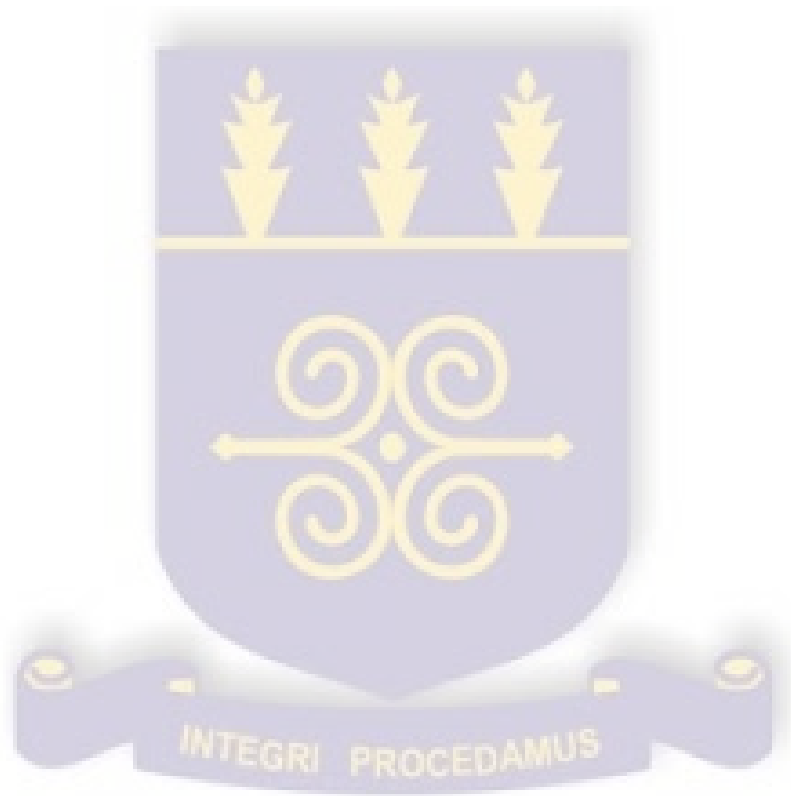
Methodology: The study was conducted at Biochemistry, Cell and Molecular Biology, University of Ghana. Subjects were recruited from Amansie Central District, Ashanti Region. Swabs of 51 BU patients were taken and immediately frozen for transport into the laboratory. Microscopy was performed using Ziehl-Neelsen and Gram staining techniques. The samples were also cultured on Luria Bertani, MacConkey, Mannitol, BPA and Sabouraud dextrose agar to identify bacteria and fungi. Antibiotic susceptibility tests were performed on selected bacteria species. DNA was extracted from the samples, after which Polymerase Chain Reaction (PCR) was performed using universal (16S rRNA), MSHA/PA (16S rRNA for mycobacterial) and *IS2404* (insertion sequence specific to mycolactone producers) primers to find the different strains of organisms. Finally sequencing was performed on the DNA amplicons that were randomly selected to identify the kinds of microorganisms causing secondary infection.

Results: All the samples were positive for bacteria. However 49 and 40 positives were obtained from PCR products using the primers MSHA/PA, and *IS2404* respectively, thus 40 BU patients were identified out of the total 51 patient samples. Majority of the bacteria identified after sequencing with universal primers for bacteria were *Staphylococcus spp* (*aureus* including MRSA, *saprophyticus*, and *lentus*), *Alcaligene spp* (*aquatilis* and *faecalis*), *Pseudomonas spp* (*aeruginosa*, *stutzeri* and *koreensis*) and *bacilli cereus* group of bacteria. Interestingly, 60% of the sequencing result for mycobacteria detected the presence of *Corynebacterium spp* (*aurimucosum*, *diphtheria* and *striatum*). Other bacteria identified were *Brevibacterium iodinum* and *Rhodococcus erythropolis*. Majority of these bacteria live in muddy areas and dirty water. The selected bacteria were less susceptible to rifampicin, clarithromycin and amikacin.

Conclusion: Other bacteria beside *M. ulcerans* colonize and proliferate on BU lesions. The selected bacteria were less susceptible to clarithromycin and amikacin and rifampicin. The pains and healing delay experienced by some BU patients could be the result of these bacteria colonizing and proliferating on the ulcer or lesions.

DEDICATION

This research work is foremost dedicated to the Almighty God for His undeserved kindness that has been showered upon me throughout my academic life. My profound appreciation goes to my mother for her love, care, support and encouragement over the years.



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LIST OF ABBREVIATIONS

μL	Micrometer
BCG	Bacille Calmette-Guerin
BD	Becton Dickinson
Bp	Base pair
BU	Buruli Ulcer
CD62	Cluster of Differentiation 62
Cm	Centimetre
EDTA	Ethylenediaminetetraacetic Acid
ER	Enoyl Reductase
g	Gram
G-C	Guanine-Cytosine
KR	Keto Reductase
L	Liter
<i>M. ulcerans</i>	<i>Mycobacterium ulcerans</i>
Mb	Megabase
MIC	Minimum Inhibitory Concentration
ml	milliliter
NCBI	National Center for Biotechnology Information
nM	nano Molar
$^{\circ}\text{C}$	Degree Celcius

PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PDT	Photo Dynamic Therapy
PLN	Peripheral Lymph node
rDNA	Ribosomal Deoxyribonucleic acid
rpm	Revolutions per minute
rRNA	Ribosomal Ribonucleic acid
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
St.	Saint
Th	Thymus- helper
UV	Ultra violet

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Buruli ulcer (BU) is a skin disease caused by *Mycobacterium ulcerans*. It usually begins as a painless nodule or papule and may progress to massive skin ulceration (Duker *et al.*, 2004b). It is the third most common mycobacterial disease after tuberculosis and leprosy (Johnson *et al.*, 2005). Buruli ulcer infection has been reported in over 30 countries globally; Australia, South America, Asia and Africa with most cases in West and Central Africa including Ghana, Cote d'Ivoire and Benin with an estimated prevalence of 22% and 16% for Cote d'Ivoire and Ghana respectively (Merritt *et al.*, 2010). The incidence of the disease keeps increasing over the last ten years but has recently started to decline.

In Ghana, the number of new cases reported was 685 in 2003, 1021 in 2004 and 1097 in 2005 and to 1010 in 2006 (Williamson *et al.*, 2008). In Ghana, active cases have been identified in all ten regions of the country and were present in 90 out of the then 110 districts of the country (Amofah *et al.*, 2002). Buruli ulcer is more prevalent in the southern belt of Ghana: Ashanti, Central, Western and Eastern Regions. The infection has been observed to affect all sexes and age groups but has been found to occur more in children less than 15 years. (Van Der Werf *et al.*, 2003). Buruli ulcer remains a priority health problem in Ghana especially in the Amansie West and Amansie Central District (Duker *et al.*, 2004a). The pathology of *M. ulcerans* is different from other mycobacterial pathogens in that it is mainly extracellular and produces a plasmid-encoded toxin with a polyketide-derived macrolide structure, mycolactone (Stinear *et al.*,

2004). Mycolactone is responsible for the pathogenesis associated with the disease. It suppresses the host's immune factors from eliciting inflammatory responses (Hong *et al.*, 2008). The ulcers are therefore, painless unless there is secondary infection (Phillips *et al.*, 2009).

In cell culture experiments, mycolactone causes apoptosis and necrosis in many human cells (George *et al.*, 2000). Destruction of subcutaneous fat of skin leads to lesion and ulcer could provide grounds for the colonization and proliferation of microorganism from normal skin flora and pathogenic species from the environment to establish secondary infection. Mycolactone, being an immunosuppressant toxin does not cause local inflammation (Van Der Werf *et al.*, 2005). Thus, painful ulcers experienced by some patients may be due to secondary infections. According to Werf *et al.* (1999), there are usually severe complications in BU such as deformity. The presence of purulent secretion (pus) or at least two of the cardinal manifestation of inflammation could provide grounds for the growth of microorganism which may cause secondary infections in BU (Ohene, 1997). Secondary infections have also been reported after surgery (Johnson *et al.*, 2005). Antibiotic treatment after confirmation of the disease is the recommended form of treatment. Wide excisional surgery may be performed if found necessary (Converse *et al.*, 2011). There are also high reported cases of antibiotic resistance of *M. ulcerans* and this has contributed to BU treatment failure (Barogui *et al.*, 2013). Unfortunately, extensive surgery may unnecessarily damage healthy tissues and it does not prevent recurrence of the disease (Merritt *et al.*, 2010). Despite these setbacks, combination of rifampicin and dapson showed marginal beneficial effect attributed to drug therapy (Espey *et al.*, 2002). Also, it has been observed that antibiotics treatment combination

containing aminoglycoside are more effective than those without (Van Der Werf *et al.*, 2005). Research in Ghana reports that patients respond to streptomycin and rifampicin and in few cases, the lesions were completely healed (Barogui *et al.*, 2013). A recent study identified the presence of secondary microbial infection associated with BU. These bacteria include *S. aureus* and *P. aeruginosa* (Yeboah-Manu *et al.*, 2013). Drug resistance in some patients as well as functional limitations after treatment serves as a hindrance to the treatment of the disease (Chauty *et al.*, 2007).

1.2. Justification/Relevance

The issue of secondary infection has not received considerable attention. The pathogenesis of Buruli ulcer is like that of other infections that break barriers for bacterial infection (Silva *et al.*, 2009). Both pathogenic and opportunistic microbes, stemming from normal skin flora or immediate environment, can colonize most skin ulcers and cause secondary infections in patients (Yeboah-Manu *et al.*, 2013). Coincidentally, long term treatment recommended may cause antibiotic resistance for other opportunistic bacteria. Secondary microbial infection and its dynamics have however not received enough attention.

Since BU was recognized as a health problem in Ghana, various researchers have investigated ways in which better treatment can be meted out to ailing persons. There have been challenges in identifying the best remedy to fight the infection due to antibiotic resistance and late case reporting. This study will aid in the identification of secondary microbial infections of BU, drug selection and the eradication of the disease.

1.3. Hypothesis

Buruli lesions may provide fertile grounds for colonization and proliferation of other microorganisms.

There is high genetic variation within isolates that cause secondary infections which can be resolved using fine genetic fingerprinting techniques.

The polymicrobial nature of secondary infected lesions confers synergy and adaptive features on these microorganisms which enable them resist some classes of antibiotics.

1.4. Aim

To detect and characterize microorganisms causing secondary infections in Buruli ulcer lesions and to determine their level of antibiotic resistance.

1.5. Objectives

- To culture and identify bacteria and other microorganisms from swabs from ulcerative lesions.
- To determine the effect of certain antimicrobial drugs on selected secondary microorganisms from BU lesions and assess the efficacy of other class of antibiotics on these microorganisms.
- To elucidate any genetic variations within isolated species causing secondary infections in BU.

CHAPTER TWO

2.0. LITERATURE REVIEW

2.1. Buruli Ulcer

Buruli ulcer is an emerging human disease caused by infection with a slow-growing pathogen, *Mycobacterium ulcerans*, which produces mycolactone, a cytotoxin with immunomodulatory properties (Demangel *et al.*, 2009). The disease is characterized by a painless nodule, papule, plaque or edema, evolving into a painless ulcer with undermined edges, often leading to invalidating sequelae. In rare cases, osteomyelitis occurs (Walsh *et al.*, 2011). These distinctive ulcers were first noticed in Uganda but described in Australia in 1948 and it was named Bairnsdale ulcer due to their focal distribution (Quek *et al.*, 2007). The exact mode of transmission of BU is unclear.

M. ulcerans is unique among mycobacterial pathogens in that it is mainly extracellular during human infection and produces a plasmid-encoded toxin with a polyketide-derived macrolide structure, named mycolactone (Stinear *et al.*, 2004). Mycolactone is believed to play a central role in determining the extracellular localization of the bacteria and modulation of immunological responses to *M. ulcerans* (Adusumilli *et al.*, 2005).

Three features of *M. ulcerans* emerges as a determinant for the pathogenesis of BU. Low optimal temperature of growth that makes the skin its preferential target, its mycobacterial pedigree that at least at some phase of its cycle in the host should lead *M. ulcerans* to behave as an intracellular microorganism triggering inflammatory cell

responses and the mycolactone-associated high cytotoxicity that contrasts with intracellular traits of its mycobacterial nature (Portaels *et al.*, 2009a).

M. ulcerans is an environmental microorganism. By using the insertion sequence *IS2404* which is found in about 200 copies in a PCR, the bacterium has been found in environmental samples and wild animals and in pathology specimens from patients (Portaels *et al.*, 2001). Interestingly, unlike the closely related *M. marinum*, it was noted that *M. ulcerans* is not capable of mounting a productive infection in fish and does not support the hypothesis based on epidemiological evidence shown by *IS2404*-PCR that fish may be a reservoir for *M. ulcerans* (Mosi *et al.*, 2012).

2.1.1. Mycolactone

Mycolactones are immunosuppressive and cytotoxic polyketides comprising five polyketide-derived macrolides. Besides its immunosuppressive effect, it usually diffuses beyond the site of *M. ulcerans* infection thus extending the lesion (Hong *et al.*, 2008). After its secretion from the bacterium, it is diffused into the affected tissues and surrounding areas. The amount and precise distribution of the toxin in the lesion is always unknown (Portaels *et al.*, 2009a). *M. ulcerans* mycolactone-negative mutants are nonvirulent and non-cytotoxic (Torrado *et al.*, 2007).

There are naturally occurring structural variants (A, B, C, D, E and F) produced by different species of very closely related mycobacteria including human pathogen. In *M. ulcerans* strain, Agy 99, mycolactone A/B is produced by 3 highly homologous type I polyketide mega synthetase (PKS) whose gene (m/s A 1:51kb, m/s A 2:7.2kb and m/s B:42kb) are found on a 174kb plasmid, known as pMUM001 (Pidot *et al.*, 2008).

Evidence suggests that *M. ulcerans* isolates from Asia, Mexico and Australia may be less virulent than isolate from Africa. This could be explained from the heterogeneity in mycolactone variants. The toxin is eliminated by BU patients in a slow rate during treatment (Mve-Obiang *et al.*, 2003).

Mycolactone induces apoptosis in human keratinocytes, thus, contributing to Buruli ulcer lesions development (Bozzo *et al.*, 2010). Mycolactone not only induces muscle damage but also prevents muscle regeneration to occur. These results may help to explain why patients with Buruli ulcer, experience muscle weakness and contracture (Houngbédji *et al.*, 2009). This molecule serves an attractive candidate target for the diagnosis and monitoring of the BU. Assay based on detection of mycolactone in serum and ulcer exudate can form the basis of BU diagnostic tests and monitoring (Sarfo *et al.*, 2011).

2.1.2. Epidemiology

After tuberculosis and leprosy, BU is the third most common mycobacterial disease (Van Der Werf *et al.*, 2005). In some West African countries, the number of BU cases may exceed those of tuberculosis and leprosy (Portaels *et al.*, 2009a). In Africa, even though it was first observed in eastern Africa (Uganda), it is more endemic in the western wing and particularly more prevalent in Benin, Cote d'Ivoire, Ghana, Guinea, Liberia, Nigeria, Sierra Leone and Togo. Ivory Coast recorded 24,000 cases, Ghana recorded 11,000, and Benin has 7,000 confirmed cases by the year 2002 (Amofah *et al.*, 2002). The overall crude national prevalence rate of active lesions was 20.7 per 100,000, but the rate was 150.8 per 100,000 in the most disease-endemic district (Amofah *et al.*, 2002). Geographical distribution prevalence and incidence of BU may vary greatly by country and in localities within a country over time. These variations may be due to multiple

factors such as geophysical activities that lead to soil turn over, climate and man-made environmental disturbances and other ecological changes such as alterations of the fauna and flora (Sopoh *et al.*, 2007).

Several studies have also suggested that there is no sex difference in the distribution of BU infection (Owusu & Adamba, 2012; Van Der Werf *et al.*, 2005). According to Hamzat & Boakye-Afram (2011), the male to female ratio is 1:1, giving more evidence to show that there is no gender pattern in the prevalence of the disease. Nevertheless, men older than 59 years may have a higher chance of developing the disease than women. This may be due to work related activities and greater access to health care than the women (Debacker *et al.*, 2004). In adults, legs are more frequently involved than arms. Children between 0 to 14 years tend to develop the disease more often on the trunk, head and neck. Lesions at other sites such as genitalia are infrequent. Men have more lesions on the legs while arms are more frequently involved in women. Lesions on the palms and soles have not been described (Debacker *et al.*, 2004).

Unfortunately, information on the focal epidemiology of BU is sparse. Incidence, prevalence and other data are usually reported at the national or district level (Amofah *et al.*, 2002). The disease is more prevalent in the tropics and the subtropics. It tends to affect people who live close to slow-moving water bodies such as swamps, rivers, ponds and lakes. However cases of the disease have been reported after flooding (Van Der Werf *et al.*, 2005).

Even though the disease is usually painless, most patients nonetheless, spend a significant amount of time in a hospital or rehabilitation setting. For instance a review of 102

patients in Ghana indicated that the average length of hospital stay was more than 100 days; and the Buruli infection led to 10 amputations, 12 joints contractures and 2 deaths arising from complications such as tetanus and sepsis of the ulcer (Amofah *et al.*, 1993). A small but increasing proportion of African patients develop osteomyelitis, a complication that usually necessitates amputation (Sopoh *et al.*, 2007). Such circumstances have economic implication which may be beyond the means of local health services in Ghana. Even in developed economies like Australia which has better access to health care, the cost and difficulty of treating *M. ulcerans* infections was observed to be considerably high (Drummond & Butler, 2004).

Some under-developed countries usually have difficulties with the clinical diagnosis of the disease or the collection of specimens for laboratory testing. Due to this, the number of BU cases may be overestimated and worse off, patients with the disease other than BU can be mistreated (Portaels *et al.*, 2009a).

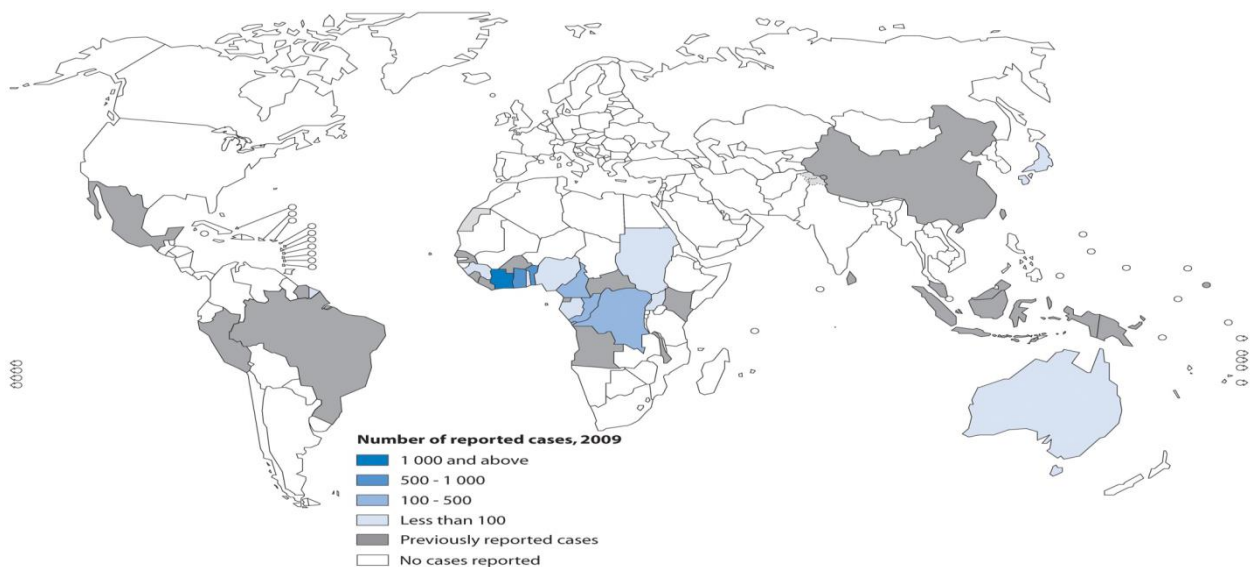


Fig. 2.1. A global map representing countries that have reported cases of Buruli ulcer disease as of 2009 (WHO) (Merritt *et al.*, 2010).

2.2. Ecology and Transmission of the Disease

Molecular studies have shown the existence of *M. ulcerans* in water, mud, fish, aquatic insects, and snails from swamps in regions endemic for Buruli ulcer (Wallace *et al.*, 2010). As most people who have Buruli ulcer live near these areas, material from swamps, ponds, or river regions may contaminate skin surfaces with *M. ulcerans* which can result in introduction of the causative agent into skin when it is broken by trauma or insect bites (Williamson *et al.*, 2014). Trauma may be as slight as a hypodermic injection or as serious as a gunshot or a landmine wound (Meyers *et al.*, 1974). Activities that are developed close to the water, such as farming constitute risk factors. A study in Uganda reported that families who use unprotected source of water like river and pond for domestic purposes had a higher prevalence rates of BU than those who used boreholes (Thangaraj, 1999). Interestingly, protective clothing appears to reduce the risk of contracting the disease (Van Der Werf *et al.*, 2005). However unlike malaria and other tropical diseases, its mode of transmission is still unclear. Earlier evidence suggested that aquatic insects (Genus: *Naucoris* and *Dyplonychus*) may be involved (Wallace *et al.*, 2010). But here still, the precise role of these water-insects, flies and mosquitoes is not known. *M. ulcerans* has been identified in the biofilms on insects. The method of transmission may likely be mechanical (Mosi *et al.*, 2008). Merritt *et al.* (2010), reported that, *M. ulcerans* is found in biofilms of aquatic habitats and contacted by grazing or filter- feeding invertebrates that are then consumed by predators known to bite humans. Few if any patient recalls having being bitten by an insect prior to the development of the disease (Van Der Werf *et al.*, 2005). Presently, it is unknown whether insect bites represent a route for transmission. In one report, severe *M. ulcerans* disease with

osteomyelitis, followed a snake bite (Hofer & Hirschel, 1993). Again, the human-environment interaction suggested is not clear. Only two cases have been reported of human to human transmission (Debacker *et al.*, 2002). Yet still, person-to-person transmission of the disease has not been properly understood, even though in some cases patients had relatives who were also infected (Sopoh *et al.*, 2010). Clustering of cases among families has occasionally been observed. This may reflect an exposure to a common source of infection and also a common genetic susceptibility to infection with *M. ulcerans* (Stienstra *et al.*, 2001). The disease has been occasionally diagnosed in tourists and other individuals who have only briefly visited countries where the disease is endemic (Semret *et al.*, 1999). Although the mode of transmission is not quite understood, there are multiple reports which show that patients have had antecedent trauma at the site where the lesion or nodule occurred (Portaels *et al.*, 2009a). Aerosols may also play a role in its transmission (Hayman, 1991).

It is possible that *M. ulcerans* inhaled or ingested is then widely disseminated in the body and reactivates in low temperature areas at sites of trauma, but direct inoculation seems most likely. The incubation period is usually 13 weeks, but much longer, and occasionally shorter, incubation periods occur (Demangel *et al.*, 2009).

2.3. Clinical Presentation of Buruli ulcer

Even though the disease can affect any part of the body, it is more often found on the extremities (especially the limbs) as compared to the other parts. Research shows that the lower limbs are more likely to be affected than the upper limbs, and this may be because the lower limbs are frequent lying in contact with soil, water, plants, and insects (Toutous *et al.*, 2013).

The disease can be divided into four histopathological stages; non-ulcerative necrotic stage, ulcerated necrotic stage, initial healing stage and late healing stage (Boleira *et al.*, 2010). Clinically, the disease can be divided into pre-ulcerative stage (papule, nodule, plaque, and diffuse oedema) as seen in Fig. 2.2A and ulcerative stage, which may be represented by ulcers smaller than 5 cm and ulcers larger than 5 cm as seen in Fig. 2.2B (Portaels *et al.*, 2009b). It can also be classified into localized disease (papule, nodule and ulcer), and disseminated disease (plaque, diffuse edema and metastasis) (Adamba & Owusu, 2011).

Patients may manifest clinical symptoms, such as plague and undermined oedema. Plaques are raised and hard, but are painless, with a certain degree of depigmentation or spotted erythema. They can be more than 2 cm in diameter, possibly reaching 15 cm. These can later develop into large ulcers with irregular borders (Boleira *et al.*, 2010). The disease can also be present with a large area of marked indurations, diffuse oedema in the legs and arms or a well-demarcated plaque (Abalos *et al.*, 2000). When there is only one oedema, the profile is more diffuse, and with poorly demarcated borders. Histological examination of excised skin lesions characteristically shows skin ulceration with extensive necrosis of subcutaneous fat. The ulcerating stage develops due to perforation of the necrosis above the epidermis. Initial ulcers may remain small, with 1 to 2 cm of diameter or become larger (larger than 5 cm) and destroy the skin around them. Necrosis frequently extends beyond areas where *M. ulcerans* is visible (Guarner *et al.*, 2003). Acid-fast bacilli are present in enormous numbers within the necrotic area and are typically arranged in spherules. These clusters of bacteria extend along thickened septa in the subcutaneous tissue. The granulomatous inflammatory response normally associated

with mycobacterial infection may be seen in long-standing lesions, but is strikingly absent in early lesions (Guarner *et al.*, 2003).

The disease may also affect the bones. Bone lesions may include osteitis or osteomyelitis beneath skin lesion or metastatic osteomyelitis from lympholematogenous spread of *M. ulcerans* (Walsh *et al.*, 2008).

More often, there are delays in reporting on the part of patients or late identification of cases. There is usually no pain and no systemic inflammatory response, although patients may complain of itch. Several factors may explain this, the main being that, the occurrence of a lesion is generally painless and with no systemic symptom (Kibadi *et al.*, 2009). This is because mycolactone produced by *M. ulcerans*, appear to play a role in inhibiting the recruitment of inflammatory cells to the site of infection, which at least explains why inflammatory responses are poor in Buruli ulcer lesions (Adusumilli *et al.*, 2005). Early stages of the disease are often ignored by the patients and may heal spontaneously. After variable periods of a week to several months, these forms ulcerate and often bring patient to the hospital (Portaels *et al.*, 2009a). As a result, in many cases the disease progresses slowly, steadily and largely unnoticed.

The type of disease that develops after the infection depends on many factors such as the immune status of the host, the size and depth of the inoculation, (superficial or deep) and the virulence of the *M. ulcerans* strain (Portaels *et al.*, 2009a).

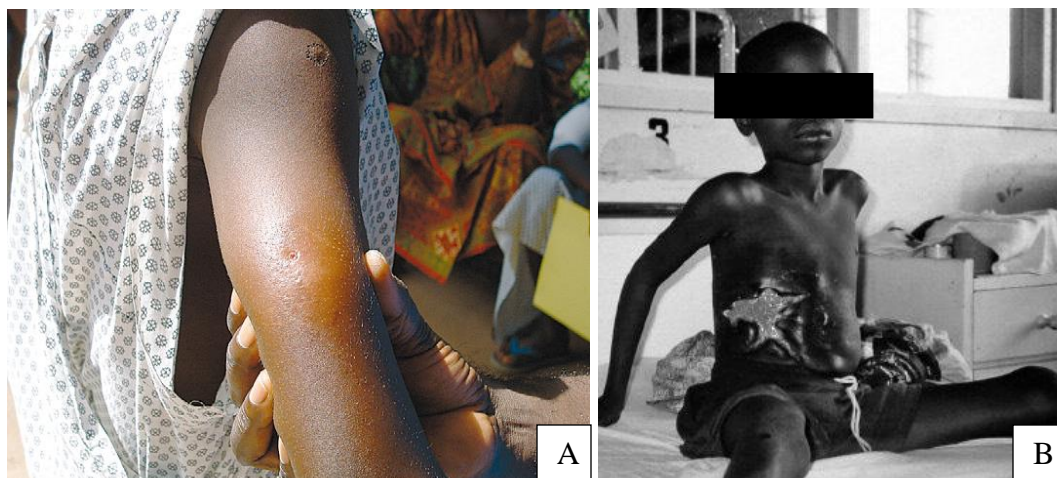


Fig. 2.2. A. Early lesion of *M. ulcerans* disease on right arm of a 12-year-old girl (Wansbrough-Jones & Phillips, 2006); B. Ghanaian boy with a well-developed plaque of Buruli ulcer on the right flank. The ulceration is remarkably “stellate,” a feature of plaque lesions (Walsh *et al.*, 2009).

2.4. Diagnosis

Microbiological staining is usually done using Ziehl-Neelsen, fluorochrome or Harris Haematoxylin and eosin staining technique. The presence of large numbers of extracellular acid-fast bacilli in a swab from a necrotic ulcer makes the diagnosis likely positive, especially in countries with a low incidence of other mycobacterial infection.

In culturing microorganisms associated with Buruli ulcer, Lowenstein-Jensen and Middlebrook 7H9, 7H10 and 7H11 agar medium can be used. *M. ulcerans* can be cultured from most cases provided the correct culture media and incubation temperature are used, and plates are kept for up to 12 weeks (Portaels *et al.*, 2008).

Microorganisms obtained can be confirmed not just by culture or staining but also through PCR (Mosi *et al.*, 2008). Loop-Mediated Isothermal Amplification (LAMP) test has been identified as a rapid and sensitive way of detecting the DNA of *M. ulcerans*

under field conditions based on the multicopy insertion sequence *IS2404*. The test is robust and specific with a detection limit equivalent to 20 copies of the target sequence (0.01 to 0.1 genome) (Njiru *et al.*, 2010). A recent report describes a new dry-reagent based PCR for Buruli ulcer that could be used in small regional centres, and reports from Ghana suggest a sensitivity of 95% or more (Babonneau *et al.*, 2015).

2.5. Treatment of Buruli Ulcer

Treatment of the disease is relatively easy if identified early. Antibiotics have the potential of curing small lesions and limiting surgery for larger lesions. It has also been found to shorten the time for healing after surgery (Converse *et al.*, 2011). Until this discovery, treatment was largely done through surgical means that often involved extensive excision, sometimes with skin transplantations. But if the identification of the disease is delayed and there is an extensive necrosis of the skin or an attack on the bone, the only option is extensive surgical treatment with skin grafting, and may involve amputation and protracted physiotherapy (Barogui *et al.*, 2009).

The use of antibiotics such as rifampicin and streptomycin is noted to have a positive impact on treatment outcomes (Etuaful *et al.*, 2005). *M. ulcerans* is usually sensitive to clarithromycin, rifampicin and ethambutol. This microorganism is susceptible to several anti-mycobacterial drugs *in vitro*, but the most promising results in the mouse footpad model were obtained with a combination of rifampicin and amikacin (Dega *et al.*, 2002). Furthermore, a human trial has recently shown that early nodular lesions may be rendered culture-negative after a minimum of four weeks therapy with rifampicin plus streptomycin (Evans *et al.*, 2003). Healing takes months and can cause deep scarring,

joint retractions, and deformity. Death is a rare complication (Sizaire, Nackers, Comte, & Portaels, 2006).

Several anti-tuberculosis agents are active against *M.ulcerans in vitro*, but experienced clinicians favour surgery rather than drug therapy in initial management. Bacille Calmette-Guerin (BCG), the current vaccine for tuberculosis, has been shown to be partially protective for a few months in the treatment of the disease (Nackers *et al.*, 2006) and in highly endemic areas BCG vaccination programs are likely to be cost-effective and relatively easy to implement (Portaels *et al.*, 2004). Even though, it has been shown to offer protection against *M. ulcerans* osteomyelitis, it has controversial results regarding protection against subcutaneous BU (Tanghe *et al.*, 2008). Several other vaccines are being put to test such as a preparation of mycolactone deficient-live *M. ulcerans* vaccine (Fraga *et al.*, 2012), mycolactone polyketide based vaccine (Roupie *et al.*, 2014), heat-shock protein based vaccine and Ag85a-based vaccine (Tanghe *et al.*, 2008). Recently, protective effect of a dewaxed whole-cell vaccine is being research on as a vaccine in curing the infection (Watanabe *et al.*, 2015). The lipidic nature of mycolactone complicates the development of immunization against the toxin. Nevertheless, an alternative would be to target enzymes involved in the biosynthesis of the toxin (Bali & Weissman, 2006).

The term “macrolide” is used to describe drugs with a macro cyclic lactone ring of 12 or more elements (Cai *et al.*, 2011). This class of compounds includes a variety of bioactive agents, including antibiotics, antifungal drugs, prokinetics, and immunosuppressant. The 14-, 15-, and 16-membered macrolides are a widely used family of antibiotics. They have excellent tissue penetration and antimicrobial activity, mainly against gram-positive cocci

and atypical pathogens and as such, may be good drugs for curing the disease (Landersdorfer *et al.*, 2009). There have been few cases of skin transplantation after treatment of the disease (Bechtle *et al.*, 2010; Sizaire *et al.*, 2006). To avoid social stigmatization, about 70% of Buruli ulcer patients in the Ga West District of Ghana consult traditional healers (Renzaho *et al.*, 2007).

Follow-up data on the rate of recurrence in hospital-treated BU patients is hardly reported. Amofah *et al.* (1998) found a local recurrence rate of 16% at the same site within a year of follow-up. Few patients had recurrence at more than one site. Others were not healed at all even after treatment. Thus treatment would require multiple interventions, including specific antibiotics, surgery, physiotherapy and follow-ups (Debacker *et al.*, 2004).

Although the disease can be managed at the early stages, beliefs and attitudes influence health-seeking behavior. Many patients and unaffected individuals in endemic areas believe that witchcraft and curses cause this indolent disease. Patients and their caregivers admit that the resulting stigma, a reluctance to seek treatment outside ones community, financial problems and fear of treatment effects, notably computation, caused delay in reporting (Stienstra *et al.*, 2002).

2.6. Suggested treatment other than antibiotics

Honey has been reported to have an inhibitory effect on aerobic and anerobic bacteria, yeast, fungi, and viruses (Al-Waili, 2004). Honey inhibited growth of *Pseudomonas aeruginosa*, *Helicobacter pylori*, *Bacteriodes*, and enteropathogens (Elbagoury & Rasmy, 1993). Honey has been used for treatment of respiratory diseases, urinary diseases,

gastrointestinal diseases, and skin diseases including ulcers, wounds, eczema, psoriasis, and Dandruff (Zaghloul *et al.*, 2001). Photo Dynamic Therapy (PDT) appears to represent an efficacious alternative modality for the treatment of localized microbial infections through the in situ application of the photosensitizer followed by irradiation of the photosensitizer- loaded infected area. Proposed clinical fields of interest of antimicrobial PDT include the treatment of chronic ulcers, infected burns, acne vulgaris, and a variety of oral infections (Maisch, 2007).

Some plants have been found to have good antimicrobial properties. Such plants include *Spanish needle tree, lipstick tree, trumpet tree, onion* and *garlic plants*. These plants have been shown to be effective against pathogenic microorganisms. It has been demonstrated that folk medicine can be as effective as modern medicine to combat pathogenic microorganisms (Srinivasan *et al.*, 2001).

Silver-based dressings appear to provide an effective alternative to antibiotics in the management of wound and ulcer infection (Chopra, 2007). Other topical treatments including application of heat (Radford, 2009), mineral clay (Haydel *et al.*, 2008) nitrite ointment and hyperbaric oxygen have been suggested (Phillips *et al.*, 2004)

2.7. Immunology of the Disease

M. ulcerans produces mycolactone; an immune suppressive toxin. This toxin is known to exert its immunosuppressive effects at the systemic level. Assays that are based on mycolactone detection on circulating blood cells may be considered for diagnostic test of early disease manifestation (Hong *et al.*, 2008). The immune mechanisms involved in protection against Buruli ulcer are also largely unknown at present. Mycolactone has

been found to inhibit the ability to activate allogenic T-cell priming and to produce inflammatory molecules (Coutanceau *et al.*, 2007). Guenin-Macé *et al.* (2011) identified that mycolactone injection led to a massive T-cell depletion in peripheral lymph nodes (PLNs) that was associated with defective expression of L-selectin (CD62-L). Pre-exposure to mycolactone impaired the capacity of T-cells to reach PLNs after adaptive transfer, response to chemotactic signals and expand upon antigenic stimulation *in vivo* in mouse.

Interestingly, peripheral blood mononuclear cells obtained from people with a past or current *M. ulcerans* infection typically show a strong T helper (Th)–2 cytokine responses when exposed *in vitro* to *M. ulcerans*. In contrast, samples obtained from their household contacts (exposed healthy controls) exhibit a Th-1 immune response, suggesting that natural resistance may be determined by cell-mediated immune mechanisms directed against intracellular organisms (Gooding *et al.*, 2002). In one fascinating case study, it has been shown that the development of ulcerative *M. ulcerans* disease is associated with a shift from the Th-1 to the Th-2 phenotype (Gooding *et al.*, 2003). Interleukin-10 may be a key cytokine that mediates local Th phenotype switching within nodules and ulcers (Pidot *et al.*, 2008). Further research can be carried out to help us clearly understand how host immunity is acquired.

2.8. Genetic Variation

2.8.1. Genetic Variation in *Mycobacterium ulcerans*

Genomic analysis has revealed that *M. ulcerans* arose from *M. marinum*, a ubiquitous fast-growing aquatic species, through horizontal transfer of a virulence plasmid pMUM001 that carries a cluster of genes for mycolactone production, followed by

reductive evolution (Demangel *et al.*, 2009). *M. ulcerans* is distinguished from *M. marinum* by three major differences; loss of approximately 1 Mb of its genome; acquisition of a large virulence plasmid which encodes a toxic immunosuppressive macrolide, mycolactone and accumulation of 771 pseudo-genes, some inactivated by acquisition of 213 and 91 copies of the insertion elements *IS2404* and *IS2606* respectively. Thus the functional *M. ulcerans* genome [5.8 MB] is about 2 MB smaller than that of *M. marinum* (6.6 MB) (Stinear *et al.*, 2007). *M. ulcerans* has two circular replicons. One is a 5,631,606-bp chromosome Agy99 and the other is a 174,155-bp plasmid, PMUM001 (Pidot *et al.*, 2008). The plasmid contains 4 copies of *IS2404* and 8 copies of *IS2606* which are both used as molecular marker (Williamson *et al.*, 2008). Interestingly, Stinear *et al.* (2007) noted that, a total of 209 copies of the insertion sequence *IS2404* and 83 copies of *IS606* have been found interspersed in the genome of the *M. ulcerans* reference strain Agy99 from Ghana.

According to Janda & Abbott (2007), the mycobacterial 16S rRNA gene (encodes 16S rRNA) has been used to trace and differentiate strains of non-tuberculosis mycobacteria. SNP typing has also been successfully used to differentiate patients' isolates of *M. ulcerans* from different regions of Ghana (Röltgen *et al.*, 2010). A novel combined 16S rRNA RT/*IS2404* qPCR assay has proved to be highly sensitive, specific, and efficient in detecting viable *M. ulcerans* in clinical samples. The assay is thus applicable for the classification of secondary lesions, monitoring of treatment success and provides a powerful tool for clinical research (Beissner *et al.*, 2013). Two enzymes involved in biosynthesis of mycolactone; enoyl reductase (ER) and keto reductase (KR) are used as

genetic markers having an average G+C content of 62.5% (Johnson *et al.*, 2005). Further research can be done to enhance the knowledge of genetic variations in *M. ulcerans*.

Although experimental infections allow the analysis of the evolution of *M. ulcerans* from the very beginning, human samples of the initial phases of the infections are not available, limiting our knowledge on the establishment of the infection (Portaels *et al.*, 2009a).

2.8.2. 16Sr RNA for Bacteria Detection

Genes used for bacteria detection include those that code for the 5S, the 16S (also called the small subunit), and the 23S rRNA and the spaces between these genes. The part of the DNA now most commonly used for taxonomic purposes for bacteria is the 16S rRNA gene (Tortoli, 2003). The 16S rRNA gene can be compared not only among all bacteria but also with the 16S rRNA gene of archeobacteria and the 18S rRNA gene of eucaryotes (Pace, 1997). The 16S rRNA gene is about 1,550bp long and is widely used as a target as it is a highly conserved gene, ubiquitous in all organisms and contains variable and hyper-variable regions of sequence. Molecular methods based on this gene are well established as a standard method for characterization and identification of bacteria (Arosio *et al.*, 2008). Variable regions within the 16S rRNA gene have been used for discrimination between species and genera (Bertilsson *et al.*, 2002). Targets containing conserved regions and areas of variability for specific identification of bacteria are the genes encoding 16S and 23S rRNA. In addition, a stretch of DNA between the 16S and 23S rDNA, the 16S-23S rDNA ISR, proved to be a comparably species specific segment. These three targets have already been used for molecular identification and differentiation of various streptococcal species (Hassan *et al.*, 2003). Universal primers

are usually chosen as complementary to the conserved regions at the beginning of the gene and at either the 540-bp region or at the end of the whole sequence (about the 1,550-bp region), and the sequence of the variable region in between is used for the comparative taxonomy (Rinttilä *et al.*, 2004). Although 500 and 1,500 bp are common lengths to sequence and compare, sequences in databases can be of various lengths. 16S rRNA gene sequences allow bacterial identification that is more robust, reproducible, and accurate than that obtained by phenotypic testing. The gene's analysis can identify uncultured bacteria, allowing independence from growth conditions (Clarridge, 2004).

2.9. Microbial infections

2.9.1 Microbial infection of wounds

Common skin lesions that can be secondary infected with bacteria are scabies, psoriasis, poison ivy, atopic dermatitis and eczema (Brook *et al.*, 1999; Brook *et al.*, 2000). Microbial infections will have little impact on a minor wound that is healing rapidly but may establish large colonies on slowly healing chronic wounds (Sibbald *et al.*, 2003). Many wound and skin infections that complicate skin lesions are caused by mixed bacterial flora. Bacteria rapidly colonize open skin wounds where they exist synergically. Microorganisms colonizing wounds usually originate from the patient's endogenous skin and gastrointestinal and respiratory flora (Erol *et al.*, 2004). Microorganisms may also be transferred to a patient's skin surface via contact with contaminated external environmental surfaces, water, fomites, air, and the soiled hands of health care workers (Weber & Sheridan, 1997). Wound colonization by yeasts and fungi usually occurs later after treatment has usually begun (Katz *et al.*, 2014). It is widely believed that aerobic or facultative pathogens such as *S. aureus*, *P. aeruginosa* and the beta-hemolytic

Streptococci are primarily responsible for delayed healing (Bowler *et al.*, 2001). Microorganisms transmitted from the hospital environment tend to be more resistant to antimicrobial agents than those originating from the patient's normal flora (Cohen *et al.*, 2004). Enteric gram-negative bacilli are usually isolated from leg and trunk lesions. Beta-haemolytic *streptococci* and *S. aureus* predominates in finger and hand lesions but *S. aureus*, the most prevalent aerobe, can be isolated from all body sites of lesions. Isolation of multiple organisms from patients illustrates the polymicrobial nature of secondary infected skin lesions and the potential for bacterial synergy between the different patients' samples. Several studies have documented the synergic effect of mixtures of aerobic and anaerobic bacteria in experimental infections. Various hypotheses have been proposed to explain such microbial synergy. It may be the result of protection from phagocytosis and intracellular killing (Ingham *et al.*, 1977), production of essential growth factors, or lowering of oxidation-reduction potentials in host tissue (Mills & O'Neill, 2013). The presence of β -lactamase-producing organisms in many secondary infected skin lesions not only survive penicillin therapy but also can protect susceptible bacteria from penicillin by releasing the enzyme into the adjacent tissues or abscess cavity (Brook *et al.*, 1984). The exact pathogenic role of the organisms isolated from secondary infected skin lesions has not yet been determined. Treatment of serious skin infection should include systemic antimicrobial therapy. This could be important in the event of poor response to therapy or spread of the infection. Antimicrobial therapy for mixed aerobic and anaerobic bacterial infections is required when polymicrobial infection is suspected (Brook, 2002).

2.9.2. Secondary infection of Buruli ulcer

Limited research has been done concerning secondary infections found in Buruli ulcer. According to Yeboah-Manu *et al.* (2013), infections from other microorganisms such as *S. aureus*, *P. aeruginosa*, *P. miriabilis* among others can be found proliferating in BU infections. After treatment with Streptomycin with Rifampicin for 8 weeks (SR8), 75% of the lesions clinically suspected were microbiologically confirmed to be under infection with *P. aeruginosa* and *P. miriabilis*. This shows high resistance to antibiotics in Ghana. *S. aureus* serves as one of the main secondary microbial infection that may infest the ulcer thus patients usually received at least one course of antibiotics other than streptomycin and rifampicin during their hospital stay (Barogui *et al.*, 2013). More research is thus needed in the field of secondary infections in BU and their susceptibility to drugs.

CHAPTER THREE

3.0 METHODOLOGY

3.1 Study Site

This study was an addition into an ongoing extensive study of zoonotic risk of Buruli ulcer between humans and mammals in Ghana and Cote d'Ivoire. For the purpose of this study, communities in the Amansie Central District of Ashanti region of Ghana which are known to be endemic for BU were used as study sites. The inhabitants are mostly farmers but some youth engage in surface mining in the Offin River basin which runs close to most of the communities. Even though there is at least one working borehole with a pump in each community, inhabitants still fetch water from surface water sources (ponds, streams, rivers) for domestic and agricultural activities. Saint Peters Hospital in Jacobu, the district capital where BU cases are usually treated was the main site for sample collection. This current study was approved by Ethical and Protocol Review Committee of the University of Ghana Medical School [MS-ET/M.11 – P 3.1/2013-2014] and permission was granted by the District Health Directorate and traditional leaders of the respective communities.

3.2 Study Design

The study was a cross-sectional study which aimed at identifying microorganisms as well as genetic variation among isolates that cause secondary infection in Buruli ulcer. Samples were taken from February 2014 to June 2014.

3.3 Sample Size Determination

The minimum samples size was determined using the equation below:

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

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

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

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

n= 2.13

The minimum sample size is approximately 2. However, a total of 51 samples were used for the study. This was to ensure statistically significant result and rule out chance variations.

3.4 Subjects

All groups of patients of all ages and both sexes were enrolled in this study. Admitted BU patients with ulcer(s)-nosocomial secondary infections, BU patients from traditional healers with ulcer(s)-community acquired secondary infections and BU self-cared patients with ulcer(s)-community acquired secondary infections. All patients and parents on behalf of their children signed a consent form before enrolling into the study. Subjects were recruited from St. Peters Hospital in Jacobu. There was also a case search to find patients who were being treated by traditional healers as well as those under self – medication.

3.4.1 Inclusion Criteria

Suspected BU patients of all ages and sexes who may or may not have experienced pain in the ulcer or lesion and have given their consent. PCR positivity of *IS2404* for BU criteria was also to be met.

3.4.2 Exclusion Criteria

BU patients who refused to consent

3.5 Data on study samples

Clinical information on the gender and age of randomly chosen subjects were recorded. Additional information on location, occupation, stage of BU and type of treatment were also taken.

3.6 Sample collection from patients

There was a case search at the hospitals and individual communities for people with signs of BU, nodule, oedema and ulcer who experienced pain. Swabs from ulcers were taken by a well-trained Laboratory Technician from the St. Peters Hospital, Jacobu, Amansie Central District. Swabs were kept in properly labelled 15 ml conical tubes. The samples were kept on ice packs in a cooler and quickly transported back to St. Peters Hospital (Jacobu) for temporal storage in a refrigerator.

3.7 Sample processing for laboratory analyses

All samples were processed prior to microscopy and DNA extraction

3.7.1 Sample Preparation

Two milliliters of 1X Phosphate Buffer Saline (PBS) was pipetted into the 15 ml Eppendorf tube. The tube was then vortexed gently for 5 minutes to dislodge microbial

cells and later transferred to a 2 ml screw-cap tube. Afterwards, 1 ml of the sample was pipetted into a new 2 ml screw-cap tube. The tube was then gently vortexed briefly and spun at 14,000 rpm for 5 minutes. Later, 800µl of supernatant was pipetted off and the remaining 200 µl (plus pellet) was used for DNA extraction. Of the remaining 1 ml, 200 µl was pipetted into another 2 ml screw-cap tube for decontamination. The remaining 800 µl was preserved at 4°C in a refrigerator for later use.

3.7.2 Gram Staining

Gram staining was performed according to the manufacturer's (BD Gram Stain Kit, New Jersey, U.S.A) protocol. Briefly, about one drop of specimen was smeared on a glass slide (frosted slide glass, Thermo Scientific, Waltham, U.S.A), allowed to dry and gently passed over flame until smear was fixed. The slide was then flooded with Gram crystal violet staining reagent and left for 1 minute. The crystal violet was then washed off with distilled water and later flooded with Gram iodine for 1 minute followed by washing off with distilled water. The slide was again flooded with decolorizing agent for 15 seconds and counterstained with Gram Safranin for 1 minute. The stain was washed off completely and the slide was air-dried. Slide was viewed under a light microscope (Leica, Wetzlar, Germany), in oil immersion at a magnification of X1000. Pictures were taken using a camera (Sony, Tokyo, Japan).

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

Acid-fast Staining was performed according to manufacturer's protocol (Oxoid, Hampshire, United Kingdom). Briefly, about two drops of specimen was smeared on a glass slide (Frosted slide glass, Thermo Scientific, Waltham, U.S.A) and gently passed over flame until smear was fixed. The slide was then flooded with carbol fuchsin and left

for 5 minutes. The carbol fuchsin was later washed off with distilled water and stain was decolorized with acid alcohol (3% hydrochloric acid). The slide was then flooded with methylene blue and left for 2 minutes. Stain was washed off completely and the slide was air-dried. Slide was viewed under a light microscope (Leica, Wetzlar, Germany), in oil immersion at a magnification of X1000. Picture was taken using a camera (Sony, Tokyo, Japan).

3.7.4 Microscopic determination of fungal elements

Fungal elements were detected as described (Garg *et al.*, 2009) with slight modification. A solution of 10% KOH containing 10% glycerol was prepared. A volume of 100 µl of the solution was pipetted into a 24 well cell culture plates (Nunc[™], New York, USA). A volume of 10 µl of each sample was added to the KOH solution. It was left to stand for 40 minutes and examined under an inverted microscope (ZEISS Promovert, Jena, Germany) at a magnification of x40 for the presence of fungal elements.

3.7.5 Culturing of bacteria

Masses of 28.0 g, 55.0 g, 111.0 g and 63 g of Luria Bertani (LB) (Oxoid, Hampshire, United Kingdom), MacConkey (Accumin[™], New York, USA), Mannitol salt (BD, New Jersey, USA) and Baird-Parker (BP) agar were weighed respectively and dispersed in 1 L deionized water. They were allowed to soak for 10 minutes, swirled to mix and then heated to boil with frequent agitation until the media dissolved completely. This was followed by autoclaving at 121°C for 15 minutes. The mixture was allowed to cool to 47°C, mixed well and a volume of 25 ml was poured into each plate. For Baird Parker agar, it was supplemented with 1 vial of Rabbit Plasma Fibrinogen reconstituted with 10 ml of sterile purified water per 90 ml before dispersing in a petri dish. Serial dilutions of

1 in 10 of each of the samples were streaked on the plate and sealed with parafilm M. This was followed by labeling and storing in an incubator (Fisher scientific, Pittsburgh, USA) at 30°C to allow growth of the bacteria. The plates were examined 16 to 48 hours for growth.

3.7.6 Culturing of fungi

A mass of 65 g of Sabouraud dextrose agar with chloramphenicol (Oxoid, Hampshire, United Kingdom) was weighed and dispersed in 1 L deionized water. It was allowed to soak for 10 minutes, swirled to mix and then heated to boil with frequent agitation until the media dissolved completely. This was followed by autoclaving at 121°C for 15 minutes. The mixture was allowed to cool to 45°C and mixed well. A volume of 25 µl was poured into each plate. Serial dilutions of 1 in 10 of each of the samples were streaked on the plate and sealed with parafilm M. This was followed by labeling and storing in an incubator (Fisher scientific, Pittsburgh, USA) at 32°C to allow growth of the bacteria. The plates were examined 2 to 7 days for growth.

3.7.7 Antimicrobial susceptibility test

This was performed according to the Kirby Bauer protocol with slight modification (Bauer *et al.*, 1966). Amikacin (30µg), Kanamycin (30µg), Clarithromycin (30µg), Hygromycin B (30µg), Streptomycin (10µg) and Rifampicin (5µg), were obtained from Mast Diagnostics (Mast Group Limited, Bootle, United Kingdom). A mass of 38g of Mueller-Hinton agar was weighed and dispersed in 1 L deionized water. It was allowed to soak for 10 minutes, swirled to mix and then heated to boil with frequent agitation until the media dissolved completely. This was followed by autoclaving at 121°C for 15 minutes. The mixture was allowed to cool to 45°C and mixed well. A volume of 25 µl

was poured into each plate. A total of 20 unique colonies of bacteria identified on the LB agar plates were subcultured on LB agar plates. Two colonies of each were picked. DNA was extracted from one set and sequenced after PCR using 16S primers. The second colony of each isolate was emulsified in 3ml sterile normal saline in bijou bottles and the density and turbidity was compared with a barium chloride standard (0.5 McFarland). Total cell count was performed before inoculation to prevent obtaining false positive and false negative results. A sterile swap was dipped into the standardized suspension of bacterial culture and used to evenly inoculate the entire surface of the Mueller-Hinton Agar (MHA) plate to obtain uniform inoculums. MH agar plates were allowed to dry. Each plate was divided into 6 sections and antimicrobial impregnated disks were placed on the middle of each section using a sterilized forceps. Each disk placed was gently pressed with the sterilized forceps to ensure complete contact with the agar surface. This was done until each sample had all antimicrobial impregnated disk placed over its plate. The plates were then covered and sealed with parafilm M, and stored at 32°C in an incubator (Fisher scientific, Pittsburgh, USA) for 18 hours, the zones of inhibition of each disk was then checked and recorded.

3.8 Molecular microbiology

3.8.1 DNA extraction

DNA extraction was performed with the Qiagen Dneasy blood and tissue kit (QIAGEN, Venlo, Netherlands) following the manufacturer's protocol. Briefly, 850 µl of 1X PBS was added to 150 µl of sample. From this, 500 µl was aliquoted into a 2 ml screw cap tube, centrifuged at 14,000 rpm for 15 minutes to pellet bacterial cells and 300 µl of supernatant was pipetted off. To the remaining solution, 180 µl of buffer ATL and 20 µl

of proteinase K were added. The mixture was vortexed briefly and incubated at 56°C for 3 hours. Later, 200 µl of buffer AL was added. The mixture was vortexed briefly and incubated at 70°C for 30 minutes. To this, 250 µl of 100% ethanol was added. The mixture was vortexed briefly and transferred to a spin column. The column was then centrifuged at 8,000 rpm for 1 minute and flow-through was discarded. It was then washed by adding 500 µl of buffer AW1, centrifuged at 8,000 rpm for 1 minute and flow-through discarded. Washing was repeated but with buffer AW2. The column was dried by spinning at 13,000 rpm for 3 minutes. It was then put into freshly labelled 1.5 ml tubes, 150 µl of buffer AE added and centrifuged at 8,000 rpm to elute DNA. The DNA was stored at -20°C until further use.

3.8.2 Polymerase chain reaction (PCR)

For each PCR run, negative and positive controls were included. A 2720 Thermal Cycler (Applied Biosystems, California, U.S.A) was used for all PCR reactions. All primers sequences used are listed in Table 1 below:

Table 3.1. List of primers used for PCR amplifications

Primers	Forward and reverse sequences	Expected sizes	References
1_16SF	F-AGGAGGTGATCCAACCGCA R-AACTGGAGGAAGGTGGGAT	350bp	(Reysenbach <i>et al.</i> , 2000)
16S rRNA	MSHA-AAAAAGCGACAAACCTACGAG PA-AGAGTTTGATCCTGGCTCAG	600bp	(Hughes <i>et al.</i> , 1993)
<i>IS2404</i> (nest 1)	pGp1: 5'AGGGCAGCGCGGTGATACGG-3' pGp2: 5'CAGTGGATTGGTGCCGATCGAG-3'	400bp	(Ablordey <i>et al.</i> , 2012)
<i>IS2404</i> (nest 2)	pGp3: 5'GGCGCAGATCAACTCGCGGT-3' pGp4: 5'CTGCGTGGTGCTTTACGCGC-3'	200bp	(Ablordey <i>et al.</i> , 2012)

3.8.2.1 Nested PCR amplification using insertion sequence *IS2404* as primers

Amplification for *IS2404* loci was performed in a nested PCR, adapted from (Ablordey *et al.*, 2012) with slight modifications. The first reaction was done in a 25 µl reaction containing 5X PCR buffer already containing 1 nM MgCl₂ (Inqaba Biotech, Pretoria, South Africa), 300 µM each of deoxyribonucleotide and primers Pg1 and Pg2 (Inqaba Biotech, Pretoria, South Africa), and 2.5 µl of genomic DNA. In the second nested reaction, 3 µl of PCR product from previous reaction was used as template in a 25 µl reaction containing similar concentrations of reagents as in the previous nested reaction except for the primers; 500 nM each of Pg3 and Pg4 (described above). Preheating was done at 95°C for 2 minutes, followed by 40 cycles each of, denaturation at 94°C for 30 seconds, annealing at 64°C for 1 minute and extension at 72°C for 1.5 minutes. Final extension was at 72°C for 10 minutes and reaction held at 4°C.

3.8.2.2 PCR amplification using primers 16S rRNA (PA/MSHA) and 1_16SR/1_16SF

Both PA/MSHA and 1_16SR/1_16SF genes were amplified separately as described (Williamson *et al.*, 2008; Reysenbach *et al.*, 2000) with slight modifications, using similar master mix concentration. This was performed in a 25 µl reaction containing 5X PCR buffer containing 2 mM MgCl₂, 400 µM each of deoxyribonucleotide, 160 nM each of forward and reverse primers, 1U GoTaq polymerase and 2.5 µl of genomic DNA. Cycling was done at 95°C for 5 minutes, followed by 34 cycles each of, denaturation at 94°C for 45 seconds, annealing at 56°C for 45 seconds and extension at 72°C for 45 seconds. Final extension was at 72°C for 10 minutes and reaction held at 4°C.

3.8.2.3 Gel electrophoresis and UV visualization

For electrophoresis, 7 µl of PCR products were run on a 2% agarose (Sigma-Aldrich, Darmstadt, Germany) gel, stained with ethidium bromide (Sigma-Aldrich, Darmstadt, Germany), using 1X Tris Acetate EDTA (Thermo Scientific, Waltham, U.S.A) as running buffer, at 100V (Biometra, Gottingen, Germany) for 50 minutes. The gel was visualized under a UV transilluminator (UVP benchtop, Cambridge, U.K). For each run, 1 kb ladder was loaded alongside. The gel picture was taken (Kodak, New York, U.S.A) and band sizes were independently scored by two Laboratory Assistants.

3.8.3 Sequence and data analyses

PCR products (30 µl), of all the DNA amplicons were sent for sequencing (Macrogen Inc, Amsterdam, Netherlands). Forward primers for all loci were used for sequencing. Sequences were aligned using NCBI basic local alignment of sequences tool (BLAST). Sequence similarity to database sequences were based on expect value (E), maximum identity and score, query coverage and total score. The nucleotide sequence was then used to construct a phylogenetic tree with the help of Chromas Lite, BioEdit and Mega 6 software.

3.9 Ethical clearance and community entry

Ethical approval with number MS-ET/M.11 – P 3.1/2013-2014 was obtained from the Ethical and Protocol Review Committee of the University of Ghana Medical School. The study was explained in the local language by the assembly man or woman to all inhabitants within each community. Permission was sought from District Health Directorate, respective chiefs and opinion leaders. All patients and parents on behalf of their children signed a consent form before enrolling into the study. Patients who were

confirmed positive for BU but were not on hospital treatment were put on 52 days of antibiotic treatment by the Amansie Central Health Directorate.

CHAPTER FOUR

4.0 RESULTS

4.1 General characteristics and demography of study population

A total of 51 participants were recruited from the Amansie Central District for the study. All participants had one or more signs indicating the presence of Buruli ulcer. All the 51 samples isolated from the participants were positive for bacterial infection. However, only 40 out of the 51 participants turned out to be positive for Buruli ulcer while 48 participants were positive for mycobacterial infection. All participants complained of pains on the lesion. A total of 26 (50.98%) of the participants were females and 25 (49.02%) were males. The participants were aged between 1-80 years. A total of 10 (19.6%) were below 18 years, 20 (39.2%) were between 18-50 years and 20 (39.2%) were above 50 years. The mean age was 38.2 with the modal age of 65 years. The youngest male and female were 1 and 10 years respectively. The oldest male and female were 80 and 75 years respectively.

Available data on the residence of the patients indicate that majority of the patients reside in Jacobu, the capital town of the Amansie Central District where the district hospital St. Peters is located. Others live in near towns and villages such as Krofom, Donkuase, Homase, Mile 14, Afoako, Biribiwomang, Sukuumu, Numereso, Fahiakobo among others.

Majority of the participants 18 (35.5%) were farmers. A total of 7 (13.7%), 4 (7.8%) and 2 (3.9%) were students, traders and miners respectively. One watchman, one seamstress

and one mason also participated with each representing 1.96%. Participants who were not working were 17 (33.3%) in number.

4.2. Isolation and characterization of bacteria isolates

4.2.1. Gram staining of colonies of bacteria identified on BU patients

For the isolation and identification of the different type of bacteria present on the lesions of BU patients, gram staining was performed on both the patients' samples as well as isolated colonies after culturing the bacteria from the swab in LB agar. The result (Fig. 4.1) below shows different types of bacteria identified after gram staining. Both gram positive and gram negative cocci and rods were identified. Majority of the bacteria identified were gram positive single cocci in the crude sample and rods shaped bacteria from the isolated colonies.

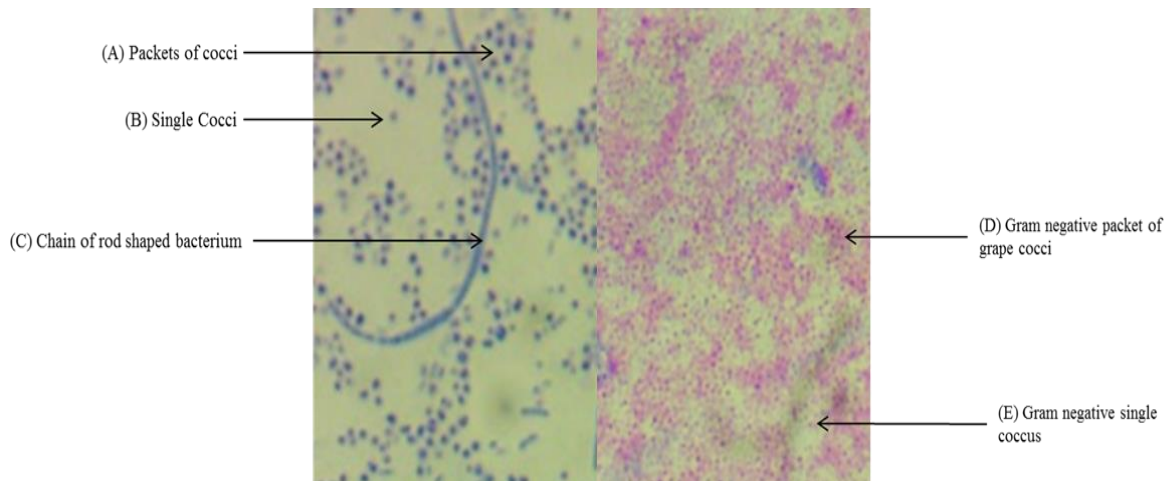


Fig. 4.1. Gram stain from a crude bacterial isolate of a BU patient

(A) Gram positive packet of cocci, (B) Gram positive single cocci, (C) Gram positive chain of rod shaped bacteria, (D) Gram negative packet of cocci and (E) Gram negative single cocci.

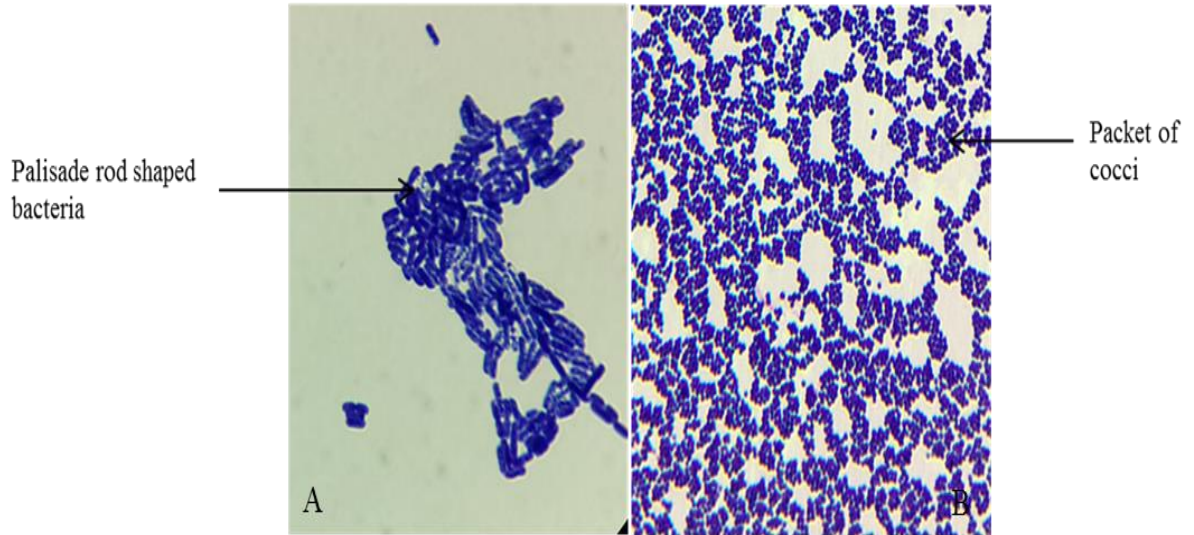


Fig. 4.2. Gram stain of unique colonies isolated from an LB plate after sub culturing of bacteria isolates from a BU patient

A percentage bar graph was plotted to determine the proportions of each type of bacteria to the total types of bacteria identified after gram staining the crude bacteria isolates of the BU patients. Majority of the bacteria identified were Gram positive 90.2% (46/51) and Gram negative cocci 52.9% (27/51). Few 5.9% (3/51) Gram positive rods-shaped bacteria and Gram negative rod-shaped bacteria 7.8% (4/51) were identified. Yeast cells were identified in 23.5% (12/51) of the crude patients' samples as shown Fig 4.3.

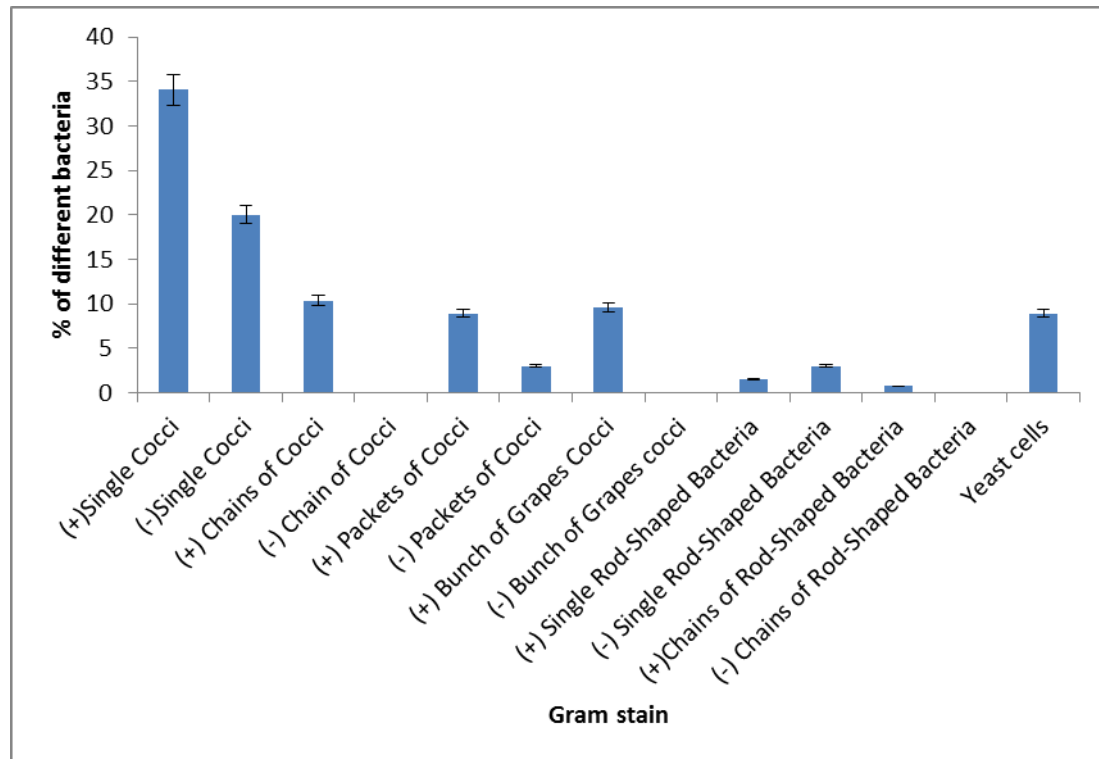


Fig. 4.3. Types of Gram positive bacteria, Gram negative bacteria and yeast cells after Gram staining of crude bacterial isolates

4.2.2 Acid-Fast Positive bacteria on BU Patient

In order to identify acid-fast bacteria such as *M. ulcerans*, Zeihl-Neelsen stain was performed on the crude bacterial isolates. Acid-fast positive cocci and rod shaped bacteria were both identified from the crude bacterial isolates of some BU patient. Fig. 4.4A shows different types of acid-fast bacteria identified after Ziehl-Neelsen stain. Fig. 4.4 B. shows acid-fast coccobacilli. This may likely be *Rhodococcus spp* confirmed after sequencing.

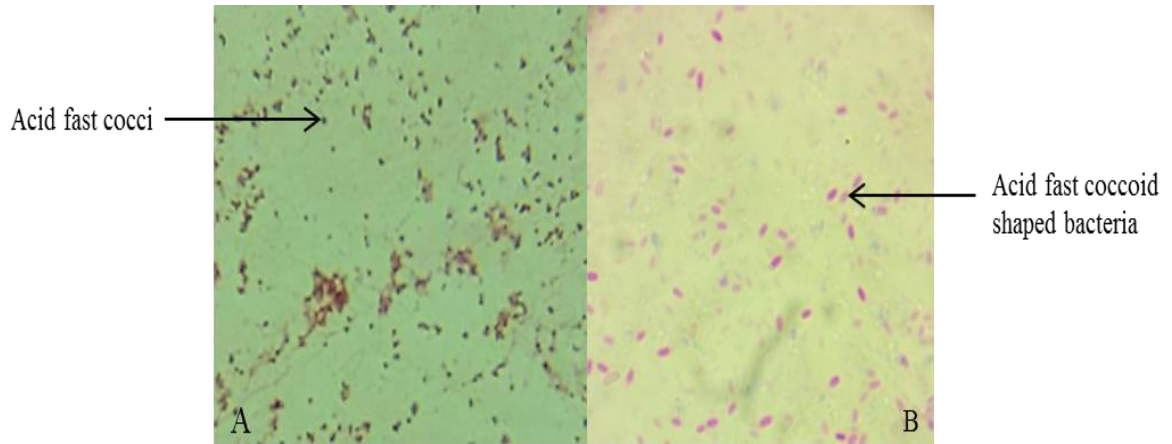


Fig. 4.4. Acid-fast bacteria identified from the crude bacteria isolate after Ziehl-Neelsen stain

A shows acid-fast single *cocci* while B shows acid-fast *coccobacilli*.

4.2.3 Non-acid fast rods identified on a crude bacterial of a BU patient

Non-acid fast rods identified from the crude patients' samples after Ziehl-Neelsen stain were very few. Unique club shaped rods were identified in two crude bacteria isolates from BU patients as shown in Fig. 4.5.

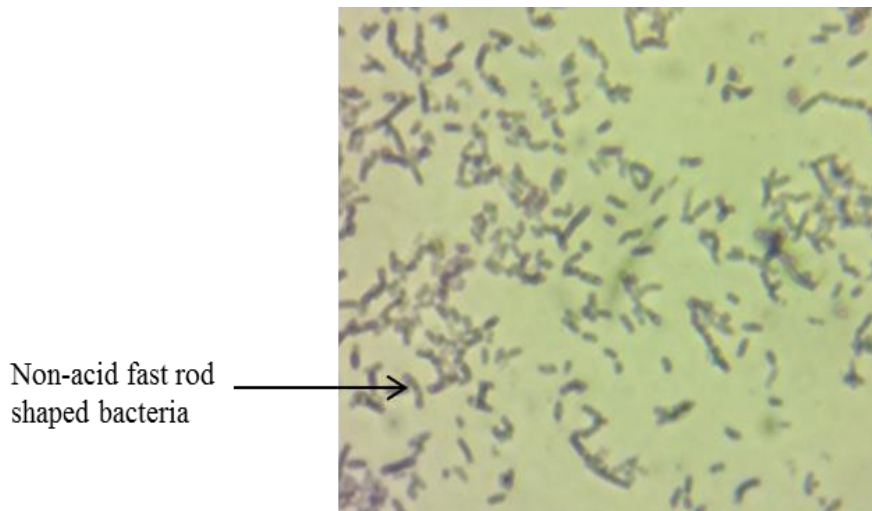


Fig. 4.5. Non-acid fast rods identified from the crude bacteria isolate of a BU patient

There are Gram positive rod-shaped bacteria that are also non-acid fast bacteria. These bacteria are usually from the suborder *Corynebacterineae*. *Rhodococcus* and *Corynebacterium* spp can be found in this group of bacteria. This club shaped bacteria shown in Fig. 4.5 above may likely be *Corynebacteria* which were confirmed after sequencing.

Computational results of Zeihl-Neelsen stain shows that majority of acid-fast bacteria identified in all patients' samples were single cocci (72.5%) representing 37 out of 51. Acid-fast rods were very few (5.9%) representing 3 out of 51 acid-fast bacteria. *Coccobacillus* (3.9%) representing 2 out of 51 and yeast cells (5.9%) representing 3 out of 51 were also identified as shown in Fig. 4.6.

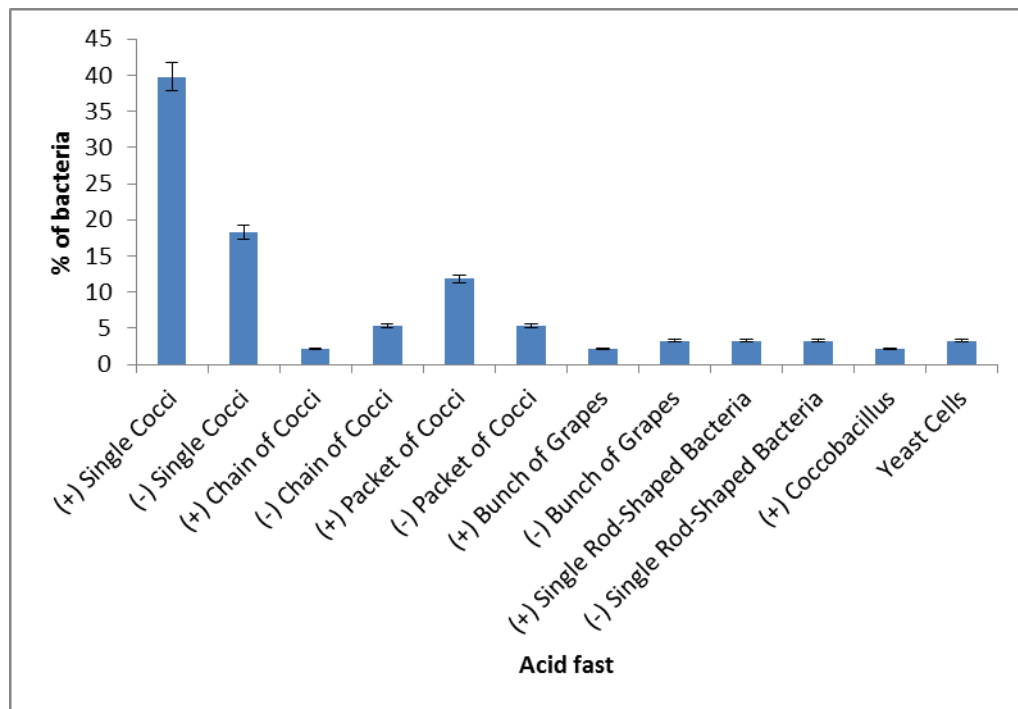


Fig. 4.6. The number and types of acid and non-acid fast bacteria present after Zeihl-Neelsen staining

4.2.4 Identification of Fungal Elements from crude microbial isolate of BU patients

Yeast cells were also identified in the patients' samples after Gram and Zeihl-Neelsen stain. Yeast cells were more prominent in Gram stains of the crude patients' samples compared to that of Zeihl-Neelsen stain

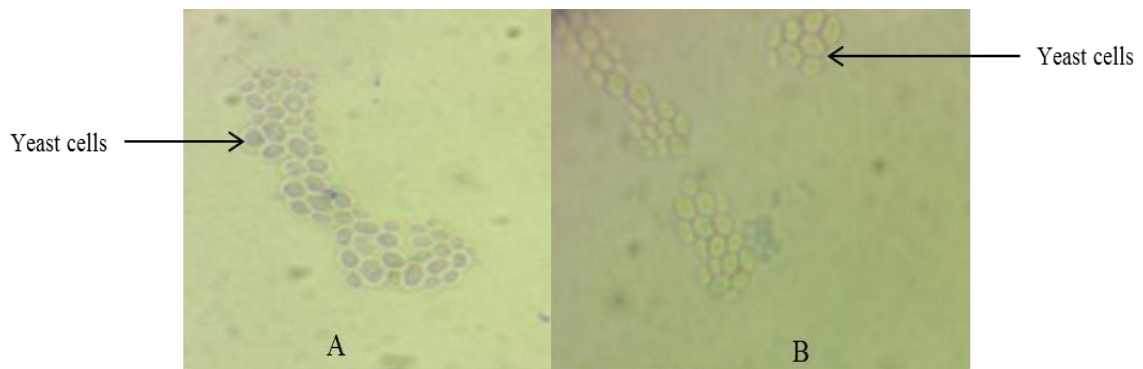


Fig. 4.7. Yeast cells identified from crude patients' samples of BU patient after Gram and Ziehl-Neelsen stain

(A) Yeast cells identified after gram stain whiles (B) Yeast cells identified after acid-fast stain.

4.2.5 Identification of Yeast Cells in 10% KOH

Yeast cells were identified from the crude patients' samples from BU patients when viewed in 10% KOH under the microscope with magnification of x40. Ten percent (10%) KOH was added to the crude microbial sample before and after culturing on Sabouraud dextrose agar. Fig. 4.8 shows yeast cells in the patients' samples before and after culturing on Sabouraud dextrose agar.

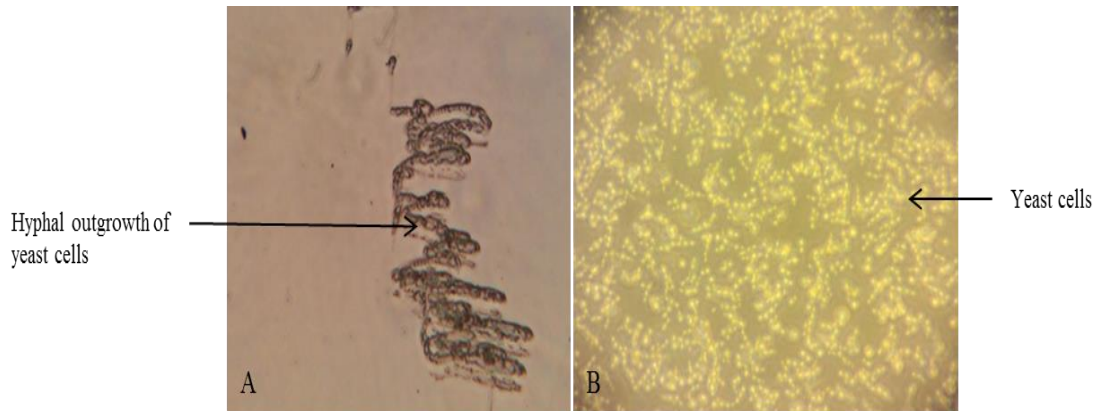


Fig. 4.8. Yeast cells viewed under an inverted microscope (x40)

(A) Hyphal outgrowth of yeast cells from crude patients' samples before culturing while (B) Yeast cells viewed after culturing on Sabouraud dextrose agar.

4.3 Culturing and Isolation of Bacteria

4.3.1 Bacteria Culture on Luria-Bertani (LB) Agar

There were growth of bacteria on the LB agar plate. Fig. 4.9 shows the growth of bacteria on an LB plate after culturing for 24 hours. White and yellow unique colonies were sub cultured and characterized for identification through sequencing.

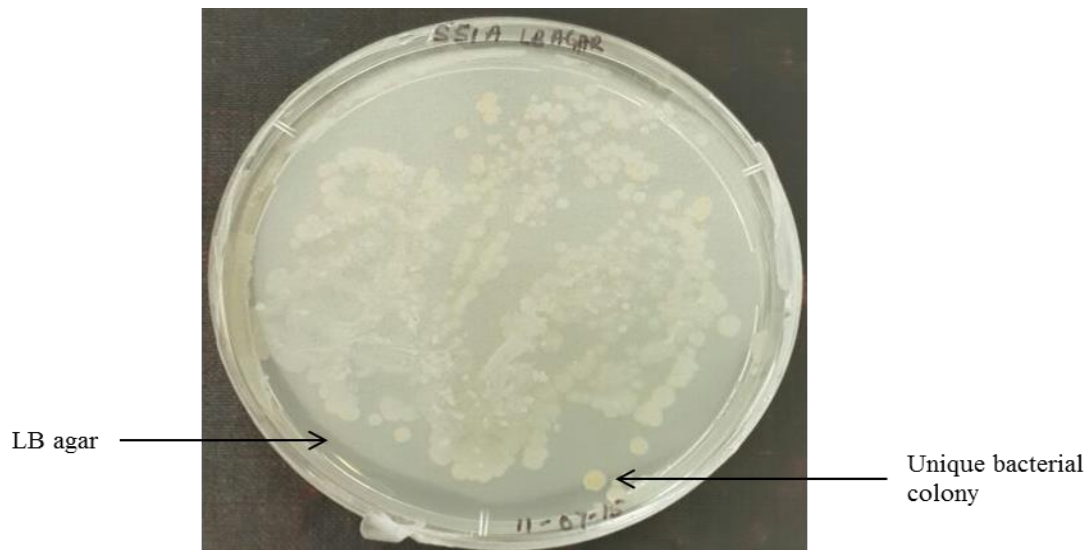


Fig. 4.9. Growth of bacteria colony on LB agar plate from a BU patient

4.3.2 Culturing and identification of *Staphylococcus* on Mannitol Salt Agar

The patients' samples were cultured on mannitol salt agar to selectively and differentially identify *staphylococcus spp* as fermenters and non-fermenters of mannitol. Those that grow as yellow colonies were mannitol fermenters while those that form pink colonies were non-fermenters of mannitol. Fig. 4.10 shows the growth of both *Staphylococci* mannitol fermenters and non-fermenter from the crude patients' samples of BU patients.

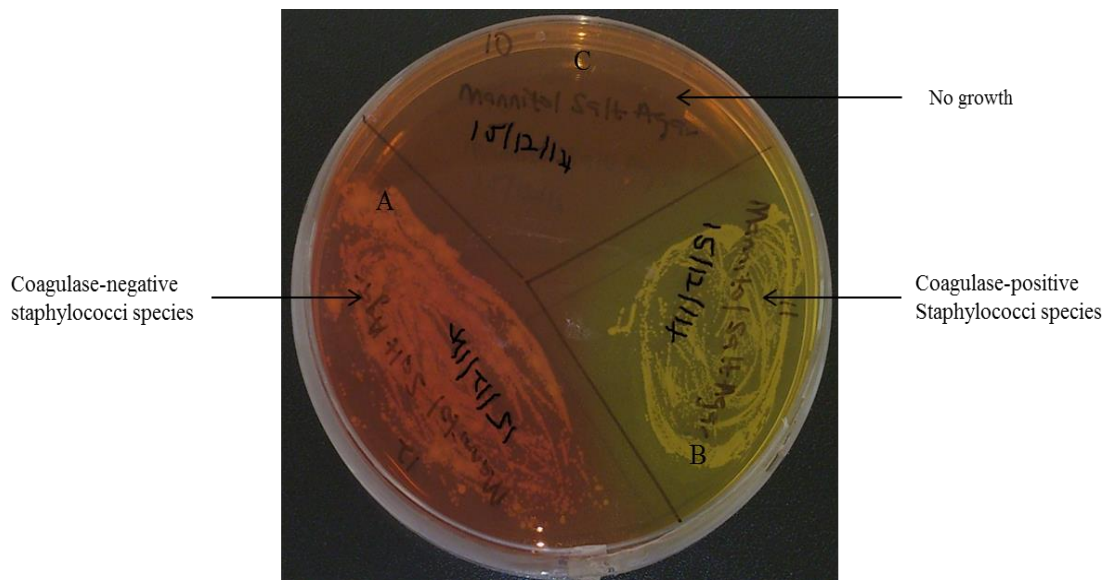


Fig. 4.10. Growth of both mannitol fermenters and non-mannitol fermenters of *staphylococci spp* from crude patients' samples from the lesions of BU patients

(A) Growth of non-fermenters of mannitol (non-pathogenic staphylococci species) while (B) Growth of mannitol fermenters (pathogenic staphylococci species). (C) Absence of *Staphylococcus*.

Fig.4.11. shows the distribution of *Staphylococci spp* from crude patients' samples of BU patients. Out of the 51 patients, mannitol fermenters (36/51) were the majority representing 76.4% of the patient. 12 out of the 51 (23.5%) patients had no growth of *Staphylococci spp*. Non-mannitol fermenters were identified in only 2 out of 51 microbial isolate representing (3.9%) of the *Staphylococci* identified.

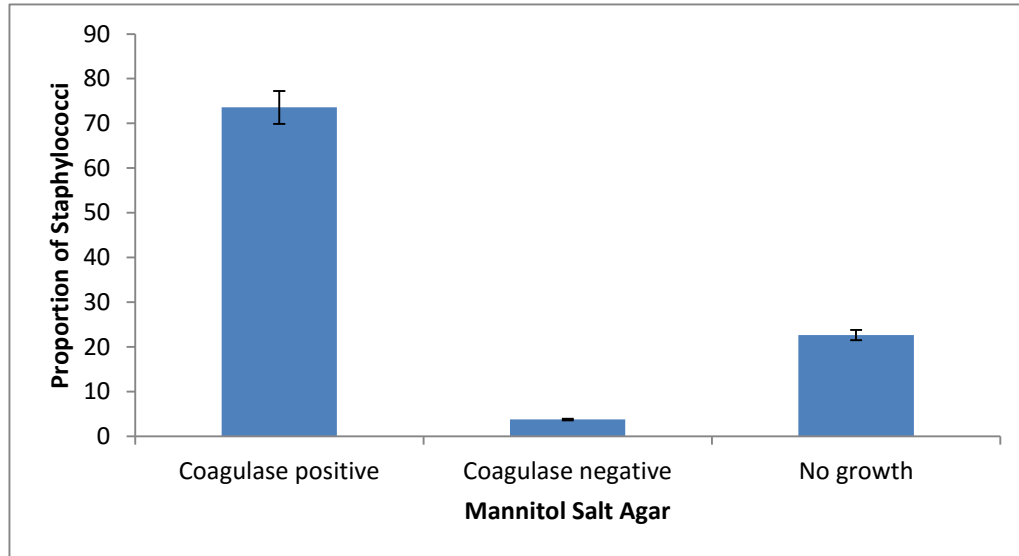


Fig. 4.11. Distribution of *Staphylococci* spp from BU patients

4.3.3 Culturing on MacConkey Agar

The patients' samples were cultured on MacConkey agar to selectively identify gram negative and enteric bacteria and differentiate them into lactose fermenter and non-lactose fermenters.

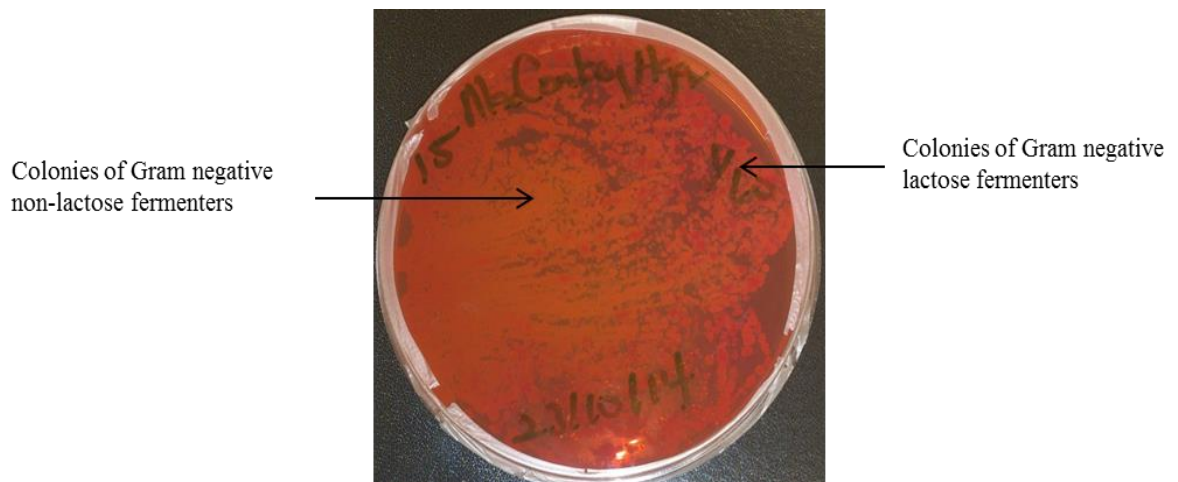


Fig. 4.12. Growth of enteric or gram negative bacteria from a crude patients' samples of a BU patient on a MacConkey plate

Fig 4.13 shows the distribution of Gram negative or enteric bacteria among the BU patients. Gram negative bacteria were detected in 48 crude patients' samples. However, Lactose fermenters were identified in 47 out of the 51 (92.2%) culture plates, non- lactose fermenters were identified in 20 (39.2%) culture plates while 4 (7.8%) plates had no growth. This shows that some BU patients had both Gram negative lactose fermenters and non-lactose on their BU lesions.

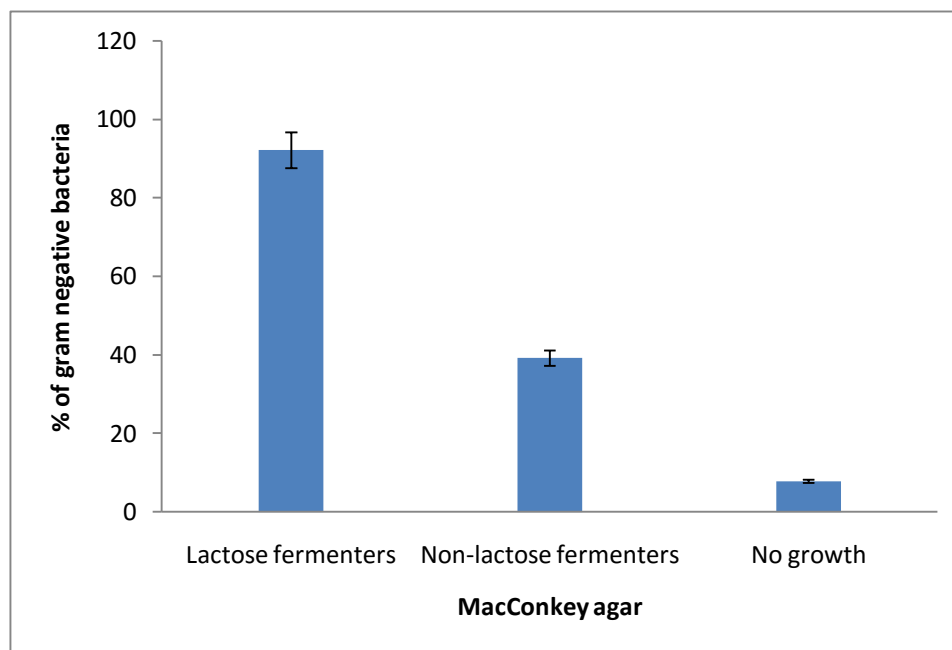


Fig. 4.13. Distribution of Gram negative or Enteric bacteria from crude isolates among the BU patients

Lactose fermenters and non- lactose fermenters were identified in 92.2% and 39.2% of the culture plates respectively while 4 (7.8%) plates had no growth.

4.3.4 Culturing on Sabouraud Dextrose Agar for Detection of Fungal Element

The patients' samples were cultured on Sabouraud dextrose agar to identify fungal elements (yeast and molds) present on the lesions of BU patients. Fig 4.14 shows the growth of fungal elements on a Sabouraud dextrose agar plate. Specimen taken from BU

patient with ID 46, had no growth of fungal element, patient 47 had growth of yeast and patient 48 had growth of both yeast and few molds. Majority (74.3%) of the fungal elements were yeast cells (39/51). Molds were also identified in 3.9% (2 out of 51) of the crude patients' samples. There was no growth in 10 patients' samples (19.6%). Fig. 4.15 shows the distribution of fungal elements identified in the crude patients' samples after culturing on Sabouraud dextrose agar.

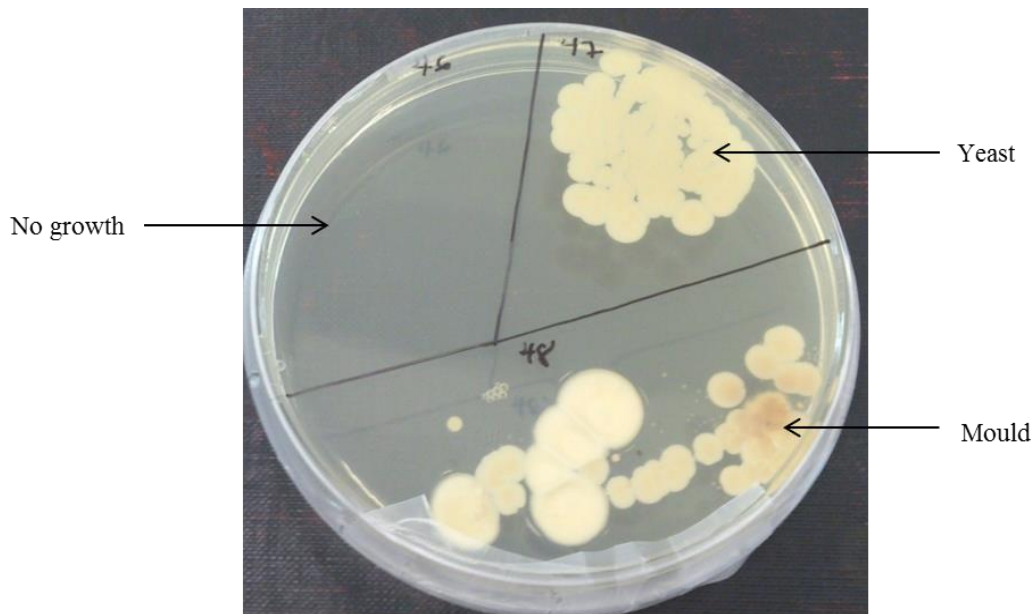


Fig. 4.14. Growth of fungal elements from patients' samples of BU patients on a Sabouraud dextrose agar plate

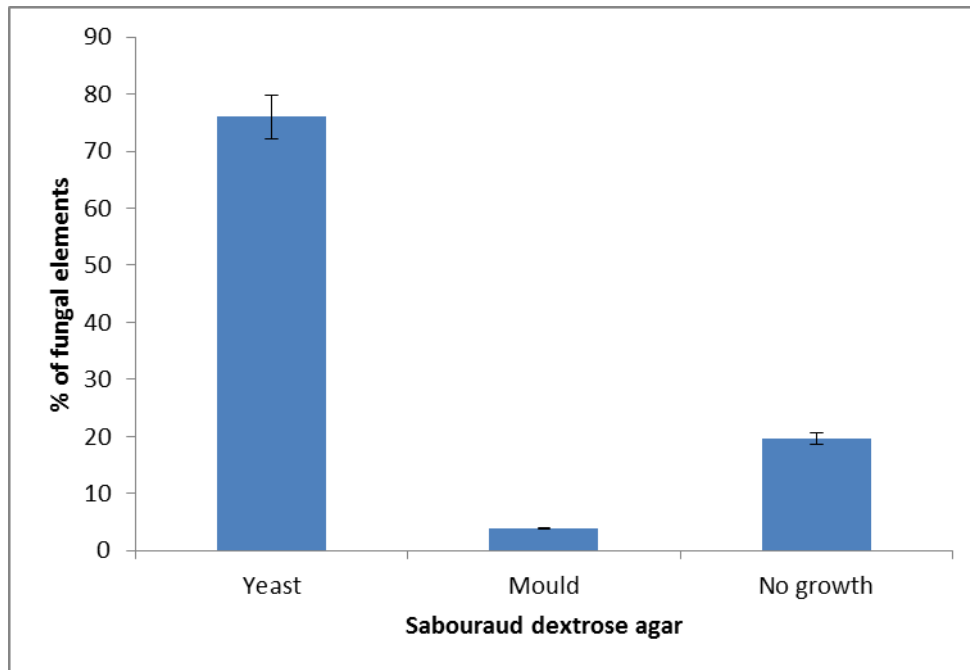


Fig. 4.15. Distribution of fungal elements present on the lesions of BU patients after culturing on Sabouraud Dextrose Agar

4.4. Antibiotic testing of selected bacteria strains after sequencing

Bacterial susceptibility of isolates to antimicrobial agents was performed by the disk diffusion method using guidelines established by the Clinical laboratory services. The concentrations used as well as the zones of inhibitions were measured according to the Clinical Laboratory Services. Antibiotic testing was performed on selected bacteria (*B. anthracis*, *B. infantis*, *B. cereus*, *B. aquimaris*, *B. thuringiensis*, *Staphylococcus. Sp M7*, *Staphylococcus sp* MRSA and *Staphylococcus aureus*). All the selected bacteria were susceptible to clarithromycin and amikacin. The *Bacillus cereus* groups bacteria were found to be resistant to rifampicin, and streptomycin whiles kanamycin had the least susceptibility.

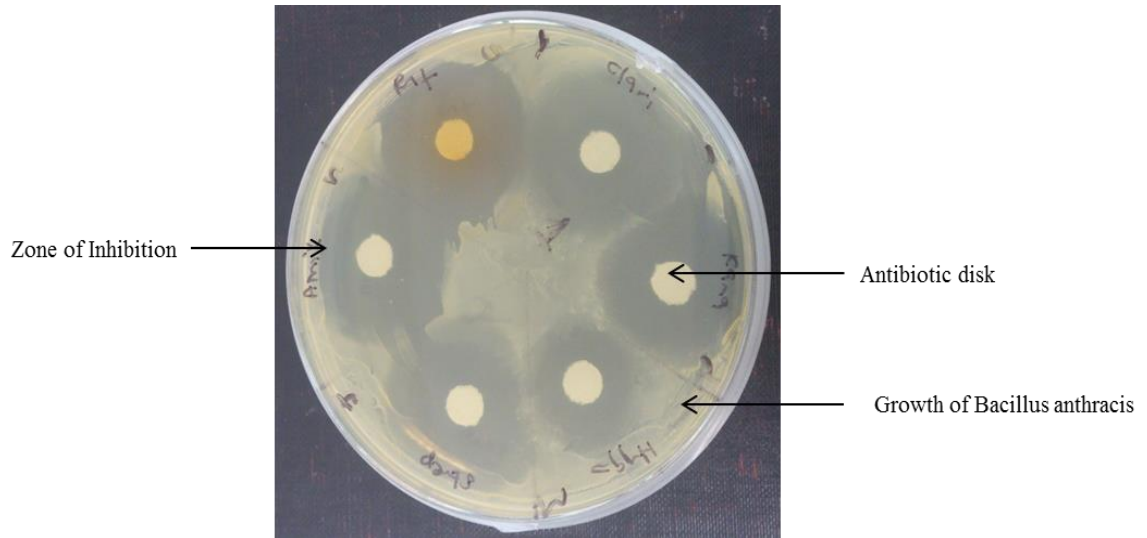


Fig. 4.16. Zones of inhibition of growth of *B. anthracis* isolated from the lesion of a BU patient using selected antibiotic

Table 4.1. The zones of inhibition (in mm) of 6 different antibiotics on susceptibility testing on selected bacteria

Bacteria	Clarithromycin (30µg)	Kanamycin (30µg)	Hygromycin B (30µg)	Streptomycin (10µg)	Amikacin (30µg)	Rifampicin (5µg)
<i>B. anthracis</i>	25mm (S)	23mm (I)	20mm (S)	25mm (I)	26mm (S)	27mm (S)
<i>Staphylococcus sp M7</i>	23mm (S)	24mm (I)	18mm (S)	24mm (I)	24mm (S)	30mm (S)
MRSA	28mm (S)	24mm (I)	18mm (S)	23mm (I)	28mm (S)	30mm (S)
<i>B. infantis</i>	18mm (S)	24mm (I)	19mm (S)	22mm (I)	21mm (S)	18mm (R)
<i>B. cereus</i>	20mm (S)	35mm (S)	22mm (S)	18mm (R)	30mm (S)	21mm (R)
<i>B. aquamaris</i>	28mm (S)	25mm (I)	20mm (S)	18mm (R)	32mm (S)	22mm (R)
<i>S. aureus</i>	30mm (S)	26mm (S)	20mm (S)	18mm (R)	28mm (S)	31mm (S)

The isolated organisms were *B. anthracis*, *B. infantis*, *B. cereus*, *B. aquimaris*, *B. thuringiesis*, *Staphylococcus. Sp M7*, *Staphylococcus sp* MRSA and *Staphylococcus aureus*. The concentrations used as well as the zones of inhibitions measured were according to the Clinical Laboratory Services standards. (R-Resistance, S-Susceptible, I- Intermediary)

4.5 Identification of bacterial strains using culture independent method

PCR was run in order to detect bacterial strains that were not identified using the culture dependent method. For the purpose of identifying universal bacteria, mycobacteria and *M. ulcerans*, primers 1_16S, MSHA/PA and *IS2404* nested (Pg1 to Pg4) with amplicon sizes 350bp, 600bp and 200bp respectively were used. All PCR reactions were carried out with at least a negative control. All samples (51) were first screened for bacteria spp using the 16S rRNA universal primers. Fig. 4.17 shows 1_16S positivity in all the patients' samples indicating the presence of bacteria in all the patients' samples. As expected, 16S positivity for mycobacteria was higher (48/51) compared to that of *IS2404* (40/51). The presence of *IS2404* discriminated Mycolactone Producing Mycobacteria (MPM) from Non-Tuberculous Mycobacteria (NTM) and hence positivity was reduced.

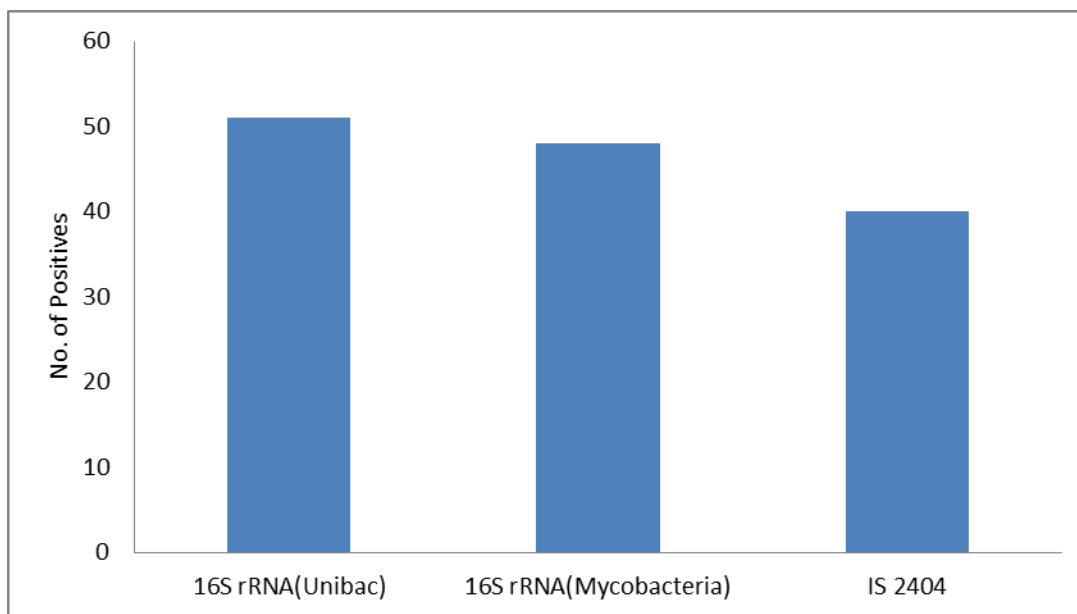


Fig. 4.17. 1_16S (universal bacteria), 16S (Mycobacteria) and *IS2404* positivity among people with signs of BU

4.5.1. Detection of bacteria strains using 1_16S rRNA (universal primers)

16S rRNA was amplified directly from all DNA of crude patients' samples as well as the 20 randomly selected bacteria colonies. As was expected, there was 100% detection of amplified DNA in all the samples showing the presence of bacteria DNA in all isolates from the BU patients. As shown in Fig 4.18 Lane 1 and 17 represents 1kb DNA ladder, lane 2-15, 18 and 19 represent PCR product from the BU patients. The size of the amplicon is 350bp. All turned out positive for bacterial infection. PC represents a positive control and NC for negative control.

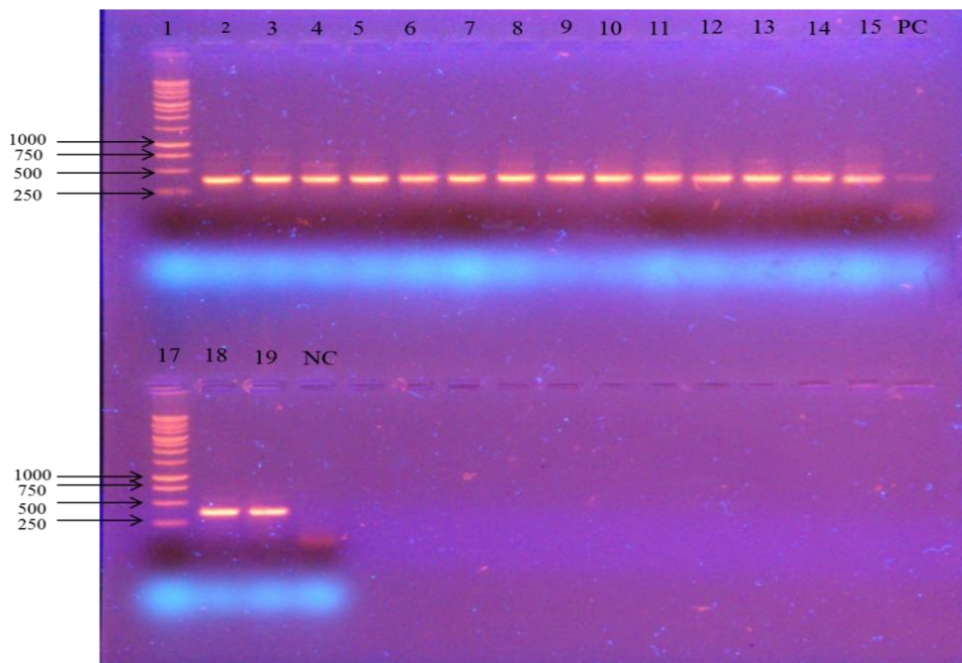


Fig. 4.18. An electrophoretogram showing the PCR product of 16 samples using 1_16S primers (universal bacteria)

4.5.2 Identification of mycobacterial strains using 16S rRNA (Sequence specific for mycobacteria)

All patients' samples were amplified with primers 16S rRNA specific for mycobacteria detection. A total of 48 were positive. However, amplification of the region and DNA sequencing led to the identification of *Corynebacterium spp* and *Rhodococcus spp*. Even though these bacteria are not mycobacteria, they have high G-C content with close phylogenetic relationship to mycobacteria. This may explain why they were amplified as mycobacteria. Fig 4.19 is a representative gel showing the results.

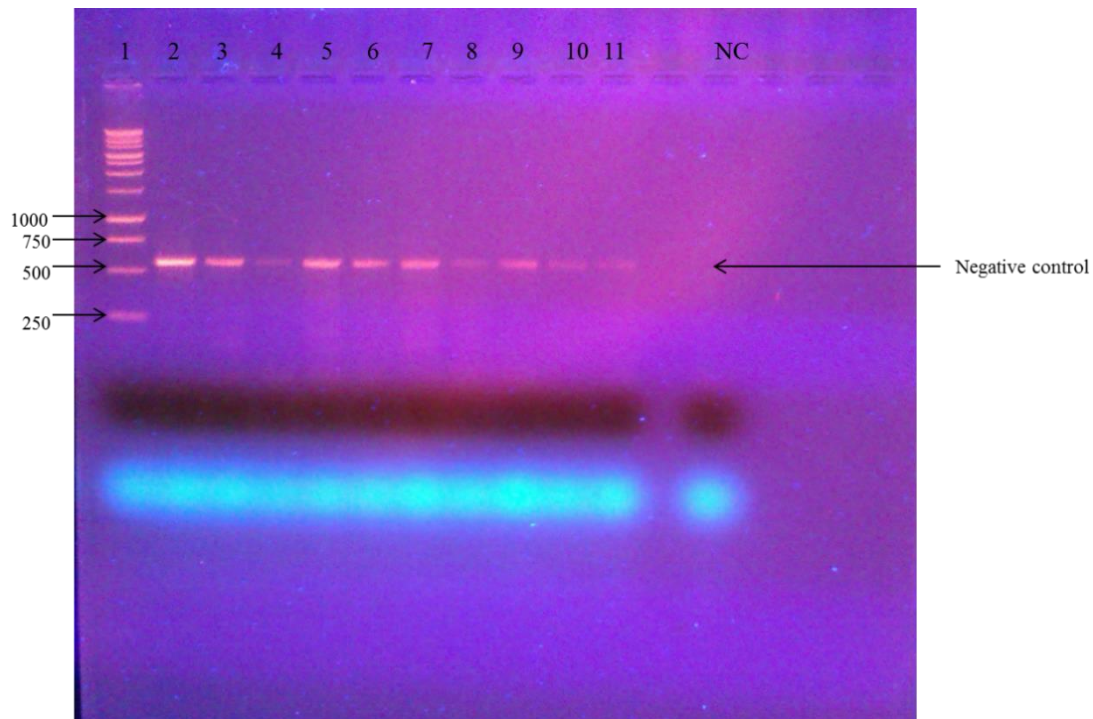


Fig. 4.19. An electrophoretogram of PCR products of 10 patients using MSHA/PA as primers for mycobacteria

Lane 1: 1kb DNA ladder (molecular weight marker); NC: negative control. Lane 2 to 11: PCR amplicons of 10 patients. The size of the expected amplicon is 600bp. All 10 patients were positive for mycobacteria.

4.5.3 Nested PCR Product of *IS2404*

The patients' samples were amplified with primers *IS2404* specific for *M. ulcerans* detection. This primer is able to distinguish between Mycolactone Producing Mycobacteria (MPM) and Non Tuberculosis Mycobacteria (NTM). Amplicons were sequenced to identify the type of MPM amplified. Fig. 4.20 shows a representative gel showing positivity for *IS2404*.

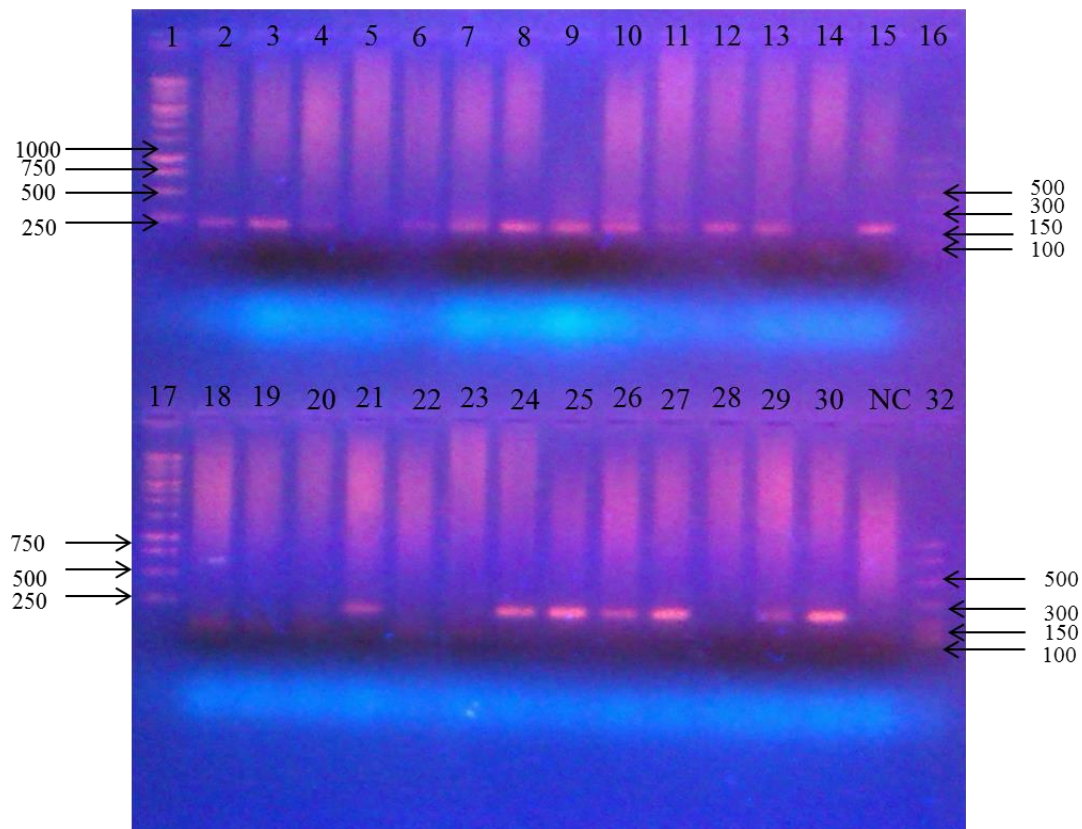


Fig. 4.20. An electrophoretogram of nested PCR product of 27 patients using *IS2404* (Pg 1 to Pg4) as primers

Lane 1 and 17: 1kb DNA ladder; lane 16 and 32: PCR marker (NEB). NC: negative control; lanes 2 to 15 and 18 to 30: patients with signs of BU.

4.6 Sequencing of 16S universal bacterial, Mycobacteria and *M. ulcerans*

Sequence data was analyzed with the help of information from National Center for Biotechnology Information. Nucleotide blast was used to determine the best similarity between the sequenced samples and data available. Comparison of the queries using percentage identities, E-value and gaps helped to determine the organism that is most similar to the subject sequence. Fig. 4.21 shows the nucleotide sequence result compared to *Pseudomonas aeruginosa* strain BN5 16S ribosomal RNA gene, partial sequence with Sequence ID: gb|KM406775.1.|Length: 791Number of Matches: 1. Comparison of the two sequences showed a gap of 0 and 100% identity. The DNA was thus identified for the bacteria *Pseudomonas aeruginosa*.

Score	Expect	Identities	Gaps	Strand
604 bits(327)	2e-169	327/327(100%)	0/327(0%)	Plus/Minus
Query 1	TGTTTCGACTTCCCCAGTCATGAATCACTCCGTGGTAACCGTCCCCCTTGCGGTTAGACT			60
Sbjct 780	TGTTTCGACTTCCCCAGTCATGAATCACTCCGTGGTAACCGTCCCCCTTGCGGTTAGACT			721
Query 61	AGCTACTTCTGGAGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGA			120
Sbjct 720	AGCTACTTCTGGAGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGA			661
Query 121	ACGTATTACCGGTGACATTCTGATTACGATTACTAGCGATTCCGACTTCACGCAGTCGA			180
Sbjct 660	ACGTATTACCGGTGACATTCTGATTACGATTACTAGCGATTCCGACTTCACGCAGTCGA			601
Query 181	GTTGCAGACTGCGATCCGGACTACGATCGGTTTTATGGGATTAGCTCCACCTCGCGGCTT			240
Sbjct 600	GTTGCAGACTGCGATCCGGACTACGATCGGTTTTATGGGATTAGCTCCACCTCGCGGCTT			541
Query 241	GGCAACCCTTTGTACCGACCATTGTAGCACGTGTGTAGCCCTGGCCGTAAGGGCCATGAT			300
Sbjct 540	GGCAACCCTTTGTACCGACCATTGTAGCACGTGTGTAGCCCTGGCCGTAAGGGCCATGAT			481
Query 301	GACTTGACGTCATCCCCACCTTCCTCC			327
Sbjct 480	GACTTGACGTCATCCCCACCTTCCTCC			454

Fig. 4.21. Sequence of one 16S universal bacteria compared to sequence nucleotide of *Pseudomonas aeruginosa* strain BN5

4.7. Phylogenetic diversity among the secondary bacterial infections of BU identified

Phylogenetic diversity of bacteria identified by 16S rRNA was analyzed. Bacteria were grouped according to their phenotypic similarity. *A. faecalis*, *A. aquatilis*, *P. stutzeri*, *P. mirabilis* and *P. aeruginosa* are all gram negative rods and thus they have a common ancestry. However, differences arise in them because the *Alcaligene spp* are under the class Beta Proteobacteriaceae whiles *Pseudomonas spp* are Gamma Proteobacteriaceae. The bacillus cereus groups of bacteria are differentiated by being gram positive rods and under the order bacillales. These are also differentiated from the *Staphylococcus spp* which are gram positive cocci and under the class coccus as shown in Fig. 4.22. The last groups of secondary bacteria are the ones in the phylum Actinobacteria. They are all under the sub order Corynebacterineae but *M. ulcerans* falls in the genus of mycobacteria. This could explain why many *Corynebacterium spp* were identified after sequencing with 16S rRNA for mycobacteria. Fig.4.23 below shows the phylogenetic tree constructed to show the relatedness of bacterial that cause secondary infections in BU patients.

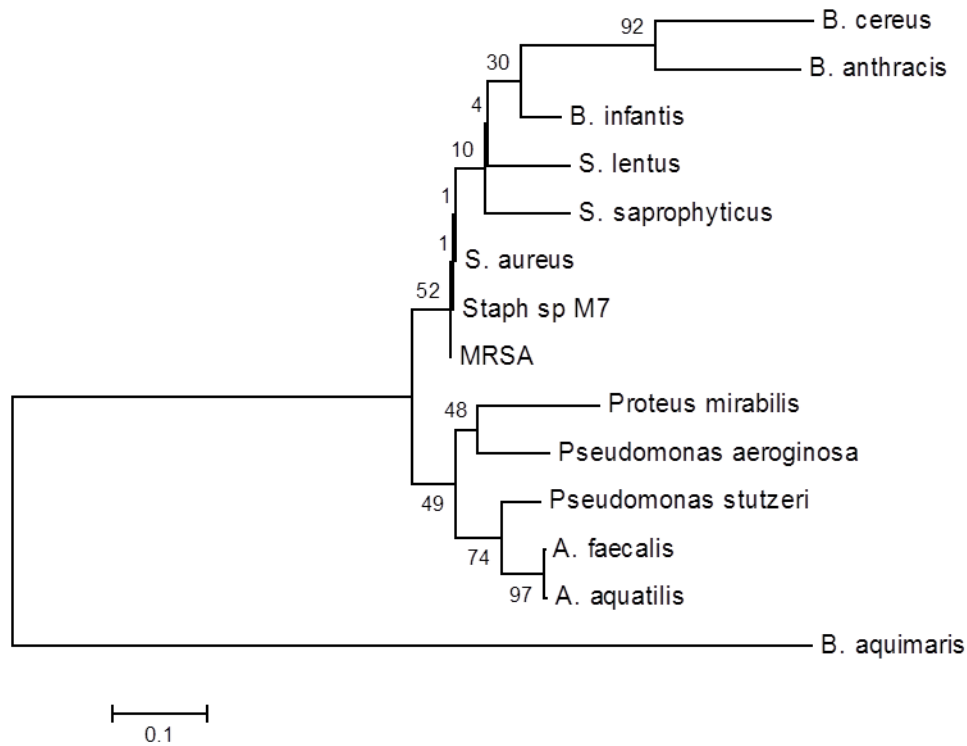


Fig. 4.22. Phylogenetic tree constructed for partial 16S rRNA gene of bacterial strains from BU patients' samples

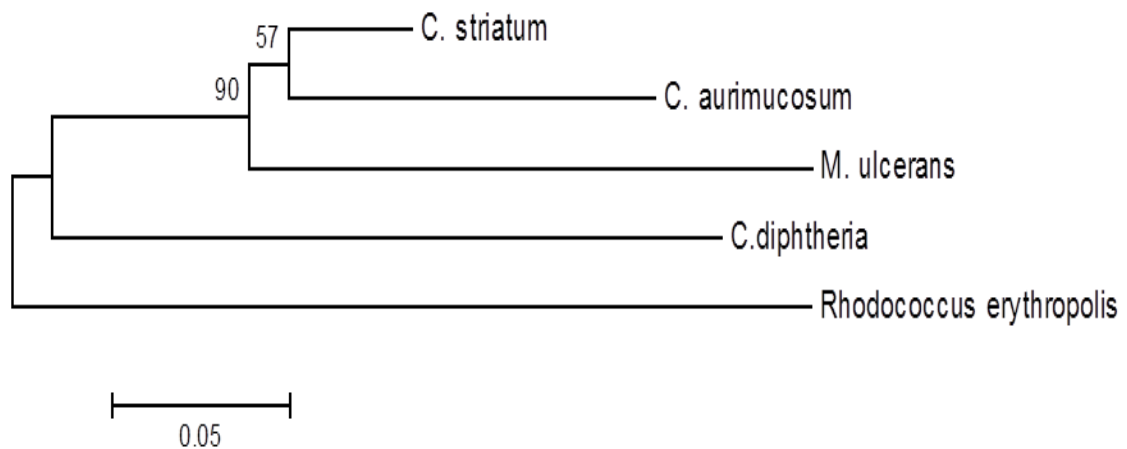


Fig. 4.23. Phylogenetic tree constructed for 16S hyper-variable region for Actinomycetales from BU patients' samples using the primer MSHA/PA

CHAPTER FIVE

5.0 DISCUSSION

Identification of secondary microbial infections of Buruli ulcer patients is crucial in helping us gain knowledge in the reason for delayed healing, the cause of pains in some BU patients, the treatment and management of the condition as a whole. Owing to the challenges associated in treating the disease with antibiotics, there is the need to identify other secondary microbial infections that colonize the wound and reduce the efficacy of the antimicrobial drugs being administered to patients (Yeboah-Manu *et al.*, 2013). Although risk factors for bacterial wound colonization have not been thoroughly studied to date, delayed treatment and insufficient wound management might contribute to colonization and prolonged wound healing (Amissah *et al.*, 2015). This study thus aimed at identifying common bacteria and mycobacteria that infest the wounds to cause pain in BU patients and delay healing of the disease. This section also discusses data from the study to support the assertion that other microbes apart from *M. ulcerans* colonize the ulcer of the patients and may be the reason for the pain experienced by some BU patients.

Debacker *et al.* (2006) reported BU prevalence to be higher among women than men and among boys than girls. For this study, there was no significant difference in the sex of the patients. A total of 50.98% were female and 49.02% were males. This is in line with a national case study conducted by (Amofah *et al.*, 2002) who found out no sex difference among BU patients. According to Debacker *et al.* (2006), Buruli ulcer commonly affects the young, even though cases are reported in all age groups. Even though majority of the participant were adults, we found no significant difference among different age groups of the participants. A total of 39.2% each were within the age groups of 18-50 years and

above 50 years while 19.6% percent were below 18 years. This information is supported by Johnson *et al.* (2007) who noted that the disease occurs in all age groups but higher attacks were with the adults and elderly. Also, Debacker *et al.* (2006) noted that even though there are no gender differences among children and adults, men older than 59 years had a higher chance of developing BU than women. In our report of the study, we noted that the oldest persons among the participants were men, with the oldest of them being 80 years.

Over 35% of respondents were farmers or practiced other agricultural activities. It has been reported that activities that take place near water bodies, such as farming, are risk factors, and wearing protective clothing appears to reduce the risk of the disease (Debacker *et al.*, 2004). These results reflect the large number of the populations that were exposed to contaminants with *M. ulcerans* from the water bodies and the working environment as a whole.

Gram staining was performed to detect gram positive and negative bacteria. For gram positive bacteria, as shown in Fig. 4.3, single positive cocci (46/51) were identified in almost all the crude patients' samples (above 90.2%). A total of 27 out of 51 patients' samples were negative single cocci (52.9%), chains of cocci (14/51) represented 27.5%, 13 out of 51 isolates were grape-like clustered cocci (25.5%), and (12/51) packets of cocci (23.5%). There were few (27/51) gram negative bacteria out of which 52.9% were single cocci and 7% were packet of cocci (4/51). Nevertheless, no gram negative chain or grape-like clustered cocci were identified. However, yeast cells were identified in 23.5% of the BU patients' samples (12/51) as shown in Fig.4.6 through gram staining. Rod-shaped bacteria identified were few, with 3.9%, 7.8% and 1.9% of the samples having

gram positive single rod-shaped bacteria (2/51), gram negative single rod-shaped bacteria (4/51) and gram positive chains of rod-shaped bacteria (1/51) respectively.

Acid-fast staining was also performed to detect the presence of acid-fast and non-acid fast bacteria among the BU patients' samples. Majority of the bacteria were acid-fast single cocci. However, very few acid-fast single rod-shaped bacteria could be noted. Detection of *M. ulcerans* through acid-fast staining on all the samples could not be attained. It has been noted that detecting *M. ulcerans* through the acid-fast (Ziehl Neelsen) stain procedure has a very low sensitivity (Lupi *et al.*, 2005). *M. ulcerans* numbers tend to decrease over time and when the patient is on chemotherapy. It is therefore, important to emphasize that the positivity of the test varies with the clinical form of the disease. It has been found to be more useful in the ulcerative stage (Van Der Werf *et al.*, 2005). If the lesion is not ulcerated, a skin biopsy may not be sufficient for the examination and confirmation of the disease. In the nodular form, positivity could reach 60% while in the edematous form, it may reach up to 80%, both in the direct examination and culture (Johnson *et al.*, 1999). Even though both *Staphylococcus* and *Streptococcus* have genera as gram positive bacteria and have spherical cell shape, they can be visually differentiated. *Streptococci* form chains of cocci due to their linear cell division while *Staphylococci* divide in various directions forming packets and grape like clusters (Brown *et al.*, 2005). There were high percentages of gram positive cocci than the other type of bacteria in almost all the samples. This is in line with a study conducted by James *et al.* (2008) who identified the predominant type of bacterial in wound to be gram positive cocci. Coccoid bacteria and gram negative rods are fairly predominant in wounds as secondary infections (James *et al.*, 2008). This shows the

presence or colonization by other microbes such as *Streptococci* and *Staphylococci* spp at site of the lesions of Buruli ulcer patients.

The patients' samples were grown on mannitol salt agar to identify *Staphylococci* spp. Even though many believe most of the bacteria that infect lesions are normal skin flora and are thus non-pathogenic (Site, 2009), majority of the *Staphylococci* identified, as shown in Fig. 4.11 were mannitol fermenters (76.5%) which are considered pathogenic. Only 2 representing (3.9%) of the patients' samples showed the growth of non-mannitol fermenters which are considered as non-pathogenic *Staphylococci* spp. Pathogenic *staphylococcus* spp can thus worsen the condition of the ulcers and delay the healing process of the lesion

The patients' samples were also cultured on MacConkey agar to detect the presence of gram negative bacteria and enterococci. Majority of the bacteria identified from the plates were lactose fermenters. Few were non-lactose fermenters. Research shows that both gram negative lactose and non-lactose fermenter such as *P. aeruginosa* and *E.coli* usually cause secondary bacterial infection especially in difficult-to-heal wounds such as ulcers (Sienkiewicz *et al.*, 2014).

A new method to identify bacteria based on color histogram and SPA LDA to classify bacterial has been proposed. This result is extremely positive from the viewpoint of routine clinical analyses, because it avoids bacterial identification based on phenotypic identification of the causative organism using gram staining, culture, and biochemical proof (de Almeida *et al.*, 2014).

Identification of yeast cells as shown in Fig 4.15 in the crude patients' samples and after culturing in Sabouraud dextrose agar was no surprise. Fungal elements have been isolated from a number of diabetic foot ulcers. The most species identified were *Candida*, *Cryptococcus*, *Trichosporon* and *Rhodotorula spp*s (Missoni *et al.*, 2006). These BU patients may have gotten these secondary fungal infections from anywhere particularly from farms, around homes and even at the place of treatment such as the hospitals or health care centers. *Candida* species have been recognized as the fourth most common cause of nosocomial (hospital-acquired) infections (Wisplinghoff *et al.*, 2004), and advanced medical procedures such as the use of catheters, neonatal intensive care, major surgery are predisposing factors to disseminated *Candida* infections. Most of the fungal secondary infections are pathogenic and opportunistic pathogens which may delay treatment of BU disease. Fungi also contribute to several other notable diseases, including disfiguring chronic subcutaneous infections as noted in some BU patients (Brown *et al.*, 2012).

Bacterial DNA was detected in all the 51 samples after PCR using primers that targets a portion of the 16s rDNA with 350bp as seen in Fig, 4.18. This shows that bacteria were present in all lesions of the patients suspected to be BU positive. PCR for 16S mycobacterium (noncoding promoter region of the 16S rRNA) revealed positivity in 48 patients reducing the number of positive cases. However nested PCR based on insertion sequence *IS2404* reduced the positive samples to 40. Thus, 40 patients were confirmed as BU positive. The *IS2404* discriminated mycolactone producing mycobacteria (MPM) from Nontuberculous Mycobacteria (NTM) and hence positivity was lowered from 48 to 40 as compared to the 16S shown in Fig. 4.17. The decrease in *IS2404*-PCR positive

samples which were previously positive for 16S rRNA suggested that the remaining were non-MPM species. *IS2404* has been reported in only MPMs but not all NTMs (Yip *et al.*, 2007). The use of the 59 noncoding promoter region of the 16S rRNA gene for the identification and differentiation of mycobacterial species was recommended because according to Chakravorty *et al.* (2007) it offers several advantages over the target sequences used thus far. This is because the 16S promoter region is more polymorphic than the entire 16S rDNA coding region or portion of the *hsp65* gene that has been used for mycobacterium detection, resulting in an increased discriminatory power (Chakravorty *et al.*, 2007). A second unrelated insertion sequence *IS2606* has been reported to be used for detection of *M. ulcerans* in both human and environmental samples (Rondini *et al.*, 2007). *IS2404* is now understood to be not specific for *M. ulcerans* because this insertion sequence has been found in a number of other aquatic mycobacterial species, including *M. marinum* (Yip *et al.*, 2007). Thus the recommended PCR is a nested PCR of a DNA repeat sequence of the *M. ulcerans* genome, *IS2404*. However, another mycobacterium, *M. liflandii* has been isolated from frogs that mimic *M. ulcerans*. This mycobacteria has been found to test positive for nested PCR *IS2404* and appears to be able to produce mycolactone (Stienstra *et al.*, 2003; Trott *et al.*, 2004). This brought about the need for sequencing for accurate confirmation.

This exploratory study suggests that combining molecular and culturing methods provides a more complete characterization of the microbial diversity of chronic wounds, and can thereby expand our understanding of how microbiology impacts chronic wound pathology and healing.

Sequencing of the *IS2404* region identified all the amplicons as *M. ulcerans*. For sequencing of 16S rRNA for universal bacteria, majority of the bacteria identified were *Staphylococcus spp* (*aureus*, *saprophyticus*, MRSA and *lentus*), *Bacillus spp* (*anthracis*, *cereus*, *infantis* and *aquimaris*), *Alcaligene spp* (*aquatis* and *faecalis*) and *Pseudomonas spp* (*aeruginosa*, *stutzeri* and *koreensis*). Other bacteria identified include *Aeromonas veronii*, *Enterobacter lignolyticus*, *Proteus mirabilis*, *Providencia stuartii*, *xenorhabdus ishibashi* and *Stenotrophomonas rhizophilia* as seen in Fig 4.22. *S. aureus* and *Alcaligene spp*s are usually identified as secondary infections in wounds. A case report of patients with cystic fibrosis indicated that *Pseudomonas spp*, *Staphylococcus spp*s and *Alcaligene spp*s were identified as nosocomial secondary infection among many of the patients with the disease (Saiman *et al.*, 2001). It exists in soil and water and has been isolated from various clinical specimens such as urine, feces, and blood. Infections due to *Alcaligenes faecalis* are opportunistic and are acquired from moist items such as nebulizers, respirators, and lavage fluid. *Alcaligenes* have also been recognized as an emerging infectious gram-negative bacterial species that can affect immunosuppressed patients (Aisenberg *et al.*, 2004). *M. ulcerans* produce mycolactone which is an immunosuppressant. This may explain why these species were found in abundant in the lesions of BU patients. Most of these bacteria that colonize and proliferate on wounds exist as biofilms. This makes them more resistant to certain classes of antibiotics (James *et al.*, 2008). *Staphylococci spp* and *Staphylococcus aureus* in particular have been implicated in delayed wound healing, although a clear correlation between the presence of *S. aureus* and wound infection has not been reported (Bowler *et al.*, 2001; Fazli *et al.*, 2009). Many *Bacillus spp* especially *anthracis* strain K3 were also identified after

sequencing. The genus *Bacillus* includes aerobic bacilli forming heat-resistant spores. *B. anthracis* are the only non-motile and the most pathogenic bacilli in this genus (Jayachandran, 2002). Also, in endemic areas in which humans and livestock interact, chronic cases of cutaneous anthrax are commonly reported. This suggests that spores of *B. anthracis* exist in the environment and are part of the ecology associated with its lifecycle (Lee *et al.*, 2007). *B. cereus* infections of human and domestic animals have also received recognition. An increasing number of infections from wounds (Kotiranta *et al.*, 2000) and insect bites (Fedhila *et al.*, 2010) have been reported. It has been noticed mainly in immunosuppressed tissues of wounds (Bottone, 2010). It is therefore not surprising to be noted as a secondary infection in BU.

Interestingly, 60% (9 out of 15) of the sequencing result for mycobacterial (PA) detected the presence of *Corynebacterium spp* (*aurimucosum*, *diphtheria* and *striatum*). Other bacteria identified were *M. ulcerans*, *Brevibacterium iodinum* and *Rhodococcus erythropolis*. *Corynebacterium*, *Brevibacterium iodinum* and *Rhodococcus erythropolis* are from the order Actinomycetales. Actinomycetales have been reported to be phylogenetically related to *Mycobacterium* genus. Due to this, *Corynebacterium* is sometimes used in the study of mycobacterium (Harper *et al.*, 2008). This could explain why many *Corynebacterium* were noticed after sequencing with MSHA/PA primers. Unlike mycobacteria, *Corynebacteria* are non-acid fast bacteria that are sometimes able to grow on Middlebrook7H9 medium. They are soil dwelling microorganisms that are usually infectious and pathogenic. Others like *C. ulcerans* are able to manifest infections on the skin that closely resembles that of *M. ulcerans*. Studies have confirmed that slow healing wounds are usually colonized by *Corynebacterium*, (Werdin *et al.*, 2009).

Majority of the bacteria identified were pathogenic and opportunistic bacteria that live in muddy areas and in dirty water bodies. Almost all the bacteria are soil dwellers that easy have access to the BU lesion during activities like playing, farming or mining. Few are normal body flora that may delay healing of the BU. The pains now being experienced by BU patients could be the result of these bacteria colonizing and proliferating on the ulcer or lesions.

WHO recommends a combination drug of rifampicin and streptomycin for 8 weeks (W.H.O Initiative, 2004). On the contrary, recommended drugs for secondary infections of BU patients have not yet been developed. Clarithromycin, kanamycin, hygromycin B, streptomycin, amikacin and rifampicin are drugs commonly used in the treatment of BU. These drugs were tested against 7 bacteria (*B. anthracis*, *B. infantis*, *B. cereus*, *B. aquamaris*, *S. aureus*, *S. species M7* and MRSA) that were identified as secondary infections. All the bacteria used for this study were susceptible to clarithromycin, hygromycin B and amikacin (100%) as seen in table 4.1. There was a susceptibility of 62.5% in rifampicin, 37.5% in kanamycin and 12.5% in streptomycin. Kanamycin and streptomycin had intermediaries of 37.5% and 62.5%. Nevertheless, resistance of some bacteria to streptomycin and rifampicin were noticed in 25% and 37.5% of the bacilli cereus group of bacteria respectively. Since these two drugs are often used in the treatment of BU, it may likely be that the secondary bacteria are developing resistance to the drugs. Generally, there were less zones of inhibition (mm) among the *bacilli* species compared to *staphylococcus* species. Resistance to streptomycin and rifampicin were more noticed in the *bacilli* species than the *staphylococcus* species. These may be due to the fact that the bacilli group of cereus bacteria form spores in harsh conditions leading to

their resistance to the drugs. It may also be due to horizontal gene transfer of antibiotic resistance between the bacteria found on the lesions of BU patients. Even though, drug resistance is a major cause for concern in methicillin-resistant *S. aureus* (MRSA) infections, (Guerin *et al.*, 2000; Liu *et al.*, 2011), MRSA were either susceptible or showed intermediate resistance for the selected drugs in this study. *M. ulcerans* has been found to be sensitive, not just to streptomycin and rifampicin but clarithromycin as well (Johnson *et al.*, 1999). Since all the bacteria were susceptible to clarithromycin, it may be recommended as a treatment for the secondary bacterial infections. A similar finding noted that eight weeks of treatment with rifampin-streptomycin sterilized *M. ulcerans* infection in mice. The bactericidal activity against *M. ulcerans* of the combination rifampin-moxifloxacin, rifampin-clarithromycin, or moxifloxacin-clarithromycin was found to be similar to that of rifampin-streptomycin. These combinations could thus be considered as alternative, orally administered combined regimens for treatment of BU in humans (Ji *et al.*, 2007).

In all, amikacin had the highest zone of inhibition. Amikacin, kanamycin and streptomycin are aminoglycosides used for the treatment of gram negative and anaerobic gram positive bacteria. Even though the bacilli species identified are gram positive, they can live in anaerobic environments. Drug treatment combination involving aminoglycosides have also been found to be more effective than those without (Dega *et al.*, 2000). This may account for the effectiveness of amikacin against the selected bacteria.

Most of the BU patients may have gotten these secondary infections through normal house and work activities. Death due to infection by *M. ulcerans* is rare, although

secondary bacterial infection may aggravate extensive areas of ulceration (Johnson *et al.*, 1999).

The type of secondary microbial infection on a BU patient may depend on several factors such as geographical area of the patient, personal hygiene, type of treatment among others. In a recent study, majority of the bacterial infection in BU patients were *S. aureus* and *P. aeruginosa* (Yeboah-Manu *et al.*, 2013). This is in line with our present study. Other bacteria such as *B. anthracis*, *Alcaligene spp* and *Corynebacterium spp* were also dominant.

The phylogenetic tree shows that majority of the bacteria identified on the BU lesions are gram positive and negative rods compared to the cocci. This may be due to the type of media used for the selection. LB, MacConkey, Mannitol salt and BP agar were the only media used for culturing the bacteria. There may also have been bias selection in the identification of unique colonies for DNA extraction and sequencing. The 16S primers used for amplifying DNA of the bacteria are considered as universal but it may have biasedly amplified certain bacteria species that were abundant in the crude patients' samples. The 3' end of the 16S rRNA was selected because it detects the hybrid region of the Shine-Dalgarno sequence which is located 8 bases upstream of the start codon of bacteria. For phylogenetic diversity, there were high frequencies of Proteobacteria among the bacteria that colonize the lesions. There were Beta Proteobacteria (*A. faecalis*, *A. aquitilis*) and Gamma Proteobacteria (*P. stutzeri*, *P. aeruginosa*). One Enterobacteriaceae was identified as *P. mirabilis*. Under the class Coccus, *S. saprophyticus*, *S. lentus*, *S. aureus*, MRSA, were identified. The bacillus cereus group of bacteria and *B. aquamaris*

were identified under the order Bacillales but *B. aquimaris* has very little phylogenetic ancestry with the other secondary bacteria identified as shown in Fig 4.22.

In Fig 4.23, the phylogenetic tree was constructed from 16S rRNA hyper-variable region for mycobacteria. However it sequenced some other classes of bacteria from the sub order Corynebacterineae. These bacteria as well as mycobacteria are in the class Actinobacteria with suborder Corynebacterineae; *C. diphtheria*, *C. striatum*, *C. aurimucosum*, *R. erythropolis* and *M. ulcerans*. Even though *Corynebacterium spp* do not cause BU, some like the *C. ulcerans* are able to give similar skin infections like the *M. ulcerans*. The phylogenetic tree shows the relationship between the bacteria.

The response of patients to the disease may also lead to secondary microbial infections on the lesions. Most of these patients reported very late for treatment. Some even compound it by using herbal medications, which are additional source of secondary microbial infections (Agbenorku *et al.*, 2012). Although risk factors for bacterial wound colonization have not been thoroughly studied to date, delayed treatment and insufficient wound management might contribute to colonization and prolonged wound healing (Amissah *et al.*, 2015). There is thus the need to seek medical help as soon as possible when signs of BU are noticed. Since many of the bacteria identified are soil dwellers, BU patients should be educated about the importance of personal hygiene to prevent the development of these secondary bacterial infections that can delay healing and probably reduce the development of pain experienced by some BU patients. Home dressing and traditional healing should be discouraged if possible.

5.1 CONCLUSION

Secondary microbial infections among BU patients are common. Most of these microbial infections are bacteria. The common bacteria identified are *Corynebacterium spp*, *Staphylococcus spp* (*aureus*, *saprophyticus*, MRSA and *lentus*), *Bacillus cereus* group (*anthracis*, *cereus*, *infantis* and *aquimaris*), *Alcaligene spp* (*aquatilis* and *faecalis*) and *Pseudomonas spp* (*aeruginosa*, *stutzeri* and *koreensis*). Fungal elements were also identified on BU lesions. All the bacteria tested were susceptible to amikacin and clarithromycin. These microbial infections may delay healing, increase resistance to antibiotic administered to BU patients and may cause the pains experienced by some BU patients.

5.2 RECOMMENDATION

- Further studies are required in a larger population of Ghana to confirm if the type of secondary infection incurred is geographically related.
- Larger sample size that can divide the secondary infection into self-medication, traditional and nosocomial can be carried out.
- Immunological changes that result in the pain experienced by some BU patients in relation to the secondary microbial infection can be researched on.
- Sequencing of fungi that causes secondary infection can be researched on.

5.3 LIMITATIONS

- Limited logistics. Eg. Financial constraints.
- Unwillingness of participants to participate unless compensated.

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APPENDICES

Appendix A

CONSENT FORM

PARENTAL CONSENT FORM TEMPLATE

Title: Genotyping and treatment of secondary infections in Buruli ulcer patients in the Amansie Central District.

Principal Investigator: Elizabeth Gyamfi

Address:

P.O.Box MS 539

Mileseven, Achimota

Accra

General Information about Research

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

Purpose of the research: Buruli ulcer is the second most common mycobacterial infection in Ghana. It is caused by *Mycobacterial ulcerans*. Effort to combat this disease has been a challenge due to the presence of mycolactone in the bacteria that makes the ulcer a painless one. Unfortunately, these microbes are becoming more and more resistance to the drugs that are being administered. Pains in the ulcers as well as resistance to

antibiotics has drawn the attention of possible secondary infection of the disease. This study aims at culturing and identifying microorganisms from tissues, biopsies and swabs from BU confirmed ulcerative lesions, to determine the effect of antibiotics on these microorganisms, and finally to elucidate any genetic variations within isolated species causing secondary infections in BU.

Procedure: Samples will be taken from patients with suspicious lesions and ulcers by trained personnel. The samples will be stored appropriately and transported for further processing. All samples will undergo case diagnosis and confirmation.

Possible Risks and Discomforts

This is no major risk associated with your participation in this study apart from the slight discomfort you may get from parts of the wound being swabbed. Samples will be taken at least three times.

Possible Benefits

Treatment of Buruli ulcer is free of charge in Hospitals. Even though there will be no direct benefit for subjects' participating in this study, patients diagnosed with secondary infection will be counseled on the treatment options accordingly. However, the main benefit of your participation is indirect as you will help us identify secondary infections in Buruli ulcer and their treatment in Ghana. At the end of the study, you will be contributing significantly in preventing the horrible effects of the disease in your district.

Confidentiality

Your records will be kept in a safe. All information collected during the study will be stored in a file which will not have your name on it, but a study number assigned to it.

Only the research team will have access to the names associated with the study numbers and for special reasons such as treatments. It is likely that data obtained from tests done on you may be published in medical journals; however, your identity will not be disclosed.

Voluntary Participation and Right to Leave the Research

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

Notification of Significant New Findings

Any new finding that would be of particular help to you will be relayed to you either personal or through the district hospital.

Contacts for Additional Information

Any question related to the study can be asked now or later. For any further information, you may contact any of the following:

Dr. Lydia Mosi

NMIMR, P.O.Box LG 581, Legon

Tel: +233 54 089 0352

Email: lmosi@noguchi.mimcom.org; lmosi@hotmail.com

Elizabeth Gyamfi (UGMS, MPhil STUDENT)

University of Ghana Medical School

Department of Medical Biochemistry

Tel: +233204803475

Email: lizgymf@gmail.com

VOLUNTEER AGREEMENT

The above document describing the benefits, risks and procedures for the research (Genotyping and Treatment of Secondary Bacterial Infection in the Amansie Central District) has been read and explained to me. I have been given an opportunity to have any questions about the research answered to my satisfaction. I agree that my child should participate as a volunteer.

Name and signature of parent/guardian

Date

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

I was present while the benefits, risks and procedures were read to the child's parent or guardian. All questions were answered and the child's parent has agreed that his or her child should take part in the research.

Name and signature of witness

Date

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

Name Signature of Person Who Obtained

Date

CHILD ASSENT FORM

Introduction

My name is Elizabeth Gyamfi and I am from the department of Medical Biochemistry at University of Ghana Medical School. I am conducting a research study entitled Detection and Treatment of Secondary Infections in Buruli ulcer Patients in the Amansie Central District. I am asking you to take part in this research study because I am trying to learn more about microorganisms as well as related isolates which have the potential of causing secondary infections in Buruli ulcer lesions and treatment. This will take 5 minutes.

General Information

If you agree to be in this study, you will be asked to answer some few questions about the infection as well as allow us to take a photograph and at most 3 swabs of the lesion.

Possible Benefits

Your participation in this study will result in free treatment and counseling at the district hospital. You would also help us identify secondary infections of the disease and how to treat this disease as a whole.

Possible Risks and Discomforts

However, the risks associated are slight discomfort you may get from parts of the wound being swabbed. Samples may be taken at most three times.

Voluntary Participation and Right to Leave the Research

You can stop participating at any time if you feel uncomfortable. No one will be angry with you if you do not want to participate.

Confidentiality

Your information will be kept confidential. No one will be able to know how you responded to the questions and your information will be anonymous.

Contacts for Additional Information

You may ask me any questions about this study. You can call me at any time 0204803475 or talk to me the next time you see me.

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

Your rights as a Participant

This research has been reviewed and approved by the Ethical and Protocol Review Committee of University of Ghana Medical School.

VOLUNTARY AGREEMENT

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

This assent form which describes the benefits, risks and procedures for the research titled Genotyping and Treatment of Secondary Infection in Buruli Ulcer Patients in the Amansie Central District has been read and or explained to me. I have been given an opportunity to have any questions about the research answered to my satisfaction. I agree to participate.

Child's Name:.....

Researcher's Name:.....

Date:

Child's Mark/Thumbprint.....

Researcher's Signature:.....

Date:

Appendix B

SOLUTION PREPARATION, SOURCE OF REAGENTS AND EQUIPMENT

C 1.1 1X TAE buffer

To prepare the working solution of 1X TAE buffer, 20 ml of the 50X TAE buffer (Thermo Scientific) was measured and diluted with distilled water to a total volume of 1 L. The pH of this solution was 8.0

C 1.2 Agarose Gel (1.5%)

To prepare 1.5% agarose solution, 1.5 g of agarose was weighed and dissolved in 100 ml 1X TAE buffer.

C 1.3 Ethidium bromide solution (10 mg/ ml)

To make 10 mg/ ml ethidium bromide solution, 0.1 g ethidium bromide tablet was weighed and dissolved in 10 ml 1X TAE buffer

C 1.4 PCR MIX FOR BACTERIA, MYCOBACTERIA AND M. ULCERANS

Reagent	1X
PCR buffer	5
10 μ M dNTPs	0.5
2.5 μ M Forward primers	0.5
2.5 μ M Reverse primers	0.5
Nuclease free water	15.87
5U/ μ l Polymerase	0.13

DNA Template	2.5
TOTAL	25μl

C 1.5 Concentrations of Antibiotic Disk prepared

Antibiotic	Disk Concentration (μ g/ μ l)
Rifampicin	5
Kanamycin	30
Amikacin	30
Streptomycin	10
Hygromycin B	50
Clarithromycin	15

C 2.0 Source of Reagents and materials used

1. Qiagen Dneasy blood and tissue kit (QIAGEN, Venlo, Netherlands)
2. 5X PCR buffer (1nM MgCl₂), dNTPs (Inqaba biotech, Pretoria, South Africa),
3. Primers, 1U GoTaq Polymerase (Thermo Scientific, Waltham, U.S.A)
4. Vortexer (Mini vortexer, VMR Scientific Products, Atlanta, U.S.A)
5. Glass beads (Sigma-Aldrich, Darmstadt, Germany)
6. Thermal Cycler (Applied Biosystems, California, U.S.A)
7. Gram Stain kit (BD Gram Stain Kit, New Jersey, U.S.A)
8. Glass slide (frosted slide glass, Thermo Scientific, Waltham, U.S.A.)
9. Light microscope (Leica, Wetzlar, Germany)
10. Inverted microscope (ZEISS Promovert, Jena, Germany)
11. Camera (Sony, Tokyo, Japan).
12. 24 well cell culture plates (Nunc[™] New York, USA)

13. Luria Bertani (LB) agar (Oxoid, Hampshire, United kingdom)
14. MacConkey agar (Accumin[™], New York, USA)
15. Mannitol salt agar (BD, New Jersey, USA)
16. Sabouraud dextrose agar with chloramphenicol (Oxoid, Hampshire, United Kingdom)
17. Incubator (Fisher scientific, Pittsburgh, USA)
18. Agarose (AGTC Bioproducts Ltd, UK)
19. 100bp DNA ladder (Promega, Madison WI, USA)
20. Electrophoresis set up
21. Ethidium bromide (Promega, Madison WI, USA)
22. Benchtop 2UV Transilluminator gel photography system (Upland, CA, USA)
23. Centrifuge (Eppendorf, Hamburg, Germany)
24. Eppendorf Mastercycler personal PCR Machine (Eppendorf, Hamburg, Germany)
25. Nuclease free water (Hyclone Lab Inc., South Logan, Utah, USA)
26. Antibiotic disks (Mast Group Limited, Bootle, United Kingdom).