KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY

COLLEGE OF HEALTH SCIENCES

SCHOOL OF MEDICAL SCIENCES

DETECTION OF VIABILITY MARKERS OF *MYCOBACTERIUM ULCERANS* AND THEIR ASSOCIATION WITH HEALING IN BURULI ULCER PATIENTS

A THESIS SUBMITTED IN FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF PHILOSOPHY, IMMUNOLOGY

IN THE

DEPARTMENT OF MOLECULAR MEDICINE

BY:

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NOVEMBER, 2016

DECLARATION

I carried out the experiment work described in this thesis at the department of Molecular Medicine, KNUST. This work has not been submitted for any other degree.

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DEDICATION

This thesis is dedicated to these wonderful people in my life: Charles Sarpong Duah, Jesse - Manuel .K. Duah, Karl - Melvin. K. Duah and all friends who supported and encouraged me throughout the study.

ACKNOWLEDGEMENT

To God be the Glory for the great things he has done. I very much appreciate the kind patience and guidance of my academic supervisor, Professor Mrs Margaret Frempong, Department of the Molecular Medicine, KNUST.

To my second supervisor, my Principal investigator (PI), Mentor and a father, Dr. Richard Odame Phillips, I say God bless you. Without you this work would not have been possible, I am deeply grateful.

I am also grateful to Dr. Mark Wansbrough-Jones and the people that made the work possible at the ST. Georges Hospital, London, Dr. Fred Stephen Sarfo at KATH for the immerse contribution in this thesis. I appreciate the support of my lecturers at the Molecular medicine department, School of medical Sciences, KNUST.

I am very grateful to the Buruli ulcer team at Agogo Presbyterian Hospital, Tepa Government Hospital, Dunkwa Government Hospital and Nkawie Toase District Hospital for their help during my sample collection and follow up of the patients.

Buruli ulcer Team at the Kumasi centre for Collaborative Research in Tropical Medicine (KCCR): Dr. Michael Frimpong, Leticia Kunaa, Aloysious Loglo, Bernadette Agbovo, Francisca Naana Sarpong and Nancy Ackon, you guys are fabulous. The laboratory work could not have been completed without your help, God bless you. Thank you to all the staff of KCCR for their support in one way or the other.

This study was funded by the Medical Research Council and I am so grateful for this.

Charles Sarpong Duah (My Husband), your immerse support and dedication towards this thesis could not have gone unnoticed, you are an inspiration and a real pillar to the success of this work. God bless you.

ABSTRACT

Background: Buruli ulcer (BU) is a neglected tropical disease caused by *Mycobacterium ulcerans* (*M. ulcerans*), an infection common in rural parts of West Africa including Ghana. It affects predominantly children between the ages of 5-15 years. Treatment of BU has changed over the past 11 years with the introduction of antibiotics (rifampicin and streptomycin) as an alternative to surgery. The aim of antibiotic treatment is that each patient receives the minimum appropriate therapy needed to achieve recurrent free cure. The current duration of antibiotic therapy (8 weeks) was based on observations in patients with early *M. ulcerans* which were excised after treatment. Thus it is likely that a shorter course of antibiotic treatment may be successful in some patients.

Aim of study: To characterise the viability of *M. ulcerans* in BU and to ascertain its association with time to/ rate of healing of lesions.

Methodology: Fine needle aspirates and swabs were obtained from patients confirmed with active BU using IS2404 PCR as a gold standard. Samples were obtained at baseline (week 0), during treatment (week 4), end of treatment (week8) and after treatment (weeks 12 and 16) for detection of viable *M. ulcerans* using combined assay 16S rRNA/IS2404 RT qPCR and culture. Patients were followed up 2 weekly with wound measurements using Silhouette required for determination of rate of healing.

Results: Of one hundred and twenty-nine patients, viable *M ulcerans* could be detected in 65% at baseline. By week 4, 20 (15.5%) of lesions had healed or 29 (22%) had undetectable viable organisms. At week 8 viable *M. ulcerans* were still detected in 43 (33%) lesions of unhealed, 15 (12%) of unhealed at week 12 and 3 (2%) of unhealed at week 16. Patients with detectable viable organisms after antibiotic treatment had significantly higher bacterial load, longer

healing time and lower healing rate at week 4 compared with those with undetectable viable organisms at baseline or by week 4.

Conclusions: We demonstrated that current antibiotic therapy for BU disease is highly successful in most patients but it may be possible to abbreviate the treatment to 4 weeks in patients with a low initial bacterial load. On the other hand, evidence has been presented that persistent infection with viable *M. ulcerans* contributes to slow healing in other patients, suggesting that those with a high bacterial load, may need antibiotics for longer than 8 weeks.

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LIST OF ABBREVIATIONS

A	adenine
AFB	acid fast bacilli
BU	Buruli ulcer
BUD	Buruli ulcer disease
BUFLS	Buruli ulcer functional limitation score
C	cytosine
DRB	dry reagent based FNA
fine needle aspirate fTLC	fluorescent thin
layer chromatography	
G	guanine
GBUI	global Buruli ulcer initiative
IS	insertion sequence
LAMP	loop mediated isothermal amplification
LJ	Lowenstein Jensen mRNA
messenger RNA	

M. ulcerans	Mycobacterium ulcerans qPCR
quantitative PCR	
ROH	rate of healing
RT	reverse transcriptase
Th	T helper tRNA
transfer RNA	
U	Uracil
WASp	Wiskott-Aldrich syndrome protein
WHO	World health organisation
ZN	Ziehl Neelsen
16S rRNA	16subunit ribosomal ribonucleic aci

CHAPTER 1

INTRODUCTION

1.0 Background

Buruli ulcer (BU) is classified as one of the neglected tropical diseases caused by *Mycobacterium ulcerans* (*M. ulcerans*), is an infection common in rural parts of West Africa including Ghana, Asia and Australia (Wansbrough-Jones and Phillips 2006). It causes large, damaging skin ulcers mainly in children aged 5 to 15 years although any age can be affected (Amofah et al. 2002). The initial lesion is a subcutaneous painless nodule tethered to the skin, an intradermal plaque or an oedema. These may enlarge over a period of days to weeks and ulcerate in the centre. Ulcers are painless and have a necrotic base and irregular, undermined edges. There is surrounding oedema in about 10% of cases. Ulcers enlarge progressively and may cover the whole of a limb or the trunk if left untreated but the patient remains systemically well unless secondary bacterial infection occurs. The mode of transmission remains unknown but there have been major advances in understanding the mechanism of disease since the establishment of the World health organisation (WHO) Buruli ulcer initiative in 1998 together with improved diagnosis and management (WHO 2016a).

Treatment of Buruli ulcer has changed considerably over the past 11 years with the introduction of antibiotics as an alternative to surgery. It has now been established that the combination of rifampicin and streptomycin daily for 8 weeks is effective in healing all forms of lesions caused by *M. ulcerans* disease and this has reduced the recurrence rate from 6-47% after surgery to 0-2% after antibiotic treatment (Chauty et al. 2007; Sarfo et al. 2010a). This treatment can be administered by village health workers and admission to hospital is rarely necessary except for cases requiring skin grafting. The current duration of antibiotic therapy (8 weeks) was based on observations in patients with early *M. ulcerans* lesions which were excised after treatment

for 2, 4, 8 or 12 weeks. All lesions remained culture positive after 2 weeks but thereafter all were culture negative (Etuaful et al. 2005). Thus it is likely that a shorter course of treatment may be successful in some patients and this shorter duration is highly desirable. This is supported by recent experience of treating *M. ulcerans* disease in Australia with antibiotic durations of less than 8 weeks suggesting that successful outcomes may be achieved in selected patients (Cowan et al. 2015). In spite of the success of rifampicin and streptomycin for 8 weeks some lesions take several months to heal. Available data from various studies similarly propose that healing of up to two thirds of patients occurs within about 25 weeks after onset of treatment (Nienhuis et al. 2010; Phillips et al. 2013; Vincent et al. 2014).

The reason for slow healing may partly be due to presence of viable organisms. A recent study has indicated that some Buruli ulcer patients treated with streptomycin and rifampicin for 8 weeks with full adherence, had persisting infection with *M. ulcerans* in some lesions at week 6 and even 4 weeks after completion of antibiotics (Sarfo et al. 2014). Mycolactone was detected sometimes in culture negative and frequently as well in culture positive samples, suggesting mycolactone may remain in tissue for some period after the demise of *M. ulcerans* or may be associated with the presence of viable organisms in some instances (Sarfo et al. 2014). It is vital to establish how often infection persists after a standard course of antibiotic treatment for Buruli ulcer.

Markers of presence of viable bacteria are based on cultures on Löwenstein Jensen medium at 32^0C which has a sensitivity of up to 60 per cent when done in a laboratory near the endemic area but it can take 6 weeks or more for a result to be obtained (Phillips et al. 2005; Portaels et al. 1996). Reverse transcriptase assays targeting 16S rRNA and mRNA were used successfully for the detection of viable mycobacteria in clinical samples from patients with tuberculosis, leprosy and recently Buruli ulcer (Desjardin et al. 1999; Martinez et al. 2009; Beissner et al. 2012) and as a surrogate for response to chemotherapy in tuberculosis (Desjardin et al. 1999).

With respect to Buruli ulcer, the assay is fast, 100% specific for *M. ulcerans* and highly sensitive with an analytical sensitivity of 6 templates. The excellent performance on clinical samples makes this tool highly promising for monitoring the therapeutic response with the goal of optimizing the duration of antimycobacterial treatment (Beissner et al. 2012). However, the healing progress of *M. ulcerans* disease using 16S rRNA as a viability marker has not yet been explored. Therefore, the main aim of the present study was to determine how soon *M. ulcerans* is killed during antibiotic treatment using a novel and sensitive 16s rRNA assay combined with qPCR for IS2404 to detect viable *M. ulcerans*.

1.1 Rationale for the study

The aim of antibiotic treatment is that each individual receives the minimum appropriate therapy needed to achieve recurrence free cure. The current duration of antibiotic therapy (8 weeks) was based on observations in patients with early Mu lesions which were excised after treatment for 2, 4, 8 or 12 weeks. All lesions remained culture positive after 2 weeks but thereafter all were culture negative (Etuaful et al. 2005). The choice of taking antibiotic treatment daily for 8 weeks was thought to be the safest for patient‟s management although lesions that received more than 4 weeks‟ treatment were all culture negative. Subsequent studies have shown that some lesions heal early whilst others heal late irrespective of their size suggesting that some may require a shorter treatment and perhaps some require longer treatment. In these studies, it was discovered that the time for healing for nodules ranged from 2 to 20 weeks, plaques from 4 to 24 weeks and ulcers from 2 to 39 weeks (Sarfo et al. 2010). In more recent studies the time for lesions to heal ranged from 4 to 36 weeks (Phillips et al. 2013).

Thus it is likely that a shorter course of treatment may be successful in some patients and this shorter duration would be highly desirable. The problem therefore is how to determine those who will require shorter treatment. Additionally, some lesions take a longer time to heal and this may be as a result of persisting viable organisms in the lesion which continue to produce mycolactone at low concentration which would inhibit production of growth factors important for wound healing (Pahlevan et al. 1999; Coutanceau et al. 2007; Torrado et al. 2007; Sarfo et al. 2010b). Therefore, it will be crucial to find out at what point the lesion become sterile in order to stop antibiotic treatment.

In a study to detect the viability of *Mycobacterium tuberculosis*, reverse transcriptase (RT) qPCR targeting 16S rRNA and mRNA were employed successfully to be good indicators for microbial viability and rapid markers for rapid assessment of response to Chemotherapy(Desjardin et al. 1999). Also RT PCR assays targeting 16S rRNA and mRNA were applied successfully for the rapid detection of viable mycobacteria in clinical samples from patients with *Mycobacterium leprae* (Martinez et al. 2009).

Similar studies have recently been undertaken by Beissner et al, (2012), in seven patients with BU disease confirmed by PCR for IS2404, whose wounds were not healed after completion of their 8 weeks‟ antibiotic treatment. Swab samples were taken for combined 16S rRNA and RT qPCR analysis. All the seven patients came out positive for IS2404 qPCR and negative for 16S rRNA. This result probably indicates that all viable bacteria in the lesion were dead after the 8 weeks antibiotic treatment proposing that 16S rRNA could be a marker for viability of *M. ulcerans*. Their results also revealed that it was positive and specific for all 29 *M. ulcerans* cultures available for the study. To further confirm the specificity of their assay, DNA extracts from closely related mycobacterial species and bacteria potentially contaminating the human skin were subjected to combined 16S rRNA RT/IS2404 qPCR viability assay and out of the 24 different species only 2 were positive for 16S rRNA (*M. ulcerans* and *M. marinum*) and 1 positive for IS2404 qPCR (*M. ulcerans*). Therefore, this combined assay could be a sensitive

and specific tool for detecting viable Mu organisms from clinical samples and culture suspensions.

The proposed study is intended to document the clearance of *M. ulcerans* during and after antibiotic treatment.

1.2 Main hypothesis

- ❖ Healing of Buruli ulcer lesions is dependent on the clearance of viable *M. ulcerans.*

1.3 Research questions

The study aims at answering the following research questions:

1. What is the sensitivity of 16S rRNA RT qPCR assay using culture as a gold standard?
2. How long does it take for a BU lesion to become sterile after commencement of antibiotics?
3. Can the quantity of bacteria load at baseline predict the presence or absence of viable *M. ulcerans*?
4. Is there a relationship between detection of viable *M. ulcerans* and healing outcome?
5. Is there a relationship between rate of healing (ROH) at week 4 and the presence of viable *M. ulcerans*?

1.4 Aim

To characterise the viability of *Mycobacterium ulcerans* in Buruli ulcer and to ascertain its association with time to / rate of healing of lesions

1.5 Objectives

- To optimize the 16S rRNA /IS2404 RT qPCR assay being employed in the study
- To determine the sensitivity of 16S rRNA RT qPCR viability assay using culture as a gold standard.
- To determine the proportion of patients with viable *M. ulcerans* and the time of disappearance of *M. ulcerans* after commencement of antibiotics.
- To establish if the bacteria load at baseline can be used to predict the presence or absence of viable *M. ulcerans*.
- To establish the relationship between the detection of viable *M. ulcerans* and healing outcome.
- To compare the rate of healing (ROH) at week 4 with the detection of viable *M. ulcerans.*

CHAPTER 2

LITERATURE REVIEW

2.1 History and Geographical distribution of Buruli ulcer disease (BUD)

About 12 decades ago, a British physician working at the Mengo Hospital in Kampala (Uganda) called Sir. Albert Cook described a skin ulcer which occurred in Uganda. The case was however not published in medical literature. In 1920, Kleinschmidt also in Uganda observed ulcers with undermining edges that contained large numbers of acid fast bacilli (Doerr, Ashwort, and Seifert 1995; W. Meyers 1994). In 1948, Peter MacCallum and his colleagues Tolhurst, Backle and Sission from Australia published the first detailed description of the infection in Australian farmers (MacCallum et al. 1948). They established the aetiology of the skin ulcer by first culturing *Mycobacterium ulcerans* (*M. ulcerans*) from leg ulcer in a child from Bairnsdale, Victoria in Australia which made them refer to the infection as "Bairnsdale ulcer". This name was after the main town in the original endemic region. The people living in the southeastern Australia still refer to the disease as Bairndale ulcer (*Estimating Buruli Ulcer Prevalence in Southwestern Ghana* 2007; Wansbrough-Jones and Phillips 2006).

In 1950, Van Oye and Ballion reported the first patient from Africa precisely Zaire, however in Meyers research in that area, *M. ulcerans* infection was observed in Zaire as far back in 1935 (Meyers et al. 1974). From 1960"s to 1970"s, there was a study into the epidemiology of the disease by Uganda Buruli group and they noted new cases in recent refugees from Rwanda who were gathered in an area close to the Nile. Many more cases were reported in Buruli County (now called Nakasongola District) in Uganda near lake kyoga, Democratic Republic of Congo, Papua New Guinea and other countries (Clancey, Dodge, and Lunn 1962). Buruli ulcer (BU) which is the most common name of the *Mycobacterium ulcerans* infection has also been referred to as Bairnsdale, Searles, Kumusi ulcer, "bile okoro" and the Mysterious disease" depending

on the geographical region where the infection was historically reported (Wansbrough-Jones & Phillips, 2006; "WHO 2015b).

In the 1980"s, BU emerged as a serious health problem, with West Africa being the most affected area and since then incidence and prevalence has been increasing. There have been advances in tackling the spreading of BUD by the World Health Organisation (WHO) after 1980. In December 1997, Dr. Hiroshi Nakagima, the then Director-general of WHO announced in Uganda that "they would take the lead to mobilize the worlds expertise and resources to fight Buruli ulcer as a serious public health problem". WHO again in February 1998, launched the Global Buruli ulcer initiative (GBUI) to coordinate control and research efforts. Through this initiative, they organised the first international conference on Buruli ulcer control and research in July the same year and since then researches from all over the world meet every 2 years in Geneva to share ideas on how to better manage the disease. Further to these initiatives to eradicate BU, in May 2004, the World Health Assembly adopted a resolution on BU which called for increasing surveillance, control and intensified research to develop tools to diagnose, treat and prevent the disease ("Estimating Buruli Ulcer Prevalence in Southwestern Ghana - Thesis.pdf" 2015; Doerr, Ashwort, and Seifert 1995;
WHO 2016b). Just recently in 2014, there were 2251 new cases of BU globally, of which 2151 were from African Region (WHO 2016a)

Currently, BU has been reported in over 33 countries globally including the Americans, Asia, Western Pacific and some countries in the west and central Africa with most of the burden in the tropical and subtropical climates (WHO 2016b) In 2014, cases were identified in 12 countries and most of these countries were in Africa which includes Ghana, Benin,
Cameroon, Côte d"Ivoire and the Democratic Republic of the Congo. Japan and Australia are the major endemic countries outside Africa. Figure 1 shows the global distribution of BUD and Table 1 also shows the distribution of Buruli ulcer cases globally from 2002 through to 2014.

BUD was first identified in Ghana in the Greater Accra region in the 1971. In the same year, more cases were found along the tributaries of the Densu river in the same region (Bayley 1971). In 1986, 96 cases were described in the Asante Akim North District in the Ashanti region by Van der Werf and his group, this was followed by reports from the Amansie West District in the Ashanti region (Bayley 1971). Since then there has been several cases reported from communities in the middle and coastal belt in Ghana. In 1993, Ghana initiated a surveillance system for reporting Buruli ulcer. This initiation resulted in 1,200 cases reported at the end of 1998, although underreporting was suspected because case reports were from remote areas. In 2002, a national case search on BUD led by Amofa, identified 5,619 patients with 6,332 clinical lesions (Amofah et al. 2002) Currently cases are still being detected in focal areas in Ghana but no case has been found in the three Northern regions.

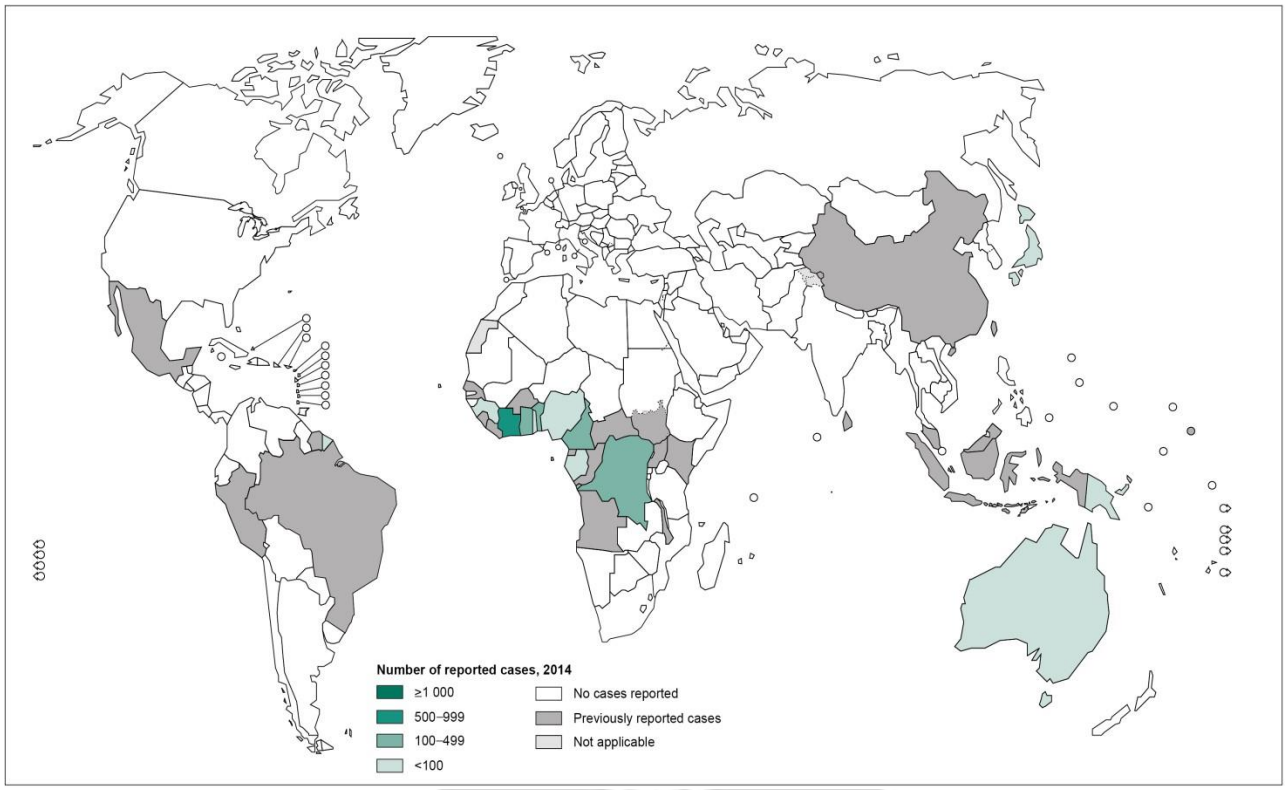


Figure 1 : Distribution of Buruli ulcer disease worldwide in 2014 (WHO, 2015)

Country	Number of new reported cases of Buruli ulcer												
	2014	**2013**	**2012**	**2011**	**2010**	**2009**	**2008**	**2007**	**2006**	**2005**	**2004**	**2003**	**2002**
Australia	89	74	105	143	42	35	40	61	72	47	34	14	32
Benin	330	378	365	492	572	674	897	1203	1195	1045	925	722	565
CÃ´te d'Ivoire	827	1039	1386	1659	2533	2679	2242	2191	1872	1564	1153	768	750
Cameroon	126	133	160	256	287	323	312	230	271	265	914	223	132
Central African Republic	No data	No data	No data	No data	No data	No data	3	No data	No data	No data	No data	No data	No data
Congo	No data	6	38	56	107	147	126	99	370	53	235	180	102
Democratic Republic of the Congo	192	214	284	209	136	172	260	340	74	51	487	119	17
Equatorial Guinea	No data	No data	No data	0	No data	No data	No data	No data	No data	3	No data	No data	No data
Gabon	47	59	45	59	65	41	53	32	54	91	43	No data	No data
Ghana	443	550	632	971	1048	853	986	668	1096	1005	1157	737	853
Guinea	54	96	82	59	24	61	80	No data	279	208	146	157	No data
Japan	7	10	4	10	9	5	2	3	1	1	1	No data	No data
Liberia	No data	8	21	No data	No data	No data	No data	No data	No data	No data	No data	No data	No data
Nigeria	65	23	40	4	7	24	No data	No data	9	No data	No data	No data	No data
Papua New Guinea	3	No data	No data	8	5	8	24	26	No data	No data	31	18	13
Sierra Leone	No data	No data	No data	28	No data	No data	1	No data	No data	No data	No data	No data	No data
South Sudan	No data	No data	No data	No data	4	5	3	8	38	24	4	360	568
Togo	67	37	51	52	67	52	95	141	40	317	800	38	96
Uganda	No data	No data	No data	No data	No data	3	24	31	5	72	7	10	117

Table 1: Buruli ulcer case detection globally from 2002 -2014 (WHO, 2015)

2.2 Mycobacterium species

M. ulcerans comes from the order *Actinomycetales*, from the phylum *Actinobacteria*. *Mycobacterium* is the only genus belonging to the family *Mycobacteriaceae* and has over 174 species of which 95 are well characterised species (Katoch 2004). Mycobacterium contains a number of strict and opportunistic pathogens affecting humans and non-humans alike. The strict pathogens affecting humans includes *Mycobacterium tuberculosis* and *Mycobacterium leprae* which are the first two commonest human Mycobacteria globally. Opportunistic pathogens from *Mycobacterium* could also be deadly and it is mostly common in immunocompromised patients. Examples of *Mycobacterium* that are opportunistic and mostly found in immunocompromised patients includes; *M. ulcerans, M. avium, M. simiae, M. kansasii and M. haemophilum* (Katoch 2004). *M. marinum* is another opportunistic pathogen and it is responsible for fish tank or swimming pool granuloma. Individuals exposed to fish or water could get this infection. Mycobacteria species that cause pulmonary diseases may include *M. scrofulaceum* and is also associated with cervical lymphadenitis in children. *M. xenopi* and *M. malnoense* have also been linked with cervical adenitis. There are other opportunistic pathogens which were recently described and they are relatively found to be associated with immunocompromised persons and these include; *M. celatum and M. genavense*. There are rapidly growing Mycobacteria which are of medical importance and they are known to be associated with traumatic and surgical wound infection, skin and soft tissue infections. These pathogens include *M. fortuitum, M. chelonae* and *M. abscessus* (Rastogi, Legrand, and Sola 2001).

The principal mycobacterial pathogens which affect animals include *M. bovis, M. paratuberculosis* and *M. avium* which is mostly associated with poultry and pigs. *M. ulcerans* has been thought to have similarities in the molecular structure with *M. marinum.* In fact, they both have 98% DNA sequence identity (Stinear et al. 2007). *Mycobacterium ulcerans (M.*

ulcerans) that causes BUD has been thought to be the third most common mycobacterial disease affecting humans making it attract a lot of attention globally but still remains a neglected tropical disease (Maudlin, Eisler, and Welburn 2009).

2.3 The causative organism of Buruli ulcer (molecular structure of *M. ulcerans*)

Mycobacterium ulcerans(M. ulcerans) is a bacterium responsible for an infection known to progressively destroy adipose and soft tissue of the skin called Buruli ulcer (WansbroughJones and Phillips 2006). *M. ulcerans* is a slow growing environmental mycobacterium that falls into a group of closely related mycobacterial pathogen (Stinear et al. 2000; Yeboahmanu et al. 2004). Because *M. Ulcerans* are slow growing organisms, they are able to facilitate growth as an endosymbiont and survive under poor conditions.(Stinear et al. 2000b). Genetic analysis indicated that *M. ulcerans* evolved from *M. marinum* by the acquisition of foreign DNA from the environment. Although these two species have different phenotypic characteristics, genetically they appear to have a close resemblance in their genetic makeup of more than 98% (Fyfe et al. 2007; Stinear et al. 2007). Figure 2 shows how *M. marinum* evolved into *M. ulcerans*.

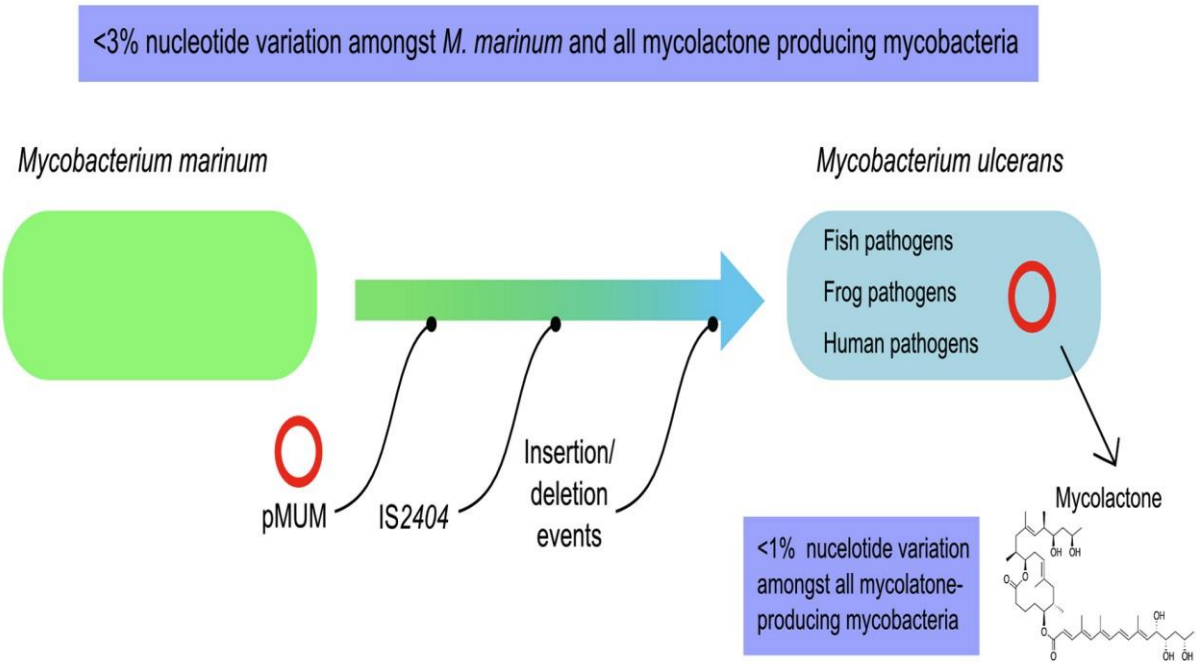


Figure 2: Overview of the evolution and principal species-defining features of *Mycobacterium ulcerans*. (Pidot et al. 2010)

Microscopically, *M. ulcerans* is an acid fast bacillus, small, cocobacillary rod, with no capsules. They grow optimally on routine mycobacteriologic media between 29 - 32^{0}C but the preferred solid media is Lewenstein Jensen (LJ) media supplemented with 0.75% glycerol. Other media like Middlebrook 7H10 and 7H11 can also be used (Françoise Portaels, Johnson, and Meyers 2001). Middlebrook 7H9 medium supplemented with oleic, albumin, dextrose and catalase (OADC) (Amofah, Asamoah, and Afram-Gyening 1998; George et al.

1999a). Also Middlebrook 7H9 (M7H9) broth supplemented with tryptose and glucose (Dobos et al. 2000; George et al. 1999). However in using BACTEC system, the preferred media is

Middlebrook 7H12B (WHO 2014). The restricted growth temperature (29-32^{o}C) of *M. ulcerans* has been thought to play a significant role in the pathogenesis of BU by limiting infection to the skin. *M. ulcerans* grows in microaerophilic condition (2.5-5%) and at a pH between 5.4 and 7.4. Isolates of *M. ulcerans* on LJ are more yellowish in African than Australian strains, rough and have well demarcated edges with the size ranging from 1-2 mm in diameter. Primary cultures are positive within 6 -12 weeks but it could take up to 9 months to achieve a positive growth (WHO 2014; Doig et al. 2012).

Genetically *M. ulcerans* has a complete 5.8Mb genome sequence which comprises of 2 circular replicons, a chromosome of 5632Kb and a large virulence plasmid (pMUM) of 174b. *M. ulcerans* has accumulated 209 copies of the Insertion Sequence (IS) *2404* with nucleotide sequence length of 1,366, 91 copies of IS*2606* (Stinear et al. 2000a), 771 pseudogenes, 2 bacteriophages and multiple DNA deletions and rearrangement. Agy99 is the most common strain of *M. ulcerans* used in molecular studies with its origin in Ghana (Stinear et al. 2007). IS*2404* expansion in *M. ulcerans* genome has led to the inactivation of many genes through disruption of coding and promoter sequence and has mediated the deletion of about 1Mb of DNA from *M. ulcerans* and also evidence of extensive loss of gene function in *M. ulcerans*.
M. ulcerans Agy99 genome has deletions or inactivation of genes that are responsible for expressing potent T-cell antigens and also genes required for pigment biosynthesis, anaerobiosis and intracellular growth (Doig et al. 2012).

2.4 Mode of transmission

Buruli ulcer disease is often probably referred to as the "mysterious disease" because its mode of transmission to humans is still unclear. Notwithstanding there has been advancement in research into how BUD is transmitted. *M. ulcerans* being an environmental pathogen, has been

reported severally to be associated with rapid environmental changes to the landscape leading to wetlands including deforestation practices and increased agriculture, dam construction, construction of agricultural irrigation systems, rice cultivation all leading to the flooding of the land. High content of arsenic in water bodies as a result of mining operations has been implicated with *M. ulcerans*. It is thought that many water bodies associated with increased sedimentation and eutrophication may enhance the growth of *M. ulcerans* due to low dissolved oxygen concentrations (Merritt et al. 2010). In a study by Australian scientists, they observed that, *M. ulcerans* enters surface waters and aquatic habitats through deforestation, erosion and run-off contamination. These environmental conditions have been known to facilitate growth and proliferation of *M. ulcerans*. People who live close to aquatic environment such as ponds, swaps, marshes and slow moving rivers are more likely to be infected with Buruli ulcer disease (Merritt et al. 2010). *M. ulcerans* DNA has been isolated in environmental samples like detritus, soil, biofilms, water filtrates, frogs and snails using the first developed PCR probe by an Australian researcher (Merritt et al. 2010).

Culture positive for *M. ulcerans* and *M. ulcerans* DNA detected in Hemiptera (Naucoridae and Belostomatidae) obtained from an endemic area in Africa suggests that aquatic bugs of the insect order may serve as a vector for *M. ulcerans. M. ulcerans* DNA was also found in the salivary glands of Naucoridae and was transmitted to laboratory mice (Johnson et al. 2005; Portaels et al. 2008).

A study led by Johnson in Australia, suggested that mosquitos predominantly Aedes camptorhynchus could be a possible vector for *M. ulcerans* after detection of *M. ulcerans IS2404* DNA in a group of mosquitos trapped. (Johnson et al. 2007). Some aquatic fishes (Poeciliidae) are implicated to be a passive reserviour of *M. ulcerans* by eating insects of species that are known to be PCR positive for *M. ulcerans* (Eddyani et al. 2004).

Currently the suggested route of transmission of *M. ulcerans* to humans include transmission through insect vector bites, direct contact with contaminated vegetation and or water, aerosol arising from contaminated water which may disseminate and infect through the respiratory tract, entrance of the bacterium through a pre-existing wound following environmental exposure and mechanical trauma. However, experiments in the guinea pig show that skin abrasion is not enough for transmission of *M. ulcerans.* (Johnson et al. 2005; Portaels et al. 2008; Williamson et al. 2014)

There is documented possible increased risk associated with one getting BUD by drinking water contaminated with arsenic, exposing the skin to contaminating sources by not wearing protective clothing, exposure to riverine bodies and young age between 2-14 years (Jacobsen and Padgett 2010; Raghunathan et al. 2005; Merritt et al. 2010)

Based on all these observations, the exact mode of transmission, and the reservoir involved in transmission of *M. ulcerans* is yet to be elucidated.

Lethocerus

These insects are aquatic bugs belonging to the genus *Naucoris* (family *Naucoridae*) and *Diplonychus* (family *Belostomatidae*).

Insectes aquatiques appartenant aux genres *Naucoris* (de la famille des *Naucoridae*) et *Diplonychus* (de la famille des *Belostomatidae*).

Plate 1: Mode of transmission of Buruli ulcer disease.

2.5 Clinical manifestation

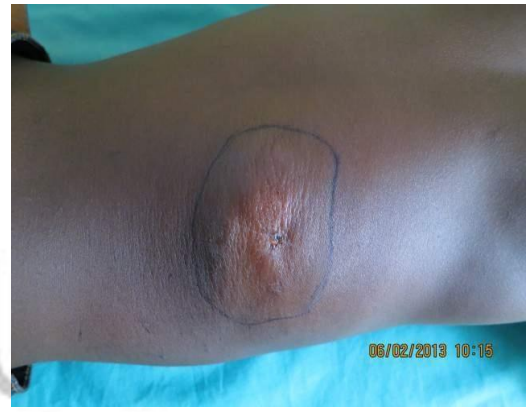
BUD presents itself in two main forms; pre ulcerative lesions and ulcerative lesions. Lesions start as a firm, painless, non-tender and non-movable subcutaneous nodule of about 1 to 2 cm in diameter tethered to the skin, or a small papule (found in the Australians) or an intradermal plaque which is indurated or an oedema which is firm and non-pitting (Williamson et al. 2008).

These pre-ulcerative forms enlarge over a period of days to weeks and then ulcerate. Ulcers are painless with a necrotic base and irregular undermined edges that often extends 15 cm or more. There is a surrounding oedema in about 10% of cases. Ulcers may enlarge progressively to cover an entire trunk or limb if left untreated. Important structures involving the eye, breast, or genitalia are sometimes severely affected and may lead to damage if proper management is not undertaken.

Patients look well and do not have a fever unless secondary bacterial infection sets in. Late presentation is common because the patients live in rural areas with poor economic situations, having no access to medical care. Again patients are sometimes taken to the traditional healer before they present for conventional medical management. Although age and gender are not known risk factors, the disease is seen in women and children aged 5 to 15 years (Amofah et al. 2002).

Buruli ulcer disease is not fatal, they heal spontaneously with antibiotic treatment with minor or no surgery. If left untreated, it causes extensive scarring leading to deformity in patients especially in areas of West Africa where treatment options are limited (Williamson et al. 2008). Some lesions also affect the bone causing osteomyelitis which could lead to amputation if care is not taken.

A B



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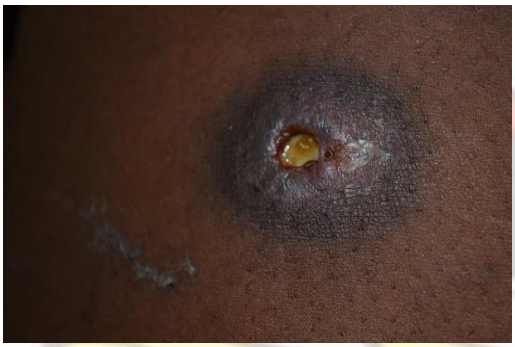
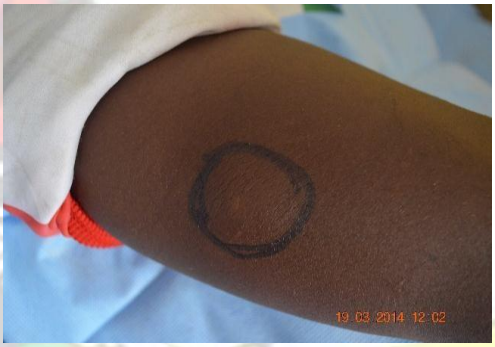




E

Figure 3: Clinical presentations of Buruli ulcer disease (Buruli_path project)

A: Patient with small ulcer on the face, B: Plaque at the back, C: Ulcer on the thigh, D: Nodule, E: Oedema of the upper limb.

2.6 Pathogenesis and pathological features of Buruli ulcer disease

Mycolactone the virulence factor for BU is responsible for the pathogenesis of BUD. Mycolactone is produced by viable *M. ulcerans*, a polyketide toxin encoded by a number of

genes on a large (174 kb) plasmid (George et al. 1999a; Sarfo et al. 2009). Animal work has shown that mycolactone which is a lipid toxin, is responsible for characteristic tissue destruction leading to ulceration (Sarfo et al. 2009). Mycolactone induces apoptosis and necrosis of many human cell types in vitro and appears to inhibit recruitment of inflammatory cells to the site of infection. It is cytotoxic to fibroblast and macrophages as well as immunosuppressive inhibiting chemotaxis at low concentration. This was confirmed by work with a guinea pig model of *M. ulcerans* infection in which either wild type *M. ulcerans* or a mycolactone negative mutant strain was injected subcutaneously. Wild type *M. ulcerans* caused lesions similar to those seen in humans but the toxin negative mutant caused granulomatous inflammation as observed in other mycobacterial infection (Adusumilli et al. 2005). Early lesions are closed but as necrosis spreads, the overlying dermis and epidermis eventually ulcerate, with undermine edges and a necrotic slough in the base of the ulcer. There is also destruction of nerves, appendages and blood vessels (WHO 2016c). The quantity of mycolactone differs in lesion forms, with significantly higher amounts in nodules and plaques compared to ulcers. Cytotoxicity were rather low for nodules and plaques but higher in ulcers and very high in oedematous lesions. Mycolactone levels were higher in the centre of the lesion but as the disease progresses, it diffuses into the undermine edges of ulcers (Sarfo et al. 2014).

There were suggestions recently that persistence of mycolactone in BU lesions could retard healing by killing keratinocytes and by inhibiting the secretion of growth factors required for wound healing (Sarfo et al. 2014). Again mycolactone has been shown to bind to WiskottAldrich syndrome protein (WASp) in epithelial cells, infuriating uncontrolled activation of Arp2/3. This disrupts assembly of actin in the cytoplasm which results in defective cell adhesion and could lead to defective wound closure (Guenin-Macé et al. 2013).

Mycolactone is intact in ulcer exudates and serum samples before antibiotic treatment and it persisted during and after treatment but as to what point this lipid toxin is eliminated from the

patient‟s body remains unknown. Mycolactone is present in all forms of Buruli ulcer disease before and after antibiotic treatment (Sarfo et al. 2010a).

Mycolactone diffuses from infectious centres into blood, where it hoards in mononuclear cell subsets. This suggests that the lipid toxin plays a role in allowing *M. ulcerans* to evade host innate immunity by suppressing inflammatory processes. Patients with active ulcers display distinctive profile of immune suppression and an impaired capacity to produce Th1 and Th2 cytokines on stimulation with mitogenic agents (Phillips et al. 2009).

Histology of *M.ulcerans* disease lesions shows clusters of acid fast bacilli in areas of subcutaneous fatty tissue necrosis accompanied by acute and chronic inflammation distant from the necrotic areas (Evans et al.2003). This histology led to the suggestion that *M. ulcerans* causes a disease by secreting a toxin which destroys human tissue, causes the extension of the lesion area, produces an intracellular infection characterised by granulomatous lesion and inhibit the development of local inflammation (Sarfo et al. 2010a). Histopathological analysis of the guinea pig infected with *M. ulcerans* show an acellular coagulation necrosis at the central area adjacent to the bacterial inoculum at 24hr of introducing *M. ulcerans* intradermally. By 10 days, there is a cluster of extracellular bacteria which is surrounded by a large area of necrosis, with the edges of the necrotic area showing infiltrate of mononuclear cells. High magnification of pyknotic cells revealed condensed nuclei characteristic of apoptosis. Inflammatory infiltrates increased throughout the period of infection, some distance away from the bacteria (Adusumilli et al. 2005).

2.7 Laboratory confirmation

Laboratory confirmation of Buruli ulcer disease is the central part in the overall management of the disease. The type of clinical sample for laboratory confirmation is dependent on the lesion type a patient is presenting with. Phillips et al (2005) evaluated the use of 4 millimetre punch biopsy specimen for diagnosing patient presenting with pre ulcerative form of the

disease. Although punch biopsy is preferred to surgical biopsy, its use is currently limited due to its association to pain, sometimes bleeding and cost involvement (Phillips et al. 2005; WHO.2014). Fine needle aspirate (FNA) sample is obtained from pre ulcerative lesions for laboratory confirmation. Since 2007, there has been advance progress in using FNA because it is easy to obtain the sample and it comes with minimal pain (Eddyani et al. 2009; Phillips et al. 2009). Swab specimens are taken in patients presenting with ulcerative form of the lesion. However, if there are no undermine edges, FNA specimens are preferred.

Currently there are four commonly used methods for laboratory confirmation of BUD and they include; Direct smear examination, PCR, culture and histopathology. However, there has been newer ones that are under investigation like the loop mediated isothermal amplification (LAMP) and fluorescent thin layer chromatograpy (fTLC) (Ablordey et al. 2012; Njiru et al. 2012; Souza et al. 2012; Wadagni et al. 2015).

As a first line of diagnosis at the district level, direct smear is stained by the Ziehl-Neelsen (ZN) for acid fast bacilli and has a sensitivity of 55% when 2 slides are read. It has the advantage of taking a short time and it is cheap. Nevertheless as any diagnostic method it requires trained personnel and regular quality control check (Frimpong et al. 2015).

Culture when employed requires decontamination of the sample and inoculating it on Lowenstein Jensen media and incubation at 30-32^{o}C. *M. ulcerans* grow very slowly on the media taking 6-8 weeks for positive culture (Phillips et al. 2005) but can take up to 6 months (Portaels et al. 1996). This technique has sensitivity between 30 to 60% (Portaels et al. 1997;

Herbinger et al. 2009). Cultures are important for identifying treatment failures and recurrences of infection. Cultures may also be necessary if drug-resistant strains of *M. ulcerans* emerge.

Histopathology on biopsy specimen has a high sensitivity of 90% but requires sophisticated laboratory and highly trained personnel. Histopathology is generally useful in establishing differential diagnosis and monitoring response to treatment (Sakyi et al. 2016).

PCR targeting the insertion sequence IS*2404* of *M. ulcerans* is the gold standard test with a sensitivity of 98%. Dry Reagent based (DRB) conventional PCR developed in order to be used in endemic countries is sensitive and well adapted in the tropical conditions (Siegmund et al. 2007)

Other tests under investigation include a novel DNA amplification method called LAMP. Though this technique is not routinely used, it has the potential to become rapid, simple and inexpensive test for *M. ulcerans* that can be implemented locally (Ablordey et al. 2012).

A more recent diagnostic test developed is the fTLC which detect mycolactone, the lipid toxin produced by *M. ulcerans*. This test showed a sensitivity of 73.2% and a specificity of 85.7%. If tested in bigger population, it could be used in the districts where BUD is endemic due to its simplicity (Wadagni et al. 2015).

2.8 16S rRNA

Ribonucleic acid (RNA) is a linear molecule composed of purines (adenine (A) and Guanine (G)) and pyrimidine (cytosine (C) and Uracil (U)) bases. Each ribonucleotide base consists of a ribose sugar, a phosphate group, and a nitrogenous base. Adjacent ribose nucleotide bases are chemically attached to each other by phosphodiester bonds. RNAs are single-stranded, more unstable especially with heat and are more prone to degradation and so cannot be stored for a longer period. When there is a need for new proteins to be formed, messenger RNA (mRNA) carries the genetic information from DNA in the form of a chain of three-base code each of which specifies a particular amino acid (Lodish et al. 2000). Transfer RNA (tRNA) which is the key to interpreting the code in mRNA, binds with the rRNA and carries the information from the DNA to the growing end of a polypeptide chain. Ribosomal RNA (rRNA) accelerates

the reaction of the assembly of amino acids into protein chains. They also bind various accessory molecules necessary for protein synthesis associated with a set of proteins to form ribosomes. The genes that encode rRNA evolve in a very unique manner that makes them powerful tools for identifying species from sequence data. There are however some micro RNAs which has been identified to be involved in molecular processes. Although some RNA molecules are passive copies of DNA, many play key, active roles in the cell. For instance, some RNA molecules are involved in switching genes on and off, while other RNA molecules make up the critical protein synthesis machinery in ribosomes. The three types of RNA participate in the essential protein-synthesizing pathway in all cells. The development of the three distinct functions of RNA was probably the molecular key to the origin of life (Lodish et al. 2000).

Ribosomes are composed of a large and small subunit, each of which composing of several different ribosomal RNA (rRNA) molecules and more than 50 proteins. With the aid of the electron microscope, ribosomes were discovered as distinct, rounded structures prominent in animal tissues secreting large amounts of protein. The small ribosomal subunit also called 30S contains a single rRNA molecule (16S rRNA) and ribosomal proteins. The large subunit also called 50S contains 23S and 5S rRNA and ribosomal proteins. The sizes of the subunits differ in prokaryotic and eukaryotic cells. The small rRNA subunit has 1500 nucleotides long in bacteria whereas in humans they are 1800. The large rRNA subunit in bacteria has 3000 nucleotides long while there are 5000 nucleotides long in humans.

16S rRNA has proven to be the most useful for establishing distinct relationship because of their high information content, conservative nature, specificity and universal distribution (Lane et al. 1985). 16S rRNA can be found in all free living organisms/ prokaryotes which has about 1.542Kb in length (Cox et al. 1991). 16S rRNA comprises of a constant regions, in which the

nucleotide sequence has been highly conserved during evolution and 8 hypervariable regions (V1-V4 and V6-V9) in which the nucleotide sequence is more variable making it useful for bacterial identification between species to species (Cox et al. 1991). These constant and variable regions in 16S rRNA has been observed in all bacteria including slow growing and fast growing mycobacteria species.

16S rRNA has been known to play several functions which include binding to protein S1 and S2 involved in the initiation of protein synthesis and also interacting with 23S, aiding in the binding of the two ribosomal subunits during protein synthesis. 16S rRNA has been successfully used as a viability marker in *M. tuberculosis* and *M.leprae* and recently in *M.ulcerans* (Juan et al. 2012; Davis et al. 2013; Beissner et al. 2012)

2.9 Management of BUD

BUD often leads to the destruction of skin tissues. Although the disease is rarely fatal, delayed treatment or management could result in deformities including limitation of movement of joints (Ruf et al. 2015)

2.9.1 Antibiotic therapy

Currently BUD is treated by administering a combination antibiotic therapy of oral rifampicin at 10mg/kg body weight daily for 8 weeks and intramuscular streptomycin at 15mg/kg body weight daily for 8 weeks. For pregnant women, streptomycin is replaced with oral clarithromycin at 7.5mg/kg body weight twice daily for 8 weeks. There have been studies to compare the effectiveness of replacing intramuscular streptomycin with clarithromycin (Chauty et al. 2007; Sarfo, et al. 2010; Phillips et al. 2013).

Currently WHO is undergoing a drug trial study to compare the efficacy of rifampicin and streptomycin with rifampicin and clarithromycin. When oral rifampicin is combined with a

second oral agent like clarithromycin, moxifloxacin or ciprofloxacin, there is avoidance of aminoglycoside toxicity and improvement of patient acceptance. The introduction of antibiotic therapy for treating BUD has reduced the recurrence rate from 35% to 1-2% (Who 2012; O"Brien et al. 2014).

2.9.2 Surgical management

Until recently, wide surgical excision was the only treatment and management option for BU patients (Schunk et al. 2009) which left patients with massive deformities and restriction of movement parts. Conservative surgery which includes debridement and skin grafting are still useful in improving wound healing and prevention of scars and deformities. Debridement is done by removing the maximum necrotic tissues while minimizing damage of healthy tissues and it is followed by primary or secondary wound closure by suturing or skin grafting. Small ulcers go on to heal without surgical interventions but larger ulcers take much longer time to heal and so require additional surgical management to hasten healing (O"Brien et al. 2014; WHO 2012).

2.10 Wound care

Wound care is very essential in the prevention of disabilities and it forms a major component in Buruli ulcer management. Caring of the wound involves; classification of the wound and preparation of the wound bed which involves cleaning of the wound surface and application of the appropriate dressing material (Velding et al. 2014).

2.10.1 Dressing

To ensure proper and higher rate of healing of Buruli ulcer wounds, daily dressing with appropriate dressing material is required. Pre ulcerative lesions (nodules and plaques) are washed with normal saline until complete healing. Ulcers are washed or cleaned with normal saline, the wound are kept moist to minimize bleeding and easy removal of dressing by applying Vaseline gauze. In an attempt to avoid longstanding complications of the wound, WHO has introduced drawtex hydroconductive dressing, this is known to facilitate autolytic debridement and an absorbent. The drawtex is placed on the wound and then finally bandaged or plastered (Treadwell and Macdonald 2012). For oedematous lesions, after the lesion has been cleaned with normal saline, short stretch compression bandages are applied.

2.10.2 Lesion measurement

During the last decade, comprehensive research and extensive clinical experience have provided evidence that the discipline of skin measurement has been a remarkable phase of expansion, improvement and acceptance in the field of wound healing (Donohue and Falanga, 2003). Being able to tell early in the treatment of wound whether a therapy is working is of extreme importance and can offer both economical and medical benefits to clinical practice and research work.

Three measurement approaches, namely; Absolute area measurement, Percent area reduction method and linear measurement techniques described by Gillman have been successfully applied in Diabetic foot ulcers and venous leg ulcers (Cardinal et al. 2008; Gilman 2004; Sheehan et al. 2003). These measurement approaches and its ability would enable the early identification of poorly healing wounds, allowing for re-evaluation, if factors beyond the therapy are causing the delay or current therapy should be altered(Donohue and Falanga, 2003). Presently there are no early predictive factors to guide clinicians to differentiate patients who will heal early from those who will have prolonged healing time. Clinicians however only

relies on experience of the disease to make decisions, thus establishing predictive factors for healing will immensely guide the decisions of clinicians in the BU clinic.

2.11 Physiotherapy and functional limitation prevention.

In rural Africa, patients tend to report to the hospital late in the course of the disease, with some presenting with extensive ulcers with joint and bone involvements leading to scaring, contractures and calcifications leading to permanent disabilities. Community based surveillance and health education programme which targeted eradication of Guinea worm may be successfully applied in BU endemic areas (Webb et al, 2009). With the introduction of early case detection programme by the WHO, there has been a reduction in functional limitation compared to decades ago (WHO 2016d). BU functional limitation score (BUFLS) was developed to assess the nature and severity of impairment caused by the disease even before starting of antibiotic treatment. This development has helped to identify patients who have or might develop functional limitation early enough to start management (Alferink et al. 2015).

CHAPTER 3

MATERIALS AND METHODS

3.1 Study area

The participants enrolled in the study were Buruli ulcer suspected patients who attended Bu clinic at Agogo Government Hospital, Tepa Government Hospital, Nkawie Toase Hospital and Dunkwa Hospital.

The Laboratory work was done at Kumasi Centre for Collaborative Research in Tropical Medicine (KCCR), a research centre at the Kwame Nkrumah University of Sciences and Technology and affiliated to the School of Medical Sciences.

3.1.1 Agogo Government Hospital

Agogo is a city in the Asante Akim North District of the Ashanti region of Ghana with the coordinates 6º48‟0„„N1º5‟0„„W. It is approximately 80km east of Kumasi, the Ashanti regional capital ("Estimating Buruli Ulcer Prevalence in Southwestern Ghana - Thesis.pdf" 2015; Agyare 2015). Agogo has a population of about 149,491 (WHO 2016e) and is surrounded by natural wall of mountains with some of the nearby towns being Hwidiem, Krodua and Ananekrom which are all Bu endemic areas ("Maps, Weather, Videos, and Airports for Agogo, Ghana" 2014).

Agogo Presbyterian hospital which is a district Hospital was established in 1931 and has been known to be the oldest mission hospital in Ghana. The hospital has staff strength of 318 with specialized care in surgery, ophthalmology, paediatrics and obstetrics. Again, there is a training centre for Buruli ulcer treatment by the national BU control programme and the WHO (WHO 2016e).

Buruli ulcer disease has been prevalent in the Asante Akim North District of Ghana since 1989 followed by detection at the Amansie West District in the same region. Since then, there has been increased case detection in and around communities of Agogo (Agyare 2015).

The Agogo Presbyterian hospital has been in the forefront of Bu management since 1990s. They were the first hospital to engage in the services of volunteers for Bu case search in their district. They have recently been embarking on early case detection activities with the

community based surveillance volunteers which has helped to reduce surgery and reporting of bigger lesions in the district (Abass et al. 2015).

3.1.2 Tepa Government Hospital

Tepa is a town and the capital of Ahafo Ano North (AAN), a district in the Ashanti region of Ghana with coordinates 7º00‟N 2º10‟„W (Ano and District 2012).

Tepa Government hospital is the only hospital in the district apart from four smaller health services facilities. AAN is one of the endemic Buruli Ulcer districts in Ghana. To help manage the disease, the Ghana Health Service has trained community based surveillance volunteers, traditional birth attendants and school health teachers on the identification of the disease at its early stage and by offering a free dressing of ulcers at the various health facilities (“Ghana » Ashanti Region » Ahafo Ano North District” 2014)

In 2005, it was discovered that AAN had the highest cases of Bu accounting for 29% of the total cases (Garriga 2014).

3.1.3 Nkawie – Toase Hospital

Nkawie is a small town, and it is the capital of Atwima Nwabiagya district, in the Ashanti Region of Ghana with the coordinates 6º40‟N 1º49‟„W. The town is bounded by two towns, Toase and Nkawie Panin (Gros 2004).

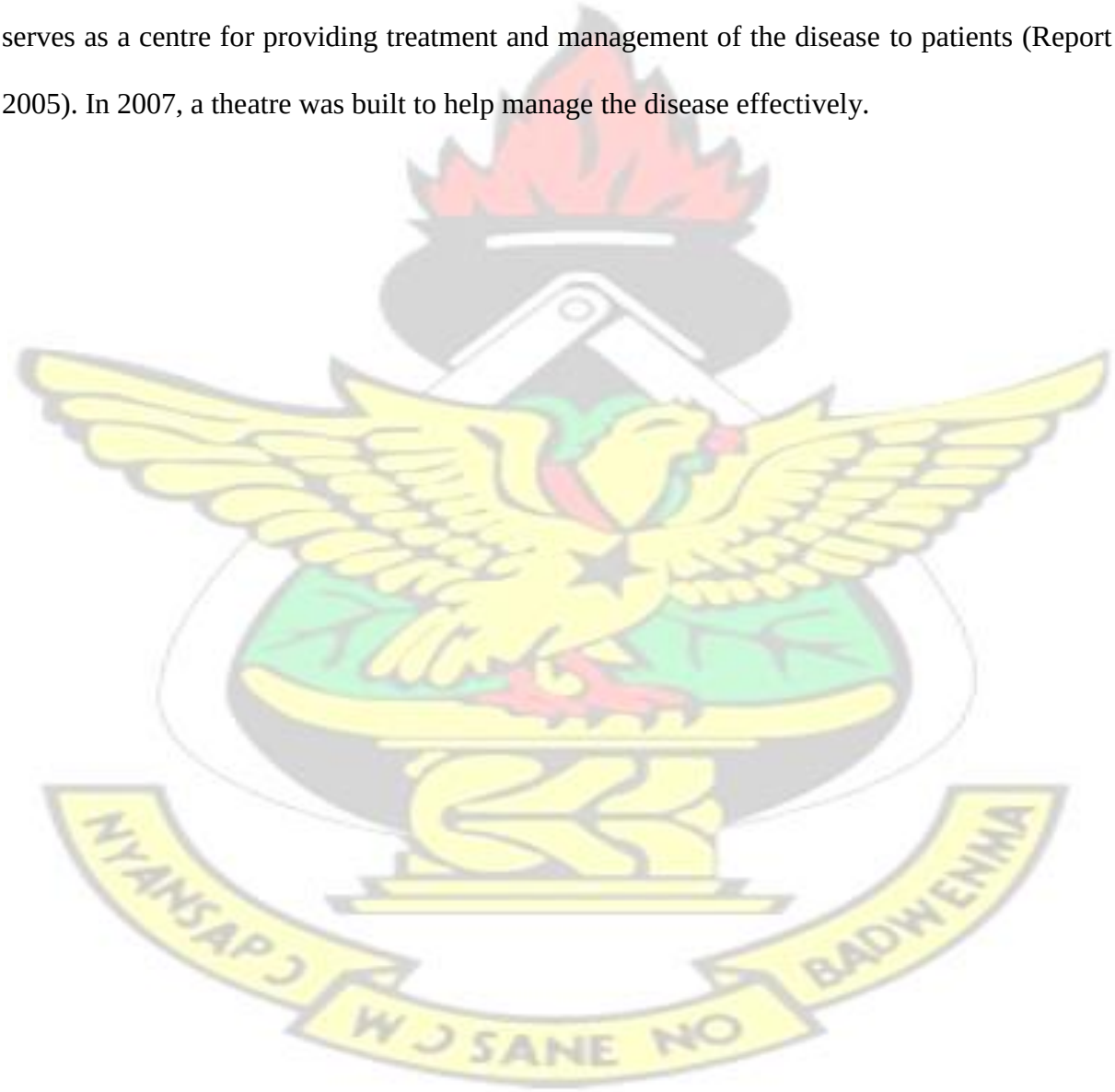
In 2007, the hospital recorded 112 cases in the district making it a highly endemic area. This discovery led to the construction of a theatre complex building at the hospital.

Nkawie –Toase is the only government hospital in the district which is located at Nkawie/Toase and it has been serving as a centre for treating and managing Bu diseases (Ghana District, 2006b).

3.1.4 Dunkwa Government Hospital

Dunkwa is the capital town of Upper Denkyira East municipal District in the central region of south Ghana. The town has a population of 33,379 which is surrounded by a number of rivers and streams including the offin river (River 2013).

Upper Denkyira is a Bu endemic area with a prevalence rate of 114.7 per 100,000 (Amofah et al. 2002). Dunkwa government hospital is the only government hospital in the district and serves as a centre for providing treatment and management of the disease to patients (Report 2005). In 2007, a theatre was built to help manage the disease effectively.



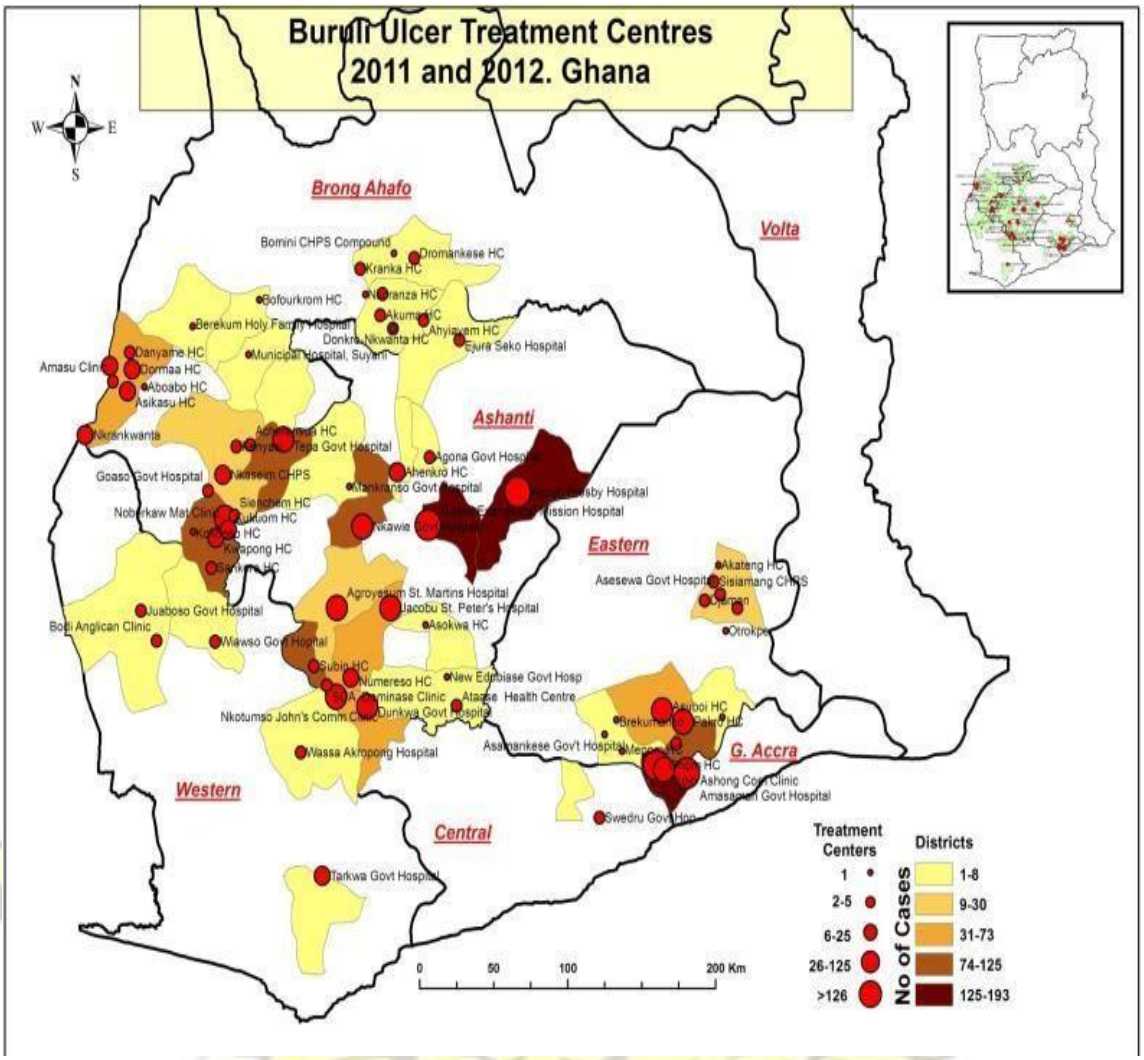


Figure 4: A map showing the various Buruli ulcer treatment centres in Ghana

3.2 Study design

This was a prospective hospital based cohort study. Patients suspected to have Buruli ulcer disease with the help of village volunteers were brought to the four BU treatment centres to be examined and managed with the help of professionals in the field. Clinically suspected cases were recruited into the study from the period June 2013 to June 2015.

3.2.1 Sample size calculation

The sample size of the study was calculated using this formula n =

$\frac{Z^2 \times P\,(1-P)}{d^2}$ (Pourhoseingholi, Vahedi, and Rahimzadeh 2013)

Where:

n = required sample size

Z = confidence level at 95% (standard value of 1.96)

P = estimated prevalence of Buruli ulcer in Ghana d

= Precision (standard value of 0.05)

The prevalence of Buruli ulcer in Ghana as at 2002 was 20.7 in 100,000 (Amofah et al. 2002)

Therefore p in percentage = $\frac{20.7}{100,000} \times 100$

P = 0.0207% n = $\frac{(1.96)^2 \times 0.0207 (1-0.0207)}{(0.05)^2}$

Therefore, the sample size without any assumptions (n) = 31.19, which is approximately 31. The time to healing of Buruli ulcer is averagely 20 weeks, which means at week 20 almost all BU lesions should be healed which is approximately 100% wound healed. Assuming that at week 16, 70% of the lesions would be healed, the sample size would reduce at week 16 by 70%. To compensate for that loss, 70% of the calculated sample size (31) was added to 31 increasing the sample size to 53.

Further to this, since the patients were being followed up to week 16 for sample collection and up to 1 year to make sure lesions heal with no complications, it was necessary to compensate for those who would be lost to follow up which was very likely. Therefore, assuming that 10% of the patients would be lost to follow up, it was compensated for by calculating 10% of the new sample size. This compensation resulted in a final sample size of 58. The smple size of 58 was considered the minimum sample size needed for the study.

Within the two years in this study, 150 patients were enrolled.

3.2.2 Inclusion criteria for enrolment of participants

- All Bu clinically confirmed patients who gave their consent to the study
- All patients older than 5years.
- All Bu confirmed patients who never took antibiotic treatment for Buruli ulcer

3.2.3 Exclusion criteria for enrolment of participants

- Patients who did not consent to the study
- Patients who started antibiotic treatment prior to the study.
- Patients who were below 5years old

3.2.4 Ethical Considerations

Ethical approval was sought from the Committee of Human Research Publication and Ethics, School of Medical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana. The study was explained to each participant in a language they understood, verbal and written consent were obtained from participants older than 18 years and parents or guardians for those participants younger. Participants who agreed to partake in the study appended their signature or thumb print on the written consent form. This procedure was done in the presence of a witness.

3.3 Study procedure

3.3.1 Patients' assessment

Patients who turn up at the various BU clinic centres were referred by health workers in the respective districts. The patients were seen on Tuesdays at Dunkwa government hospital,

Wednesdays at Agogo Government hospital, Thursdays at Tepa government hospital and on Fridays at Nkawie-Toase government hospital.

Upon arrival at the various clinics, patients were clinically examined by a team comprising of specialised Doctors from KATH, and experienced Nurses, disease control officer and biomedical scientist by looking for clinical features of Buruli ulcer. An agreement was finally attained by the team.

The patients‟ vital information (which included height, weight, temperature and blood pressure) were obtained. Demographic data which included age, sex, lesion form, category of lesion were also obtained using the WHO BU01 form (Appendix 1) with the help of the nurses and sometimes biomedical scientist.

Anonymous codes were assigned to each participant to mask the identity of them. The patients‟ lesions were grouped into categories based on the size and location of the lesion as displayed on the BU01 form. A lesion whose diameter was less than 5cm was considered category I lesion, lesions whose diameter was between 5-15cm and those whose lesions were more than 15cm were considered categories II and III lesions respectively according to WHO standards. Lesions which were on critical sites like the eye, genitalia, and breast were classified as category III lesions. Patients who presented with more than one lesion either on the same site or different site of the body was also classified as category III lesion all according to WHO recommendations.

Digital photographs of the lesions with their codes were taken at all-time visits until complete healing. Digital planimetry using Silhouette with capture image (ARANZ Medical 2016) was used to capture and measure nodular, plaque and ulcer lesions, oedematous lesions healing were monitored by digital photographs and measuring of the length of oedema and circumference with tape measure. The parameters measured included the widest width, area,

volume, and perimeter which were used to calculate the rate of healing of the various lesions. Fine needle aspirate (FNA) and swab samples for diagnostic confirmation were taken from non-ulcerative lesions and ulcerative lesions respectively.

Patients were administered a standard combination antibiotic therapy in the Bu clinics comprising of 10mg/kg Rifampicin and 15mg/kg streptomycin for 8weeks.

3.3.2 Sample collection

Clinical samples were taken for diagnostic confirmation by doing Dry reagent based PCR and microscopy. Extra samples were taken at baseline week 0, week 4, week 8, week 12 and at week 16.

For pre-ulcerative lesions, 3 FNA or swab samples were taken for combined 16S rRNA/IS2404 qPCR analysis and culture for non-ulcerative lesions and ulcerative lesions respectively. 2 FNA was put into 2 separate tubes for the 16S rRNA/IS2404 qPCR analysis and 1 FNA into another tube for culture.

3.3.3 Sampling method

3.3.3.1 FNA procedure

FNA samples were taken with the help of Doctors at the various Bu clinics by inserting a 21gauge needle into the centre of a non-ulcerated lesion or into a viable inflamed skin immediately adjacent to ulcers and moving it back and forth within the subcutaneous tissue. The needle tip was then flushed seperately into a 500µl RNA protect and 1ml PANTA media for DNA and RNA extraction and culture respectively using a 5ml syringe (Phillips et al. 2009).

3.3.3.2 Swab taking procedure.

Swab samples were obtained by circling the entire undermined edge of the ulcers using sterile swab sticks. The swab sticks were then placed separately in 500µl RNA protect for DNA and RNA extraction and 1ml PANTA media for culture respectively (Siegmund et al. 2007).

3.3.3.3 Sample transport and storage

To experiment on the type of appropriate transport media to use in 16S rRNA/IS2404 RT qPCR assay, two different transport media were used for each patient. One sample was transported in a 500µl PANTA media and another in 500µl RNA protect (Qiagen, UK) from the Bu treatment centres to KCCR where the samples were processed and analysed.

For 16S rRNA /IS2404 RT qPCR assay:

Upon arrival in the laboratory, the samples in the PANTA transport media were stabilized by adding 1ml of RNA protect reagent to the sample. This was vortexed for 10sec.

The samples were incubated for 5min at ambient temperature and vortexed interspersed 10 seconds every 1 min. The samples were incubated on ice for 5min to increase the precipitation of the samples. The samples were pelleted by centrifugation at 5000g at ambient temperature for 7min. For swab samples, the swab sticks were carefully taken from the solution before gently decanting the supernatant while leaving the pellet at the bottom of the tubes.

The samples in the 500µl RNA protect bacteria reagent were incubated at ambient temperature for 5min while vortexing 10sec for every 1 minute within the 5min incubation period followed by centrifugation at 5000g at ambient temperature for 5min. For swab samples, the sticks were carefully taken from the solution and supernatant gently decanted leaving the pellets at the bottom of the tubes. Both samples transported in PANTA media and

RNA protect bacteria reagents were stored at -70^{o}C prior to extraction of DNA and RNA.

For culture samples:

Samples for culture were transported in 1ml or 4mls PANTA media for FNA and swab samples respectively. In the laboratory, the samples were kept at 4ºC overnight before they were decontaminated using the modified Petroff"s method and subsequent inoculation of the pellets unto LJ media.

3.3.4 Laboratory Assessment

3.3.4.1 Diagnostic confirmation

For diagnostic confirmation of patients suspected with Buruli ulcer disease, direct smear on a well labelled slide was prepared and air dried on the field for ZN microscopy using the Ziehl Neelsen stain. 2 FNA and 2 swab samples were placed in 300µl and 700µl of cell lysis solution respectively for extraction of DNA and Dry Reagent Based (DRB) PCR.

3.3.4.1.1 Microscopy

In the laboratory, the slides were heat fixed by passing them rapidly several times through the flame of a Bunsen burner, allowed to cool and arranged on a staining rack. The slides were flooded with Carbol fuchsin in 5% Phenol solution, cotton wool soaked in 70% ethanol was flamed and placed under the slides to heat the stain until vapour, allowed to stain for 5min and gently washed with water. The slides were then flooded with 20% H_2SO_4 for another 5 min, washed and the extra water tipped off making sure all H_2SO_4 was washed off. 0.3% methylene blue was added for 1 min, washed and dried on a rack. The stained slides were examined under ×100 oil immersion objective. Positive slides showing red rod-like bacilli were graded based on the number of acid fast bacilli (AFB) seen according to WHO standards (WHO 2014) as follows, more than 10 AFB/ field for at least 20 fields were reported as +++positive, 1 to 10 AFB per field were reported as ++positive, 10 to 99 AFB per 100 fields were reported as +positive and 1-9 AFB per 100 fields were reported indicating the exact number of AFB. At least 100 fields showing no AFB were examined to declare a slide negative.

3.3.4.1.2 DNA extraction and DRB PCR

In the laboratory, the activity of the mycobacteria in the samples were immediately inactivated by incubation in a thermomixer at 95ºC for 15min and then kept in the fridge at 4ºC until extraction. The DNA was extracted using the Puregene DNA isolation kit (Genomic DNA Purification kit, Gentra systems). The extraction procedure involved cell lysis, Protein precipitation, DNA precipitation and DNA hydration.

Cell lysis

For swab samples, the *Mycobacterium ulcerans* cells were first lysed with 15µl lysozyme (10mg/ml), incubated while shaking gently at 37ºC for 1 hour in a thermomixer. 10µl Proteinase K (20mg/ml) was added and incubated while shaking at 55ºC for 4hours in a thermomixer followed by inactivation at 80ºC for 20min.

On the other hand, 10µl Proteinase K (20mg/ml) was added to the FNA samples first, incubated while gently shaking at 55ºC for 4 hours in the thermomixer, Proteinase K inactivated at 80ºC for 20min and allowed to cool down to room temperature. 15µl lysozyme (10mg/ml) was added and incubated while shaking gently at 37ºC for 1 hour in the thermomixer.

Protein Precipitation

The proteins in both the FNA and swab samples were precipitated by firstly incubating them on ice for 5 min followed by addition of 230µl and 100µl Protein Precipitation Solution (PPS) to Swab and FNA samples respectively. The samples were then vortexed vigorously at high speed for 20 seconds and then incubated on ice for 5 min. The samples were then centrifuged

at 13,000xg for 5min. The supernatants were transferred into a labelled 2ml tube containing 700µl isopropanol (>99.7%) with 2µl glycogen.

DNA Precipitation

The tube contents were mixed by inverting gently about 50 times and then centrifuged at 13,000 xg for 5min. The supernatant was poured off gently and 700µl of 70% ethanol added to the pellet and mixed gently. The solution was centrifuged at 13,000 xg for 5min and the ethanol poured off carefully. The pellets were dried by inverting the tube on 45^{o}angled clean absorbent paper for 1hour.

DNA hydration

Dried DNA was solubilised in 200 µl for swab and 50µl hydration solution (Qiagen, Germany) for FNA respectively by pipetting up and down for about 20 times followed by incubating in thermomixer for 1hour at 65^{o}C.

DRB- PCR

DNA obtained for each patient sample was amplified in DRB-PCR targeting the *M. ulcerans* IS2404 repeat sequence. Prior to the DRB-PCR, 1.25 µl each of the oligonucleotides MU5 forward primer (10uM) (5‟ AGCGACCCCAGTGGATTGGT 3‟) and MU6 reverse primer (10uM) (5‟ CGGTGATCAAGCGTTCACGA 3‟) (Herbinger et al. 2010; Frimpong et al. 2015), were lyophilized in 0.2ml PCR reaction tubes using a RVC 2-25 vacuum concentrator (Christ, Osterode, Germany).

PuReTaq Ready-To-Go PCR beads (GE Healthcare Life Sciences, UK) were added to the reaction tube containing the lyophilized primers and dissolved in 22.5µl DNAse free water.

2.5 µl of extracted patients‟ DNA were then added. For quality control purposes, negative extraction control (a tube was taken through the extraction process but did not contain clinical

sample), inhibition control (patients‟ DNA was spiked with culture suspension) and positive control (culture suspension confirmed to be *M. ulcerans*) were included.

The amplification protocol was as follows: 94^{o}C for 10 min, followed by 40 cycles at 94^{o}C for 10s, 58^{o}C for 10 s, and 72^{o}C for 30 s, with a final cycle at 72^{o}C for 15 min. Amplification products were kept at 4^{o}C until they were processed further by agarose gel electrophoresis and visualized by UV trans illumination and photo documented.

Agarose gel electrophoresis

To visualize and interpret the amplification products, the PCR reaction products were separated by gel electrophoresis. 1.5% agarose gel was prepared by dissolving 1.8mg of agarose powder in 120ml 1xTBE buffer. This was heated in microwave until completely dissolved and allowed to cool down to 50^{o}C. 10µl of Gel red was added to the agarose, mixed and poured into a gel electrophoresis tray with well comb placed in it. The liquid gel was allowed to cool to room temperature, transferred into a gel electrophoresis chamber and 1x

TBE was poured into the chamber to cover the gel. 25µl of each product was mixed with 3µl 6x loading dye and loaded into the gel wells. 6µl of 100 bp DNA ladder was placed in the first well and included in the run. 100V voltage was applied and electrophoresis was run for 45 min. The gel with the DNA fragment was visualized using UV trans illuminator connected to a computer. The bands corresponding to each patient‟s sample were compared to that of the positive control band and reported. The samples were reported as either positive, negative or inhibited.

3.3.4.2 Viability assays

3.3.4.2.1 Culture

Samples in PANTA media were decontaminated using the modified Petroff method (Françoise Portaels, Johnson, and Meyers 2001) by shaking the samples on a shaker for 5min, transferred into a new labelled 50ml falcon tube and mixed with an equal volumes of 4% NaOH (1M NaOH) for 15 min on a shaker. The samples were then centrifuged at 1421xg for 15min, the supernatant discarded and the pellets washed with 15ml of 0.9% NaCl (0.15M NaCl) and centrifuged at 1421xg for 15min. The sediment was suspended in 0.5ml of 0.15M NaCl and 0.25 ml inoculated on each of two well-labelled Lowenstein-Jensen media and incubated at 32ºC for up to 6 months. Cultures were read weekly for growth. Positive growths were examined first by ZN staining and specificity confirmed by PCR targeting the insertion sequence IS2404. Contaminated cultures were autoclaved before discarding.

3.3.4.2.1. Combined 16S rRNA RT / IS2404 qPCR

To increase the specificity of the test, a combined extracted *M. ulcerans* RNA and DNA from the same sample was amplified by qPCR after reverse transcription of 16s rRNA to cDNA or directly in the case of DNA.

DNA and RNA isolation

The DNA and RNA were extracted from the pellets stored at -70ºC using the AllPrep DNA/RNA Micro kit (Qiagen, UK) as previously described (Beissner et al. 2012). The samples were lysed in 100µl of lysozyme in 15mg/ml cell lysis solution (Qiagen, UK) and 20µl of 20mg/ml Proteinase K. The samples were placed on ice for 5min interspersed with occasional 15 seconds vortexing after every 1minute and then incubation at 45ºC for 5min while shaking vigorously. 350µl RLTplus buffer (Qiagen, UK) with added β-mercaptoethanol was added to the samples and vigorously vortexed for 15 seconds and homogenized according to manufacturer‟s instructions using QiaShredder (Qiagen, UK). Homogenates were transferred into a labelled All Prep DNA spin column with collection tubes,

centrifuged at 9000x g for 30seconds and the column containing the DNA placed in a new labelled 2ml Eppendorf tubes for further purification. The flow through containing RNA were mixed with 350µl of 70% ethanol, transferred onto RNeasy spin column on a new collection tube and centrifuged at 9000x g for 15 sec. The spin column containing the RNA were subjected to three washing steps with 700µl buffer RW1, 500µl of RPE buffer and 500µl 80% ethanol. To elute the RNA on the spin column membrane, 50µl of RNAse free water was carefully pipetted onto the spin column with an attached collection tube and centrifuged for 1 min at 9000xg. The total RNA eluted was immediately placed on ice for further procedures.

Purification of the DNA was achieved by two separate washes with 500µl of buffer AW1, followed by 15 seconds centrifugation at 9000xg and then with 500µl of buffer AW2 followed by 2min centrifugation at 9000x g. The DNA were eluted with 50µl of already warmed elution buffer at 70^{o}C into a labelled 1.5ml eppendorf tubes after allowing the column to sit for 2min before centrifuging at 9000x g for 1min to get a total elution of about
50µl. The eluted DNA was kept in the fridge for IS2404 qPCR

Reverse transcription

To remove contaminating genomic DNA, 2µl gDNA wipe out buffer (Qiagen, UK) was added to 12µl of the total RNA extracts, incubated for 5min at 42^{o}C and the reaction stopped by incubating at 95^{o}C for 3min. 2µl of the sample was taken as wipe out control. *M. ulcerans* whole transcriptome RNAs were reverse transcribed into cDNA using Quantitect Reverse transcription kit (Qiagen, UK) by adding 2µl of RNAs free water, 5µl of premixed RT reaction buffer and 1µl of reverse transcriptase (RT), incubated at 42^{o}C for 15min and then inactivated at 95^{o}C for 3min according to the manufacturer‟s instructions as previously described elsewhere (Beissner et al. 2012).

Quantitative PCR.

The purified DNA were amplified by adding 2µl of the DNA to a master mix containing 8.6µl of Diethyl procarbonate (DEPC) treated water, 1µl each of forward primer IS2404 TF (5‟AAA GCA CCA CGC AGC ATC T 3‟), reverse primer IS2404 TR (5‟AGC GAC CCC AGT GGA TTG 3‟) and probe IS2404 TP2 (5‟ FAM-CCG TCC AAC GCG ATC GGC ABBQ‟3) (TibMolBiol), 4µl of 5x HOT FIREPol Probe qPCR plus containing DNA polymerase, 5x probe qPCR buffer, 15mM $MgCl_2$, deoxynucleotide triphosphates (SolisBioDyne, Estonia), 0.4ul of Exogenous IPC DNA (Invitrogen, UK) and 2µl of exogenous internal positive control (IPC) reagent containing corresponding primers and 5‟VIC-3‟ TAMRA labelled probe (Invitrogen, UK).

2µl of cDNA were amplified in master mix containing 8.6µl of Diethyl procarbonate (DEPC) treated water 1ul of MU16S TF (5'-cgatctgccctgcacttc-3'), MU16S TR (5'-ccacaccgcaaaagctt-3') and MU 16S TP (5' FAM-cacaggacatgaatcccgtggtc-BBQ 3') each (TibMolBiol), 4µl of 5x HOT FIREPol Probe qPCR plus, 2µl of exogenous internal positive control (IPC) reagent containing corresponding primers and 5‟VIC-3‟ TAMRA labelled probe (Invitrogen, UK) and 0.4µl of Exogenous IPC DNA (Invitrogen, UK). Amplification of DNA for IS2404 and cDNA for 16S rRNA target was carried out at 95^{o}C for 15 min, and then 40 cycles of 95^{o}C for 15 sec and 60^{o}C for 60 sec in a BioRad CFX 96 real time PCR detection system (BIORAD, Singapore). 10 fold dilutions of known amounts of cloned IS2404 (99bp) and 16S rRNA (147bp) (eurofins mwg operon, Germany) ranging from 300000 and 3 copies were included with PCR amplification for preparation of a standard curve and to know the limit of detection (LOD) of the assay. Templates were calculated based on the known mass of one template. Quantification of IS2404 and 16S rRNA serial standards were prepared in dilutions ranging from 3 copies to 300000 copies. The threshold cycles values obtained from the BioRad analyser were translated into bacillary load.

3.3.5 Lesion measurement Approach

The widest width of the lesions was measured at baseline and at week 4 using digital planimetry Silhouette (ARANZ Medical, Christchurch, New Zealand). Using the linear measurement approach, the ROH was calculated by first calculating the healing progress which is the change in widest width divided by 2 ($\Delta(W_w/2)$ in millimetres. The ROH was then calculated by dividing the healing progress by the number of weeks lesion was monitored which is 4 ((($\Delta(W_w/2)$)/t) (Gilman 2004).

3.3.6 Data Management and statistical analysis

The raw data generated from the study was entered in Microsoft excel and analysed using Graph Pad Prism 5.0 version and Microsoft excel. Excel was used to calculate the proportions of viable organisms with treatment. The rest of the analysis was performed using Graph pad prism. Descriptive statistics was used to obtain general description and the parameters used included median, minimum, maximum ranges and total numbers. Mann Whitney test was used to compare the quantities of bacillary load at baseline with the presence or absence of viable organisms and also to compare the relation between rate of healing at week 4 and detection of viable organisms. Fisher‟s exact test was used to test the correlation in the positive results of 16S rRNA assay with culture. A paired student‟s t test was used to compare the two transport media used. Survival analysis was done to know the extent of presence or absence of viable organisms on healing. P value < 0.05 was considered statistically significant in all the analysis.

CHAPTER 4

RESULTS

4.1 Patients characteristics

Patient demographics were obtained using the WHO BU01 form as described under the methods (Section page 37-38). Of 150 patients presenting to treatment centres with clinically suspected disease, 129 were confirmed as Buruli ulcer. Table 2 shows the characteristics of 129 patients of which 61 were males and 68 females with median age of 14 years (range 5 to 85 years). There were nearly equal proportion of pre-ulcerative lesion forms (44%), comprising of 29 (22%) nodules, 24 (19%) plaque, 4 (3%) oedema and 72 (56%) ulcerative forms of which 4 (3%) were oedema that has ulcerated. 113 (88%) of the lesions were less than 15 cm in maximum diameter (category I), but there were also 16 (12%) larger lesions (category III). 63 (49%) of the samples taken were FNA while 66 (51%) were swabs.

Table 2: Characteristics of Participants enrolled in the study

	No. of Participants N (%) n = 129
Age(years)	
Median(Range)	14(5-85)
Sex	
Male: Female	61: 68
Lesion Form	
Nodule	29 (22)
Plaque	24 (19)
Oedema	4 (3)
Ulcer	68* (53)
Ulcerated oedema	4** (3)
Category of lesion	
I (<=5cm)	57 (44)
II(5-15cm)	56 (44)
III(>15cm)	16(12)
Sample type	
FNA	63(49)
Swab	66(51)

*FNA samples were taken in 3 patients presenting with ulcers because they had no undermined edges.

**FNA sample was taken in 1 patient presenting with ulcerated oedema.

4.2 Diagnostic confirmation

Tests performed to confirm BU disease were ZN microscopy, culture, DRB-PCR and qPCR. Samples taken were either swab or FNA depending on the type of lesion the patient presented

with as described earlier (page 38-39, 40-44.). All patients had a positive result for quantitative PCR targeting insertion sequence (IS) 2404. 50 out of 125 (40%) were smear positive for AFB‟s, 44 (34%) subjects of 129 were culture positive and 104 of 127 (82%) were positive for DRB-PCR. Table 3 shows the distribution of positive and negative results of the laboratory results performed among the clinical forms and the lesion size. Patients presenting with pre ulcerative lesions had a significantly higher positive result compared to ulcerative forms in ZN microscopy (p=0.0426) and culture (p= 0.0007), however there was no significant difference in the DRB-PCR results among the pre ulcerative and ulcerative lesions (p=0.3565). The category of the lesion however, did not affect the positivity of all the laboratory tests performed. These results suggest that; qPCR was the most sensitive laboratory test for confirmation. District hospitals can however perform ZN microscopy first before sending to reference laboratory for PCR especially for pre ulcerative forms of the

lesion.

Table 3: Comparing the positivity of laboratory results with clinical forms and categories of lesion.

	Pre ulcerative (N=57)			**Ulcerative (N=72)**			**Lesion size up to 15cm (N= 113)**		**>15cm (N=16)**	
	Nodule (n=29)	Plaque (n=24)	Oedema (n=4)	Ulcerated Oedema (n=4)	Ulcer (n=68)	**p value**	Category I (n=57)	Category II (n=56)	Category III (n=16)	**p value**
Microscopy										
Positive	14	13	1	1	21	**0.0426[a]**	22	22	6	1.000[d]
Negative	15	9	3	3	45		33	33	9	
Not done	0	2	0	0	2		2	1	1	
Culture										
Positive	13	15	1	0	15	**0.0007[b]**	18	21	5	1.000 [e]
Negative	16	9	3	4	53		37	35	11	
Not done	0	0	0	0	0		0	0	0	
DRB-PCR										
Positive	26	21	2	2	53	0.3565[c]	50	44	10	0.2809[f]
Negative	3	3	2	2	13		7	12	4	

Not done	0	0	0	0	2	0	0	2	
qPCR									
Positive	29	24	4	4	68		57	56	16	
Negative	0	0	0	0	0	NA	0	0	0	NA

[a, b, c] Fisher"s exact test was used to compare pre ulcerative and ulcerative lesions microscopy, culture and DRB-PCR results.

[d, e, f] Chi square test was used to compare categories of lesions" microscopy, culture and DRB-PCR result

4.3 Optimizing 16S rRNA assay.

Three initial optimization experiments were performed to:

(i) establish whether the 16S rRNA gene could only detect viable organisms and not dead organisms.

(ii) determine the appropriate transport media needed to transport clinical sample for the extraction and quantification of combined 16S rRNA gene and insertion sequence 2404

(iii) determine the limit of detection using cloned standards of known concentration of 16S rRNA gene and insertion sequence 2404

4.3.1 Establishing the presence of 16S rRNA gene in viable Mu bacteria

To establish whether the 16S rRNA gene could only detect viable organisms and not dead organisms; 8 samples were used for this experiment and consisted of; 5 viable *M. ulcerans* dissolved in RNA protect, 1 viable *M. ulcerans* dissolved in RNA protect, Only RNA protect, 100 heat killed *M. ulcerans* and 10 heat killed *M. ulcerans*. RNA was extracted and quantified using 16S rRNA/ IS2404 RT qPCR as described in the method section (section pages 39-40, 44 - 47).

Table 4 shows that, 16S rRNA was detected in samples that contained 5 viable *M. ulcerans* and 1 viable *M. ulcerans.* However, 16S rRNA could not be detected in samples that contained 100 heat killed *M. ulcerans*, 10 heat killed *M. ulcerans* and only RNA protect. These results therefore suggested that, 16S rRNA could only be detected in viable *M. ulcerans* culture but not in killed *M. ulcerans* or RNA protect.

Table 4: Establishing the presence of 16S rRNA in viable *M. ulcerans*.

Sample type	16S rRNA detection	Ct*-value (16S rRNA
5 viable *M. ulcerans* bacteria	Positive	22.03
5 viable *M. ulcerans* bacteria	Positive	21.9
1 viable *M. ulcerans* bacterium	Positive	33.2
1 viable *M. ulcerans* bacterium	Positive	33.05
RNA protect	Negative	N/A
RNA protect	Negative	N/A
100 heat killed *M. ulcerans* bacteria	Negative	N/A
10 heat killed *M. ulcerans* bacteria	Negative	N/A

* Ct (threshold cycle); the cycle at which the 16S rRNA gene was detected

N/A means not amplified

4.3.2. Survival of *M. ulcerans* in PANTA and RNA protect transport media

To determine the appropriate transport media needed to transport clinical sample for the extraction and quantification of combined 16S rRNA gene and insertion sequence 2404

To achieve this objective, Buruli patient samples obtained from 18 patients were placed in tubes containing PANTA media and separately into tubes containing RNA protect (in duplicate). Samples were transported to KCCR for extraction and quantification of 16S rRNA gene as described elsewhere in the methods (Section pages 39-40, 44 - 47.).

Figure 5 shows that four samples transported in RNA protect were positive for 16S rRNA whilst their corresponding samples in PANTA media were not detected. There were 2 samples that were positive for 16S rRNA gene when transported in PANTA media, but their Ct values were high indicating that the quantity of viable organisms was low. Overall there was no

significant difference between the threshold cycle (Ct) values (p=0.1514, Wilcoxon matched pair test) when samples placed into PANTA media were compared with those placed in RNA protect from the same patient.

For this reason, RNA protect was the preferred media to transport patients sample for extraction and quantification of 16S rRNA gene.

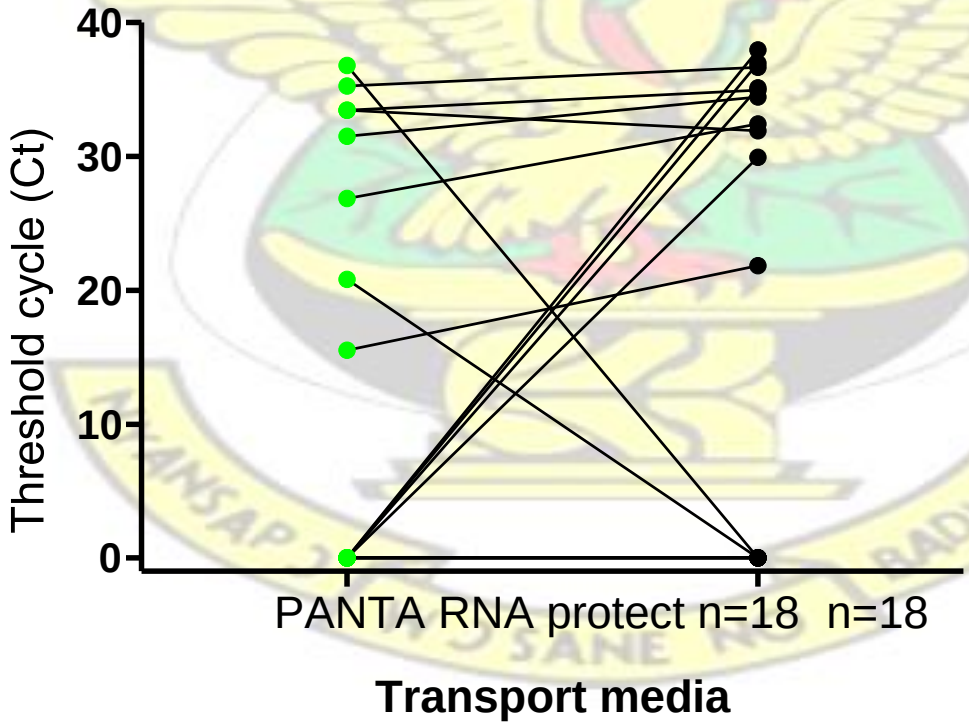


Figure 5: Determination of the survival of *M. ulcerans* viability using PANTA media and RNA protect.

4.3.3 Determination of the standard curve and analytical sensitivity of 16S rRNA qPCR and IS2404 qPCR.

The analytical sensitivity was determined as the lower limit of detection (LOD, lowest template concentration rendering amplification (Dijkman et al. 2010) for qPCR components.

To establish the analytical sensitivity of the qPCR 10-fold serial dilutions of cloned IS2404 (eurofins mwg operon, Germany) and cloned 16S rRNA templates (eurofins mwg operon, Germany) with known copy number were amplified.

The LOD was one template for 16S rRNA and one template for IS2404 (Table 5a, b and figure 6A and B).

This suggest that the assay was efficient and sensitive enough to detect one viable *M. ulcerans* by the 16S rRNA qPCR or one IS2404 molecule by the IS2404qPCR if present in the patient‟s sample extracted, for amplification.

Table 5a: Quantification of *M. ulcerans* 16S rRNA using cloned plasmid.

Sample ID	Copy Number	Ct value
Standard 1	3.07E+05	15.65
Standard 2	3.07E+04	19.32
Standard 3	3.07E+03	22.88
Standard 4	3.07E+02	25.99
Standard 5	3.07E+01	29.45
Standard 6	3.07E+00	32.75
Standard 7	2	34.43
Standard 8	1	35.57

Standard curve for 16S rRNA (A)

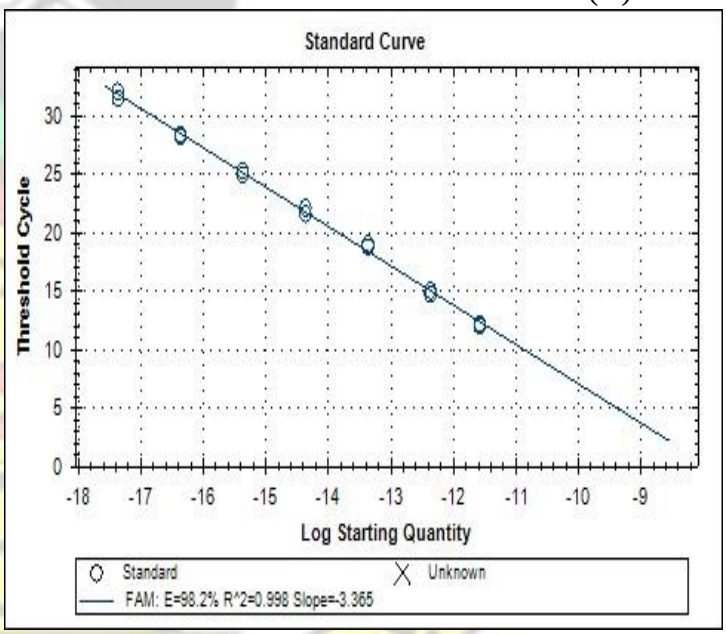


Figure 6A: Standard curve of *M. ulcerans* 16S rRNA using cloned plasmid. The efficiency (E) was 98.2%, R^2 was 0.998.

Table 5b: Quantification of *M. ulcerans* IS2404 using cloned plasmid.

Sample ID	Copy Number	Ct value
Standard 1	3.00E+05	14.9
Standard 2	3.00E+04	18.72
Standard 3	3.00E+03	22.26
Standard 4	3.00E+02	25.79
Standard 5	3.00E+01	28.81
Standard 6	3.00E+00	32.04
Standard 7	2	34.01
Standard 8	1	35.44

Standard curve for IS2404 (B)

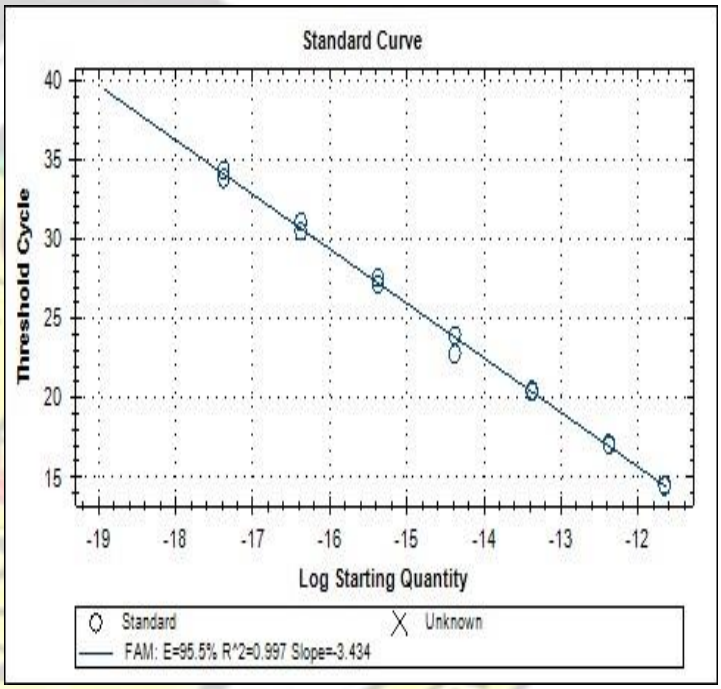


Figure 6B: Standard curve of *M. ulcerans* IS2404 using cloned plasmid. The efficiency (E) was 95.5%, R^2 was 0.997.

4.4 Determination of the clinical sensitivity of 16S rRNA RT qPCR viability assay using culture as a gold standard

To determine the sensitivity of the 16S rRNA RT qPCR in clinical practice, samples were obtained from 129 patients‟ samples before antibiotic commencement. One sample was processed for viability by culture on LJ slopes and another by the combined 16S rRNA RT /IS2404 real time PCR assay as described earlier (section pages 38-40, 44 - 47).

Assuming all untreated PCR positive lesions contained viable *M. ulcerans*, the 16S rRNA assay was 65% sensitive. 44 of 129 (34%) had viable organisms demonstrable by *M. ulcerans* culture on Lowenstein Jensen slopes. 42 out of 44 (95%) culture positives had viable organisms detected by *M. ulcerans* 16S rRNA detection at week 0 (Table 6).

The concordance between culture and *M ulcerans* 16SrRNA assay positive results was 95% (95% CI 85-99%). (Table 6). One of the two negative 16S rRNA result lesion was almost healed at week 4 so sample was not obtained to repeat the assay while the other negative 16S rRNA result was positive at week 8, indicating that the negative results could probably be due to sampling error or low bacterial load at the lesion sites. 48 further patients of 90 that had a positive result by the *M. ulcerans* 16S rRNA assay had a negative culture result. In this study the sensitivity of culture was 34%.

Table 6: Sensitivity of *M ulcerans* 16SrRNA assay detection

	Culture (No of patients) N=129		
***Mu* 16S rRNA**	Positive (n=44)	Negative (n= 85)	Sensitivity(95%CI) %
Positive (n=90)	42	48	95(85-99)
Negative (n=39)	2	37	

4.5 Determination of the proportion of patients with viable *M. ulcerans* with antibiotic treatment.

To determine the proportion of patients with viable *M. ulcerans* with antibiotic treatment, patients‟ samples were taken before antibiotic treatment commencement (week 0), during antibiotic treatment (weeks 4 and 8) and after antibiotic treatment (weeks 12 and 16). 16S rRNA/IS2404 RT qPCR assay was performed on these samples to know at what point the lesion became sterile as described in the method section page 38-39.

Figure 7 shows the proportion of lesions in which viable organisms were detected before (week 0), during (week 4) and after antibiotic treatment (weeks 8, 12 and week 16). Viable *M ulcerans* could be detected by combined 16S rRNA reverse transcriptase / IS*2404* Real-Time qPCR assay in 84 (65%) of samples at baseline (week 0). By week 4, 20 (15.5%) lesions had healed and a further 29 (22%) had undetectable viable organisms. However viable *M. ulcerans* were still detected in 43 (33%) unhealed lesions at week 8 and in 15 (12%) unhealed lesions at week 12, and in 3 (2%) unhealed lesions at week 16. At week 8, 36 (28%) lesions had healed while 31 (24%) had undetectable viable organisms. 41 (32%) lesions healed at week 12 and a further 48 (37%) had undetectable viable organisms. There were some lesions that were negative at early sampling times but became positive at the next sample taking. 6 (5%) of the lesions that were negative at week 0, became positive at week 4, and 12 (9%) of the lesions that were negative at week 4, became positive at week 8. Again 2 (1.5%) lesions that were negative at week 8 became positive at week 12.

These results suggest that, some lesions may be suitable for shorter antibiotic treatment while some may require longer antibiotic treatment.

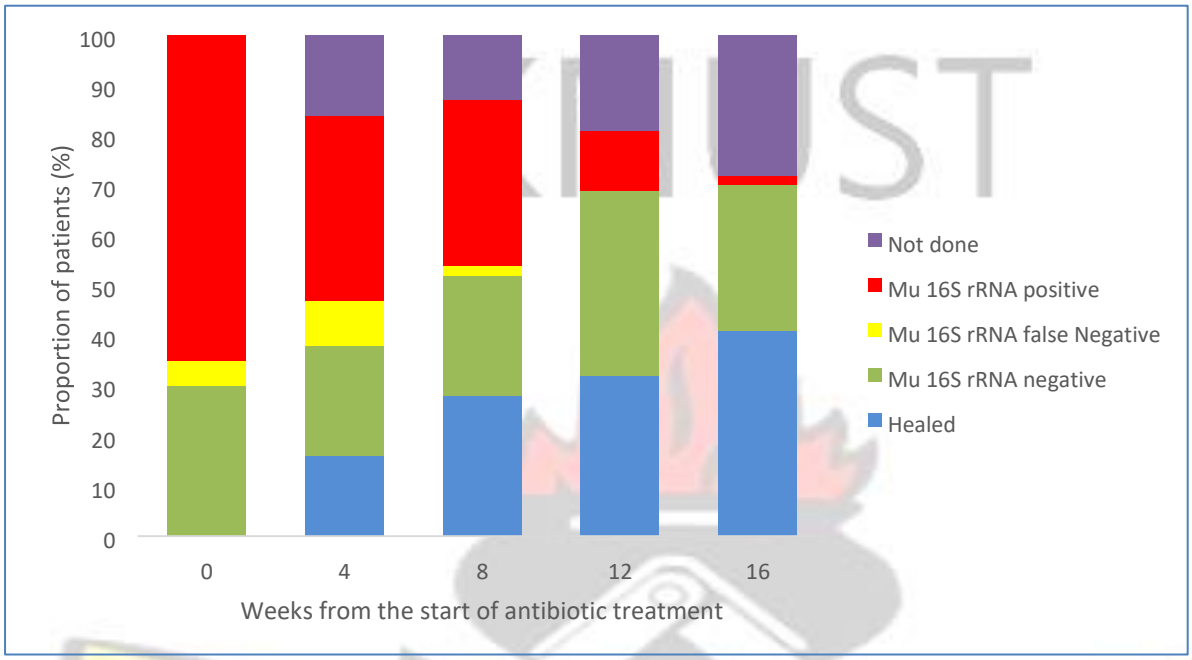


Figure 7: Proportion of patients with or without detectable viable organisms demonstrable by detection of *M ulcerans* 16S rRNA RT qPCR at baseline (week 0), during antibiotic treatment (week 4) and after antibiotic treatment (weeks 8, 12 and 16).

Patients whose lesions had attained 100% closure for ulcerative lesions or complete skin reepithelialisation were considered healed. Yellow indicates patients with negative 16S rRNA at the time point which was later positive. Purple indicates patients who did not attend on that occasion or who could not be sampled.

4.6 Disappearance of viable *M. ulcerans* after commencement of antibiotic treatment

To achieve this objective, the viability results from participants under section 4.6 were put into 5 groups; patients whose lesions did not have viable organisms at week 4, and those whose lesions had viable organisms only up to weeks 4 or week 8 or week 12 or week 16.

Figure 8 shows the rate of disappearance of viable *M. ulcerans* with treatment in the five different groups of patients. *M. ulcerans* 16S rRNA was detectable at baseline (week 0) but not at week 4 or subsequently in 27 (21%) (Figure 8a). Similarly, 22 (17%) patients had detectable viable organisms until week 8 (Figure 8b) or 29 (22.5%) until week 12 (figure 9c). 4 lesions negative at baseline, were positive at week 4 and 8 but not subsequently. Furthermore, 10 lesions that were negative at week 4, became positive at week 8 but remained negative thereafter (figure 8c). Again 12 (9 %) patients had detectable viable organisms until week 16 (figure 8d). One of the week 12 positives had undetectable viability at week 4 and week 8. Also 3 (2%) had detectable organisms even 8 weeks after end of antibiotic treatment (figure 8e).

The results show that patients have variable rate of *M. ulcerans* disappearance ranging from 4 weeks to possibly after 16 weeks.

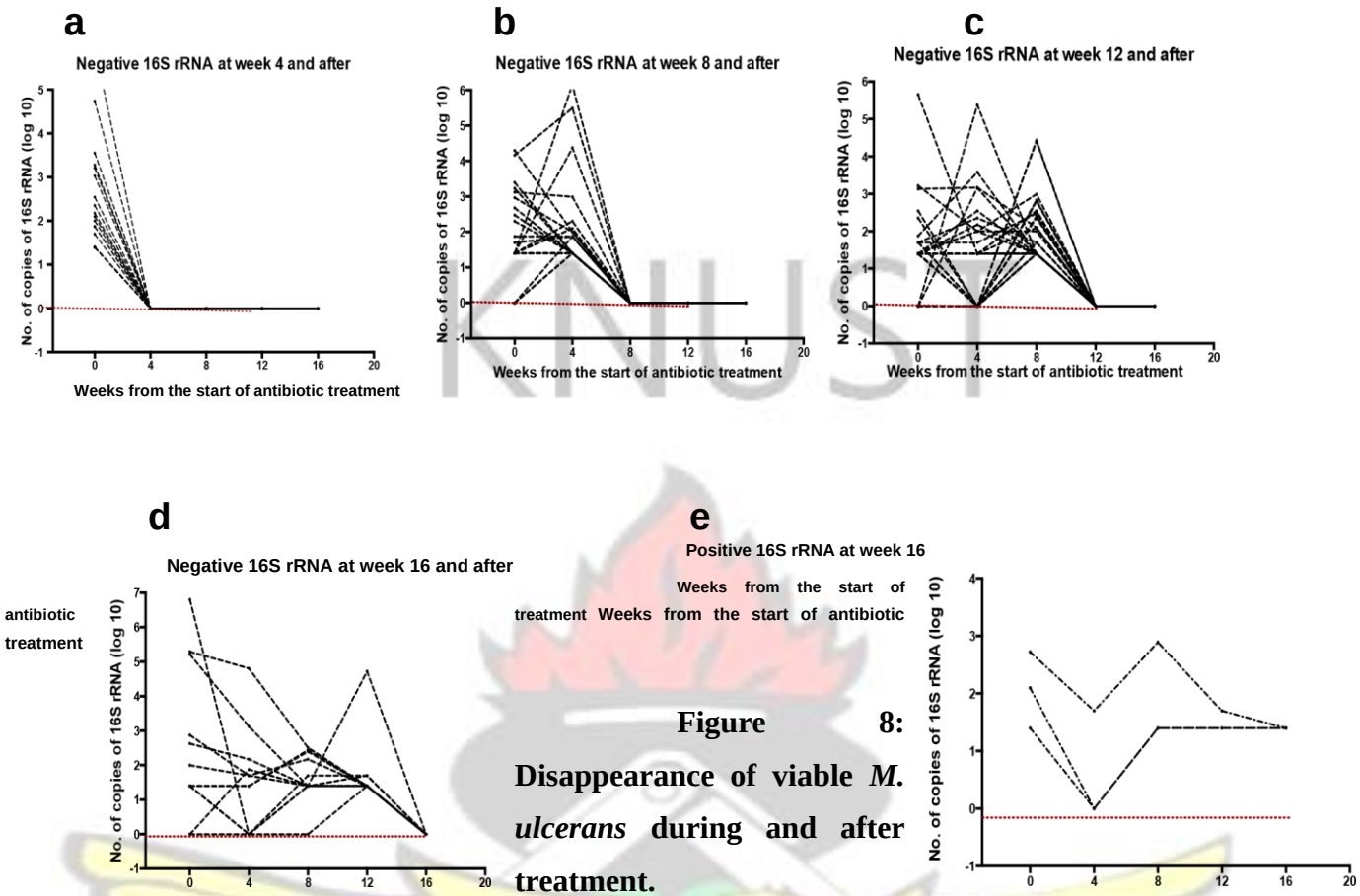


Figure 8: Disappearance of viable *M. ulcerans* during and after treatment.

Patients whose lesions had detectable viable organisms using 16S rRNA combined with RT qPCR for IS2404 at week 0 but had no viable organisms subsequently (a), patients with presence of viable organisms at week 4 but no viable organisms at weeks 8, 12 or 16 (b), patients with presence of viable organisms at week 8 but not at week 12 or 16 (c), patients with presence of viable organisms at week 12 but not at week 16 (d), patients with persistent viable organisms at week 16 (e).

4.7 Predicting the presence or absence of viable *M. ulcerans* using bacillary load at baseline.

To determine if the presence or absence of viable organisms after treatment initiation can be predicted from initial bacillary load, IS2404 quantified at baseline (week 0) for all participants were correlated with the presence of viable organism demonstrable by detectable 16S rRNA at week 4, 8, 12 or 16. Patients were grouped as patients whose lesions did not have viable organisms at week 0 or week 4, and those whose lesions had viable organisms only up to weeks 4 or week 8 or week 12 or week 16.

Figure 9a shows that, before antibiotic treatment, 36 participant lesions in which 16S rRNA was negative had a significantly lower bacterial load than the 93 lesions with detectable 16S rRNA (p=0.0004; Mann Whitney). This was consistently the case when lesions negative at baseline were compared to those having detectable viable organisms only at week 0 (n=27; p=0.006), only up to week 4 (n=22; p=0.0002), only up to week 8 (n=29; p=0.002), only up to week 12 (n=12; p=0.003) and up to week 16 (n=3; p=0.02). (Figure 9b).

These findings suggest that, the lower the bacillary load in the lesion, the less likely to detect viable *M. ulcerans* and vice versa

a **b**

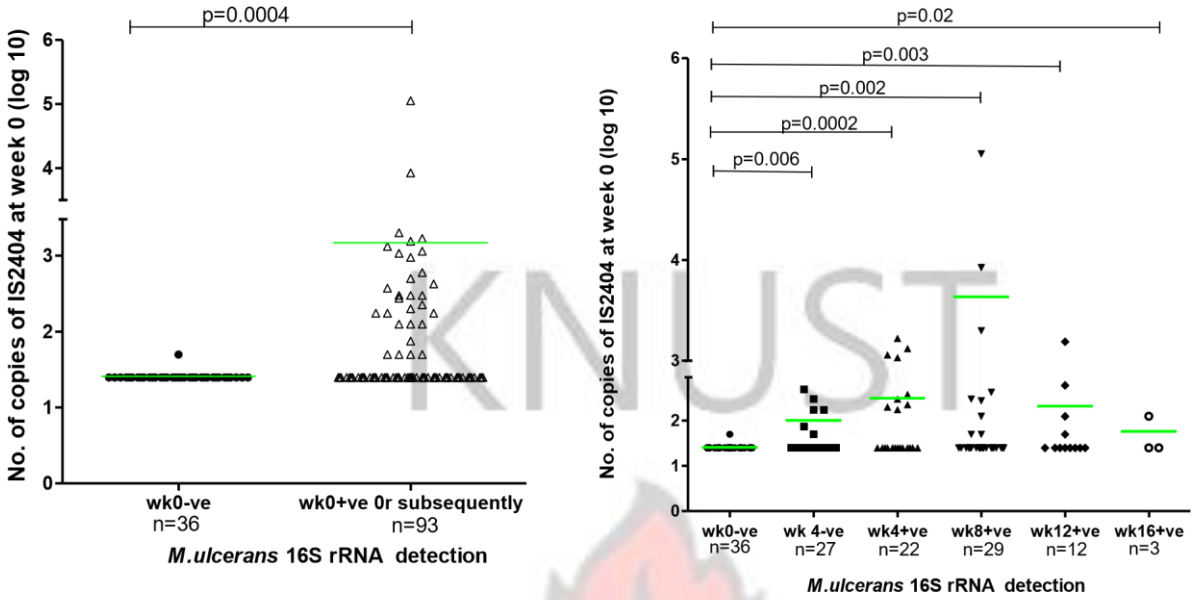


Figure 9**:** Comparison of initial bacterial load based on quantification of *M. ulcerans IS2404* by qPCR with absence of viable organisms determined by *M ulcerans*16S rRNA RT qPCR at baseline and week 4 or presence of viable organisms at week 4, 8, 12 and 16.

Molecules of IS2404 were generated from standard curve. Horizontal lines represent means and each point represents one patient.

Wk 0 -ve: Viable organisms were not detected at weeks 0, 4,8,12 and 16.

Wk 0 +ve or subsequently: Viable organisms detected at week 0 or subsequently

Wk 4 -ve: Viable organisms were detected only at week 0

Wk 4 +ve: Viable organisms were detected at week 4 but undetectable by week 8, 12 and 16 Wk

8 +ve: Viable organisms were detected at week 8 but not at weeks 12 or 16.

Wk 12 +ve: Viable organisms were detected at week 12 but not at week 16.

Wk 16 +ve: Viable organisms were detected at week 16

4.8 Detection of viable *M. ulcerans* and healing outcome

4.8.1 Establishing the relationship between detection of viable *M. ulcerans* and time to complete healing

In order to achieve this objective, the documented time to complete healing for all the 129 patients were compared with the absence or presence of viable organisms with treatment. Patients were grouped as patients whose lesions did not have viable organisms at week 0 or week 4, and those whose lesions had viable organisms only up to weeks 4 or week 8 or week 12 or week 16.

The results showed a significant difference between patients with undetectable viable organisms at baseline when compared with those with viable organisms only up to 4 weeks (p=0.001, Mantel cox test), 8 weeks (p=0.0001), 12 weeks (p=0.0004) and 16 weeks (p= 0.004) (Figure 10). Again, there was a significant difference in the time to healing between patients with undetectable viable organisms at week 4 when compared with those with viable organisms up to weeks 4 (p=0.03, Gehan-Beslow Wilcoxon test), 8 (p=0.005), 12 (p=0.01) and 16 (p=0.03) (Fig 10). At week 8, 58% and 44% of patients with undetectable viable organisms at week 0 and 4 had healed respectively compared to 9% of those with viable organisms at week 4 and none healed for patients with viable organisms at weeks 8, 12 or 16 (Figure 10). This was not attributable to lesion size because there was no significant difference in size of lesions with detectable 16S rRNA at week 12 or 16. Figure 11 shows the relationship between time to complete healing of individual lesions in relation to their initial size and the duration of 16S rRNA positivity. Lesions with persistently positive 16S rRNA were more likely to fall below the trend line for healing in those with negative 16S rRNA at baseline or week 4. The median time to complete healing was 8 weeks in patients with negative 16S rRNA at week 0 and 12 weeks in those negative at week 4, compared with 28 weeks for patients with detectable 16S

rRNA at week 4, 24 weeks for those positive at week 8 and 31 weeks for those positive at week 12.

The results suggest that the time to complete healing for patients with undetectable viable organisms at baseline was shorter when compared with those with viable organisms only up to 4 weeks 8 weeks 12 weeks and 16 weeks. Again, patients with undetectable viable organisms at week 4 after treatment initiation had shorter healing times than patients with viable organisms at weeks 4, 8, 12 and 16.

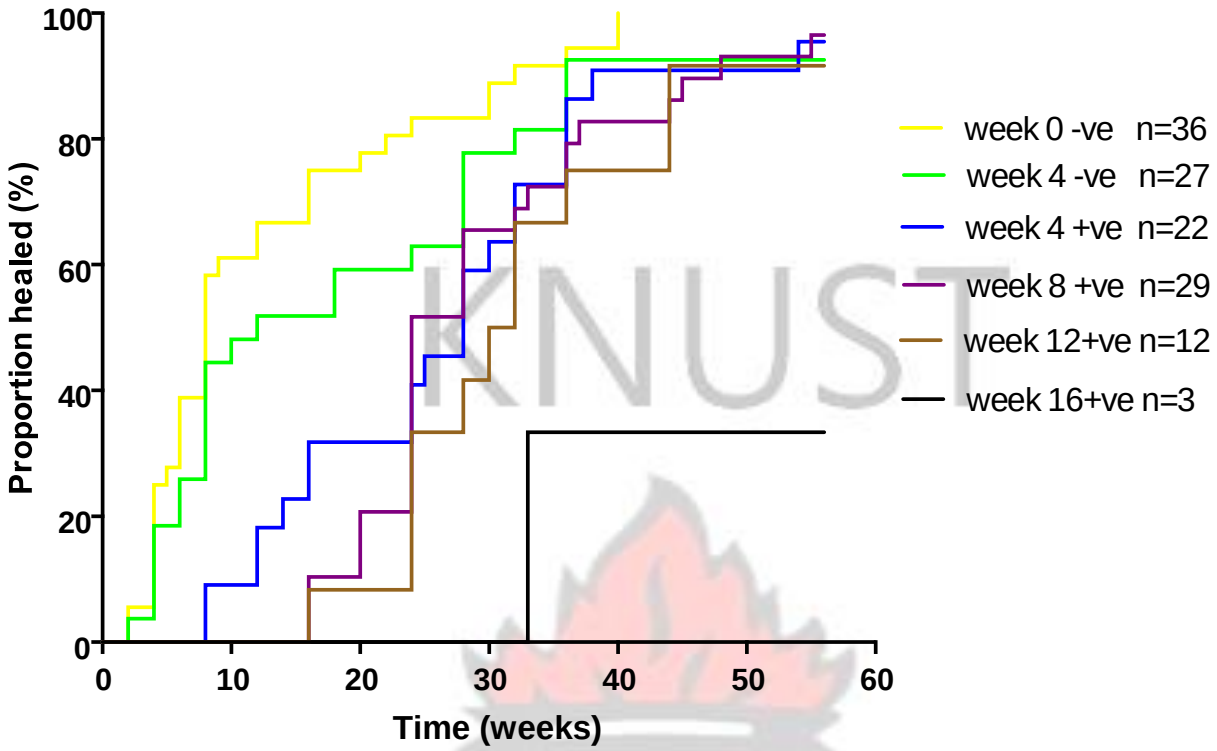


Figure 10: Detection of viable organisms and healing outcome.

Survival analysis curve of cumulative healing of lesions of patients with viable *M. ulcerans* detection. Log rank test was used to test for significance difference between survival curves.

Week 0-ve: Viable organisms were not detected at weeks 0, 4,8,12 and 16.

Week 4-ve: Viable organisms were detected only at week 0. One patient was lost to follow up and second had secondary osteomyelitis not healed at week 56.

Week 4+ve: Viable organisms were detected at week 4 but undetectable by week 8, 12 and 16.

One patient had healed at week 24 but lesion traumatized at week 28 and had not healed at week 56.

Week 8+ve: Viable organisms were detected at week 8 but not at weeks 12 or 16. One patient was lost to follow up.

Week 12+ve: Viable organisms were detected at week 12 but not at week 16. One patient was lost to follow up.

Week 16+ve: Viable organisms were detected at week 16. Two patients‟ lesions not completely healed at week 56.

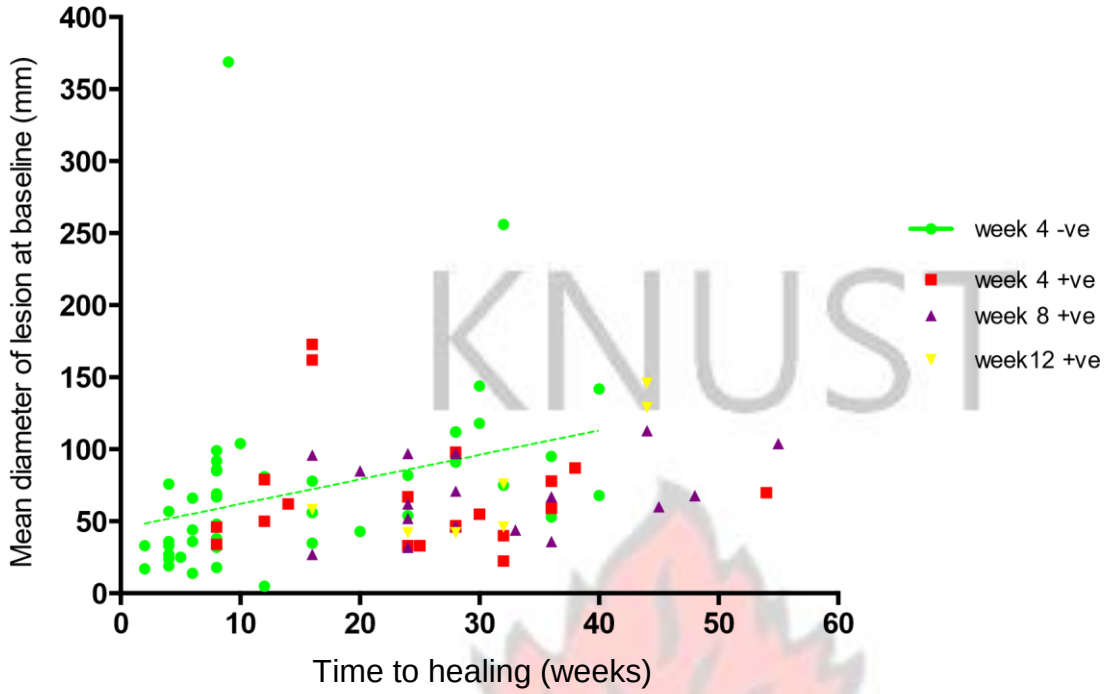


Figure 11: Time to complete healing is dependent on clearance of *M. ulcerans*

Each point represents one individual showing the correlation between initial size of lesion and the time to complete healing. The colours (red, purple and yellow) indicate the time at which samples for 16S rRNA were last positive. The green colour represents patients whose lesions were 16S rRNA negative before antibiotic treatment and at week 4 who had a low bacterial load (figure 4) with trend line for healing rate.

4.8.2 Effect of detection of viable M. ulcerans on rate of healing at week

All 129 patients wound sizes were measured before antibiotic treatment (week 0) and during antibiotic treatment (week 4) using digital camera (Silhouette Aranz). The rate of healing (ROH) was computed in millimetres per week by subtracting the mean diameter of the lesion in mm determined at week 4 from that determined at week 0 and dividing this result by 4. Mean

diameter is the mean of the maximum diameter and the greatest diameter at right angles to that as described elsewhere (page 38, 47). Presence of viable organisms was determined at weeks 0, 4, 8, 12 using the *M. ulcerans* 16S rRNA RT qPCR assay.

Figure 12 shows that, lesions with undetectable viable organisms at baseline had significantly higher rate of healing when compared with lesions with detectable viable organisms at week 8 (p= 0.002) and week 12 (p= 0.04) (Figure 12). Similarly, lesions with undetectable viable organisms at week 4 had significantly higher rate of healing when compared with detectable viable organisms at week 8 (p= 0.003) and week 12 (p= 0.04). This finding suggest that, the rate of healing is highest when viable organisms are not detected at baseline or become undetectable 4 weeks after starting antibiotic treatment. The presence of viable *M. ulcerans* has a negative effect on the rate at which the lesion healed per week, which in turn affect the overall healing success of BU lesions.

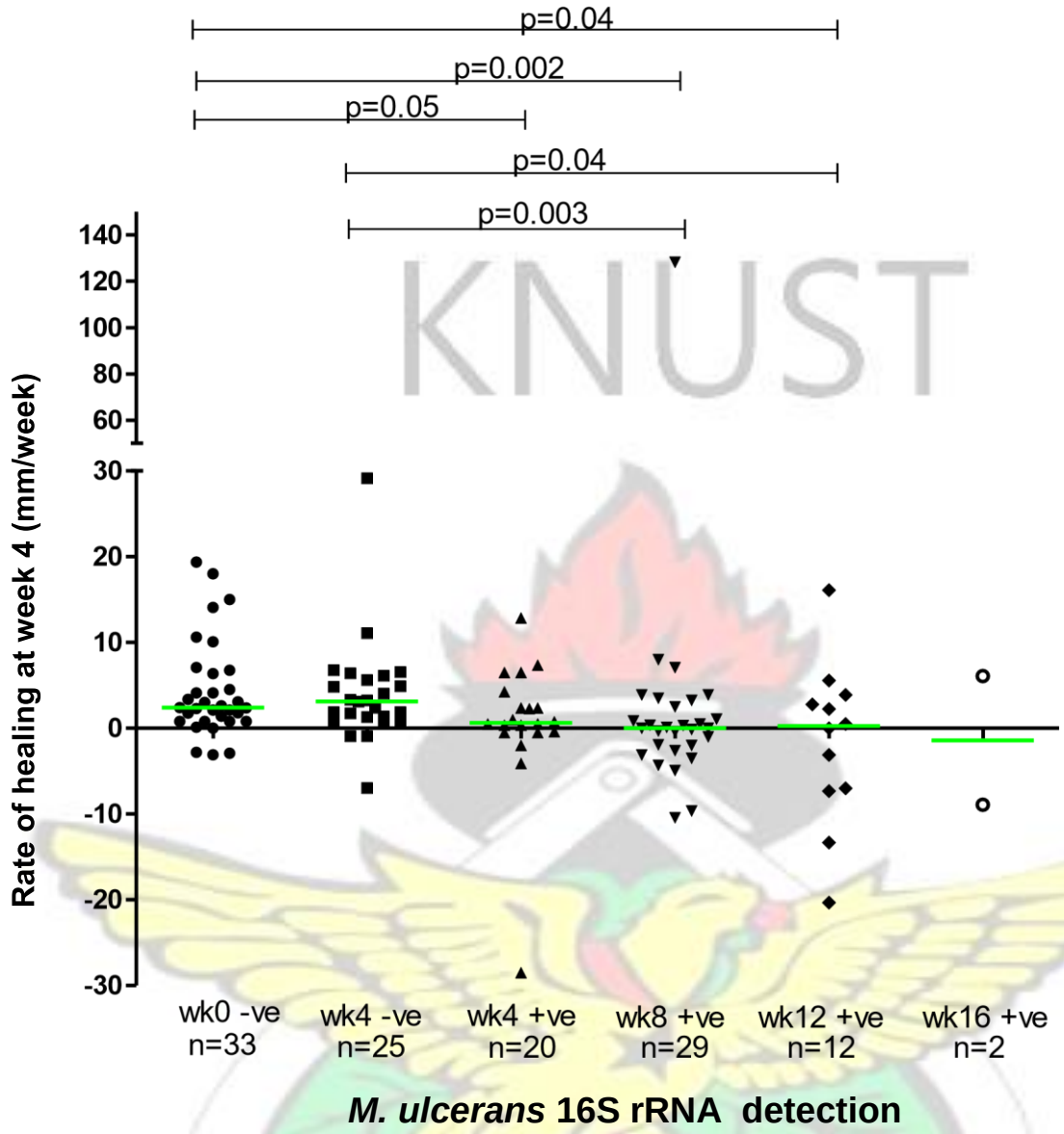


Figure 12: Effect of detection of viable *M. ulcerans* on rate of healing at week 4.

Horizontal lines represent medians and each dot represents each participant lesion. ROH was calculated for nodules, plaques and ulcers. 5 patients with oedema, 2 patients with negative viability at baseline and 1 patient with positive viability at week 4 did not have a ROH measurement.

CHAPTER 5

5.0 DISCUSSION

5.1 Optimization experiments.

In every experiment, there is the need to confirm the efficiency and improve the sensitivity of the assay before implementing the study. In this study, experiments were conducted to establish 16S rRNA as a viability marker. We also explored on the appropriate transport media for the assay and determined its analytical sensitivity.

Determination of bacterial viability is critical to monitoring the bactericidal activity of antibiotics in vitro and in vivo as well as assessing the presence of viable pathogens in samples. Molecular markers of viability have been shown to be useful for rapid appraisal of drug efficacy (Aellen et al. 2006). Microbiologists define viable organisms as those that can multiply to form colonies on solid media or liquid media. However, metabolic indicators such as membrane potential, the ability to generate reducing power or to undertake DNA synthesis are useful indicators of viability of cells in the natural environment (Sheridan et al. 1998). In this study, the argument whether 16S rRNA could be a useful marker of viability was investigated. Our study confirmed that, the 16S rRNA assay as set up by Beissner could only detect *M. ulcerans* in pure cultures but was not heat killed *M. ulcerans,* contrary to the suggestion that 16S rRNA was not a useful indicator of viability in *Escherichia coli* and that it was present in heat killed organisms (Sheridan et al. 1998). However, our finding was in line with another study, where 16S rRNA as a viability marker was able to differentiate between live and drug treated *Streptococcus gordonii* (Aellen et al. 2006). 16S rRNA when subjected to harsh conditions like physical, chemical or higher temperatures above 80^{o}C makes them loose their physical integrity and stability. This allows their destruction by

RNases (Aellen et al. 2006).

Transport media such as PANTA preserve the viability of organisms and help maintain the original ratio of organism in the clinical sample. RNA protect on the other hand, preserves the

RNA in the clinical sample from nucleases. For the purpose of our study, it was necessary to preserve the RNA from degrading in order to detect 16S rRNA. In this study, it was established that there was no statistical difference in using either PANTA or RNA protect as a transport media. However, it was interesting to note that, using RNA protect increased the detection of *M. ulcerans* 16S rRNA in patients‟ sample. Using PANTA media as a transport media also require stabilizing the RNA by adding RNA protect upon arrival at the laboratory (Beissner et al. 2012). The use of RNA protect as a transport media doubles its function as a stabilization solution for RNA thereby saving cost and time. Implementing RNA protect as a transport media is easy and it does not require extensive training. Again finding that using RNA protect increased the detection of 16S rRNA needs to be assessed in a larger sample size.

Analytical sensitivity is the smallest amount of substance or concentration of an analyte that can be reliably measured by an analytical procedure (Saah and Hoover 1997; Alankar and Vipin 2011). Our study showed an analytical sensitivity of 1 copy for both *M. ulcerans* 16S rRNA and IS2404. In this study there was slight improvement showing that the assay was more sensitive and efficient to use when compared to an earlier study that had analytical sensitivity of 2 copies for *M. ulcerans* IS2404 using cloned standard and 6 copies for *M. ulcerans* 16S rRNA using whole genome of *M. ulcerans.*

5.2 Sensitivity of 16S rRNA using culture as gold standard.

Detection of viable *M. ulcerans* by a combined assay for 16S rRNA and IS2404 by qPCR has been shown to be specific (Beissner et al. 2012). In this study the sensitivity of 16S rRNA was 65% when compared with another study which had a sensitivity of 83%. However, the sample size of 18 by the earlier study was rather small by contrast with 129 in this study, indicating that sample size plays a role in the sensitivity of an assay. The sensitivity of the culture in this study was 34% and this

confirms the limitation of using culture as a viability assay. In this study, 95% of the samples that were culture positive were also positive for 16S rRNA, indicating a high sensitivity of 16S rRNA. Although two samples that were positive for cultures were negative for 16S rRNA at week 0, the study admits that it could be due to sampling error. Therefore, 16S rRNA assay has been shown to be more sensitive than culture.

5.3 Detection of viable *M. ulcerans* and its disappearance after commencement of antibiotics

In this study, we have shown the rate at which viable bacteria were cleared from Buruli ulcer lesions during antibiotic treatment for 8 weeks. There were several striking findings, the first of which was that, after only 4 weeks of treatment, 20 (15.5%) lesions had healed or 29 (22%) had no detectable viable *M. ulcerans* in the lesion. If these lesions could be identified before or during the early stages of treatment it is possible that the course of antibiotics could be shortened substantially with considerable benefit to patients as well as a reduction in the cost of management. If lesions could be shown to be sterile at 4 weeks, it would be justified to abbreviate the course of antibiotics. This would need to be assessed in a clinical trial. The recommendation that patients receive treatment for 8 weeks was derived from the finding that early lesions excised after 2 weeks‟ antibiotic treatment were still culture positive but those excised after 4 weeks were all negative (Etuaful et al. 2005). Evidence for shorter treatment for selected patients is supported by recent data from Australia which showed that complete healing was achieved after 14 to 28 days of antibiotics in selected patients but this was a retrospective study and most of the patients had received early surgical treatment in addition to antibiotics (Cowan et al. 2015). Clearly there is a problem with sampling error in this study, which used small samples from wound swabs or FNAs, as demonstrated by the finding that a few samples became positive later having been negative at week 4. The presence of detectable

M ulcerans 16S rRNA after chemotherapy with rifampicin and streptomycin may be indicative sometimes of a persistent altered physiological state of *M ulcerans* such that it can reactivate to cause recurrent disease later. An analogous situation arises when *M. tuberculosis* is treated with rifampicin and pyrazinamide. Subpopulations consisting of dormant or semi-dormant, antibiotic tolerant persisters survive longest during chemotherapy and are difficult to kill with any new antibacterial drug. They are thought to be responsible for the prolonged period required for effective chemotherapy in tuberculosis (Yanmin, Coates, and Mitchiso 2003; Coates and Hu 2007; Hu et al. 2000) . In human *M. ulcerans* disease, lesions with persistent viable organisms still go on to heal, albeit slowly, presumably due to immune clearance of the organism whereas in tuberculosis, residual live organisms invariably cause disease. It is not known whether antibiotic tolerant persisters cause relapse in *M. ulcerans* disease but current evidence does not support this. In another study by our group, it was found that there is a partial effect of presence of viable organism on the immune system indicating that clearance of viable organism leads to immune system recovery.

5.4 Prediction of the presence or absence of viable M. ulcerans using bacillary load at baseline

The cost and skill requirement for the 16S rRNA assay would prohibit its routine use in most countries where Buruli ulcer is endemic but it may be possible to predict rapid responders in other ways. This is the subject of ongoing studies.

Several observations infer that the initial bacterial load may determine the time to total clearance of viable bacteria from BU lesions. A crude estimate of bacterial load was made by quantifying the number of copies of IS2404 using qPCR. A better estimate could be made by taking multiple samples or biopsies but this was not possible because it is considered ethically unacceptable. Given the limitations of the data it is not surprising that there was not a

significant correlation between initial bacterial load and the time for which viable bacteria remained detectable but figure 9 illustrates that they are probably related since the bacterial load in lesions with negative 16S rRNA at week 0 was significantly lower than that in all other groups.

5.5 Detection of viable M. ulcerans and healing outcome

In this study, there was a faster healing rate over the first 4 weeks in patients who were clear of active infection by 4 weeks (figure 6) than in the other patients and the time to complete healing was significantly longer in those with persistent infection independently of the initial lesion size (figure 5). There has been speculation about why some lesions heal slower than others despite appearing identical before treatment and the findings from this study suggest that persistent infection is an important contributing factor. At the end of the standard 8 weeks‟ period of antibiotic treatment, 52% of lesions had healed or were 16S rRNA negative but, surprisingly, 35% were still actively infected raising the question whether antibiotic treatment should be prolonged for a selected subgroup of patients. In another study, it was observed that some patients‟ lesions were not healing though they did not have viable organisms. This finding was speculated to be due to drug resistant M. ulcerans strain (Beissner et al. 2012). The finding that healing was delayed in the groups that had detectable viable *M. ulcerans* compared with those with negative 16S rRNA supports the idea of continuing antibiotics, perhaps for a further 4 weeks but against this is the fact that all the lesions healed eventually without further antibiotics. There is also the difficulty of identifying such lesions except within the context of a research study since this assay is expensive and impractical for routine use. At present a judgement would have to be made on purely clinical grounds. Recurrent *M. ulcerans* disease was fairly common before the antibiotic era when 618% of patients experienced relapse after surgical treatment alone (Amofah, Asamoah, and Afram-Gyening 1998; Debacker et al. 2005),

probably because there were residual *M. ulcerans* in apparently healthy tissue at resection margins (Rondini, Mensah-Quainoo, Junghanss, and Pluschke 2006). However, since observed antibiotic therapy was introduced, reported series have shown relapse rates below 2%. Individuals with a deeply compromised immune system such as those co-infected with HIV are at risk of relapse or overwhelming disseminated disease but this is more likely due to the need for a competent immune response to clear infection. The presence of *M. ulcerans* 16SrRNA indicates persistence of viable organisms in the tissue. This is supported by our previous findings that mycolactone can be detected in some patients after they finish antibiotics as can positive cultures for *M. ulcerans* (Sarfo, et al. 2010b). The presence of mycolactone, the toxin secreted by *M. ulcerans*, probably indicates that viable organisms are still extant but the pharmacokinetics of mycolactone is not known and it could persist after the demise of the organisms. Mycolactone is a powerful inhibitor of many growth factors and if it persists in a Buruli ulcer it is likely to retard healing. Further investigations are ongoing to identify lesions containing the toxin after the end of treatment in the present study.

CHAPTER 6

6.0 CONCLUSION AND RECOMMENDATION

6.1 Conclusion

This study has shown from the optimization experiments that, 16S rRNA can only be detected in viable *M. ulcerans* but not in heat killed *M. ulcerans*. This confirms that 16S rRNA is a viability marker for *M. ulcerans*. To increase the positivity of 16S rRNA, the study recommends using RNA protect as a transport media.

For the detection of viable M. ulcerans, 16S rRNA has been shown in this study to be more sensitive than culture. The study has further demonstrated that current antibiotic therapy for

BU disease is highly successful in most patients but it may be possible to shorten the treatment to 4 weeks in patients with a low initial bacterial load. Again the study has established that the quantity of bacteria load at baseline could be used to predict the presence or absence of viable organism before or after commencement of antibiotic treatment. On the other hand, evidence has been presented that persistent infection contributes to slow healing in other patients, probably those with a high bacterial load, who may need extended antibiotics treatment to increase the rate of healing.

6.2 Recommendation

There is the need for further work to be done to see if there is an association between *M. ulcerans* 16S rRNA and mRNA detection suggestive of transcriptional activity which would indicate that the organisms are in a replicative state.

There is the need to correlate the presence of mycolactone detection with 16S rRNA to know whether the slow healing of some lesions is not just due to the persistence of viable *M. ulcerans* but also persistence of mycolactone.

Using RNA protect as a transport media needs to be assessed in a larger sample size.

Establishing of predictive factors for healing using rate of healing at week 4 needs to be addressed.

Furthermore, there is the need to establish the presence of serum markers associated with slow healing.

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APPENDIX 1

Buruli ulcer clinical and treatment form – New case

BU 01.N

Health facility:

Name of health worker: _____

Date of diagnosis or admission (dd/mm/yy) ___/___/___

Date of complete healing (dd/mm/yy) ____/____/____

Name of patient _____

Age (yrs): _____ Sex : Male ☐ Female ☐

Weight (Kg): _____ Profession: _____

_____ ID#: Address (village or town): _____ District: _____

CLINICAL HISTORY AT DIAGNOSIS

Duration of illness before seeking care (weeks)_

Use of traditional treatment: Yes ☐ No ☐

Limitation of movement at any joint Yes ☐ No ☐

Previous treatment with streptomycin Yes (Duration in days:_____) ☐ No ☐

REFERRED BY:

Self -referral ☐ Village health worker ☐

Family member ☐ Former patient ☐

Health worker ☐ School teacher ☐

CLINICAL FORMS

Nodule (N) ☐ Plaque (Q) ☐

Oedema (E) ☐ Ulcer (U) ☐

Osteomyelitis (O) ☐ Papule (P) ☐

CATEGORIES

Category I: A single lesion ≤ 5 cm in diameter ☐

Category II: A single lesion 5 – 15 cm in diameter ☐

Category III: A single lesion >15 cm in diameter , multiple lesions, lesions at critical sites, osteomyelitis ☐

LOCATION OF LESION(S)

Upper Limb (UL) ☐ Abdomen (AB) ☐ Buttocks and Perineum (BP) ☐

Lower limb (LL) ☐ Back (BK) ☐ Thorax (TH) ☐ Head and Neck (HN) ☐

CRITICAL SITES

Eye ☐ Breast ☐ Genitalia ☐

LABORATORY CONFIRMATION

Specimen taken: **Yes** ☐ **No** ☐ Date specimen taken: ____/____/____

Specimen type: Swab ☐ FNA ☐ Biospy ☐

Results

ZN	Positive ☐	Negative ☐	Date ____/____/____
PCR	Positive ☐	Negative ☐	Date ____/____/____
Histo	Positive ☐	Negative ☐	Date ____/____/____

TREATMENT TYPE (Tick all applicable) Dressings ☐ Antibiotics ☐ Surgery (Date:_____/_____/_____) ☐ POD ☐

DOSAGES **Rifampicin:** _____(mg) **Streptomycin:** _____(mg) **Other (name):**_____:_____(mg)

Cross out each day (X) after administering the antibiotics; If antibiotics not taken, indicate with a symbol (Ø)

Day / Month	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	Total Doses

TREATMENT OUTCOME

1a: Antibiotic treatment completed ☐ 2a: Healed without surgery ☐ 3a: Healed without limitation of movement at any joint ☐ 4: Referred for further treatment ☐

1b: Antibiotic treatment **not** completed ☐ 2b: Healed with surgery ☐ 3b: Healed with limitation of movement at any joint ☐ 5: Lost to follow up ☐ Died ☐

Buruli ulcer clinical and treatment form – New case BU 01.N

Dosage Guide						
Weight of patient (kg	Rifampicin (300 mg/tablet)		Streptomycin (1gm/2ml)		Other: ……	
	Dose (mg)	Number of tablets	Dose (g)	Volume (ml)	Dose (mg)	Number of tablets
5 – 10	75	0.25	0.25	0.50		
11 – 20	150	0.50	0.33	0.70		
21 – 30	300	1.00	0.50	1.00		
31 – 39	300	1.00	0.50	1.00		
40 – 54	450	1.50	0.75	1.50		
>54	600	2.00	1.00	2.00		
If Streptomycin is contraindicated (e.g. pregnancy, previous treatment with streptomycin), contact the national programme manager or designated referral treatment centres.						

FOLLOW UP APPOINTMENTS AFTER TREATMENT	
DATE	**COMMENTS**
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