KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY

COLLEGE OF HEALTH SCIENCES

SCHOOL OF MEDICAL SCIENCES

CYTOKINE (IFN- γ, IL-5) RESPONSE TO INFECTION WITH *MYCOBACTERIUM ULCERANS*

A THESIS SUBMITTED IN FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF PHILOSOPHY, IMMUNOLOGY

In the

Department of Molecular Medicine

BY:

ALOYSIUS KODJO DZIGBORDI LOGLO

NOVEMBER,2016

KNUST

KNUST

DECLARATION

I, Aloysius Kodjo Dzigbordi Loglo, hereby declare that this work is the result of my own research, except for the references to other people's works, which have been cited and acknowledged accordingly, and that it has not been presented or submitted for any other degree. This work is submitted to Department of Molecular Medicine, KNUST for the award of a Master of Philosophy, Immunology.

Aloysius K. D. Loglo ……..…… ……..

(Student) Signature Date

Certified by:

Prof.(Mrs.) Margaret Frempong ……..…… ……..

(Supervisor) Signature Date

KNUST

Dr Richard Odame Phillips ……..…… ……..
(Supervisor) Signature Date

Certified by:

Prof. F. A. Yeboah ……..…… ……..

(Head of Department) Signature Date

DEDICATION

I dedicate this work to my parents Mr. Aloysius Kodjo Loglo and Mrs. Regina Lily Loglo, my sister Francisca Sena Loglo and my brother John Yao Loglo.

KNUST

ACKNOWLEDGEMENT

First and foremost, I give thanks to the Almighty God who gave me life and has sustained me through thick and thin to see this day.

The writing of this research work could not have been done without the help and cooperation of other people. I am, therefore, grateful to Prof.(Mrs) Margaret Frempong and Dr Richard Phillips who supervised this work to completion. I am also greatly indebted to my mentor, Richard O. Phillips, for exposing me to the world of medical research and project management. I also wish to say thank you to all others whose work are cited in this research and to those whose ideas sneaked in unrecognized.

I am very grateful to Dr Fred Stephen Sarfo, Dr Yaw Amoako, Dr Elliot Tannor, Dr Justice Boakye-Appiah for their assistance in patient recruitment and management. I also want to acknowledge the immense contribution of the clinical team at Agogo Presbyterian Hospital, Tepa Government Hospital, Dunkwa Municipal Hospital and Nkawie-Toase Hospital. Abass Kabiru Mohammed, Justice Abotsi, Samuel Mireku, Mathias Forson, Thomas Berko, George Amofa and Tuah Wilson.

Finally, I wish to say a big thank you to my team members at KCCR, Dr Michael Frimpong, Mrs Mabel Duah,Francisca Afia Sarpong,Leticia Kunna and Benedette Agbavor for your dedicated assistance.

ABSTRACT

Background: Buruli ulcer is a disease of the skin and soft tissue caused by the pathogen *Mycobacterium ulcerans*. Mycolactone, a lipid toxin has been identified as the main bacterial virulence factor for the disease. This toxin has been shown to be responsible for the immunosuppression and tissue necrosis which is characteristic of the disease. There is currently no vaccine for Buruli ulcer disease. In this study, the systemic immune responses of *M. ulcerans* infected patients to polyketide synthase domains were investigated. The effect of paradoxical reactions on the Th1 and Th2 responses of *M. ulcerans* infected patients were also evaluated.

Methods: This was a prospective observational study. All clinically suspected cases of Buruli ulcer were confirmed by standard PCR. Interferon gamma (interferon-γ) secretion following whole blood stimulation with polyketide synthase domains(PKS) using heparinised blood samples from patients with PCR confirmed Buruli ulcer, endemic controls and non-endemic controls were investigated using the ELISA. The effectiveness of 12 recombinant antigens and Ag85Aulc as potential vaccines were tested using the ELISA method. Also, concentrations of cytokines (interferon-γ, IL-5) in overnight supernates of whole blood assays in Buruli ulcer patients who developed paradoxical reaction, after stimulation with *M. ulcerans* sonicate antigens were measured using ELISA.

Results: The results show that responses to antigens were generally high (above 80% responders) with the exception of ACP3 where active Buruli ulcer cases had 47% and endemic controls 71% responders. The highest percentage responders in both participant groups were observed in reaction to Ksalt (100% responders) and ER (100%

responders) antigens. A higher proportion of endemic controls responded with higher interferon-γ responses than the Buruli ulcer cases to all the PKS domain antigens and to DNA encoding mycolyltransferase Ag85A of *M. ulcerans* (Ag85Aulc). There was no response to any of the antigens in all but one of the control participants from nonendemic areas. There was a trend to increasing interferon-γ responses with treatment; this was not statistically significant except for ACP2 and ATac2. Patients with nonulcerative lesions mounted generally higher interferon-γ responses than those with ulcerative disease. There was a positive but not statistically significant association between interferon-γ responses and time to healing of the patients to the PKS antigens.

Although patients who developed paradoxical reactions mounted a lower interferon-γ response [median (range) 754.32 (50.93-4190.4) pg/ml] at baseline compared to patients who had no paradoxical reactions [1246.93 (81.11-6969) pg/ml], there was no statistically significant difference between the two groups. By contrast, these two groups of patients elicited comparable median interleukin-5 (IL-5) response levels 35.61 (24.79-67.83) pg/ml vs 35.52 (11.82-993.90) pg/ml). Patients who developed paradoxical reaction mounted a consistently low Th1 and Th2 responses when compared with patients who did not develop paradoxical reactions. Th1 and Th2 responses of patients who developed paradoxical reaction improved with treatment.

Conclusion: These results suggests that the immune response of patients to PKS domains was lower compared to that of the contacts, which could be suggestive to the immunosuppressive effect of mycolactone on immune response. ER and Ksalt were the most immunogenic antigens. A vaccine made up of the most immunogenic plasmid DNA encoding mycolactone polyketide synthase domains and Ag85Aulc is an

interesting possibility that require further study. We have confirmed that paradoxical reaction has an effect on the immune response of patients.

KNUST

TABLE OF CONTENTS

KNUST

LIST OF TABLES

KNUST

LIST OF PLATES AND FIGURES

KNUST

ABBREVIATIONS AND ACRONYMS

ABBREVIATION	NAME
ACP	Acyl carrier protein
ATP	Acyltransferase with propionate specificity
ART	Antiretroviral therapy
Ag85	Mycolyl transferase antigen
Ag85ulc	Antigen 85A from *M. ulcerans*
ATp	Acyltransferase with propionate specificity
ANOVA	Analysis of variance
BCG	*Bacillus Calmette-Guerin*
BUD	Buruli ulcer disease
BU01	Buruli Ulcer treatment form
CLS	Cell Lysis Solution
DEPC	Diethylpyrocarbonate
DRB	Dry reagent base
DH	Dehydratase
ELISA	Enzyme linked immunosorbent assay
ER	Enoylreductase
FNA	Fine Needle Aspiration
Hsp65	Heat shock protein-65

KNUST

HIV	Immunodeficiency virus
IL	Interleukin
IS2404	Insertion sequence 2404
Interferon-γ	Interferon gamma
IFN- γ	Interferon gamma
IRIS syndrome	Immune reconstitution inflammatory
IQR	Interquartile range
KS	Ketosynthase
KR	Ketoreductase
LPS	Lipopolysaccharide
ML	Mycolactone
Mu sonicate	*Mycobacterium ulcerans* sonicate
Mu	*Mycobacterium ulcerans*
MM	*Mycobacterium marimum*
M. bovis	*Mycobacterium bovis*
PMA+Io ionomycin	Phorbol 1-myristate 1-acetate plus
PCR	Polymerase chain reaction
PR	Paradoxical reaction

KNUST

Non-PR patients	Patients who did not develop paradoxical reaction
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PKS	Polyketide synthase domains
RPMI medium	Rosewell Park Memorial Institute
TBE	Tris Borate EDTA buffer
Th1	T helper 1 Th2
T helper 2 tPA plasminogen activator TST	Tissue
Tuberculin skin test	
TNF-α	Tumor necrosis factor alpha
WHO	World Health Organisation

KNUST

CHAPTER 1

INTRODUCTION

KNUST

1.0 Background

Buruli ulcer is a skin disease which affects the soft tissues and is caused by infection with a slow growing pathogen, *Mycobacterium ulcerans (*Mu*)* (Demangel *et al*., 2009). Cases have been reported from 33 countries from tropical, subtropical and temperate climates in Africa, South America and the Western pacific regions respectively (WHO, 2014). Globally, over 5000 cases are consistently reported annually from 15 out of the 33 countries. Of the number, children below the age of 15 years are most infected with 48% from Africa, 10% from Australia, and 19% from Japan (WHO, 2014). Majority of the cases reported from sub-Saharan Africa are from poor rural communities (WHO, 2014). Even though, there is a strong association between incidence rate of the disease with stagnant or flowing water bodies, the specific mode of transmission remains elusive (Bratschi *et al.*, 2014). The disease usually manifests itself as a painless nodule, a firm plaque, or oedematous lesion which soon ulcerates with characteristic undermined edges (Etuaful *et al*., 2005). However, late presentation of lesion results in scars with contractures and disabilities when found over joints and at times amputation (Huygen *et al*., 2009).

Reliable diagnosis of Mu infection is of significant importance to the success of clinical studies of Buruli ulcer. The gold standard confirmatory test for Mu is Polymerase chain reaction (PCR) for the *IS2404* repeat sequence. This is undertaken in the Buruli ulcer laboratory at the Kumasi Centre for Collaborative Research in Tropical Medicine

(KCCR) on routine bases. Studies have been published proving the high sensitivity of PCR on DNA extracted from punch biopsies and subsequently from fine needle aspirate samples (Phillips *et al*., 2005; Phillips *et al*., 2009; Eddyani *et al*., 2009).

Treatment has shifted from surgery to antibiotic therapy with the combination of rifampicin and streptomycin for 56 days which is more efficacious in healing all lesions caused by Mu disease. This therapy has been reported to reduce the recurrence rate from 6-47% after surgery to 0-2% after antibiotic treatment (Chauty *et al*., 2007; Sarfo *et al*., 2010). Although antibiotic therapy was shown to be effective in treatment of Mu infection (Gordon *et al*., 2010), a phenomenon called paradoxical reaction, has been reported in some of the patients. In this situation, patients develop clinical deterioration of lesion(s) following initiation of antibiotic therapy. It is characterized by the rapid worsening of ulcers and progression of non-ulcerated lesion forms into ulcers. This can also be reported when there is a formation of a new lesion. Paradoxical reaction occur during antibiotic treatment (Beissner *et al*., 2010). This reaction have now been proved to complicate up to 20% of patients on antibiotic therapy for Mu infection and can cause significant secondary tissue necrosis (Wanda *et al.*, 2014). Exposure to mycobacterial antigens, a reduction in suppressor mechanisms, or improved host cell–mediated immunity have been implicated as responsible for this reaction (Nienhuis *et al*., 2012).

Research has shown that Human Immunodeficiency virus (HIV)-positive individuals on highly active antiretroviral therapy experience the worst episode of paradoxical reactions (Beissner *et al*., 2010). Buruli ulcer-HIV co-infection is an emerging management challenge for Buruli ulcer disease (O'Brien *et al*., 2014a). There is an increased incidence of several forms of Buruli ulcer disease in HIV infected individuals (Wanda *et al*., 2014). Co-infection with HIV is thought to result in more severe disease and slower healing times following treatment (O'Brien *et al*., 2014a). Nevertheless, the

World Health Organisation (WHO) recommends that all co-infected patients be actively screened for tuberculosis before commencing Buruli ulcer disease treatment and before starting antiretroviral therapy (ART).

Additionally,cotrimoxazole preventive therapy followed by the combination antibiotic therapy for 8 weeks and ART should be administered to HIV co-infected patients living in an area with established prevalence of malaria and or bacterial infections (O'Brien *et al*., 2014a).

Through research there have been some advancements over the years to better understand Mu infection with respect to immunology. Previous studies have used whole bacteria or burulin, which is a crude, heat-killed bacterial sonicate as well as culture filtrate proteins to elucidate the cellular immune response against Mu (Huygen *et al.*, 2009).

Patients with early Buruli ulcer disease elicited a delayed hypersensitivity response following intradermal injection of a crude preparation of burulin resulting in no immune response. However, 45 (76%) patients with healed lesions who were initially nonreactive, mounted favourable responses which is an indication of T-cell responsiveness (Dobos *et al*., 2000).

A Study was conducted on ten individuals with a history of previous Buruli ulcer disease and four patients with active disease in Australia. Peripheral blood mononuclear cells (PBMC`s) of test subjects was stimulated for 6 days with live *M. ulcerans* or live *Mycobacterium bovis*. Low levels of interferon gamma was produced compared to PBMCs from healthy tuberculin-positive study participants indicating that there was Tcell immune unresponsiveness to mycobacterial antigens (Gooding *et al*., 2001). In a similar study in French Guyana, PBMCs from patients were stimulated with whole

killed *M. ulcerans* or *M. bovis*. Five patients presenting with early nodules showed more profound Th1 cytokine profile while those with ulcers had a Th2 cytokine profile (Bourreau *et al*., 2004).

In Ghana, a larger study was carried out using an overnight stimulation of whole blood with *M ulcerans* sonicate among BU patients and the pattern of interferon gamma (interferon-γ) production was consistent with the hypothesis that the development of a Th-1 response is a slowly developing process (Phillips *et al*., 2006). Using similar methods, it was shown in a subsequent study that a gradual but significant recovery of the interferon-γ response emerged with antibiotic treatment at week 4 and week 8 compared with baseline (Sarfo *et al*., 2009).

Culture filtrate antigens 423 and 425 induced a similar pattern but lower interferon-γ response than those against Mu sonicate (Phillips *et al*., 2006). An essential fraction of the secreted proteins in mycobacterial culture filtrates is the mycolyl transferase antigen 85 (Ag85). Antigen 85 is a 30-32 kDa family of three proteins (Ag85A, Ag85B and Ag85C) (Fakult and Sch, 2009) , which all have a mycolyl transferase enzymatic activity needed for the integrity of the cell wall. This cross-reactive antigen has been researched in detail (Tanghe *et al*., 2008). Most of the healthy subjects infected with *M. tuberculosis* or *M. leprae* and in BCG vaccinated mice after stimulation with purified antigen-85 from BCG elicited a profound T-cell proliferation and interferon-γ secretion (Wiker and Harboe, 1992). Also, in patients with Buruli ulcer, their PBMCs produced lower interferon-γ responses against Ag85 purified from BCG compared to that from healthy BCG vaccinated participants (Gooding *et al*., 2001).

1.1 Rationale of the study

Currently, there is no vaccine for Buruli ulcer disease. Numerous studies have shown that *Mycobacterium bovis(M. bovis)* BCG vaccine is only protective for 6 months after vaccination in children (Nackers *et al*., 2006, Tanghe *et al*., 2007, Portaels *et al*., 2004, Huygen *et al*., 2009). A vaccine against Mu infection will be helpful in protecting children in areas with evidence of disease endemicity. It will have an effect on the length of antibiotic therapy by shortening the duration and ultimately preventing recurrence and severe forms of the disease. There is the need to understand the protective and favourable immune response to Mu infection.

Out of the family of mycobacteria, Mu is the only one that manufacture a lipid toxin as its main bacterial virulence factor. Three large polyketide synthases encoded by three large genes (mlsA1, mlsA2 and mlsB) localized on the 174kb pMUM001 virulence plasmid is responsible for biosynthesis of mycolactone (ML) (Stinear *et al*., 2004). These synthases are made up of unique modules, which each have a specific sequence of enzymatic domains. At non-toxic levels, mycolactone which is a polyketide-derived macrolide has the ability to locally restrain T-cell responses. The reason why mycolactone can cause the suppression of T-cell production is unclear, but it has been established that it can block cytokine responses at a post-transcriptional level and change both early signaling at the T cell receptor level by activation of the Src-family kinase Lck (Silva *et al*., 2009). ML is cytotoxic at higher concentrations (Phillips *et al.*, 2009).

Although patients with Mu infection present with some form of immunosuppression, through antibiotic therapy the whole process is reversed. Following a proliferation phase within macrophages, Mu induces the lysis of the infected host cells through its mycolactone synthesis and the bacteria become extracellular (Coutanceau *et al.*, 2005).

This extracellular phase suggests that humoral responses may be more important for protection against Mu than against other mycobacterial diseases, such as tuberculosis and leprosy.

The potential candidate for a Buruli ulcer vaccine should target mycolactone since Mu pathology is closely linked with this toxin, but its chemical structure makes it poorly immunogenic coupled with the inability to detect ML specific antibodies in both infected mice and humans. Nonetheless, antibodies against some of the polyketide synthase domains involved in the synthesis of ML have been identified in Buruli ulcer patients and healthy controls from an area where the disease is prevalent. This findings suggest that these domains are highly immunogenic (Pidot *et al.*, 2010). Recently published studies indicate that these domains exhibit immunogenicity in mice but their effect in humans have not been explored (Roupie *et al.*, 2014).

Interferon-γ is protective against *M. ulcerans* infection (Bieri *et al.*, 2016; Torrado *et al.*, 2010). Therefore, quantifying this cytokine in humans after exposure to the plasmid antigens will provide insight into their potential usefulness as vaccine candidates for Buruli ulcer disease (Roupie *et al.*, 2014).

Interleukin 5(IL-5) is one of the immunological markers that can be used to understand paradoxical reaction in Buruli ulcer patients. Apart from being an anti-inflammatory cytokine, it stimulates both activated B and T cell proliferation and differentiation of helper CD4+ T cells (Sarfo, 2014). The recovery of IL-5 responses in whole blood assays of patients reflect the reversal of mycolactone-mediated local immunosuppression and restoration of active humoral and cellular immune responses (Sarfo, 2014).

1.2 Study hypothesis

- We hypothesize that some of the plasmid DNA encoding mycolactone polyketide synthase domains are immunogenic and are able to induce an appropriate Th1 type immune response demonstrable by interferon-γ production by patients with Buruli ulcer disease and their household contacts.
- We hypothesize that patients with lower Th1 responses and Th2 responses were more likely to develop a paradoxical reaction with treatment.

1.3 Research questions

This thesis answers the following research questions:

- Are the plasmid DNA encoding mycolactone polyketide synthase domains (Plasmid antigens) immunogenic?
- Are the interferon-γ responses to the plasmid antigens specific to *M. ulcerans* infection?
- Does the interferon-γ response elicited by the patients change with treatment?
- Is there evidence of changes in interferon-γ response associated with different forms of the disease?
- Is the level of interferon-γ response to the plasmid antigens associated with healing of Mu lesions?
- How does the Th1 and Th2 responses at baseline compare for patients with Buruli ulcer disease with or without paradoxical reaction after initiation of treatment?
- Is there a unique trend in the development of the Th1 and Th2 immune response of patients with Buruli ulcer disease with or without paradoxical reaction after initiation of treatment?

1.4 Aim of the study

To study the systemic immune response of *M. ulcerans* disease patients to plasmid DNA encoding mycolactone polyketide synthase domains.

1.5 Specific objectives:

- To determine if the plasmid DNA encoding mycolactone polyketide synthase domains (Plasmid antigens) are immunogenic.
- To determine if the interferon-γ responses to the plasmid antigens specific to *M. ulcerans* infection.
- To determine if the interferon-γ response elicited by the patients change with treatment.
- To determine if the interferon-γ response change with different forms of the disease.
- To determine if interferon-γ response to plasmid antigens is associated with healing of *M. ulcerans* lesion
- To determine if there are differences in the Th1 and Th2 responses at baseline in patient with Buruli ulcer disease with or without paradoxical reaction after initiation of treatment
- To determine if there is a unique trend in the development of the Th1 and Th2 immune response of patients with Buruli ulcer disease with or without paradoxical reaction after initiation of treatment.

CHAPTER 2

LITERATURE REVIEW

2.1 History of the disease

Buruli ulcer disease is a skin infection which also affects the soft tissues and is caused by *Mycobacterium ulcerans (M. ulcerans).* It was first identified in Australia (Demangel *et al.,* 2009). First case of Buruli ulcer ulcer disease was reported in a farming community in Bairnsdale district in South-east Australia. The ulcers identified were painless among patients from the community. Thirteen years later, researchers in Australia identified the aetiological agent of the then Bairnsdale ulcer; which was named *M. ulcerans* (Maccullum, 1948).

In Africa, the first case of *M. ulcerans* infection was reported in the 1960`s. Many cases of the infection were reported in Uganda especially the Buruli county which led to the disease being called Buruli ulcer. Currently, the disease is more prevalent in West and Central Africa. It is also widespread in subtropical countries (Demangel *et al.,* 2009). Mikoshiba and others in 1982 identified the first case in Japan (Yotsu *et al*., 2012). The patient had no international travel which implied that it was an endemic infection. Another research team identified a close resemblance of the *M. ulcerans* species to the pre-existing one. In 1989, the *M. ulcerans* identified subspecies from Japan was called *M. ulcerans* ssp. Shinsluense (Yotsu *et al*., 2012).

2.2 Epidemiology

Buruli ulcer is the third commonest neglected tropical disease out of the family of mycobacteria (WHO, 2014). Thirty three countries from tropical, subtropical and temperate climates in Africa, South America and Western Pacific regions report a greater number of the cases (WHO, 2014). Annually, over 5000 cases are reported globally from 15 countries. Children below the age of 15 years in poor rural communities are the most infected (WHO, 2014). Out of the number of cases reported

annually, a proportion of 48% in Africa, 10% in Australia, 19% in Japan are children below the ages of 15 years (WHO, 2014).

According to the WHO, in 2014, 12 out of the 33 countries reported 2200 new cases which indicates 50% reduction compared to 5000 cases reported in the year 2009. There is no documented reason for the reduction in numbers (WHO, 2014). Majority of the cases are reported from sub-Saharan Africa by some countries like Cote d`Ivoire, Ghana, Benin, Cameroun and Democratic Republic of Congo (WHO, 2014). After Cote d`Ivoire, Ghana is the next country to report majority of the cases (WHO, 2014) with the disease being mostly found in the Greater Accra region along the tributaries of the Densu River (Adu and Ampadu, 2015). It can also be found in the Asante Akim North, Amansie West, Atwima Mponua, Ahafo Ano districts of the Ashanti region.The disease is also prevalent in the Asutifi district in Brong Ahafo and Upper Denkyira district of the Central region (Adu and Ampadu, 2015).

In terms of prevalence rate of the disease, a study in Benin reported a prevalence rate of 21.5/100000 per year which was higher than detection rate of people infected with tuberculosis or leprosy (Portaels *et al*., 2004).

Furthermore, Amofa et al established that highly endemic districts in Ghana had an estimated point prevalence rate that was as high as 150.8/100000 individuals (Amofah *et al.*, 2002) with prevalence per 100000 population in the Amansie West, Asante Akim north and Upper Denkyira districts of 151,132 and 115 respectively (Amofah *et al.*, 2002).

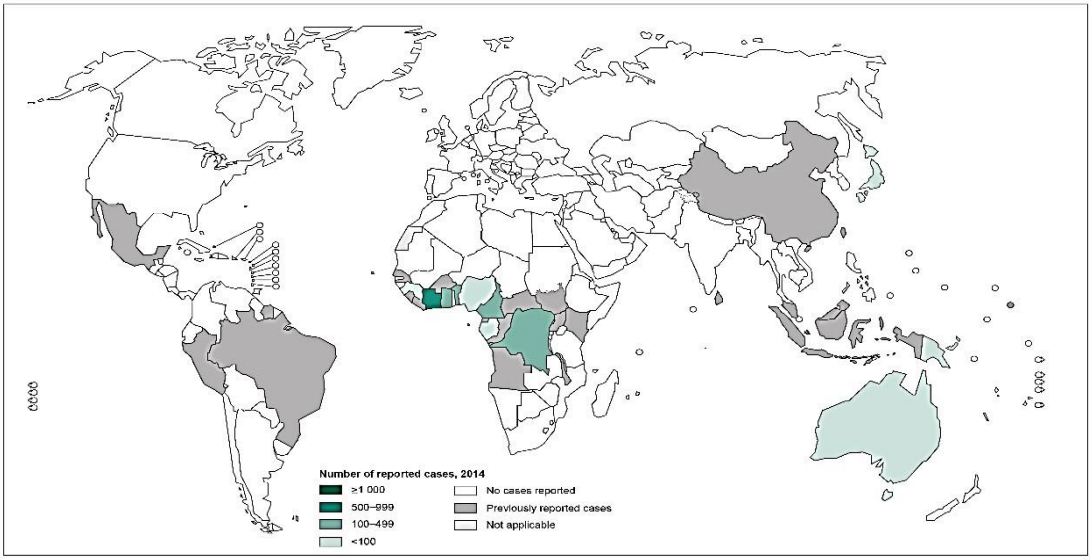


PLATE 1.0 WORLDWIDE DISTRIBUTION OF BURULI ULCER DISEASE.

SOURCE: WORLD HEALTH ORGANIZATION 2014.

2.3 Mode(s) of transmission

The method of transmission of *M. ulcerans* infection has not been fully elucidated. However, there have been some improvement in understanding the mode(s) of transmission of *M. ulcerans* infection based on studies carried out in countries such as Australia, Mali, Cameroon, Benin (Röltgen & Pluschke, 2015; Bessis *et al.*, 2014; Bratschi *et al.*, 2014; Garchitorena *et al.*, 2014; Ebong *et al.*, 2012; Merritt *et al.*, 2010; Boyd *et al.*, 2012; Fakult, 2012 ; Coleman *et al.*, 2004). Buruli ulcer disease can be found in places where there are water bodies (Willson *et al.*, 2013). Even though there is an established link of the occurrence of the disease with stagnant or flowing water bodies, the medium of transmission seems elusive (Bratschi *et al.*, 2014).

As a result of its slow growth rate, culturing *M. ulcerans* samples have been difficult. But, a major development occurred with the first PCR probes for *M. ulcerans* based on the detection of IS2404.This was preceded by the discovery of *M. ulcerans* DNA in environmental samples including detritus, soil, biofilms, water filtrates, fish, frogs,

snails, insects and invertebrates (Merritt *et al.*, 2010). Another study in Ghana and Benin identified many IS2404 positives from environmental samples in both environments where the Buruli ulcer was proven to be prevalent and in places it does not exist (Willson *et al.*, 2013).

A previous study suggested that fishes were passive reservoirs of the *M. ulcerans* as they may concentrate the bacterium in their gills and intestines. Gills and intestines of several fishes were found to be positive for IS2404, which is specific for Mu (Willson *et al.*, 2013). Another group also used enoyl reductase(ER) PCR targets for the ER domain of mls A, the polyketide synthase that encodes the lactone core of mycolactone. ER-PCR is less sensitive but more specific than IS2404 PCR because its target has four copies (Willson *et al.*, 2013). The ER positivity rate was 39% which was higher in this study than IS2404 positivity rates in 2004 by Eddyani *et al* and Kotlowski *et al* (10 and 20% respectively) (Eddyani *et al*., 2004; Kotlowski *et al*., 2004; Willson *et al.*, 2013). However, the challenge was that although *IS2404* PCR detects DNA, the death of infected organisms leads to the release of *M. ulcerans* DNA into the environment where it may stick to a number of substrates. As such, PCR methodology detects DNA, but it does not show definite proof for the presence of intact bacteria in a matrix. Eventually, *M. ulcerans* was cultured from an aquatic water bug in Benin (Portaels *et al*., 2008).

In addition, predaceous aquatic organisms have been implicated as the host of *M. ulcerans* in Australia (Marsollier *et al*., 2002). Willson *et al* in 2013, suggested that the potential reservoir for *M. ulcerans* in West Africa which needs careful examination was the adult amphibians, and *H. binaculatus* may be useful as an indicator of habitats likely to support mycolactone producing mycobacteria. A feeding linkage within aquatic habitats was established considering that *M. ulcerans* has been detected in biofilms on aquatic plants and in many different aquatic invertebrate taxa (Willson *et al*., 2013).

Aquatic bugs (Hemiptera) and water strider (Hemiptera:Gerridae,Gerris sp.) were shown to be possible reservoirs of *M. ulcerans* through isolation of pure cultures in a study undertaken in Cameroon (Ebong *et al*., 2012). Others also showed that, *M. ulcerans* could be acquired from feeding on inoculated insect prey (a blow fly maggot), transmitted to mice via biting, and that infected mice eventually developed clinical Buruli ulcer (Merritt *et al*., 2010). These experiments actually are in line with the idea that predaceous aquatic insects may play an essential role in maintaining *M. ulcerans* within food webs in the aquatic habitats, but their role in actual transmission to humans remains elusive. A study in Benin reported the possibility of non-genetic familial person-to-person infection (Merritt *et al*., 2010;Sopoh *et al*. 2010) by contrast with another study that did not support person-to-person transmission (Coleman *et al*., 2004).

2.3.1 Risk factors

Buruli ulcer disease is characterized by highly focal transmission clusters. *M. ulcerans* infection revealed a number of factors linked with the disease. In Africa and Australia people residing or working near water bodies, poor wound management, not wearing protective clothing are at a greater risks for contracting *M. ulcerans* infection (Röltgen and Pluschke, 2015).

In Australia, a case-control study demonstrated that exposure to mosquitoes is a risk factor. It was reported that more patients as compared to control individuals recalled being bitten by mosquitoes on the lower limb (Röltgen and Pluschke, 2015). In Ghana, swimming in rivers on a habitual basis was identified as the only significant water associated risk factors for *M. ulcerans* infection (Coleman *et al*., 2004). Conversely, a study in Cote d`Ivoire asserted that swimming in rivers was not associated with development of Buruli ulcer (Marston *et al*., 1995).

2.4 Clinical manifestation

There are two clinical stages of *M. ulcerans* infection: preulcerative and ulcerative stages of the disease (Boyd *et al*., 2012). The pre-ulcerative forms of the disease are nodule, plaque and oedema.

Initially the lesion is a painless subcutaneous nodule (Plate 2.0), usually less than 5 cm in diameter and under the skin, which breaks down centrally after days to weeks forming an ulcer with characteristic undermined edges (Plate 5.0). This makes the lesion look smaller than its true size. Buruli ulcer lesions are painless except for when there is a secondary bacterial infection (Wansbrough-Jones and Phillips, 2006). A plaque is a firm, raised lesion of more than 3 cm in diameter with ill-defined edges (Plate 3.0). Patients may also present with the oedematous form, which is diffuse, nonpitting swelling with ill-defined edges that affects large areas of their limb or other parts of their body (Plate 4.0). Oedema may also develop after ulceration. The skin surrounding the lesion may be reddened or discoloration may be observed. Usually, there are no general symptoms but mild fever may occur with edematous forms (Yotsu *et al.*, 2015; WHO, 2014).

The last stage of the disease is characterized by the destruction of the skin due to necrosis of the dermis and subcutaneous adipose tissue (Yotsu *et al.*, 2015; WHO, 2014). This is the ulcerative stage where deep ulcers extend into the subcutaneous adipose tissue, with undermined wound edges and thick necrotic tissue at the wound base (Plate 5.0) (Yotsu *et al*., 2015).

Buruli ulcer lesions are often present on uncovered areas of the body, such as the limbs and the face. Over 50% of the lesions are situated on the lower limbs (Kumar *et al.*, 2015). Apart from the skin the disease can affect the bone and cause osteomyelitis

(Yotsu *et al.*, 2015; WHO, 2014). In most cases the disease does not progress in a systematic order, in that, there are patients whose lesions can progress from nodular stages directly to ulcer or from a plaque to an ulcerative stage.

The World Health Organisation has standardized the way of classifying the lesions based on their diameter and multiplicity (WHO, 2014). Category I(one) is made up of a single lesion less than 5 cm in diameter. A single lesion measuring between 5 and 15 cm in diameter is considered to be in category II(two), whereas category III(three) may include a single lesion more than 15 cm in diameter or multiple lesions or lesion(s) at a critical site (eye, breast, and genitalia) and osteomyelitis. Category III is further subdivided into the following: 3a: a single lesion more than 15 cm in diameter with osteomyelitis; 3b: lesion(s) at critical sites (eye, breast, genitalia); and 3c: small multiple lesions (WHO, 2014) (Kumar *et al*., 2015).

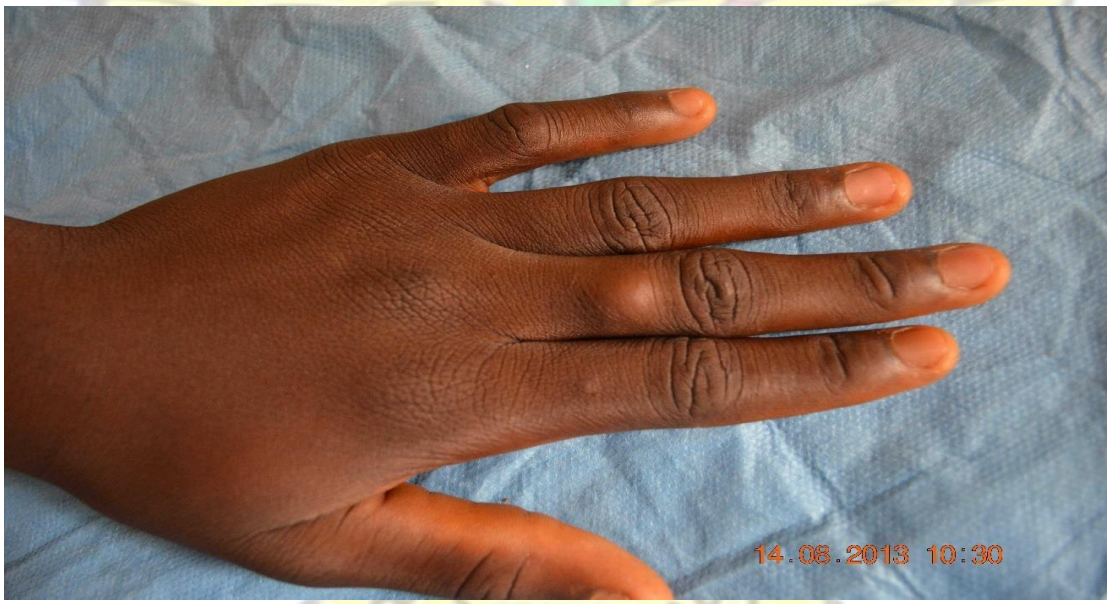


PLATE 2.0 A NODULAR LESION FORM PRESENTED AT 8 WEEKS LOCATED ON THE UPPER LIMB OF A 12-YEAR-OLD GIRL.

SOURCE: AGOGO PRESBYTERIAN HOSPITAL, GHANA.

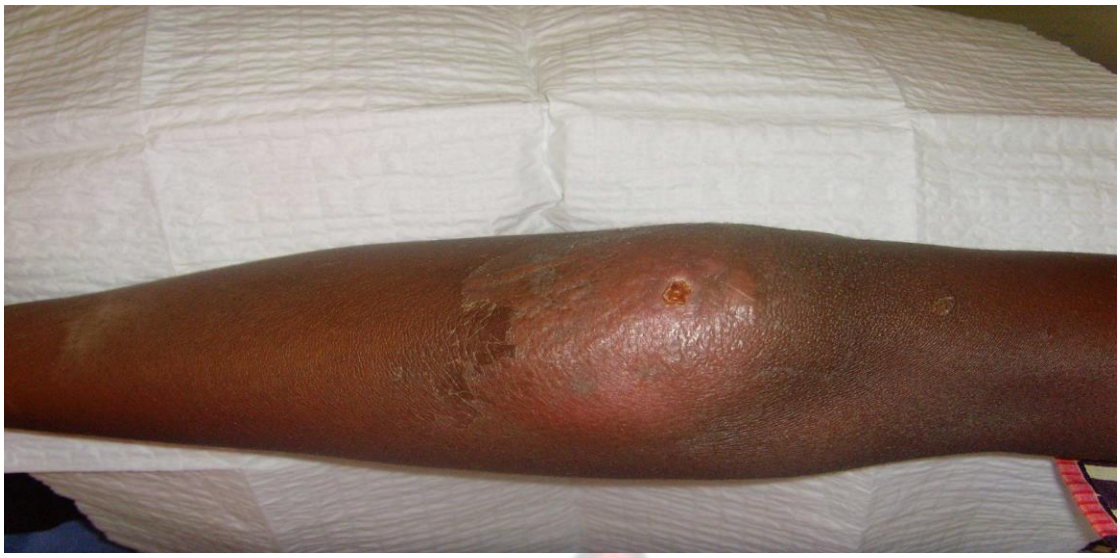


PLATE 3.0 A PLAQUE PRESENTED AT WEEK 0(BASELINE) LOCATED ON THE UPPER LIMB OF A 16-YEAR-OLD BOY.

SOURCE: TEPA GOVERNMENT HOSPITAL, GHANA.

PLATE 4.0 TYPICAL OEDEMATOUS FORM PRESENTED AT WEEK 0(BASELINE) LOCATED ON THE LOWER LIMB OF A 13-YEAR-OLD MALE.

SOURCE: AGOGO PRESBYTERIAN HOSPITAL, GHANA.

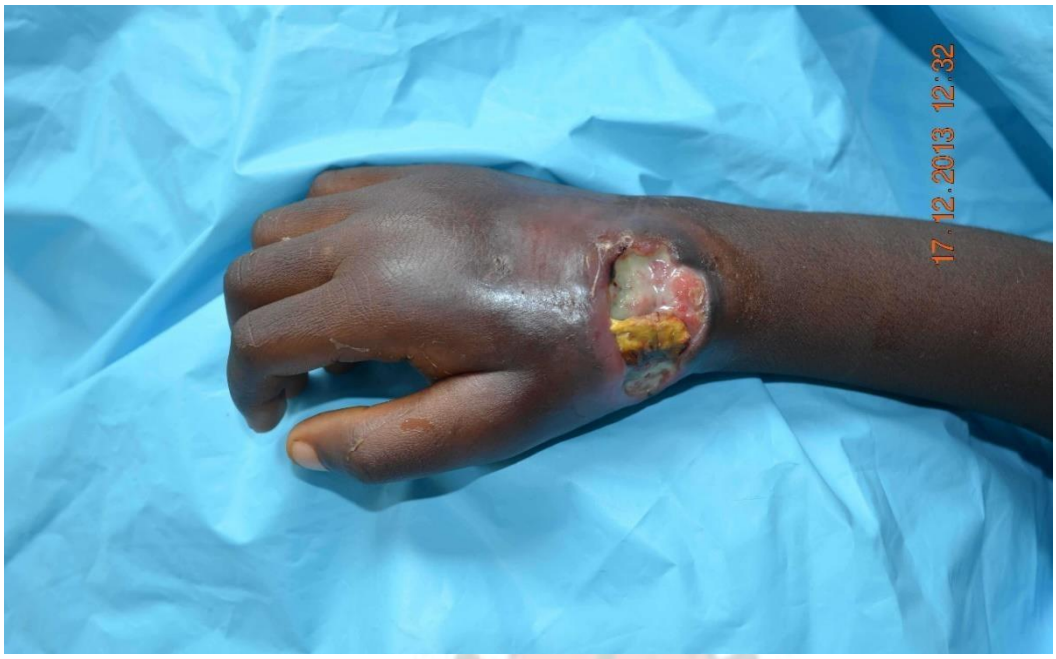


PLATE 5.0 AN ULCERATIVE LESION FORM PRESENTED AT WEEK 0(BASELINE) LOCATED ON THE UPPER LIMB OF A 5-YEAR-OLD GIRL.

SOURCE: DUNKWA GOVERNMENT HOSPITAL, GHANA.

2.4.1 Paradoxical reactions

Antibiotic therapy as the standard means of treatment for Buruli ulcer disease have been shown to produce remarkable outcomes (Phillips *et al*., 2014). Antibiotic treatment results in superior clinical outcomes compared to surgery (Nielsen *et al*., 2013). However, there have been some outliers as to the way lesions of patients are supposed to heal when antibiotic therapy commences. These outliers exhibit a phenomenon called paradoxical reactions. This is described as a deteriorating response to treatment of an infection after initial improvement. Some mycobacterial species tuberculosis, aviumintracellulae complex and leprosy have also been shown to cause paradoxical reactions after initiation of treatment (Nielsen *et al*., 2013). Paradoxical reaction can be identified by the clinical deterioration of Buruli ulcer lesions into severe forms of the disease after antibiotic treatment (Beissner *et al*., 2010). A proportion of 20% of Buruli ulcer disease patients experience complications as a result of paradoxical reactions

when receiving antibiotics which may lead to significant secondary tissue damage (Wanda *et al.*, 2014).

In Australia, two cases of paradoxical reactions were reported in 2009. In the first place there is improvement of antibiotic treatment, was followed by a worsening of the clinical symptoms of the presence of a lesion. It was first interpreted as treatment failure for both cases. As a result, the antibiotic regimen of the patients was changed. But, this was later understood as immune-mediated reaction to effective antibiotic therapy (O'Brien *et al*., 2009). Another study used prednisone therapy for 4-6 weeks for patients with paradoxical reactions, which yielded an improved clinical outcome. The lesions healed 9-12 months after initial treatment (Friedman *et al*., 2012). Prednisone was used as an adjunctive treatment to end the severe immune-mediated reaction and check secondary tissue necrosis. The host immune response to *M. ulcerans* may be suppressed by the use of Prednisone. It can also result in reduced blood serum levels which may result from an interaction with rifampicin (McAllister *et al*., 1983; Finch *et al.*, 2002).

In Ghana, a study reported that more than 30% of patients showed an increase in lesion size compared to previous lesion size and 9 patients developed new lesions either during or after completion of the antibiotic treatment (Nienhuis *et al*., 2012). Paradoxical reactions in this study occurred towards the end of 56 days of antimicrobial treatment. All lesions also healed during follow up and were not misinterpreted as treatment failure (Nienhuis *et al.*, 2012).

It was shown in a case series that some patients developed new skin lesions 12-409 days after completion of antibiotic treatment. Concentration of mycolactone plays a role during paradoxical reactions (Yeboah-Manu *et al*., 2013).

The immune system is an essential element during paradoxical reactions. Co-infection with HIV is perceived to make Buruli ulcer disease more severe and negatively affect healing times following treatment. Moreover, it is not known whether HIV patients with *M. ulcerans* infection are more likely to experience episodes of paradoxical reaction or whether this is affected by the initial level of immune suppression or whether it is further potentiated when the generalised immune suppressed state is partially reversed by ART. However, it is known that paradoxical reactions are common in HIV infected individuals starting ART with a variety of other microorganisms such as tuberculosis, Cryptococcus and *Mycobacterium avium* complex and that the rate increases with increasing immunosuppression at ART baseline (Wanda *et al*., 2014).

2.5 Laboratory diagnosis

Clinical suspicion of *M. ulcerans* infection needs to be confirmed through laboratory tests. This serves as a guide in assessing accuracy of case identification and positivity rate in the long term.

Laboratory confirmation is essential for several reasons:

1. to determine that the disease is Buruli ulcer;
2. to determine the precise prevalence and incidence of Buruli ulcer in a given area;
3. to confirm new foci;
4. to appropriately manage the disease using anti-mycobacterial therapy with or without surgery;
5. to confirm the failure of treatment, or relapse or reinfection after treatment. Currently, there is no point-of-care rapid diagnostic test but there are four

accepted laboratory tests available for confirmation of clinical suspicion (Beissner *et al*., 2010). These are direct smear examination for acid-fast bacilli (AFB), bacterial culture, polymerase chain reaction (PCR) targeting genomic region *IS2404* and

histopathological examination (Beissner *et al*., 2010).

KNUST

2.6 Treatment of Buruli ulcer disease

Historically, surgery was the standard method of treatment for Buruli ulcer disease (Walsh *et al*., 2008).

The curative method used for papules and nodules was wide excision and primary closure of the lesion (Uganda Buruli Group, 1970). Plaques and oedematous lesions were excised widely down or through the fascia if it was necrotic. Exploratory incisions and blunt dissection may help determine the limit of induration and necrosis (Walsh *et al*., 2008). Small lesions were excised and closed first while larger lesions were more often excised, with borders determined by lateral excision and blunt dissection. Splitskin autografts were then normally applied after a bed of granulation lesion (Walsh *et al*., 2008).

However, other researchers found alternate ways of treatment. Glynn *et al* in 1972 discovered the localized use of heat to lesions helped healing with or without surgery (Glynn *et al*., 1972). In contrast, others found this treatment to be inefficient and the advanced nature of the devices and dressings made it impossible to be used routinely (Thangaraj *et al*., 1999). Furthermore, Adjei *et al* in 1998 showed that phenytoin, an anti-convulsant, when used topically to ulcers enhanced healing without the disfiguring and contractures that are normally characteristic of Buruli ulcer (Adjei *et al*.,1998). The treatment turnout to be costly as patients needed to be in the health facility for over 100

days. Since Buruli ulcer patients are mostly from poor rural vicinities, it was costly for patients to be managed in case of recurrence after a prolonged surgery (Asiedu *et al*., 1998).

In 2004, an alternative Buruli ulcer treatment was proposed by WHO. It was recommended that a combination antibiotic regimen of oral rifampicin (10mg/kg) and intramuscular streptomycin (15mg/kg) given daily for at least 8 weeks (WHO, 2014) was to be used to treat Buruli ulcer.

2.7 Pathogenesis of *M. ulcerans*

Consistent progress in Buruli ulcer disease research have led to clearer grasp of the pathogenesis of the disease. A study into the genome of *M. ulcerans* have led to a major discovery. A virulence plasmid (Plate 6.0) responsible for the pathogenesis of the disease was discovered (George *et al*., 1999). Later on, it was shown that there was 98% DNA sequence identity between *M. ulcerans* and *Mycobacterium marimum(MM)*. Furthermore, there was a couple of differences between the species. *M. ulcerans* is mainly found extracellularly, while *MM* produces a granulomatous intracellular lesion. Genomic subtraction experiments were undertaken and these led to the recognition of fragments of Mu-specific polyketide synthases genes. A lipid toxin was responsible for the unique characteristics of Buruli ulcer disease. This lipid toxin is known as Mycolactone. Mycolactone is a lipid toxin which was established to be responsible for immunosuppression and tissue necrosis in patients infected with *M. ulcerans* (Stinear *et al.*, 2004).

Mycolactone plays a key role in the pathogenesis of *M. ulcerans* infection. A temperature of 30-33°C is required for optimal growth and augmentation of the toxin, mycolactone. The temperature enables progression of lesions in the skin and

subcutaneous tissue. This enables mycolactone to destroy tissues and suppress host immune response (Ashworth *et al*., 1995; Walsh *et al.*, 2008).

M. ulcerans infection starts at a latent phase with inoculation deep into the skin or subcutaneous tissue. This stage is characterized by slow proliferation of *M. ulcerans*, possibly intracellularly at first, followed by the production of small amounts of mycolactone resulting in tissue necrosis, especially of the adipose tissues (Walsh *et al.*, 2008). The quantity of mycolactone synthesized is increased as a result of the microaerophilic environment and perhaps nutrients favourable for tissue necrosis. Cellular immune response is inhibited at the necrotic stage as a result of immunosuppression (Walsh *et al.*, 2008). Lesions of patients with a high level of resistance may heal on its own without progression to the ulcerative forms or it can also develop into a small ulcer.

In other patients the lesion is undermined as a result of tissue necrosis and will ultimately breakdown into larger ulcers with characteristic undermine edges on the skin. In humans with least resistance, a nodule hardly develops and the necrosis extends swiftly and widely to cover large body surface areas (Walsh *et al.*, 2008).

Eventually, there is reduction in immunosuppression and viable *M. ulcerans* causing the necrotic stage to halt in most patients. This is preceded by healing and scarring which marks the beginning of the granulomatous stage. The plasmid encoding the production of mycolactone and heterogeneity in *M. ulcerans* genome, is responsible for the variations in clinical presentation and progression of the disease (Ablordey *et al*., 2015).

Types, structure and synthesis of mycolactone

After the discovery of mycolactone, a lot of research work went into its structure and how it is synthesized. Five structurally distinct mycolactones, namely A/B, C, D, E and F are produced by several strains of *M. ulcerans*. Mycolactone A/B strains are isolated from Africa, Malaysia and Japan. Mycolactone strains have been isolated in other nations. *M. ulcerans* from Australia produces mycolactone C, Chinese strains produce mycolactone D and mycolactones E produced by *Mycobacterium liflandii* and *Mycobacterium pseudoshottsii* produce the mycolactones F. Little is known about the South American strains of mycolactone. Out of the five different strains, mycolactone A/B is the most potent, while F is the least potent. This was quantified through immunosuppressive and cytotoxic activity measurement which showed a convenient 'alphabetical' gradient. Interestingly, all mycolactones have a preserved core structure, and any variation occurs in the length, methyl branching, oxidation state and stereochemistry of the acyl side chain (Demangel *et al*., 2009).

The *M. ulcerans* plasmid, pMUM001 which carry an array of genes has abundance of insertion sequences (IS). It consists of 4 copies of IS2404 and 8 copies of IS2606 (Stinear *et al.*, 2004). A 105 kb of the plasmid contains six genes coding for proteins involved in mycolactone production (Plate 6.0). Mycolactone core-producing PKS are encoded by mlsA1 (50,973 bp), mlsA2 (7,233 bp) and the side chain enzyme by mlsB (42,393 bp). The three PKS genes are highly related, with stretches of up to 27 kb of near identical nucleotide sequence (99.7%). The whole 105-kb mycolactone locus essentially consist of only 9.5 kb of uncommon, non-repetitive DNA sequence (Stinear *et al.*, 2004).

Multiple enzymatic activities that are found in type I PKSs are needed for one round of chain extension and alteration in a single polypeptide like type I fatty acid synthases. Thorough assessment of their predicted module and domain design strongly implied

that these PKSs produce mycolactone, and this was afterwards confirmed by transposon mutagenesis. MlsA1 and MlsA2 together constitute a loading module and nine extension modules that synthesize the macrolactone core and upper side chain, whereas MlsB, with its loading module and seven extension modules, produces the acyl side chain. The process of synthesis of mycolactone is shown in Plate 7.0, and highlights the sequential fusion and alteration of either acetate or propionate subunits at each extension module. PMUM001 also harbours three additional CDSs that encode putative auxiliary enzymes for mycolactone synthesis. Cyp140A7 is a cytochrome P450 hydroxylase that probably hydroxylates C-12′ of the mycolactone side chain.

Experimental evidence is lacking for the remaining two enzymes, a type II thioesterase (locus tag MUP_038) and a FabH- like ketosynthase (locus tag MUP_045), but they may play a part in chain termination and transfer of the mycolactone acyl side chain to the core15 (Plate 7.0) (Demangel *et al.*, 2009).

The minimum set of enzymatic domains required for PKS activity includes ketosynthase (KS), acyltransferase (AT) and an acyl carrier protein (ACP) domain (Jenke-kodama *et al.* 2003). Ketoreductase (KR), dehydratase (DH), and enoylreductase (ER) domains are also commonly found in modules and form a socalled reductive loop, providing reducing enzyme activities that modify the two or three-carbon unit being added to the polyketide (Jenke-kodama *et al*. 2003).

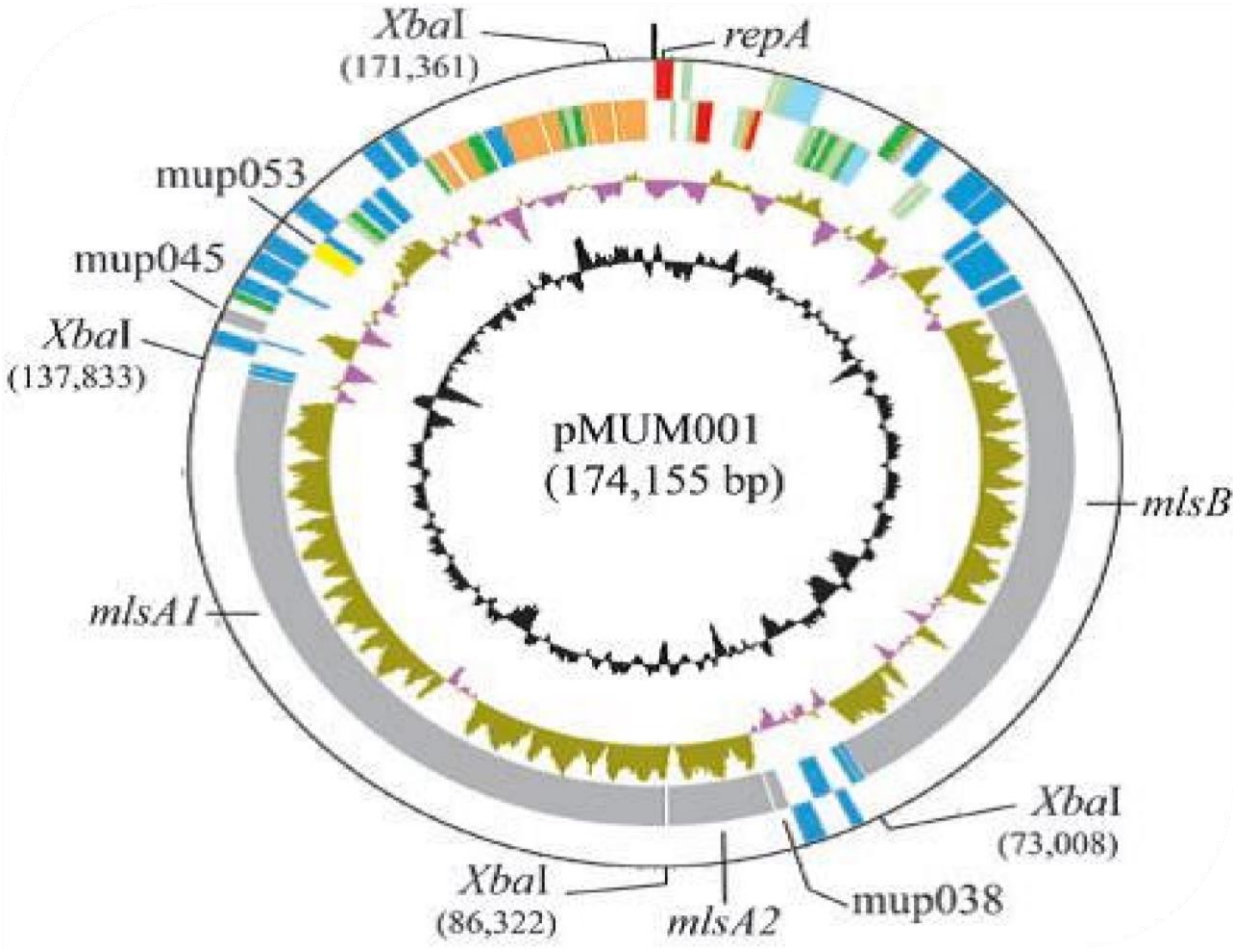


PLATE 6.0 CIRCULAR REPRESENTATION OF PMUM001.

SOURCE: STINEAR *ET AL*. 2004

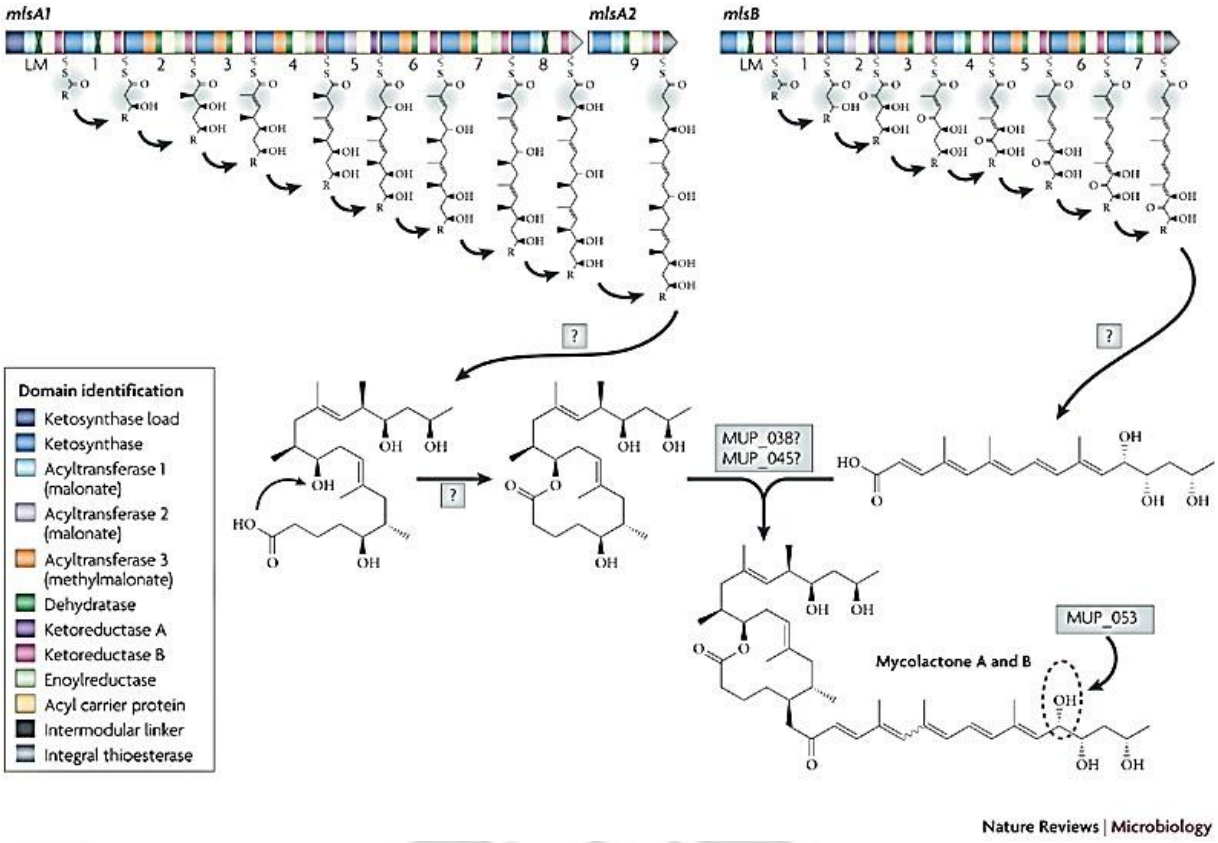


PLATE 7.0 PROPOSED PATHWAY FOR THE BIOSYNTHESIS OF MYCOLACTONE A AND B.

SOURCE: DEMANGEL *ET AL*. 2009

2.8 Immune response to *M. ulcerans* infection

The immune system protects the organism against infection. The innate immunity is the organism's first line of defence against pathogens. When the organism is antigenically challenged, the adaptive immunity takes over. It responds with a high degree of specificity and is able to develop immunological memory. The adaptive immunity with the development of immunological memory is able to respond to a primary infection by five to six days. A secondary infection is swiftly dealt with as a result of developed immunological memory from previous exposure. Lymphocytes and the molecules that are produced are the key players in adaptive immunity (Kindt *et al*., 2007).

Animal model and human studies have led to a lot of development in Buruli ulcer immunology. Cell-mediated immunity plays a part in healing (Phillips *et al.*, 2006). Immunosuppressive and immunomodulatory properties of mycolactone have a major effect on host cells that are key to both innate and adaptive immune response to *M. ulcerans.*

In an animal model study, rodents were infected with mycolactone positive and mycolactone negative *M. ulcerans* strains. Results showed that inflammatory cells are killed by necrosis when encountering high toxin concentrations. Inflammatory cells more unique from area of necrosis were thought to be killed by apoptosis. On the other hand, granulomatous lesions with the ability to self-heal were observed with mycolactone negative mutants (Schu *et al*., 2007). Within 24 hours of infection with *M. ulcerans*, wild-type *M. ulcerans* was taken up by neutrophils and occasionally MHC class II. This followed its transportation to local lymph nodes at where viable organisms were discovered 7 days after infection. The bacteria at the infection foci were entirely extracellular beyond 8 weeks.

Another group demonstrated that non-poisonous amount of mycolactone are immunosuppressive on professional antigen presenting cells. Production of tumour necrosis factor by monocytes and macrophages were halted as a result of infection with *M. ulcerans* or incubation with exogenous mycolactone. Production of mycolactone suppressed the ability of the dendritic cells to prime cellular responses and secrete chemotactic signals that are vital for inflammatory responses. Immature dendritic cells are not able to efficiently secrete chemo attractants for monocytes and T helper 1(Th1) lymphocytes. In ulcerative lesions, due to mycolactone secretion in the skin, trafficking of inflammatory cells to site of infection is halted (Demangel *et al*., 2009).

In Buruli ulcer research, there have been varying views as to whether the patients can elicit a T Helper 1 (Th1) response to *M. ulcerans.* Although, Th1 response is downregulated by overproduction of interleukin 10, there is a unanimous agreement that mycolactone plays an essential role when one takes the Th1 and T Helper 2 (Th2) responses to *M. ulcerans* infection into perspective.

In showing the extent of T cell sensitization in Buruli ulcer disease, patients were intradermally injected with a crude preparation of *M. ulcerans* sonicate. Patients presenting with early disease did not respond to the antigen. However, 45(76 %) of the patients with healing lesions elicited an immune response to the *M. ulcerans* sonicate (Stanford *et al.*, 1975; Wansbrough-Jones and Phillips, 2006).

T-cell non-responsiveness to mycobacterial antigens was reported in another study. In this study, peripheral blood mononuclear cells (PBMC) were stimulated and incubated for 6 days with live *M. ulcerans or M. bovis.* Lower interferon-γ levels in patients compared to that of healthy tuberculin-positive individuals was reported. A Th1 cytokine profile in patients with early nodular lesions and a Th2 cytokine profile from patients with ulcers were reported in another study where PBMC`s of patients were stimulated with whole killed *M. ulcerans* or *M. bovis* (Gooding *et al.*, 2001; Wansbrough-Jones and Phillips, 2006).

Whole blood stimulation assay was undertaken to understand Th1 and Th2 responses. In Ghana, whole blood samples of patients were stimulated with *M. ulcerans* sonicate antigen and incubated overnight. Median interferon gamma responses between patients with ulcers and those with healed Buruli ulcers were identical while those with nodular forms showed lower responses. In addition, interleukin-10 responses were more

profound in patients with active *M. ulcerans* disease than in those with healed lesions, and the pattern of response was similar to that seen in tuberculosis.

One-third of early lesions healed spontaneously, which could imply that there was a favourable immune response or *M. ulcerans* failed to synthesize sufficient mycolactone to establish active infection (Phillips *et al*., 2006a; Wansbrough-Jones and Phillips, 2006).

KNUST

Through the use of a semi-quantitative reverse transcriptase PCR, levels of mRNA for interferon gamma and interleukin 10 showed that patients with longer established ulcers show low levels of interferon gamma and higher levels of interleukin 10 whilst in contrast high interferon gamma levels and low interleukin 10 levels were measured in nodular forms (Phillips *et al*., 2006b; Wansbrough-Jones and Phillips, 2006).

In Ghana, both T-helper 1 and T-helper 2 cytokine production in both nodules and ulcers was studied. A higher trend towards expression of mRNA for interferon gamma, Tumour necrosis factor α, interleukin 12p40, interleukin 1β, interleukin 10 was reported. The effect of mycolactone is restricted to tissue destruction and halting inflammation in its immediate infection foci and that, in adjacent tissues where mycolactone has not diffused a typical mycobacterial immune response can start (Phillips *et al*., 2006; Wansbrough-Jones and Phillips, 2006).

These findings imply that patients infected with *M. ulcerans* develop a Th1 immune response but the rate of development differs between individuals. Notwithstanding, the fact that severity of the disease could have an effect on immune response. As result of that a means of preventing the onset of the disease will be favourable.

2.8.1 Prevention of M. ulcerans disease

Even though, the mode of transmission of Buruli ulcer disease is unknown, studies have recommended ways of preventing the disease.

From epidemiological studies, *M. ulcerans* transmission is restricted to a specific geographical area. Therefore, risk of infection is non-existent in non-endemic areas. Other biting insects for instance mosquitoes may spread the infection (Johnson *et al.*, 2007). As a result of those findings, it is advised that the regular use of insect repellents or bed nets for sleeping reduce chances of infection. An Australian study recommends the use of insect repellents, cleaning of the skin or wounds after soil exposure, and mosquito control are logical preventive strategies that should be considered by individuals and public health authorities (O'Brien *et al*., 2014b).

Evading contact with *M. ulcerans* infected areas is very unlikely in tropical rural localities as scantily dressed people play and work. Wearing protective clothing when farming and prompt cleansing of any skin injury may lower rates of infection, but accomplishing these measures is not applicable (Huygen *et al*., 2009).

In Africa, the current mode of prevention is through active case search by trained health workers. Even though active case search seems to provide favourable results, the cultural beliefs of people living in endemic communities seem to be a hindrance as to when or if they will report suspected cases to the nearest health facility (Stienstra *et al*., 2002). Most patients report to hospital as a last resort when the traditional methods of treatment fails (Huygen *et al*., 2009).

A sure alternative to prevention of *M. ulcerans* infection is through the use of a vaccine. Since the era of Edward Jenner the development of vaccines against diseases such as whooping cough, poliomyelitis, tetanus, etc have shown that vaccines have immense

effects on disease eradication than any approach (Kindt *et al*., 2007). Presently, there is no vaccine for Buruli ulcer disease. A vaccine for the disease will be a favourable alternative.

2.9 *M. ulcerans* vaccine candidate

Buruli ulcer vaccine immunology have gone through several years of evolution. Progress made so far in finding vaccine candidates for the disease will be reviewed in this section.

Bacillus Calmette-Guerin (BCG) vaccine: There is currently no vaccine against *M. ulcerans* infection. It was suggested that the cross-reactive role of the *Mycobacterium bovis (M. bovis)* vaccine for Tuberculosis could be used for Buruli ulcer prevention as a vaccine. But later studies provided contrasting results.

During the late 1960`s and early 1970`s, two randomised controlled trials of BCG vaccination for the prevention of Buruli ulcer disease have been conducted. Study participants were grouped according to those who had previously had Buruli ulcer disease, previous BCG or no previous BCG. Those without earlier BCG were randomised to be given or not to be given a dose of BCG, if their tuberculin skin test (TST) results was less than 6mm. Participants were followed up for 68 weeks. In the first six months BCG had a protection rate of 72% with a total efficacy rate of 47%. On the second 6 months, this level of protection was almost 0%. In non-randomised subjects with prior Buruli ulcer disease with healed lesions and a positive TST had protective immunity. The protection decreased as TST status declined (Smith *et al.*, 1976; Huygen *et al*., 2009).

In central Uganda in the South-east bank of the Nile, the study participants were surveyed and offered TST. BCG was administered randomly to 50% of all these

participants, regardless of the past Buruli ulcer disease, BCG scar or tuberculin status. The overall protection of 47% just as the first study. It was proven that protection seems to decline with time. BCG only provided protection in those with initial TST less than 4mm and a reduction in the size of the Buruli ulcer lesion on patients with BCG scar, but successive BCG vaccination did not show additional protection. Both studies showed that BCG produced a significant but only short-lived protection against *M. ulcerans* infection. Results from the tuberculin-positive subjects suggested that past exposure to *M. tuberculosis* induced a cross reactive immune response (Smith *et al.*, 1976). In Kuwait, where the disease was known to be non-endemic, there was a crossreactive skin test response to *M. ulcerans* in BCG vaccinated school children. This was suggestive of the close phylogenetic relationship of mycobacteria from the *M. tuberculosis* complex on the one hand and *M. ulcerans –M. marinum* on the other (Huygen *et al.*, 2009). It was also reported in Benin that children affected by *M. ulcerans* who have had BCG vaccination were protected against osteomyelitis (Portaels *et al.*, 2002).

A recent case control study undertaken in Democratic Republic of the Congo, Ghana and Togo from 2010 through 2013,demonstrated that there was no significant proof of a protective effect of routine BCG vaccination on the risk of developing either Buruli ulcer disease or severity of the disease (Phillips *et al.*, 2015).

In an animal model study of mice infected with *M. ulcerans, M. bovis* BCG vaccine offered a short lived protection which remained unchanged after adding a booster dose (Tanghe *et al.*, 2007).

In conclusion, *M. bovis* BCG vaccine could only provide short-lived protection against Buruli ulcer disease and the cross reactive immune response induced by *M. bovis* BCG

vaccine is not sufficient. Therefore, a species specific immune response is required to effectively control *M. ulcerans* infection.

Mycolactone: Mycolactone was seen as the next potential vaccine candidate. The chemical makeup and possibly because of its immunosuppressive properties, makes it poorly immunogenic in both humans and mice (Huygen *et al.*, 2009).Thus, others turned to look into mycolactone deficient *M. ulcerans.*

Mycolactone-negative M. ulcerans: It is believed that the bacteria express all antigens expressed by virulent *M. ulcerans* bacteria, except mycolactone. Therefore, they can induce an adaptive immune response. Stinear and team showed that Mycolactonenegative *M. ulcerans* have been developed either spontaneously or through random transposon mutagenesis (Stinear *et al.*, 2004). Through random transposon mutagenesis several genes are involved in mycolactone synthesis (Stinear *et al.*, 2004; Adusumilli *et al.*, 2005). Mutations of some of these genes ablate toxin production completely, while strains with mutations in other genes produce incomplete toxin (Adusumilli *et al.*, 2005). To guarantee the safety of this vaccine candidate especially in case of immunocompromised individuals, it has been proposed that at least two targeted gene deletions should be introduced into live attenuated mycobacterial vaccines (Huygen *et al*., 2009). This can eventually affect the persistence and immunogenicity of bacteria negatively by over-attenuating the vaccine strain, it is essential that safety precautions are taken as the bacteria are viable and could possibly return to a virulent state.

In 2012, an animal model study characterised the histological and cytokine profiles triggered by vaccination with either BCG or mycolactone-negative *M. ulcerans*, followed by footpad infection with virulent *M. ulcerans*. It was reported that BCG

vaccination had a positive effect on how long it took for the onset of *M. ulcerans* growth. This was evident through the induction of an earlier and consistent interferonγ T cell response in the draining lymph nodes. *M. ulcerans* - infected footpads with BCG vaccination resulted in cell-mediated immunity with a predominant chronic mononuclear infiltrates with increased and sustained levels of interferon-γ and TNF-α mRNA. IL-4, IL-17 or IL-10 elicited no significant responses in the footpad or the draining lymph node, in either infected or vaccinated mice (Fraga *et al*., 2012). Although with this protective Th1 response, BCG vaccination did not prevent the later advancement of *M. ulcerans* infection, an observation which had been reported by Converse et al (Converse *et al*., 2011). Another animal model study showed that mycolactone deficient *M. ulcerans* progression in mice was significantly slow compared to *M. ulcerans* positive infected mice (Fraga *et al.,* 2012). This could be attributed to the protective Th1 recall responses against *M. ulcerans*. Precisely how this cell-mediated immunity induced by vaccination is compromised remains to be understood. As a result of the poor immunogenicity of mycolactone and the potential of mycolactone negative *M. ulcerans* to later on turn pathogenic a new approach to find an appropriate vaccine candidate was explored.

In vaccine development, research has shown that the best approach to manufacture a vaccine is through using DNA vaccine development platforms (Sumithra *et al*., 2013). Its suggested that DNA immunization have the potential of being the best approach to combat bacterial infections especially intracellular bacteria. It must be subjected to further testing including various second generation DNA vaccines optimization strategies. Immunogenicity of second generation DNA vaccines have progressed in vector and antigen composition, upgraded formulations and methods in the incorporation of adjuvants and prime boosting strategy. This advancement has spurred

revived interest in DNA platforms which is evident of the many ongoing experiments on bacterial DNA vaccines (Sumithra *et al*., 2013). DNA vaccines offer the advantage that they can be formulated to target specific cell compartments for antigenic processing. Plasmids encoding a secreted form of the protein by fusing it to the signal sequence of human tissue plasminogen activator (tPA) are generally more immunogenic, for both B and T cells, than plasmids encoding a mature form (Huygen, 2003). In Buruli ulcer vaccine research several DNA vaccine platforms have been used to find an efficacious vaccine for the disease. Results of those studies will be reviewed as follows.

***Antigen 85A from M. ulcerans (Subunit vaccines)*:** Subunit vaccines are safe since they are well characterized and can be applied especially in people living with HIV without causing any danger. On the other hand, repeated booster doses are required as their antigenic repertoire is limited, and the induced immune response and memory are generally weak (Sarfo, 2014).

Tanghe et al identified the possibility of vaccination with plasmid DNA encoding the mycolyl-transferase 85A from BCG. Results from the study showed that the bacterial load in the foot pads of *M. ulcerans* infected mice was significantly reduced Vaccination with Antigen 85A (Ag85A) protein from *M. ulcerans* provided some level of protection. A DNA prime-protein boost immunization protocol produced a protective efficacy similar to the one induced by the BCG vaccine (Tanghe *et al.*, 2001). An improved potency of Species-Specific DNA Vaccine encoding MycolylTransferase Ag85A from *M. ulcerans* by Homologous Protein Boosting has been reported. However, this cross-reactive protection was inadequate to totally control the infection in mice vaccinated with plasmid DNA encoding Ag85A from *M. bovis* BCG. Immunogenicity and protective efficacy of Ag85A from *M. tuberculosis* and *M.*

ulcerans, administered as a plasmid DNA vaccine, as a recombinant protein vaccine in adjuvant or as a combined DNA prime-protein boost vaccine were compared in mice. All three vaccination formulations induced cross-reactive humoral and cell-mediated immune responses, although species-specific Th1 type T cell epitopes were shown in both the NH2- terminal region and the COOH-terminal region of the antigens. This partial species-specificity was reflected in a higher albeit not sustained protective efficacy of the *M. ulcerans* than of the *M. tuberculosis* vaccine, particularly when administered using the DNA prime-protein boost protocol (Tanghe *et al*., 2008).

Until recently, a study used live-recombinant strains of BCG and *M. smegmatis* which express the immunodominant MUAg85A in mice. Priming with BCG MU- Ag85A followed by an *M. smegmatis* MU-Ag85A boost strongly induced murine antigen - specific CD4+ T cells and elicited functional interferon gamma producing splenocytes which recognized MU-Ag85A peptide and whole *M. ulcerans* better than a BCG primeboost vaccination. It was shown that mice vaccinated with a single subcutaneous dose of BCG MU-Ag85A or prime-boost displayed significantly enhanced survival, reduced tissue pathology, and lower bacterial load compared to mice vaccinated with BCG. Importantly, this level of superior protection against experimental Buruli ulcer compared to BCG has not previously been achieved (Hart *et al*., 2015).

Heat shock protein-65 (Hsp65) from M. ulcerans: In 2006, another group researched into the potential of another subunit protein. They researched into the immunogenicity of *M. ulcerans* Heat shock protein -65 (Hsp65) and protective efficacy of a *Mycobacterium leprae (M. leprae)* Hsp65-based DNA vaccine against Buruli ulcer. Genes encoding the GroEL-2 protein Hsp65 are highly conserved among mycobacterial species, with 96 and 95% identity at the amino acid level between the *M. ulcerans* antigen and the homologous proteins in *M. tuberculosis* and *M. leprae*, respectively.

Hsp65 was shown to be very immunogenic. Outcome of vaccination in mice with plasmid encoding Hsp65 encoding DNA vaccine was also significantly protective against *M. ulcerans* infection, which was indicative of the reduced bacterial loads in tails of infected mice. This study showed that DNA vaccine expressing Hsp65 and Ag85B provided comparable levels of protection which was lower to the one afforded by vaccination with BCG (Coutanceau et al., 2006). But as a result of exhibiting strong homology with human Hsp60 and the risk of developing autoimmune diseases, Hsp65 was not an appropriate vaccine candidate (Huygen *et al.*, 2009).

Later on, studies into the complete genome sequence of *M. ulcerans* revealed the loss of other immunodominant mycobacterial proteins such as ESAT-6, CFP-10 and Hspx which were also potential sub-unit vaccine candidates. The loss of the immunodominant proteins by *M. ulcerans* was an evolutionary strategy to help the organism to evade the host`s immunological responses and may represent part of an ongoing adaptation of *M. ulcerans* to survive in host environment that are screened by immunological defence mechanisms (Huber *et al*., 2008).

Polyketide synthase domains: Huygen et al has suggested that enzymes involved in the synthesis of mycolactone are the potential vaccine candidates (Huygen *et al.*, 2009). Pidot *et al* in 2010 showed that patients with Buruli ulcer and their contacts can produce antibodies against three (AT-propionate, ER and KR-A) of the polyketide synthase domains. Four years later, a mouse model study researched into the vaccine potential of nine of the polyketide synthase domains (Pidot *et al.*, 2010). This study used a larger number of the PKS which will provide a clearer illustration of their performance. The overall findings showed that the strongest antigen specific antibodies could be detected in response to acyl transferase (propionate) and enoylreductase. Acyltransferase

(proprionate) induced a more profound interleukin-2 or interferon gamma responses. But eventually, protection provided by Ag85A or *M. bovis* BCG was more favourable than the one conferred by vaccination (Roupie *et al*., 2014). But these PKS domains have not been tested in humans. A study in human subjects will be beneficial as the next step to help prevent Buruli ulcer disease.

Monovalent vaccine: In 2015, a mouse model study, vesicular stomatitis virus-based RNA replicon particles encoding the *M. ulcerans* proteins MUL2232 and MUL3720 were generated and the expression of the recombinant antigens characterized. The recombinant replicon particles elicited antibodies that reacted with the endogenous antigens of *M. ulcerans* cells. A prime-boost immunization regimen with MUL2232recombinant replicon particles and recombinant MUL2232 protein induced a strong immune response but only slightly reduced bacterial multiplication in a mouse model of *M. ulcerans* infection. It was shown that a monovalent vaccine based on the MUL2232 antigen will probably not sufficiently control *M. ulcerans* infection in humans (Bolz *et al*., 2015). This study is yet to be replicated in human subjects.

CHAPTER 3

MATERIALS AND METHODS

3.0 Study area

Out of the 10 regions of Ghana majority of the Buruli ulcer cases are reported in the Ashanti Region (Owusu-Sekyere *et al*., 2014). This study was carried out at the 4

different sites as a result of endemicity of the disease in those districts; three of the chosen sites namely Agogo Presbyterian Hospital, Tepa Government hospital and Nkawie-Toase Government Hospital, are located in the Ashanti Region while Dunkwa Government hospital is located in the Central region.

Agogo Presbyterian Hospital is located in the Asante-Akim-North district of Ashanti Region. The disease has been prevalent in the district since the early 1970s (Agogo Prebyterian Hospital, 2015). It is the designated training centre for Buruli ulcer treatment by the National Buruli ulcer control programme and WHO (WHO, 2014). The hospital boasts of 50 trained Bu volunteers in the surrounding communities. The Buruli ulcer clinic day for the hospital is Wednesday. The district is approximately 80 km east of the Ashanti region. It covers a total surface area of 1217.7 sq. Km with a population of 170,882. Farming is the major occupation of its inhabitants (Ghana Districts, 2015).

Dunkwa Government Hospital is the main referral centre for Buruli ulcer cases in the Upper Denkyira East of the Central region. The Buruli ulcer Clinic day for the hospital is Tuesday. The district is located in the south of Ghana. It covers an estimated area of 1020sq Km with a settlement population of 33,379 people. The Dunkwa on Offin town is drained by the Offin river and other small streams. Small scale gold mining and farming serve as the source of livelihood to its inhabitants (Ghana Districts, 2015).

Tepa Government Hospital serves the Ahafo-Ano district situated in the north-western part of the Ashanti Region. Tepa Government Hospital is the district's referral centre for Buruli ulcer cases. The Ghana Health Service report of 2005 indicated 186 cases in the district. The Buruli ulcer clinic day for the hospital is Thursday. Farming is the main occupation of the populace. The district is bounded to the south by Atwima district to

the east by Ahafo Ano south and to the north by Tano South district and West by Asutifi district (Ghana Districts, 2015).

Nkawie-Toase Government Hospital serves the Atwima Nwabiagya district in the Ashanti Region. The Hospital also serves as the surrounding districts' referral centre for Buruli ulcer treatment. In 2007, 112 Buruli ulcer cases were reported from the district (Atwima Nwabiagya, 2015). The Buruli ulcer Clinic day for the hospital is Friday. The district is located in the Western part of the region and shares common boundaries with Ahafo-Ano-South and Atwima Mponua districts. It covers an estimated area of 294.84sq km with a projected population of 179,753. There are several streams in the district namely, Offin, Owabi and Tano rivers. Small scale farming and fishing serves as a source of livelihood to its inhabitants (Ghana Districts, 2015).

3.1 Study design

This was a hospital based study and a prospective, observational study.

3.1.1 Sample size calculation

From an earlier study on dynamics of the cytokine response, the median interferon-γ was 498.4+/-88 pg/ml in patients compared to 78.25+/-9.1 in controls (Sarfo *et al.*, 2009). It was assumed that the cytokine levels expected from patients in the study will be less than 8% since the immunogenicity of the antigens in humans have not being ascertained.

From that assumption 3% was used for the targeted population for this study.

Therefore

To detect this difference at the 3% level of significance,

The sample size "n" was computed using the formula,

n = $\frac{z^2pq}{\delta^2}$

Where:

n=the desired sample z=The standard normal deviate, usually set at 1.96, which corresponded to the 95 percent confidence level.

p=The proportion in the target population estimated to have recurrence of Buruli ulcer

q=The proportion in the target population who do not have Buruli ulcer,1.0-p

δ= degree of accuracy desired, usually set at 0.05

Assuming cytokine levels was expected to be at 3% in the study population.

n=

n=45.6

n=46 The sample size was increased to 51 to account for 10% loss to follow up

in the

study and

to

demonstr

ate the

expected

effect.

KNUST

3.2 Ethical clearance

Ethical approval was obtained from the Committee on Human Research, Publications and Ethics (CHRPE/AP/229/12), at School of Medical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana. Permission to conduct the study at all study sites was sought from the respective hospital management and the district health directorate. Consent was read in the appropriate local language (Twi) to study participants. Information gathered was treated as confidential. Patients agreed to participate in the study by signing the consent forms. They were made to understand they had the right to withdraw consent to participate in part or fully at any time during the study without giving any reason. Their withdrawal of consent did in no way negatively affect their further management. Consent was sought from household contacts of patients who have never had Buruli ulcer disease to be recruited as controls.

3.3 Patient recruitment Procedures

Active case search was undertaken by experienced health workers in endemic communities. These health workers have been managing the Buruli ulcer clinic and consistently undergo training from WHO Buruli ulcer disease management experts. At

the treatment centre, patients were screened by physicians and nurses to clinically confirm *M. ulcerans* (Mu) infection.

All eligible patients were screened for HIV infection and pregnancy. The clinical forms and dimensions of their BU lesions were also assessed. This was by observation if it meets the aforementioned clinical forms for its appearance and palpation for induration and when a patient present with a non-ulcerative form. Study specific data collection forms as well as standard Buruli Ulcer treatment forms (BU01 appendix III) were used for collecting demographic and clinical data.

Patients' lesions were grouped into one of three categories according to lesion size and photographs taken with a Nikon D5100 digital camera (Nikon Corp., Japan) and with Aranz silhouette device (Aranz Medical Limited, New Zealand). The Aranz silhouette device provided the surface area, volume and diameter of the lesion when pictures were taken to aid monitoring disease progress. Oedematous lesions however were monitored with tape measure and digital photographs. Nodules and plaques were traced out with a marker for easy identification. Nurses applied dressing to ulcerated lesions according to the WHO recommendation.

A visit card indicating the name, unique study identification number and visit dates up to 52 weeks was given to newly recruited patients. Patients were reviewed at 2 weekly intervals during antibiotic treatment of Rifampicin and Streptomycin or Rifampicin and Clarithromycin coupled with routine update of clinical data.

PLATE 8.0 ARANZ SILHOUETTE DEVICE BEING USED TO FOLLOW-UP ON DISEASE PROGRESSION

3.4 Study population

A total of 62 patients from four study sites were enrolled into the study. From the study sites, Agogo recruited 37(60%), Tepa recruited 11(18%), Nkawie enrolled 8(13%) and Dunkwa recruited 6(9%) patients. Of this,30 were males while 32 were females.

Out of that number, 24 patients were recruited for the Plasmid antigen study, 38 into the paradoxical reaction study.

For controls, 37 age-matched contacts from the 4 study sites were enrolled together with twelve (12) non-endemic controls. Among the 37 contacts of the patients,14(38%) were from Agogo,11(30%) from Tepa,5(13%) from Nkawie and 7(19%) from Dunkwa study site.

A total of 111 participants were enrolled into the study

3.4.1 Inclusion criteria

- All patients 5 years and above clinically diagnosed as having Buruli ulcer. Participants not previously treated for Buruli ulcer disease
- Participants who gave informed consent or guardian in the case of minors.

3.4.2 Exclusion criteria

- Patients not willing to consent to the study.
- Participants below the age of 5 years
- Evidence of clinical significant neurological, cardiac, pulmonary, hepatic, or renal disease by history, physical examination and/ or laboratory tests.

3.5 Specimen collection

3.5.1 Swab

Swab samples were taken from underneath the undermined edges by twirling the swab (silver health diagnostics) and circling the entire undermined edge of the ulcer. The swab was placed into labelled cryovials containing 700µl Cell Lysis Solution (CLS) (Qiagen, USA) for conventional PCR.

3.5.2 Fine Needle Aspiration (FNA)

The lesion was inspected, carefully palpated and the site disinfected using sterile alcohol Preps (Tyco healthcare group LP, U.S.A) or an alcohol soaked swab. The FNA was obtained from the centre of the lesion or via a weak area in the case of non-ulcerated oedematous lesions. A 5ml syringe and a 21-gauge needle (Neomedic limited, United Kingdom) were used for plaques and nodules, the pre-ulcerative lesions without undermined edges. The lesion was held with one hand, the needle was inserted into the estimated centre of the lesion and moved back and forth (about 4 times) within the subcutaneous adipose tissue in different directions without withdrawing. Suction was

applied each time the needle was moved forth. Finally, the needle was withdrawn and its content flashed in and out of a 2ml cryovial containing 300µl Cell Lysis Solution (CLS).

3.5.3 Blood samples for immunological assays

Prior to taking blood, the process was explained to the patient. A tourniquet was wrapped around the upper arm near the medial cubital vein at the elbow and disinfected with sterile alcohol Preps. Approximately 6mls of blood was drawn from the vein into a heparin tube (BD vacutainer systems, UK). The tourniquet was removed, the needle withdrawn and a pad pressed for some minutes to halt bleeding. The heparin tube was gently turned upside down twice to ensure even mixing of the blood with anticoagulant to avoid clotting, labelled and placed in a stable rack.

3.5.4 Sample transport

Containers (vials) were uniquely labelled to show the type of samples taken, for swab and FNA. The specimen collection bag was labelled with the packing date of the bag to confirm the expiry date of the reagents since they were discarded if unused after 6 months. All collected samples were placed in the specimen collection bag and tightly sealed. They were then transported to Kumasi Centre for Collaborative Research in Tropical Medicine (KCCR) Laboratories for further processing of the samples.

3.6 Laboratory assessments - diagnostic confirmation

All required tests were performed at KCCR Laboratories. A number of laboratory diagnostic tests were performed to confirm the presence of *Mycobacterium ulcerans* from samples taken from patients.

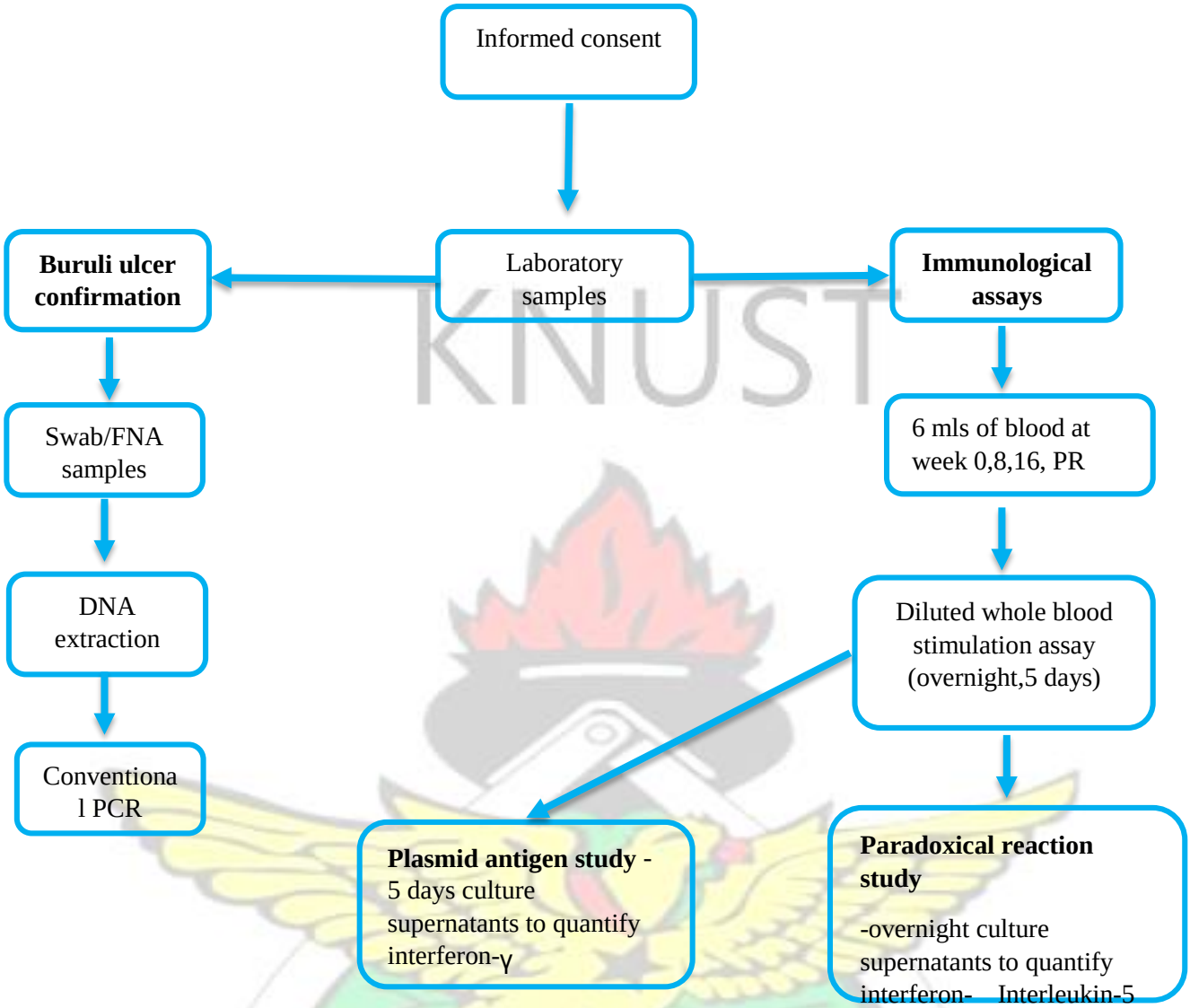


FIGURE 1.0 FLOWCHART TO STUDY PROCEDURE.

PR- REPRESENTS PARADOXICAL REACTION

3.6.1 DNA extraction

On arrival of samples from the study sites, the samples were heat-inactivated by incubation at 95˚C for 15 minutes in a thermomixer. There were four stages in the DNA

extraction, namely: Cell Lysis, Protein Precipitation, DNA Precipitation and DNA Hydration.

Cell lysis was achieved by adding 10µl proteinase K (20mg/ml) to 700µl cell lysis buffer containing swab sample and incubated in a thermomixer at 55ºC for 4 hours or overnight. The Proteinase K was inactivated by incubation in the thermomixer at 80ºC for 20 minutes. The sample was then allowed to cool down at room temperature (1 hour). A volume of 15µl Lysozyme was added and incubated at 37˚C in a thermomixer for an hour. The samples were placed on ice for 5 minutes.

Protein precipitation was attained by adding 100µl or 230µl of protein precipitation solution (PPS) (Qiagen, USA) for FNA and swab samples respectively. This was vortexed at high speed for 20 seconds. Samples were placed back on ice for 5 minutes after which they were centrifuged at 13,000 rpm for 5 minutes. During centrifugation, a 2ml reaction tube was prepared containing 700µl of 100% Propan-2-ol and 2 µl of glycogen.

DNA precipitation started when the supernatant was poured (leaving behind the precipitated protein pellet) into the already prepared 2ml reaction tube and mixed by inverting gently for 10 times. The samples were then centrifuged at 13,000 rpm for 5 minutes and the supernatant discarded. A volume of 700µl of 70% ethanol was added and the tube inverted 4 times to wash the DNA pellet. The sample was centrifuged at 13,000 rpm for 5 minutes. Carefully the ethanol was slowly poured off to avoid losing the precipitated DNA pellet. The tube was then inverted and drained on a clean tissue paper towel and allowed to air dry for an hour or overnight.

Hydration was attained by adding 50µl or 200 µl DNA hydration solution (Qiagen, USA) was added to the sample; FNA and swabs respectively and rehydrated by

carefully pipetting up until pellet have formed an aqueous solution. The solution was incubated at 65˚C for 30 minutes for FNA and 60 minutes for swabs. Hydrated DNA extracts were kept at 2-8º C until conventional PCR was set up.

3.6.2 Dry reagent base (DRB) PCR for IS2404

One (1) pure Taq ready-to-go PCR bead was added to each 0.2ml tube containing lyophilized primers (MU5 and MU6, 1.25µl each,10µM). For the DRB – PCR, oligonucleotides MU5 (5' aaagcaccacgcagcatct 3') (TibMolbiol, Berlin, Germany) and MU6 (5' cggtgatcaagcgttcacga 3') (TibMolbiol, Berlin, Germany) were lyophilized in 200ul reaction tubes. Lyophilisation was carried out in an RCV 2-25 vacuum concentrator (Christ, Osterode, Germany).

A volume of 22.5µl of Diethyl pyro carbonate (DEPC) water was added to each patient sample, the negative extraction control tube and positive control tube. A volume of 25µl of the water was added to the negative PCR control tube. To the corresponding PCR tubes, 2.5µl DNA extract or 2.5µl extraction control extract was added. Finally, 2.5µl of the positive control extract was added to the positive control sample tube. The final mixture was used for PCR.

3.6.3 Thermal cycling

The cycling conditions for the insertion sequence IS*2404* PCR are:

Denaturing at 95˚C for 10 minutes, 40 cycles at 95˚C for 10 seconds (sec), 58 ˚C for 10 secs, 72˚c for 30 seconds and One (1) cycle at 72˚C for 10 minutes. Samples were held at 8˚C.

3.6.4 Gel electrophoresis

A 1.5% agarose gel was prepared by weighing 1.8 g of agarose. Into 120ml of 0.5X Tris Borate EDTA buffer (TBE). The final content was mixed gently and heated in a microwave for 4 minutes. The cooked gel was allowed to cool at RT, 10µl of gel red added and gently swirled to mix.

Afterwards, an adhesive cellotape was used to seal the open ends of gel electrophoresis tray. The spacer comb was fixed (the number of samples being worked was directly proportional to the number of spacer comb used). The gel was poured into the prepared tray and allowed to solidify.

TBE-Buffer at 0.5x was poured into the electrophoresis chamber up to the maximum mark. The adhesive tapes on the ends of the casting tray were removed as well as the spacer combs and then placed into the gel electrophoresis chamber. This was followed by adding 15µl of each DRB-PCR sample mixed with 3µl loading dye and pipetted into the gel slots. DNA ladder of a 100 base pair (bp) was loaded into one gel slot at a volume of 6µl. The gel chamber was connected to a power supply for 45 minutes at 100 Volts.

The agarose gel was photographed using the video camera integration (inqababiotic, South Africa) on 1.50s, UV 100%, exposure time of 1/8 sec, zoom 12.5 and focus 1 meter under UV illumination.

Under UV light, if a 492 base pair corresponds to each patient sample compared with the 100 base pair DNA ladder, this is indicative of *M. ulcerans* DNA in the test sample. Each test included a positive, inhibition and negative controls. Samples were reported as positive, negative or inhibited.

3.5 Immune response of study participants

3.5.1 Antigens selected for ex-vivo cell stimulation

Antigens used included 1 µg/mL phorbol 1-myristate 1-acetate plus ionomycin (PMA+Io) and 5 µg/mL lipopolysaccharide (Sigma-Aldrich, UK) as a positive control and RPMI medium as a negative control. Plasmid DNA encoding 11 Mycolactone polyketide synthase domains namely, acyl carrier protein (ACP) types 2 and 3, acyltransferase with acetate specificity types 1 and 2 (ATac 1, ATac 2), acyltransferase with propionate specificity (ATp), enoylreducatse (ER), type A and B ketoreductase (KR A, KR B), ketosynthase type C (KS C), load module ketosynthase domain (ksalt) and dehydratase (DH) were provided by Dr. K. Huygen (WIV-ISP Site Ukkel, Belgium). The genes encoding all 11 enzymatic modules of the polyketide synthase were cloned in eukaryotic expression vector pV1. Jns-tPA. In this plasmid, the genes were known to be expressed under the control of the promoter of IE1 antigen from cytomegalovirus preceded by the signal sequence of human tissue plasminogen activator (Roupie *et al*., 2014). All antigens were used at a final concentration of 5 µg/mL based on an early study (Phillips *et al*., 2006)

Mu sonicate antigen was prepared from Mu isolates of African origin by sonification. The antigen was prepared as follows: A loopful of Mu colonies cultivated on Lowenstein Jensen slopes was transferred into 10 ml of sauton's medium (2mM $MgSo_4$, 10 mM citric acid, 3 mM K_2HPO_4, 30 mM asparagine, 0.005% ferric ammonium citrate, 520mM glycerol, pH adjusted to 7.2 with ammonia, autoclaved for 20 minutes at 121ºC) and incubated at 30ºC overnight. The 10-ml *M. ulcerans* culture was passed into 50 ml and subsequently into 500ml of Sauton`s medium with shaking (speed,100 rpm; New Brunswick Scientific Co., Inc., Edison, N.J.). After 4 weeks, the bacterial pellet, obtained after centrifugation of *M. ulcerans* cultures at 4ºC for 30 minutes at 18,000 x

g, was washed twice with 1x Phosphate Buffered Saline (PBS) in 500 ml polycarbonate tubes and centrifuged at 4ºC for 30 minutes at 18,000xg (10,500 rpm) (Sorvall Plus centrifuge). The pellet was suspended in 35 ml sterile water and sonicated with a Branson 250 Sonifier at 50% duty cycle using a small probe in a cup-horn container: four cycles of 15 minutes continuous cooling, interspersed with 5-minutes breaks of cooling on ice. The Mu sonicate was aliquoted in 2 ml portions and lyophilized (Phillips *et al*., 2006).

3.6 Diluted Whole Blood Stimulation Assay

Heparinized blood samples were taken at baseline (week 0), after treatment (week 8), week 16 and in case the patient developed a paradoxical reaction. The diluted whole blood stimulation assay was carried out under aseptic conditions. A portion, 4ml of the whole blood was diluted in a ratio of 1:10 in sterile RPMI medium in 50 mls falcon tubes, mixed gently and distributed, 1ml per well of sample (Plate 10.0), into prelabelled 24-well culture plates (Nunclon Surface culture plates). Two plates were used for each patient for 20 hours stimulation and 5 days stimulation respectively. The required volume of the plasmid antigens (stimulus) was added; one well each was left unstimulated. The stimulus was added respectively as follows: PMA, LPS, ATp, Ksalt, ACP2, KRA, KSc, DH, KRA, ATac1, Ag85Aulc, KRB, ER, ACP3. The culture plates were gently swirled 10 times clockwise and anticlockwise in the laminar flow cabinet after the addition of the stimulus.

For the paradoxical reaction, the antigens used were PMA, Ag85A and / or *M. ulcerans* sonicate. Culture plates were incubated at 37°C and 5% CO_2 overnight (20 hours) and 5 days in a CO_2 incubator (Thermo Scientific, Germany). Supernatants (200-500µl per well) were collected into cyrovials under sterile conditions and stored at -80°C and later

assayed for Interferon-γ and Interleukin-5 using enzyme linked immunosorbent assay (ELISA).

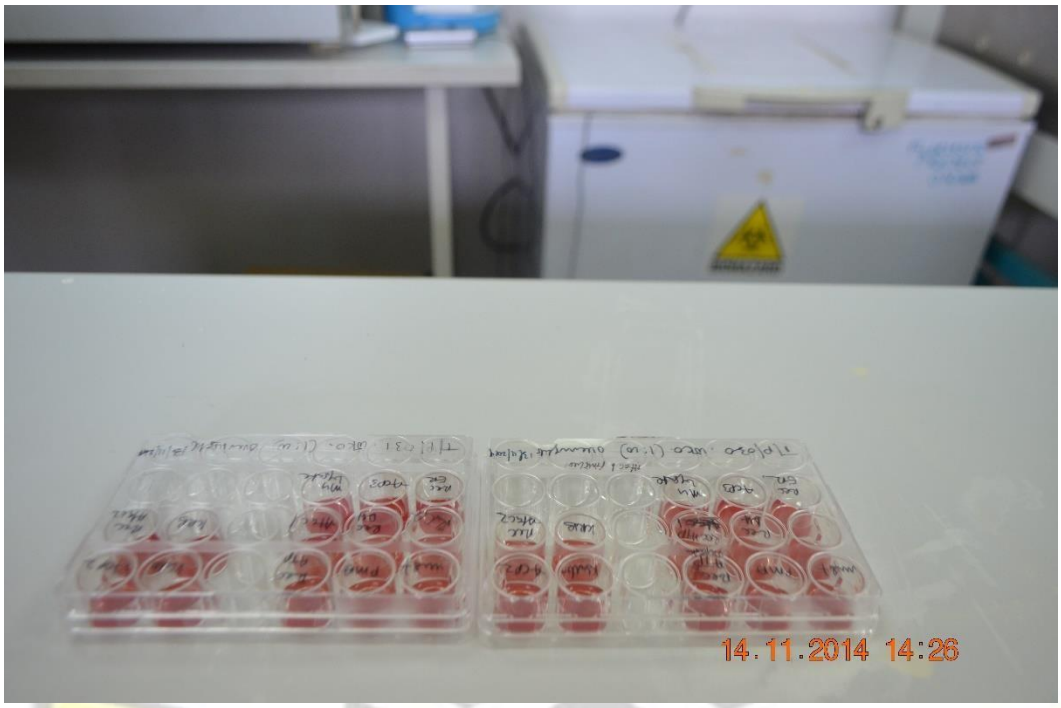


PLATE 9.0 CULTURE PLATES ABOUT TO BE INCUBATED AFTER WHOLE BLOOD STIMULATION ASSAY.

3.7 Cytokine quantification by Enzyme linked Immunosorbent Assay (ELISA)

Interferon-γ and Interleukin-5 were the cytokines measured in this study. Their quantification was determined using OptEIA set for human interferon-γ and IL-5 (BD Biosciences, Pharmingen, U.S. A) as described below.

All assays were performed using a high protein binding ELISA plate (Greiner bio-one, Germany). The plates were coated with anti-human monoclonal antibody in a coating buffer of 7.13g $NaHCO_3$, 1.59g Na_2CO_3; q.s. to 1.0L with 10N NaOH at a pH of 9.5. The plates were sealed with parafilm (American can, U.S. A) and incubated overnight at 4 ºC and washed using an ELISA plate washer (Gentaur/GDMS Belgium) for 3 wash cycles. The wash buffer used was made up of Phosphate buffered saline (PBS) with 0.05% Tween-20 (Sigma-Aldrich Inc., Germany). Unspecific binding sites were

blocked using assay diluent (blocking buffer), the plates were then incubated at room temperature for an hour. After which, contents per well was aspirated and washed 3 times.

A serial dilution of the initial standard with concentration of 300 pg/ml was prepared and 100 µl per well was added to the first pair of wells in duplicates. The test samples (supernatants) and negative control (blank) were then added. The plates were incubated for 2 hours at room temperature and washed for 5 wash cycles.

A working detector solution consisting of biotinylated anti-human monoclonal antibody, streptavin-horse radish peroxidase conjugate and sample diluent (1:250) was added into each well at a volume of 100 µl. After incubation for an hour, the plates were washed for 7 times. Equal volumes of substrate solution A (tetramethyl benzidine) and B (hydrogen peroxide). were mixed and 100 µl of the final mix was pipetted into each well. The plates were incubated in the dark for 30 minutes.

To stop the activity of the substrate solution (Plate 11.0), 50µl of concentrated hydrogen sulphate (2M H_2SO_4) was pipetted into each well.

3.8. Reading and calculation

The absorbance was read within 30 minutes. The optical densities of the standards and samples were measured using TECAN Sunrise ELISA reader (Tecan Group Ltd. Switzerland). The mean absorbance of duplicate standards, samples, and controls were calculated for each plate, and the mean zero standard absorbance was subtracted.

Results were analysed with GraphPad Prism 6 software (GraphPad Software, Inc., USA) and a standard (best-fit) curve was plotted. Values for unstimulated cultures were subtracted from those for stimulated cultures. The lower detection limit used was 4.7pg/ml.

PLATE 10.0 ADDITION OF STOP SOLUTION TO HALT ACTIVITY OF SUBSTRATE DURING ELISA ASSAY.

3.9 Statistical analysis

Descriptive statistics were used to obtain general descriptive information such as the mean and standard deviation from the data. One sample analysis (Fisher's exact test) was used to compare two proportions or age distribution, sex distribution of study participants. One-way ANOVA (Kruskal-Wallis test) was used to compare group means (age distribution, sex distribution of study) participants. Descriptive results of cytokine levels were expressed as medians and ranges. Effect of antibiotic treatment on responses, comparing non-ulcerative and ulcerative clinical forms, responses of patient with paradoxical reactions versus patients without paradoxical reaction were analyzed using Mann-Whitney U test. The cytokine levels of patients were evaluated for association or correlation with their time to healing with the Spearman r. A $p \leq 0.05$ was considered statistically significant.

CHAPTER 4

RESULTS

4.1 Characteristics of immune response to plasmid antigen study participants.

To determine the systemic immune response of BUD patients to plasmid antigens, 73 participants were enrolled.

Table 1.0, shows the demographics of the participants. There were 24 participants with active Buruli ulcer disease confirmed by *M. ulcerans* IS2404 PCR. The 37 endemic controls had known contact to Bu patients, and 12 non endemic controls were nonresidents of the disease endemic communities. The median (IQR) age was 14 years (range 8-35) for active lesions, 24 years (range 16-29) for endemic controls and 25years (range 22-28) for non-endemic controls. There were 11 non-ulcer forms (4 nodules and 7 plaques) and 13 ulcers. Ten lesions were category I, 10 category II and 4 category III.

TABLE 1.0 CHARACTERISTICS OF 73 STUDY PARTICIPANTS.

Parameters	Active Buruli ulcer	Endemic controls*	Nonendemic controls**	Total	P value
	n=24	n=37	n=12	73	
Age median(IQR)	14(8-35)	24(16-29)	25(22-28)	22(14-29)	0.0852
Age median(range)	14(5-70)	24(9-58)	25(19-31)	22(5-70)	0.0852
Sex					
Male	13(54%)	18(49%)	7(58%)	38(52%)	
Female	11(46%)	19(51%)	5(42%)	35(48%)	
BCG Scar					
Yes	13(54%)	18(49%)	7(58%)	38(52%)	
No	11(46%)	19(51%)	5(42%)	35(48%)	
Lesion form					
Nodule	4(17%)	-	-	4	
Plaque	7(29%)	-	-	7	
Ulcer	13(54%)	-	-	14	
Category of lesion					
Category I	10(42%)	-	-	10	

KNUST

Category II	10(42%)	-	-	10
Category III	4(16%)	-	-	4

4.2 Immunogenicity of Plasmid DNA encoding mycolactone polyketide synthase domains.

To determine the immunogenicity of the plasmid antigens, patients with active Buruli ulcer disease and controls shown in Table 1.0 were studied. The controls were made up of endemic controls and non-endemic controls. Venous blood obtained from patients with *M. ulcerans* disease and controls into sodium heparin vacutainer tubes were used to measure their immune response. Their immune responses were determined by stimulating their whole blood with the 13 plasmid antigens and incubated for 5 days. A whole blood stimulation assay was used because its more appropriate for field studies. Culture supernates were tested for the presence of interferon-γ indicative of a Th1 immune response responsible for protection in mycobacterial disease. Using a response cut-off of 37 pg/ml interferon-γ to define a responder, the proportion of subjects making a positive response to each of the antigens was calculated.

Figure 2.0 shows that using a cut off of 37 pg/ml interferon-γ to define a responder, more than 80% of patients and participants from endemic areas responded to all the antigens except ACP3 where 47% of active Buruli ulcer cases and 71% of endemic controls responded. The highest proportion of responders in both participant groups was observed in reaction to Ksalt (100%) and ER (100%). Overall a higher proportion of endemic controls responded with higher interferon-γ responses than the Buruli ulcer cases to all the PKS domain antigens and to DNA encoding mycolyltransferase Ag85A of *M. ulcerans* (Ag85Aulc). There was no response to any of the antigens in all but one

of the control participants from non-endemic areas [Figure 3.0(A), (B), (C)]. The exception was the same participant for all antigens. This person was a European who had previously carried out focus group discussions in a Buruli ulcer endemic village on patients with Buruli ulcer and had assisted with obtaining their vital signs at the health centre for 3 months. These results above suggest that the antigens are immunogenic. Interestingly ER and Ksalt were the most immunogenic antigens in both cases and endemic controls.

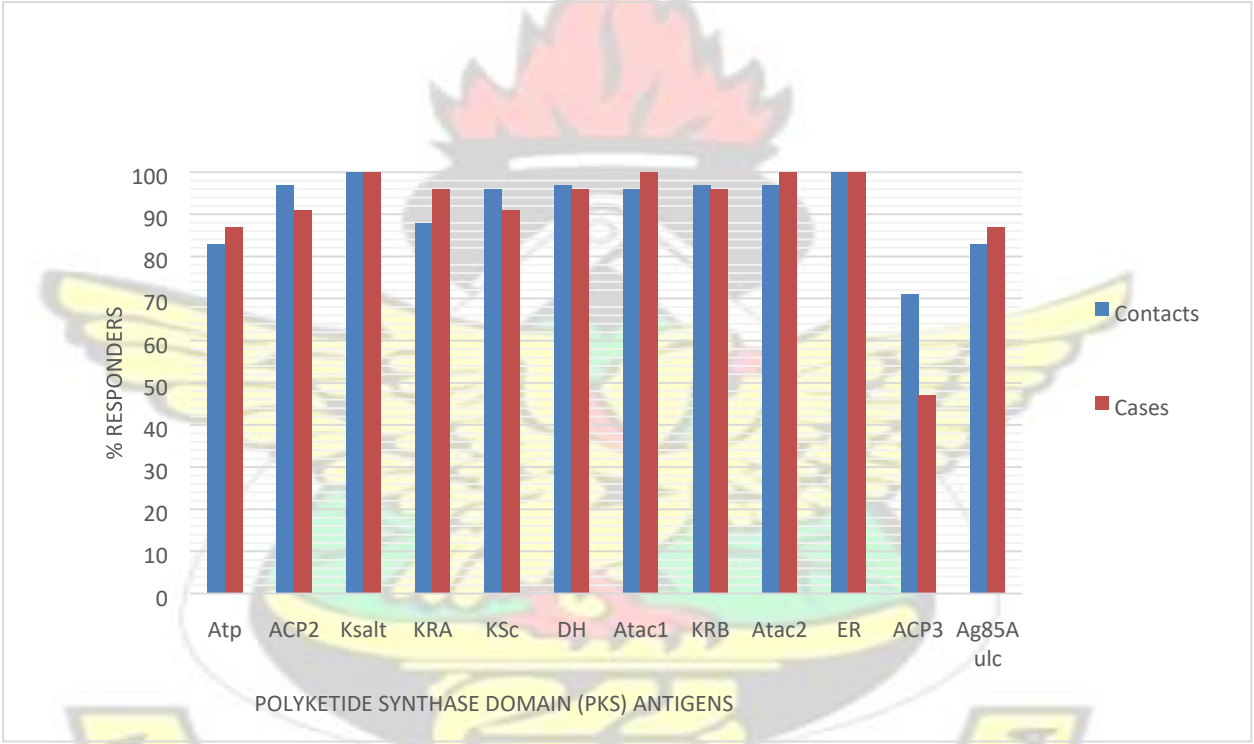


FIGURE 2.0 FREQUENCY OF INTERFERON-Ɣ RESPONDERS TO A PANEL OF PKS ANTIGENS AND AG85AULC AFTER DILUTED WHOLE BLOOD STIMULATION FOR 5 DAYS OF BUD CASES AND HEALTHY ENDEMIC CONTROLS (CONTACTS). A RESPONSE CUTOFF OF 37 PG/ML INTERFERON-Ɣ WAS USED TO DEFINE A RESPONDER.

4.2.1 Specificity of the plasmid antigens

To determine the specificity of the plasmid antigens, patients confirmed with Buruli ulcer disease and controls recruited had their immune responses determined by stimulating their whole blood obtained at baseline with plasmid antigens and incubated for 5 days. Culture supernates were tested for interferon-γ. The interferon-γ responses were compared for the various plasmid antigens in patient and control participants.

When individual responses were compared, contacts of Buruli ulcer patients produced significantly higher interferon-γ responses compared with those of patients for ATp (median 449.39 vs 3415.58 pg/ml), Ksalt (521.24 vs 4610.81) pg/ml), ACP2 (319.09 vs 3791.51 pg/ml), KS C (618.37 vs 3944.93 pg/ml), DH (median 671.20 vs 4596.45 pg/ml), ER (3009.53 vs 5024.38 pg/ml), KRB (1004.00 vs 5261.07 pg/ml), Atac2 (1215.08 vs 4900.18 pg/ml) ($P < 0.05$). Although there was a trend to higher IFN-γ responses in contacts compared with cases it did not reach significance with ATac1, ACP3, KR A and *M. ulcerans* Ag 85A [Figure 3.0(A),(B),(C)]

These results above suggest that endemic controls generally mounted significantly higher interferon-γ responses compared to diseased patients. However, the responses were discriminatory for those living in non-endemic areas who are known to have no contact with *M. ulcerans*.

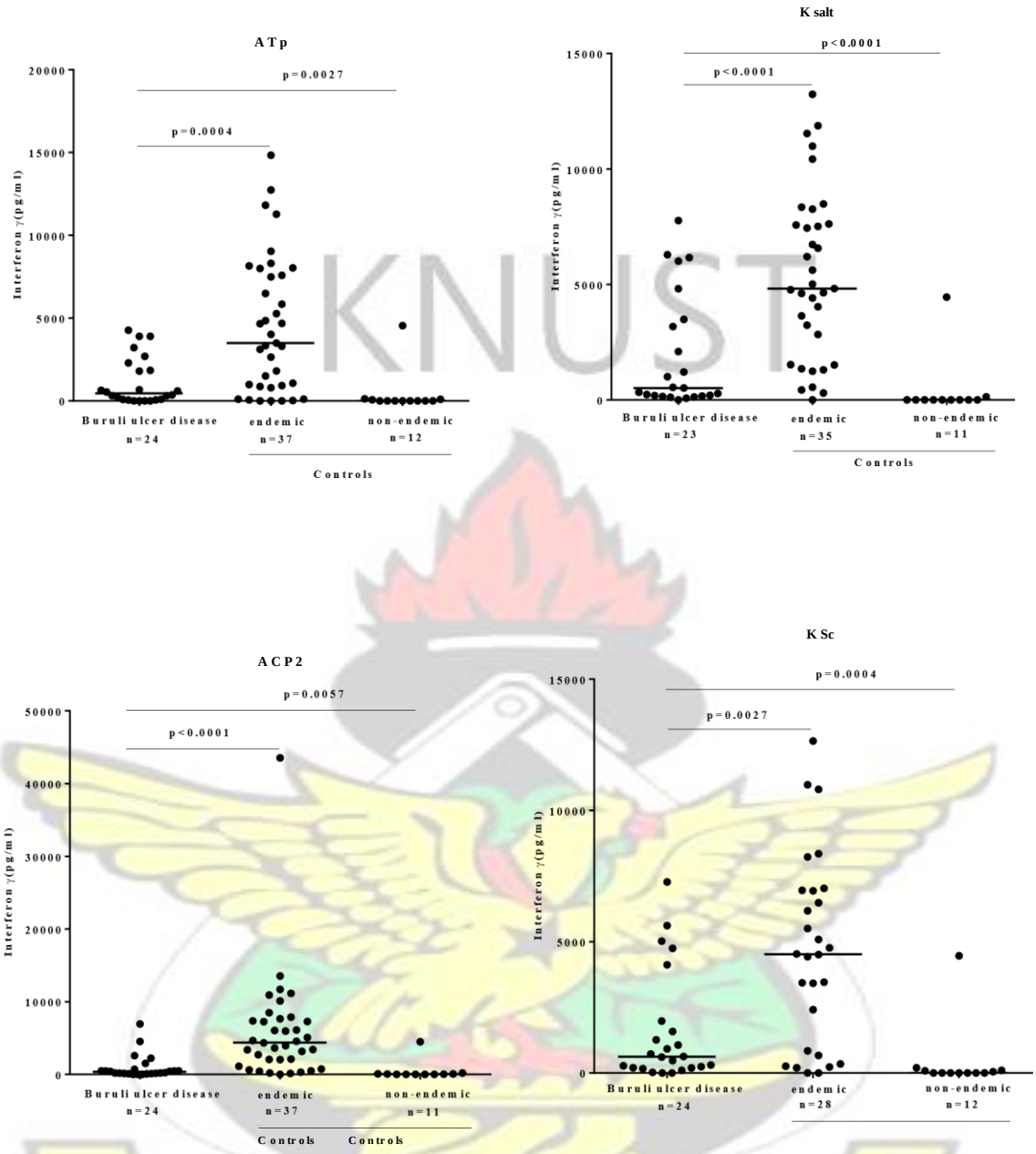


FIGURE 11.0(A) INTERFERON-Ɣ RESPONSES TO A PANEL OF PLASMID DNA ENCODING MYCOLACTONE POLYKETIDE SYNTHASE ANTIGENS AFTER DILUTED WHOLE BLOOD STIMULATION FOR 5 DAYS OF *MYCOBACTERIUM ULCERANS* DISEASE CASES, HEALTHY ENDEMIC CONTROLS AND HEALTHY NON-ENDEMIC CONTROLS. EACH DOT REPRESENTS RESPONSE OF ONE STUDY PARTICIPANT. THE HORIZONTAL LINES REPRESENT THE MEDIANS.

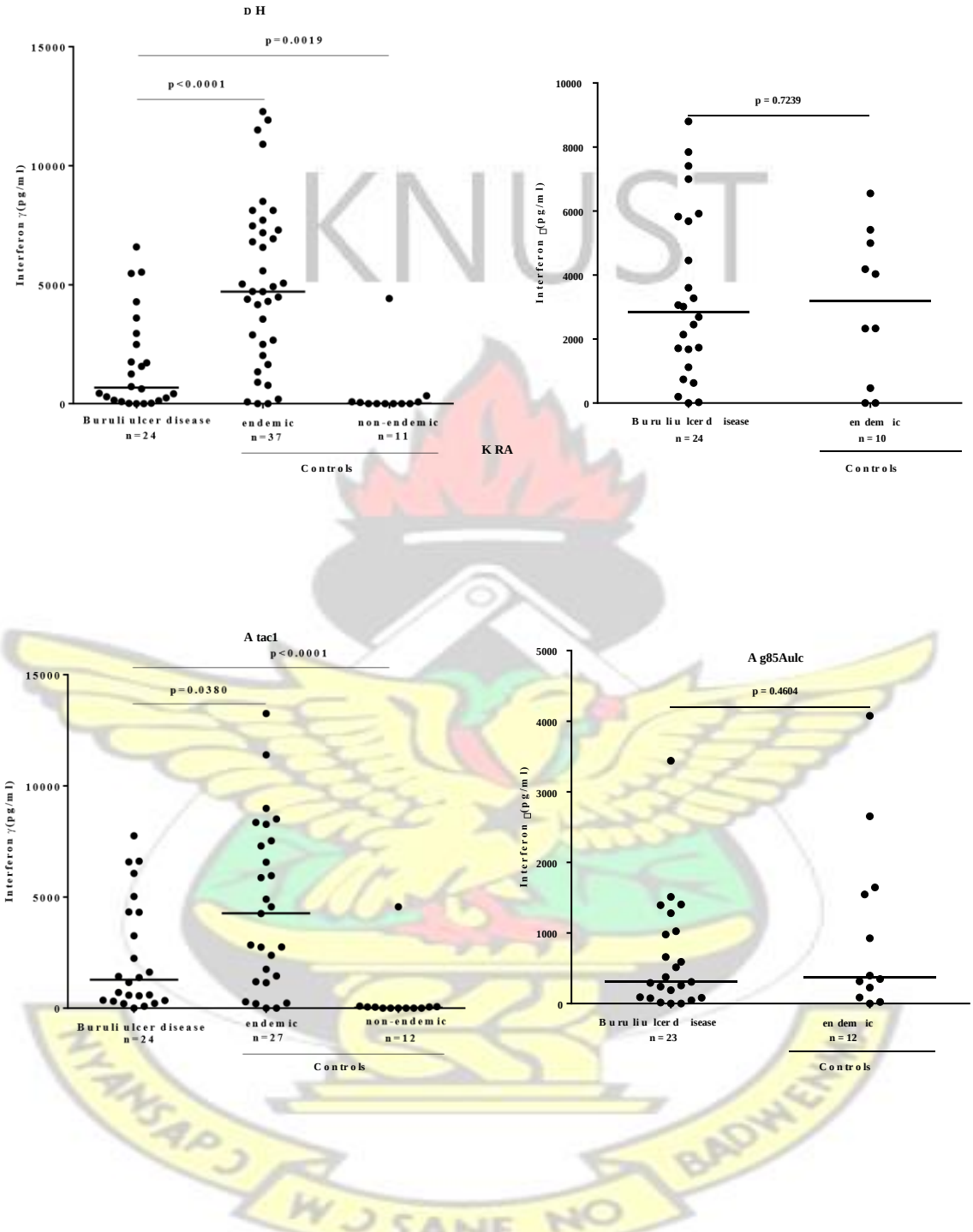
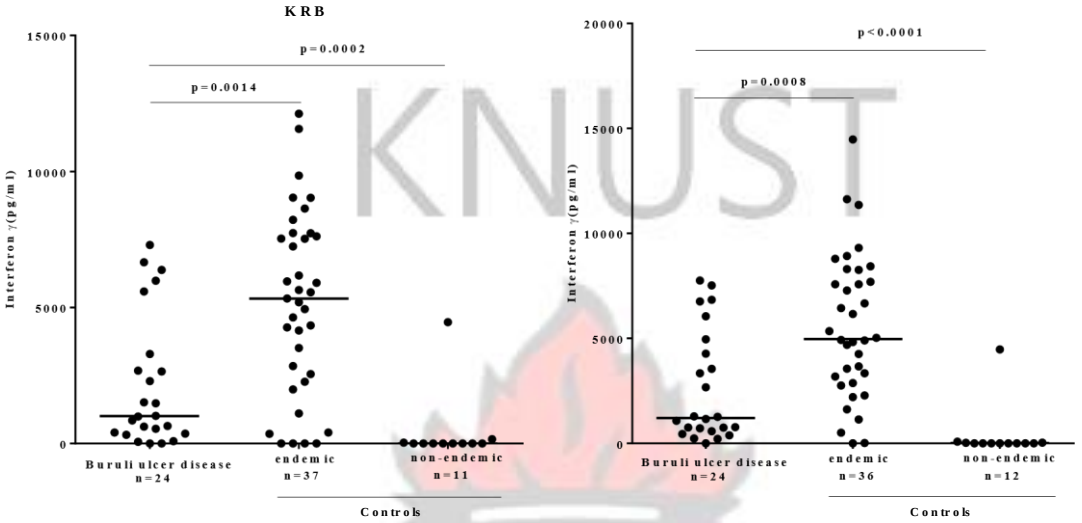
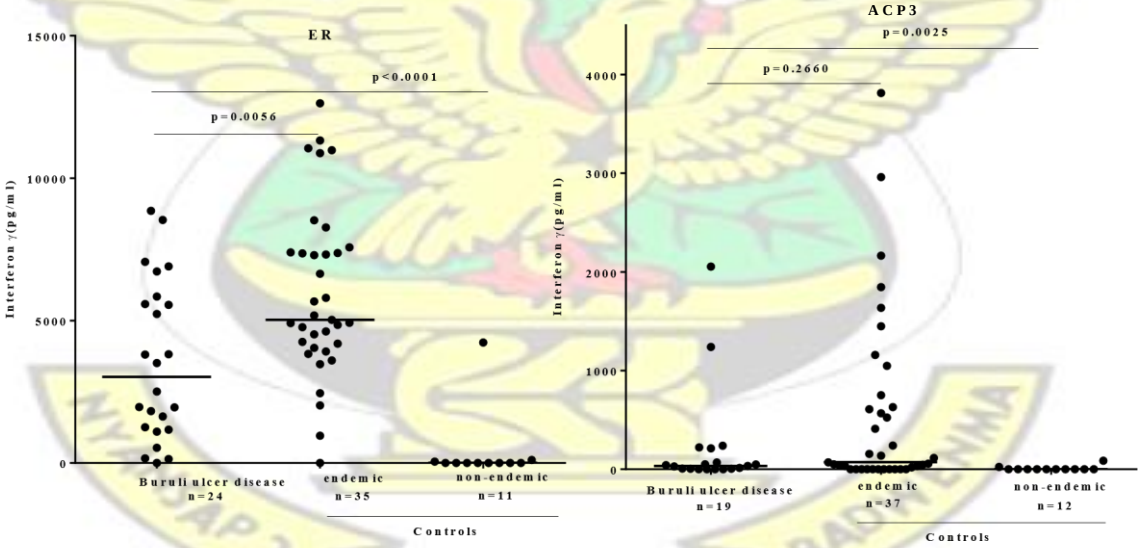


FIGURE 12.0(B) INTERFERON-Ɣ RESPONSES TO A PANEL OF PLASMID DNA ENCODING MYCOLACTONE POLYKETIDE SYNTHASE ANTIGENS AFTER DILUTED WHOLE BLOOD STIMULATION FOR 5 DAYS OF *MYCOBACTERIUM ULCERANS* DISEASE CASES, HEALTHY ENDEMIC CONTROLS AND HEALTHY NON-ENDEMIC CONTROLS. EACH DOT REPRESENTS RESPONSE OF ONE STUDY PARTICIPANT. THE HORIZONTAL LINES REPRESENT THE MEDIANS.


KRB
p=0.0002
p=0.0014
Interferon γ(pg/ml)
15000
10000
5000
0
Buruli ulcer disease
n=24
endemic
n=37
non-endemic
n=11
Controls
A tac2
p<0.0001
p=0.0008
Interferon γ(pg/ml)
20000
15000
10000
5000
0
Buruli ulcer disease
n=24
endemic
n=36
non-endemic
n=12
Controls


ER
p<0.0001
p=0.0056
Interferon γ(pg/ml)
15000
10000
5000
0
Buruli ulcer disease
n=24
endemic
n=35
non-endemic
n=11
Controls
ACP3
p=0.0025
p=0.2660
Interferon γ(pg/ml)
4000
3000
2000
1000
0
Buruli ulcer disease
n=19
endemic
n=37
non-endemic
n=12
Controls

KNUST

FIGURE 13.0(C) INTERFERON-Ɣ RESPONSES TO A PANEL OF PLASMID DNA ENCODING MYCOLACTONE POLYKETIDE SYNTHASE ANTIGENS AFTER DILUTED WHOLE BLOOD STIMULATION FOR 5 DAYS OF *MYCOBACTERIUM ULCERANS* DISEASE CASES, HEALTHY ENDEMIC CONTROLS AND HEALTHY NON-ENDEMIC CONTROLS. EACH DOT REPRESENTS RESPONSE OF ONE STUDY PARTICIPANT. THE HORIZONTAL LINES REPRESENT THE MEDIANS.

4.2.2 Interferon gamma responses of patients with Buruli ulcer disease before and after treatment.

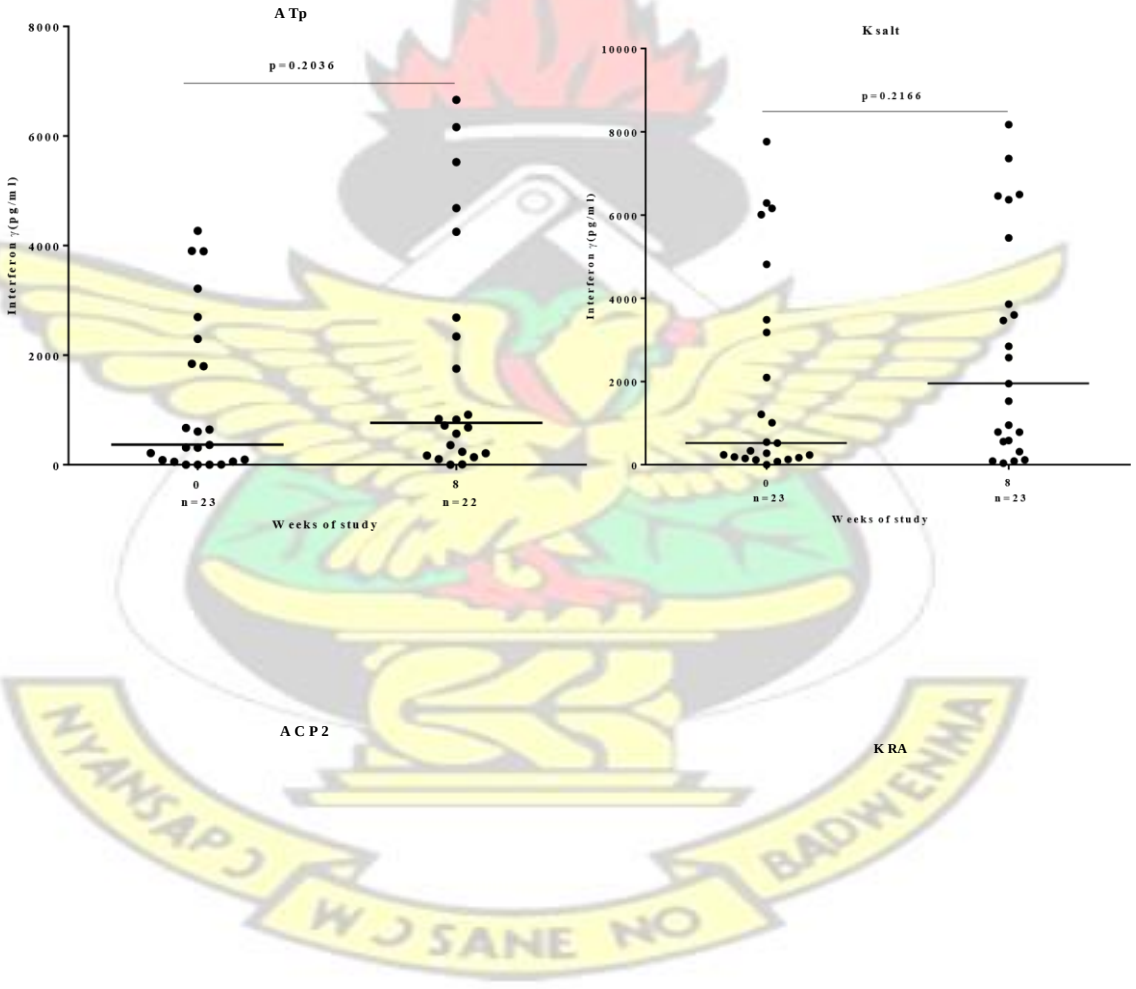
To determine if the interferon-γ responses change with treatment, patients with active Buruli ulcer disease (see table 1.0) were studied. Treatment with combination rifampicin and streptomycin or rifampicin and clarithromycin was initiated by the attending physician and patients followed up 2 weekly. Treatment of patients strictly followed the treating clinician's discretion according to WHO guidelines (WHO, 2014). Whole blood obtained from such patients at baseline, week 8 and week 16 were stimulated using the plasmid antigens at a concentration of 5 □g/ml and plasma supernates collected after 5 days incubation (see methods section 3.6). Plasma supernates were used to quantify interferon gamma secretion.

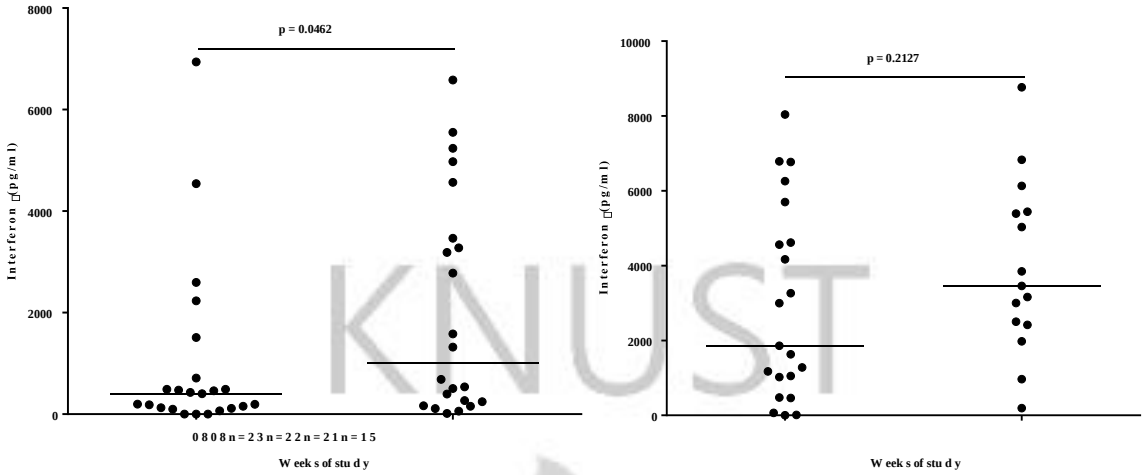
Figure 4.0(A), (B), (C), (D) shows the interferon-γ response of 24 patients to the plasmid antigens. Significantly higher responses to recombinant antigens ACP2 and ATac2 (P = 0.046 and P = 0.049 respectively) were observed after 8 weeks standard antibiotic treatment. There was no significant difference in response to the other candidate antigens before and after 8 weeks treatment (Table 2.0).

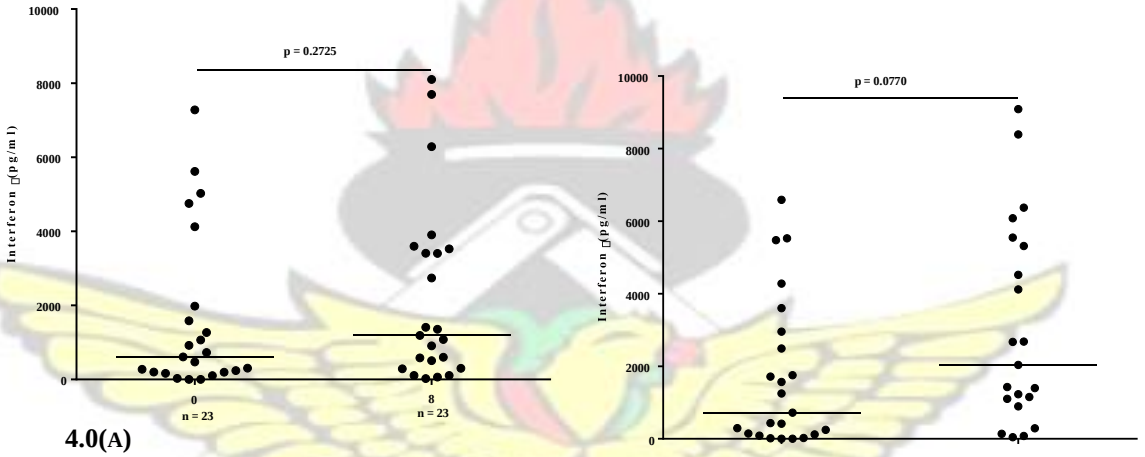
For three of the most immunogenic antigens: ATp [1544 (61.26-11066) p=0.03], Ksalt [3803 (136.1-11229) p=0.02] and ER [7662(63.80-11103) p=0.0003] profound interferon-γ responses were made eight weeks after completion of antibiotics (week 16) and were significantly higher than those at baseline. Further, interferon-γ responses to

FIGURE INTERFERON-Ɣ RESPONSES BEFORE (WEEK 0) AND AT COMPLETION (WEEK 8) OF ANTIBIOTICS TO PLASMID DNA ENCODING MYCOLACTONE POLYKETIDE SYNTHASE DILUTED WHOLE BLOOD STIMULATION FOR 5 DAYS OF *MYCOBACTERIUM ULCERANS* DOT REPRESENT RESPON THE HORIZONTAL LINES MEDIANS.

ER at week 16 were significantly higher than those at week 8 (p=0.001) (figure 5.0). There was no correlation between clinical form of disease, duration of lesion, category of lesion, time to healing and level of interferon-γ responses to Ksalt and ER antigen.





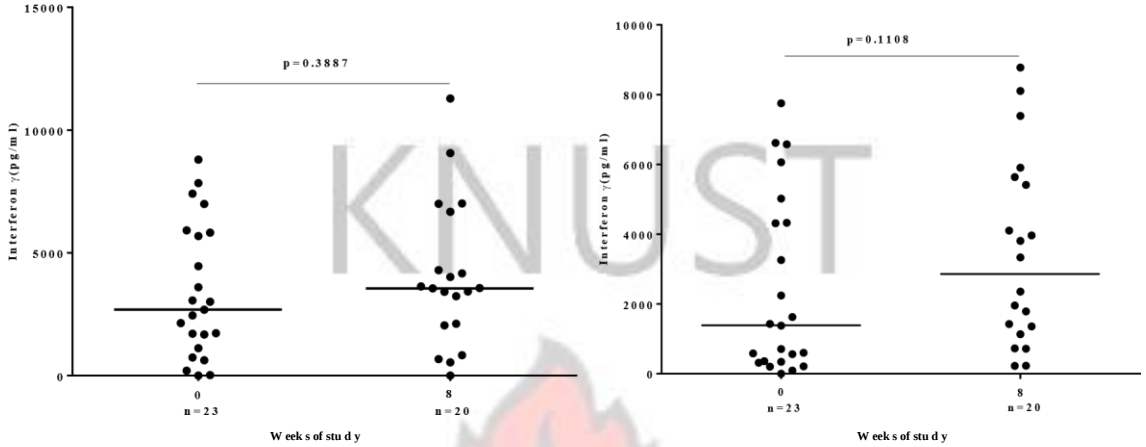


4.0(A)

ANTIGENS AFTER DISEASE CASES. EACH SE FOR ONE STUDY PARTICIPANT. REPRESENT THE

KNUST
NYANSAPO WOSANE NO BADWENMA

FIGURE INTERFERON-Ɣ RESPONSES BEFORE (WEEK 0) AND AT COMPLETION (WEEK 8) OF ANTIBIOTICS TO PLASMID DNA ENCODING MYCOLACTONE POLYKETIDE SYNTHASE DILUTED WHOLE BLOOD STIMULATION FOR 5 DAYS OF *MYCOBACTERIUM ULCERANS* DOT REPRESENT RESPON THE HORIZONTAL LINES MEDIANS.



KNUST

FIGURE INTERFERON-Ɣ RESPONSES BEFORE (WEEK 0) AND AT COMPLETION (WEEK 8) OF ANTIBIOTICS TO PLASMID DNA ENCODING MYCOLACTONE POLYKETIDE SYNTHASE DILUTED WHOLE BLOOD STIMULATION FOR 5 DAYS OF *MYCOBACTERIUM ULCERANS* DOT REPRESENT RESPON THE HORIZONTAL LINES MEDIANS.

4.0(B)

ANTIGENS AFTER DISEASE CASES. EACH SE FOR ONE STUDY PARTICIPANT. REPRESENT THE

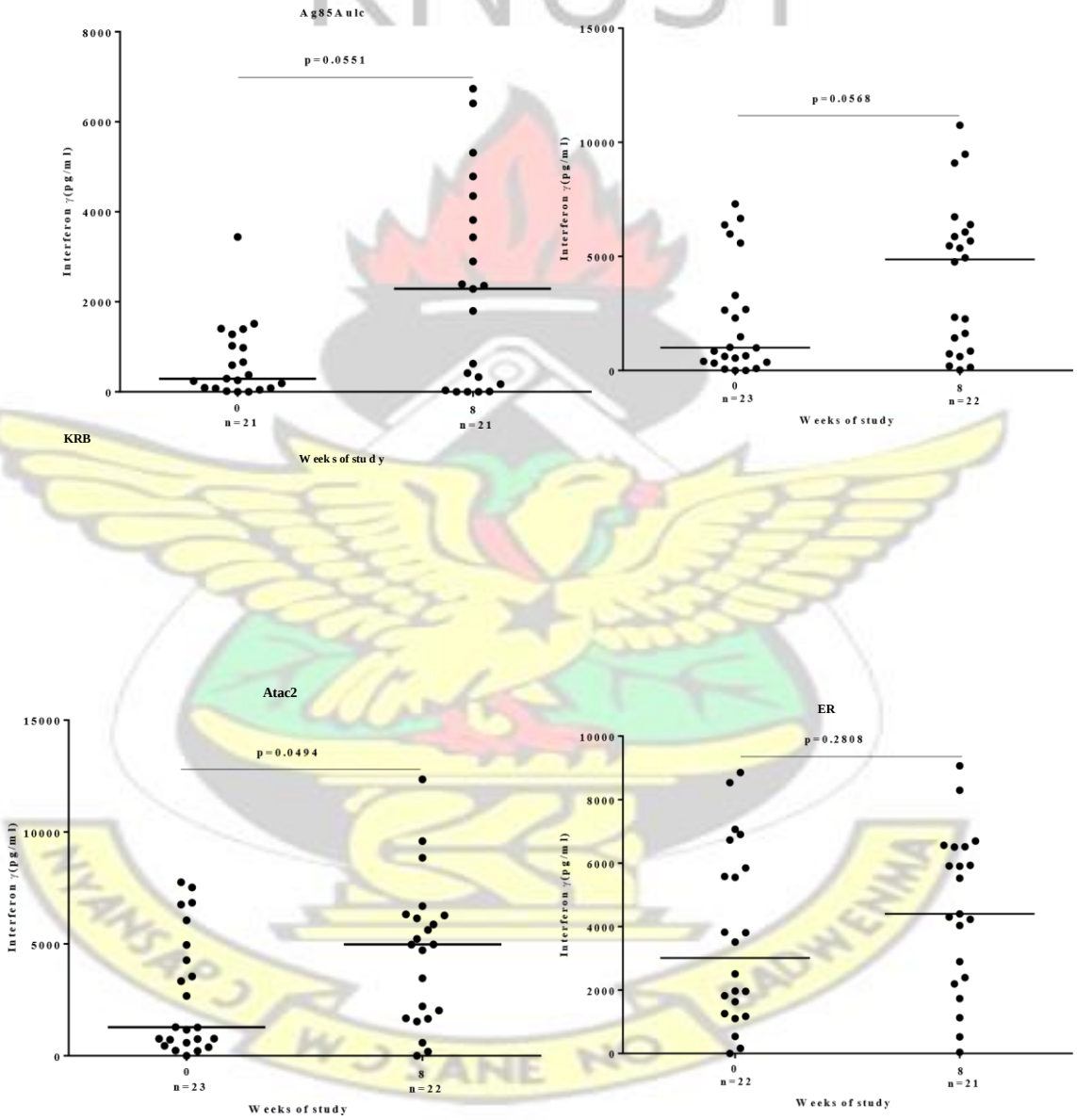
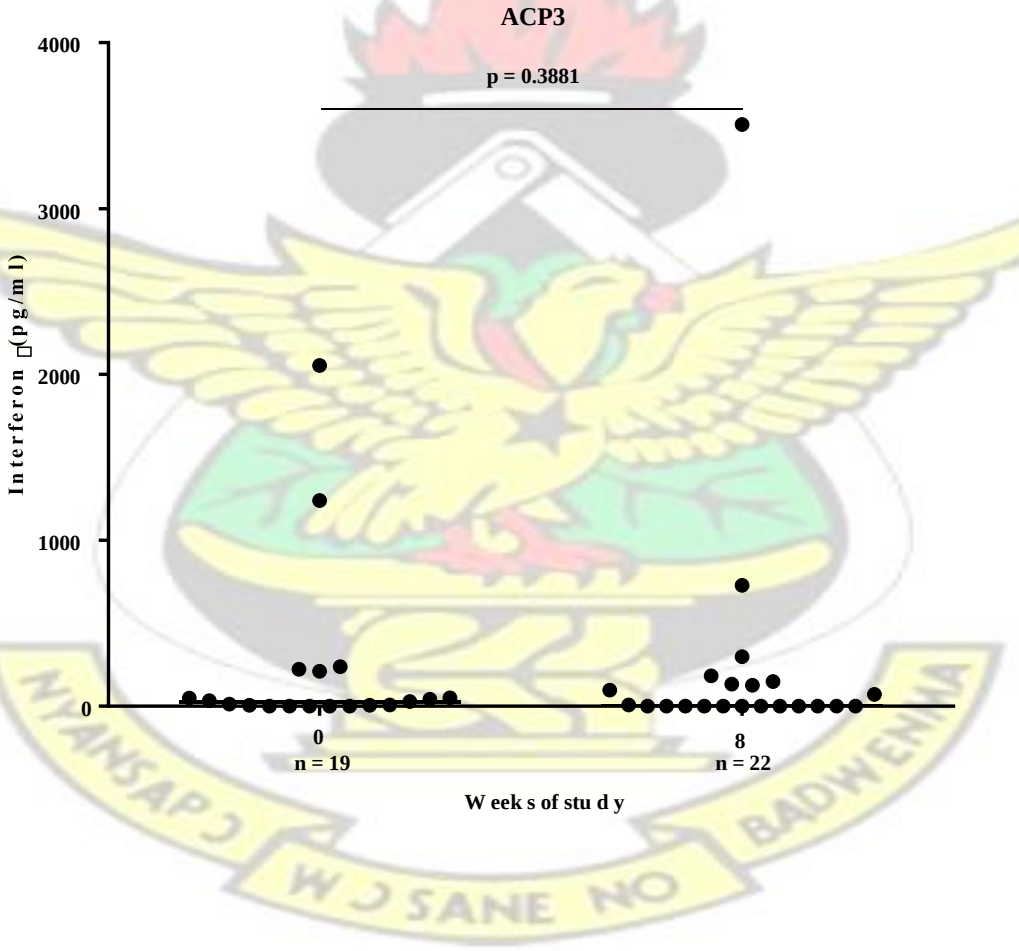


FIGURE INTERFERON-Ɣ RESPONSES BEFORE (WEEK 0) AND AT COMPLETION (WEEK 8) OF ANTIBIOTICS TO PLASMID DNA ENCODING MYCOLACTONE POLYKETIDE SYNTHASE DILUTED WHOLE BLOOD STIMULATION FOR 5 DAYS OF *MYCOBACTERIUM ULCERANS* DOT REPRESENT RESPON THE HORIZONTAL LINES MEDIANS.

4.0(C)

ANTIGENS AFTER DISEASE CASES. EACH SE FOR ONE STUDY PARTICIPANT. REPRESENT THE



KNUST
NYANSAPƆ WƆ SANE NO BADWENKA

FIGURE 4.0(D) INTERFERON-Ɣ RESPONSES BEFORE (WEEK 0) AND AT COMPLETION (WEEK 8) OF ANTIBIOTICS TO PLASMID DNA ENCODING MYCOLACTONE POLYKETIDE SYNTHASE ANTIGENS AFTER DILUTED WHOLE BLOOD STIMULATION FOR 5 DAYS OF *MYCOBACTERIUM ULCERANS* DISEASE CASES. EACH DOT REPRESENT RESPONSE FOR ONE STUDY PARTICIPANT. THE HORIZONTAL LINES REPRESENT THE MEDIANS.

KNUST

TABLE 2.0 INTERFERON-Ɣ RESPONSES TO A PANEL OF PKS ANTIGENS AFTER DILUTED WHOLE BLOOD STIMULATION FOR 5 DAYS OF 24 *MYCOBACTERIUM ULCERANS* DISEASE CASES.

	Week 0	Week 8	
Antigens	Median interferon-γ range (pg/ml)	Median interferon-γ range (pg/ml)	p-value
ATp	363.6(0.00-4269)	770.2(0.00-6659)	0.2036
Ksalt	533.0(0.00-7764)	1950(36.94-8173)	0.2166
ACP2	402.0(0.00-6939)	1004(13.18-6584)	0.0462*
KSC	609.1(0.00-7277)	1190(24.50-8101)	0.2725
DH	721.8(0.00-6587)	2037(44.06-9086)	0.077

FIGURE INTERFERON-Ɣ RESPONSES BEFORE (WEEK 0) AND AT COMPLETION (WEEK 8) OF ANTIBIOTICS TO PLASMID DNA ENCODING MYCOLACTONE POLYKETIDE SYNTHASE DILUTED WHOLE BLOOD STIMULATION FOR 5 DAYS OF *MYCOBACTERIUM ULCERANS* DOT REPRESENT RESPON THE HORIZONTAL LINES MEDIANS.

KRA	2688(0.00-8797)	3561(4.394-11288)	0.3887
Rec KRA	1858(0.00-8041)	3458(193-8765)	0.2127
ATac1	1379(0.00-7753)	28439(225.7-8776)	0.1108
Ag85A ulc	293.2(0.00-3440)	2287(0.00-6735)	0.0551
KRB	993.1(0.00-7299)	4851(25.18-10745)	0.0568
ATac2	1264(0.00-7753)	4973(0.00-12355)	0.0494*

KNUST

ER	3010(0.00-8855)	4397(37.58-9064)	0.2808
ACP3	28.78(0.00-2054)	0.6633(0.00-3507)	0.3881

* Mann Whitney test p-value < 0.05 indicate a significant difference

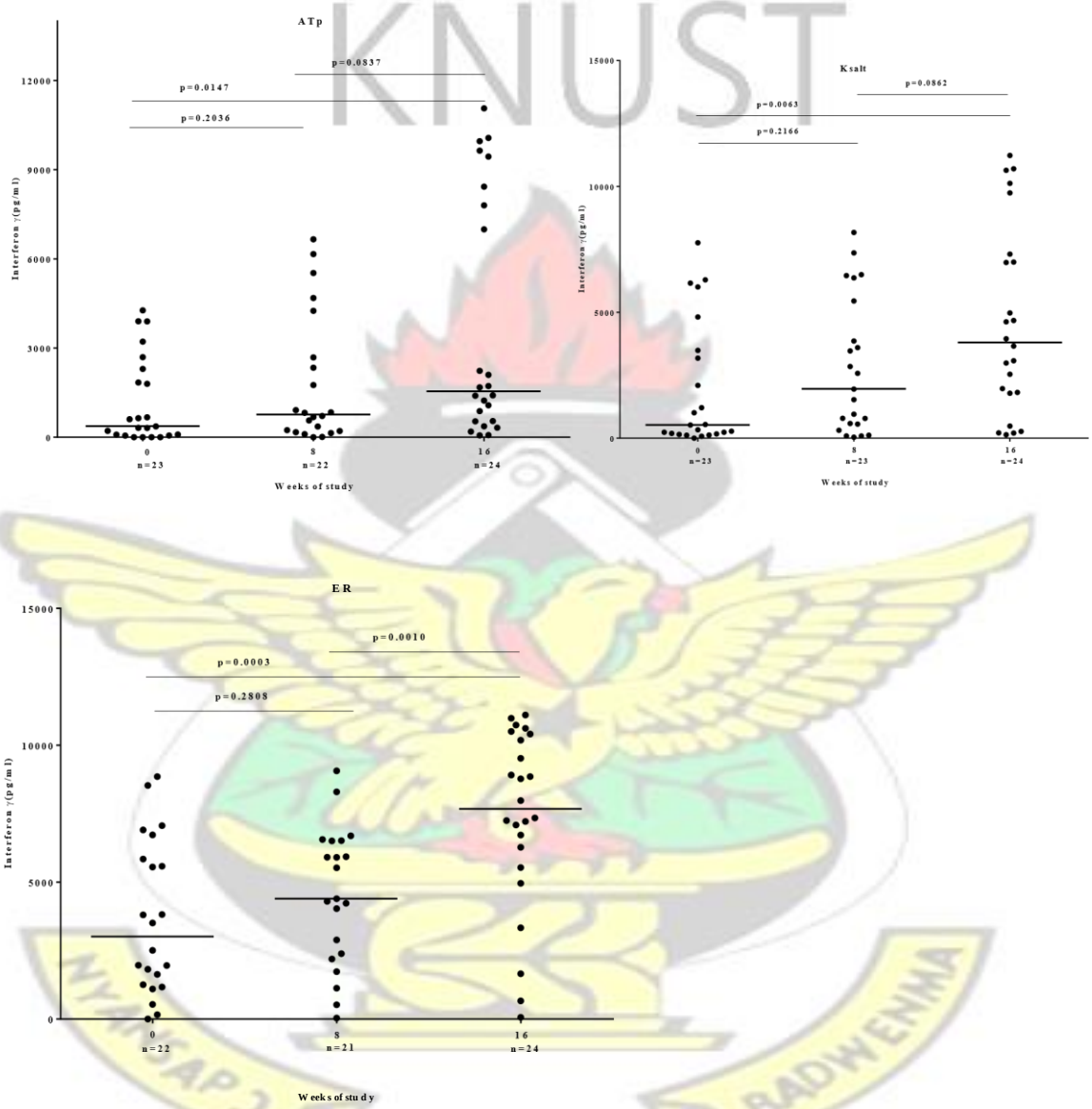


FIGURE 14.0 INTERFERON-Ɣ RESPONSES BEFORE (WEEK 0), AT COMPLETION (WEEK 8) OF ANTIBIOTICS AND 8 WEEKS AFTER ANTIBIOTIC COMPLETION (WEEK 16) TO PLASMID DNA ENCODING MYCOLACTONE

POLYKETIDE SYNTHASE ANTIGENS ATP, KSALT AND ER AFTER DILUTED WHOLE BLOOD STIMULATION FOR 5 DAYS OF *MYCOBACTERIUM ULCERANS* DISEASE CASES. EACH DOT REPRESENT RESPONSE OF ONE STUDY PARTICIPANT. THE HORIZONTAL LINES REPRESENT THE MEDIANS.

KNUST

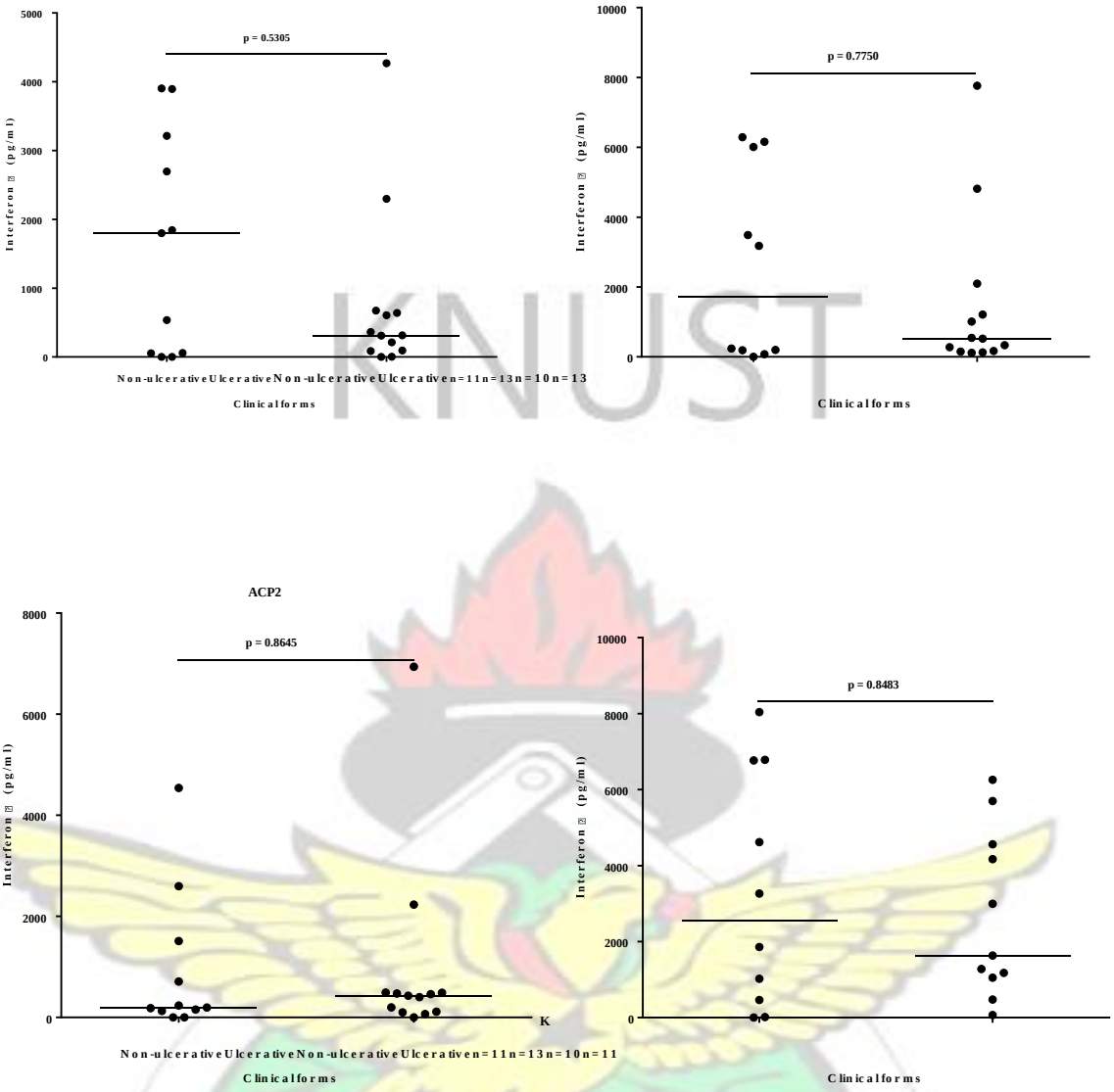
4.3.5 Interferon gamma response of patients with non-ulcerative versus ulcerative forms of the disease.

To determine if interferon-γ response of patients presenting with non-ulcerative and ulcerative lesion forms vary, the interferon-γ responses of patients with BU at baseline was determined by stimulating their whole blood with the plasmid antigens and incubated for 5 days. Culture supernates were tested for interferon gamma. Interferonγ responses of patients with ulcerated disease and non-ulcerated disease were compared.

Figure 6.0(A), (B), (C), (D) shows that, 11 patients with non-ulcerative forms mounted stronger responses to the PKS antigens compared to those of 13 patients with ulcerative forms. Although ACP3 induced a significantly higher response from non-ulcerative lesion forms compared to ulcerative forms, responses to this antigen were generally low.

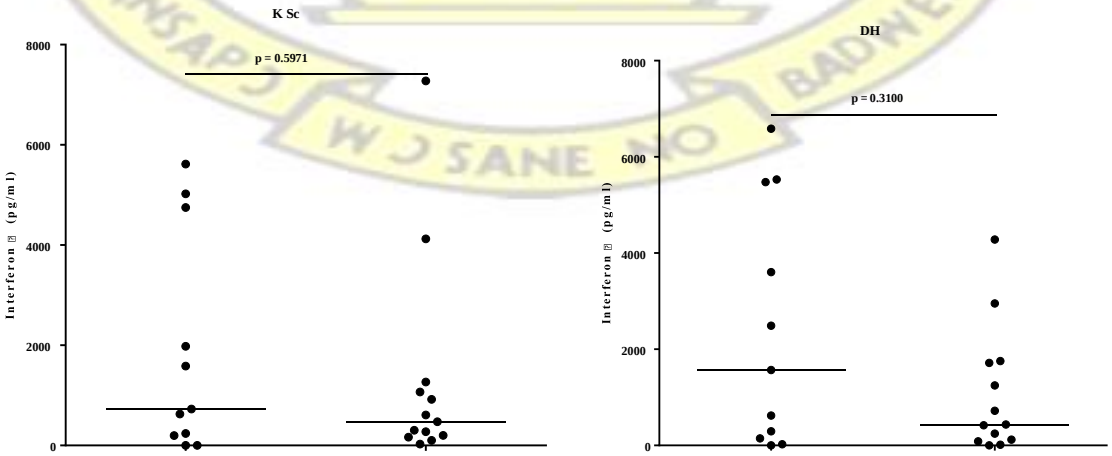
These results suggest that the severe form of Buruli ulcer disease have much more impaired interferon-γ responses to most plasmid antigens.

A T p **K salt**



R A

FIGURE 15.0(A) INTERFERON-γ RESPONSE AT ULCERATIVE AND NON-ULCERATIVE STAGES OF THE DISEASE. EACH DOT REPRESENTS RESPONSE OF ONE STUDY PARTICIPANT. THE HORIZONTAL LINES REPRESENT THE MEDIANS.



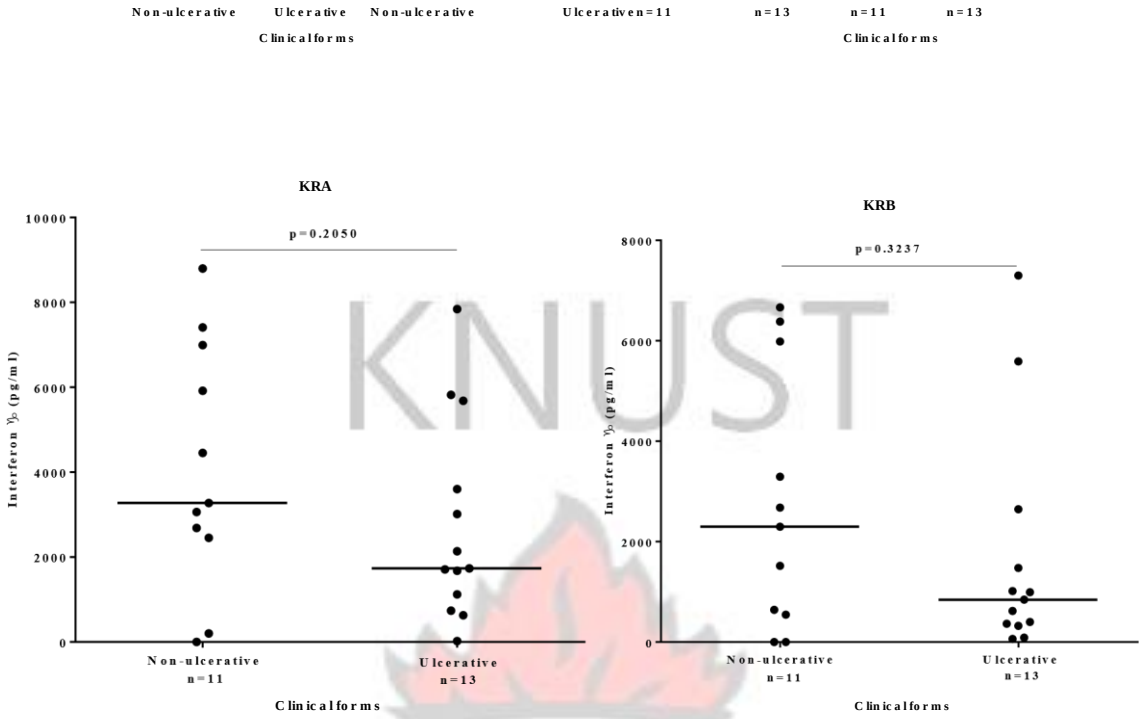
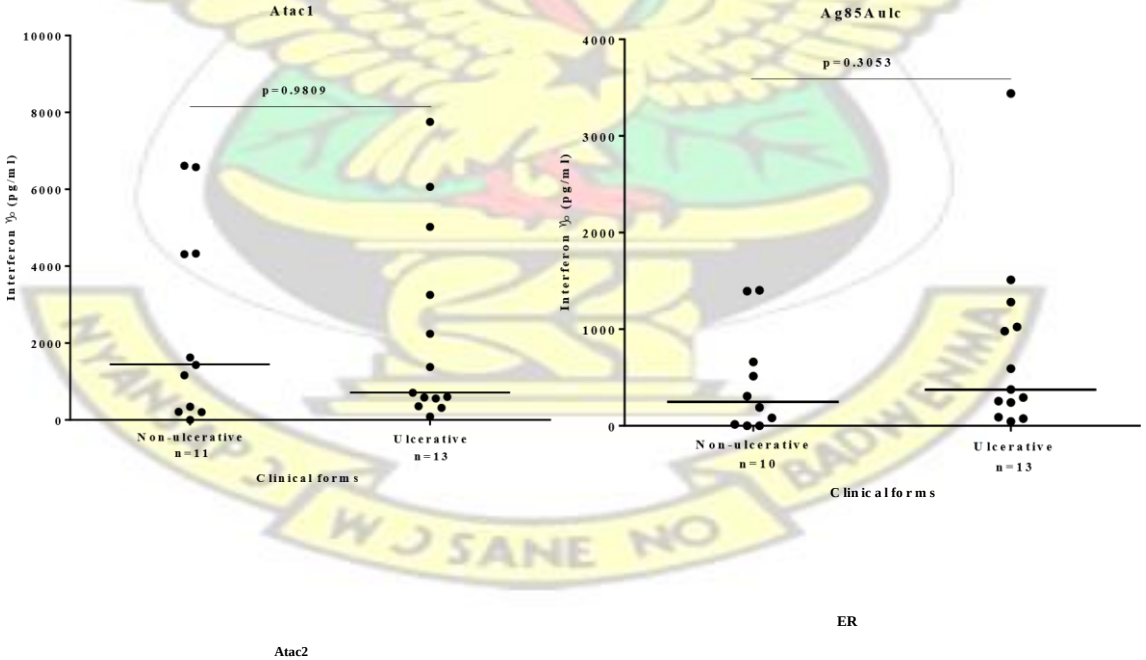


FIGURE 16.0(B) INTERFERON-Ɣ RESPONSE AT ULCERATIVE AND NON-ULCERATIVE STAGES OF THE DISEASE. EACH DOT REPRESENTS RESPONSE OF ONE STUDY PARTICIPANT. THE HORIZONTAL LINES REPRESENT THE MEDIANS.



Atac2

ER

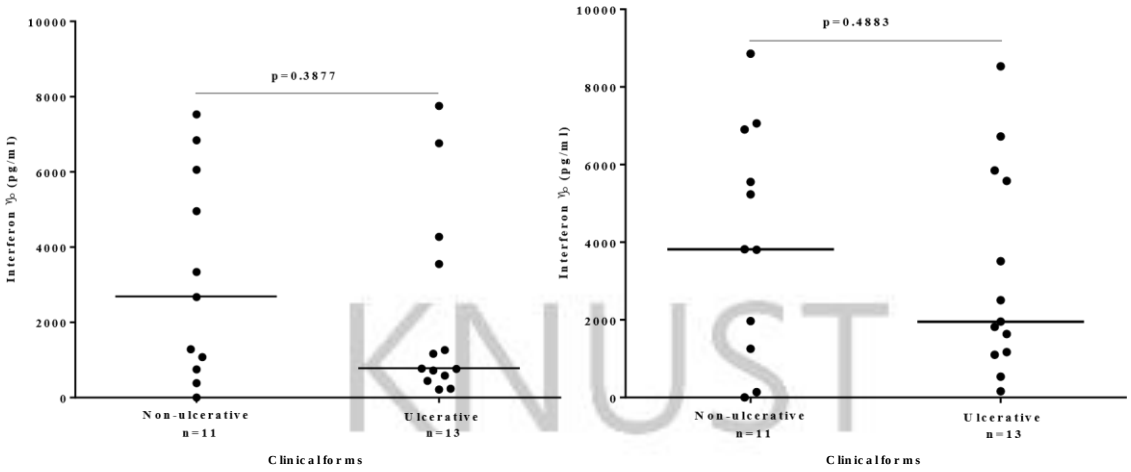


FIGURE 17.0(C) INTERFERON-ɣ RESPONSE AT ULCERATIVE AND NON-ULCERATIVE STAGES OF THE DISEASE. EACH DOT REPRESENTS RESPONSE OF ONE STUDY PARTICIPANT. THE HORIZONTAL LINES REPRESENT THE MEDIANS.

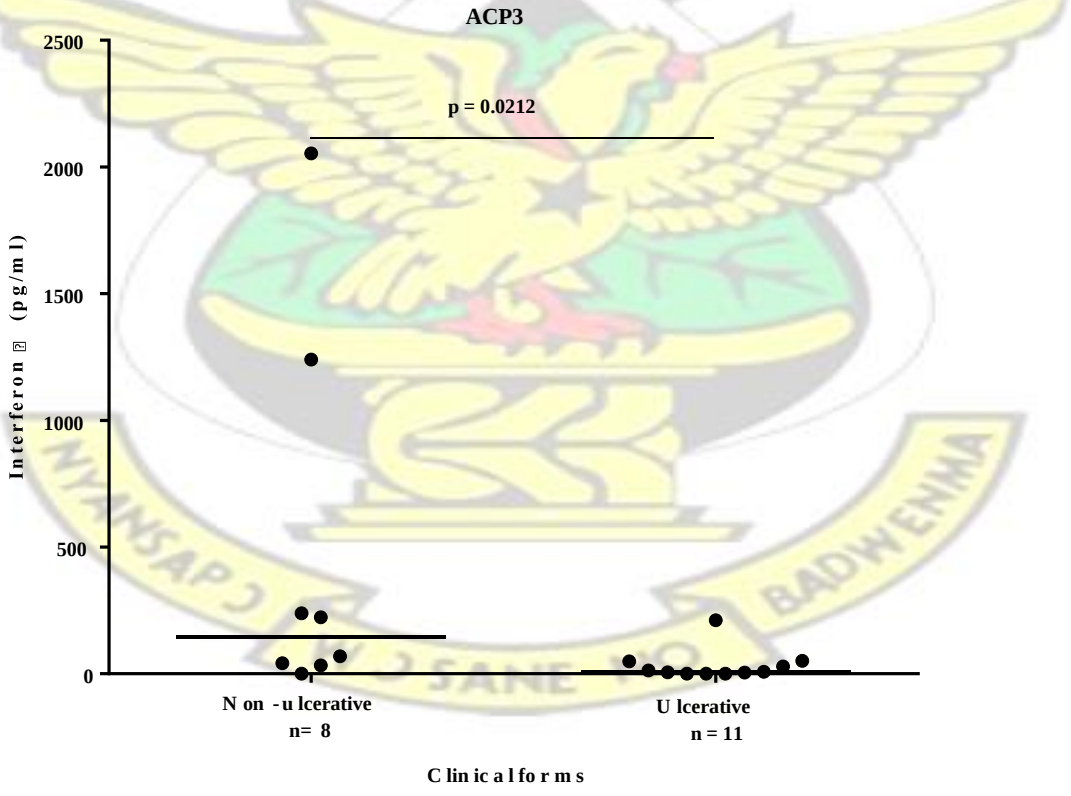


FIGURE 18.0(D) INTERFERON-ɣ RESPONSE AT ULCERATIVE AND NON-ULCERATIVE STAGES OF THE DISEASE. EACH DOT REPRESENTS RESPONSE OF ONE STUDY PARTICIPANT. THE HORIZONTAL LINES REPRESENT THE MEDIANS.

KNUST

4.2.3 Correlation between interferon gamma levels and time to healing.

To determine if there was a correlation between interferon-γ responses of patients to the PKS antigens and the time at which their lesions heal (time to healing), interferonγ responses determined for patients with active Buruli ulcer disease were compared with documented time to healing.

Figure 7.0(A), (B), (C), (D), shows that there was no demonstrable correlation between interferon-γ responses and the time to healing.

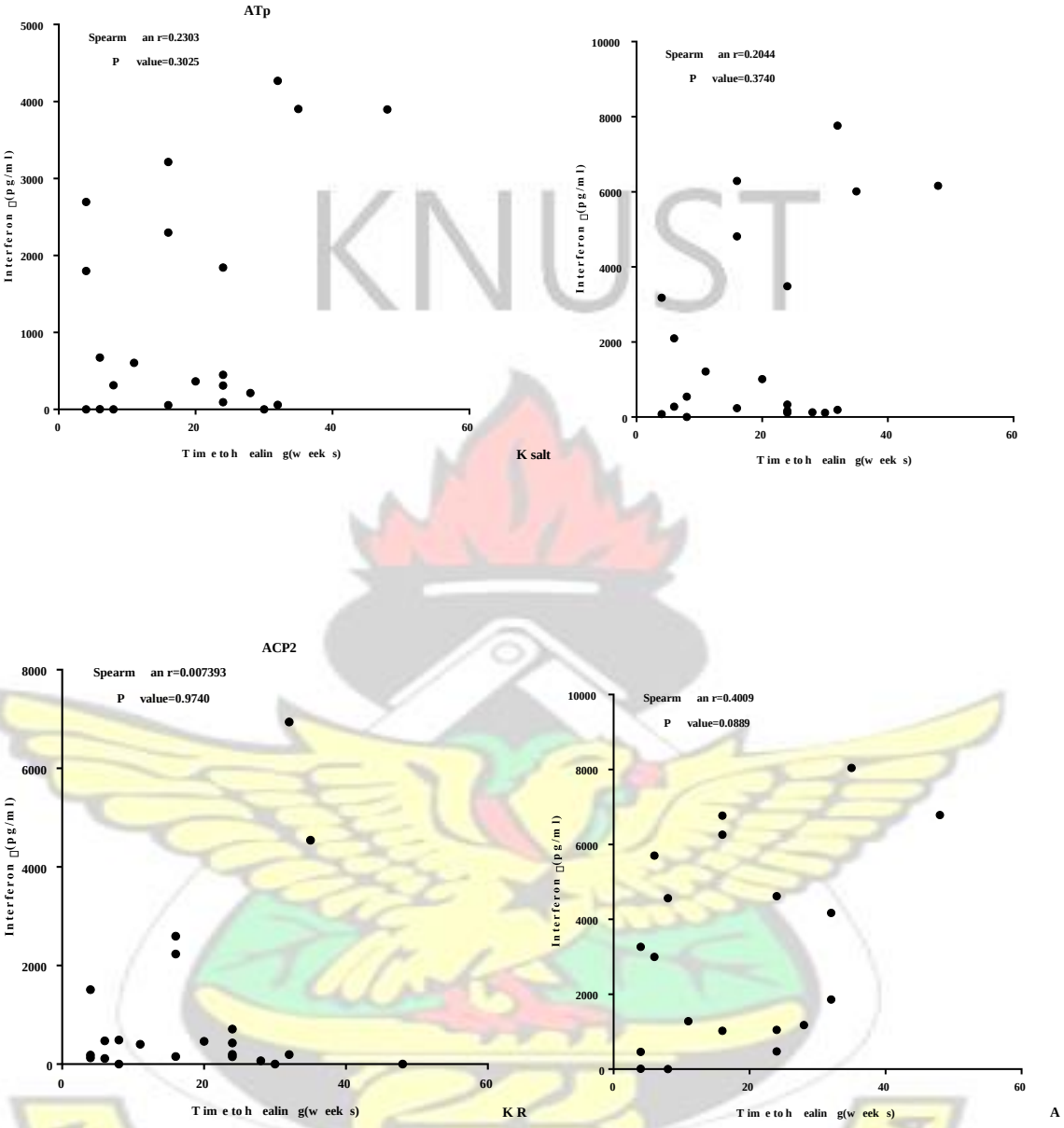


FIGURE 19.0(A) CORRELATION BETWEEN INTERFERON-Ɣ RESPONSE AND TIME TO HEALING OF PARTICIPANTS WITH BURULI ULCER DISEASE.

KNUST
NYANSAPO SANE NO BADWENMA

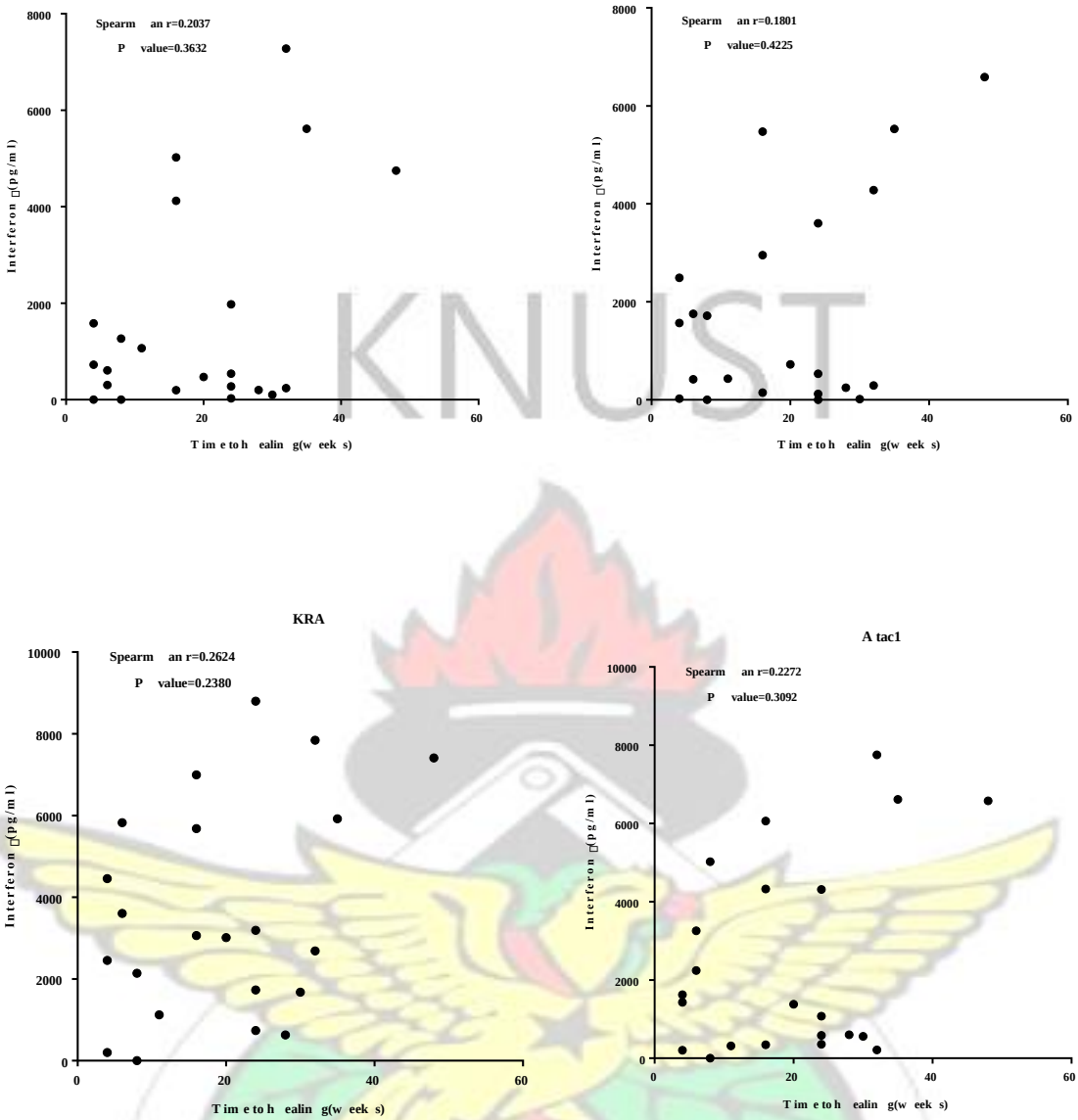


FIGURE 20.0(B) CORRELATION BETWEEN INTERFERON-γ RESPONSE AND TIME TO HEALING OF PARTICIPANTS WITH BURULI ULCER DISEASE.

KNUST
NYANSAPOW WO SANE NO BADWENMA

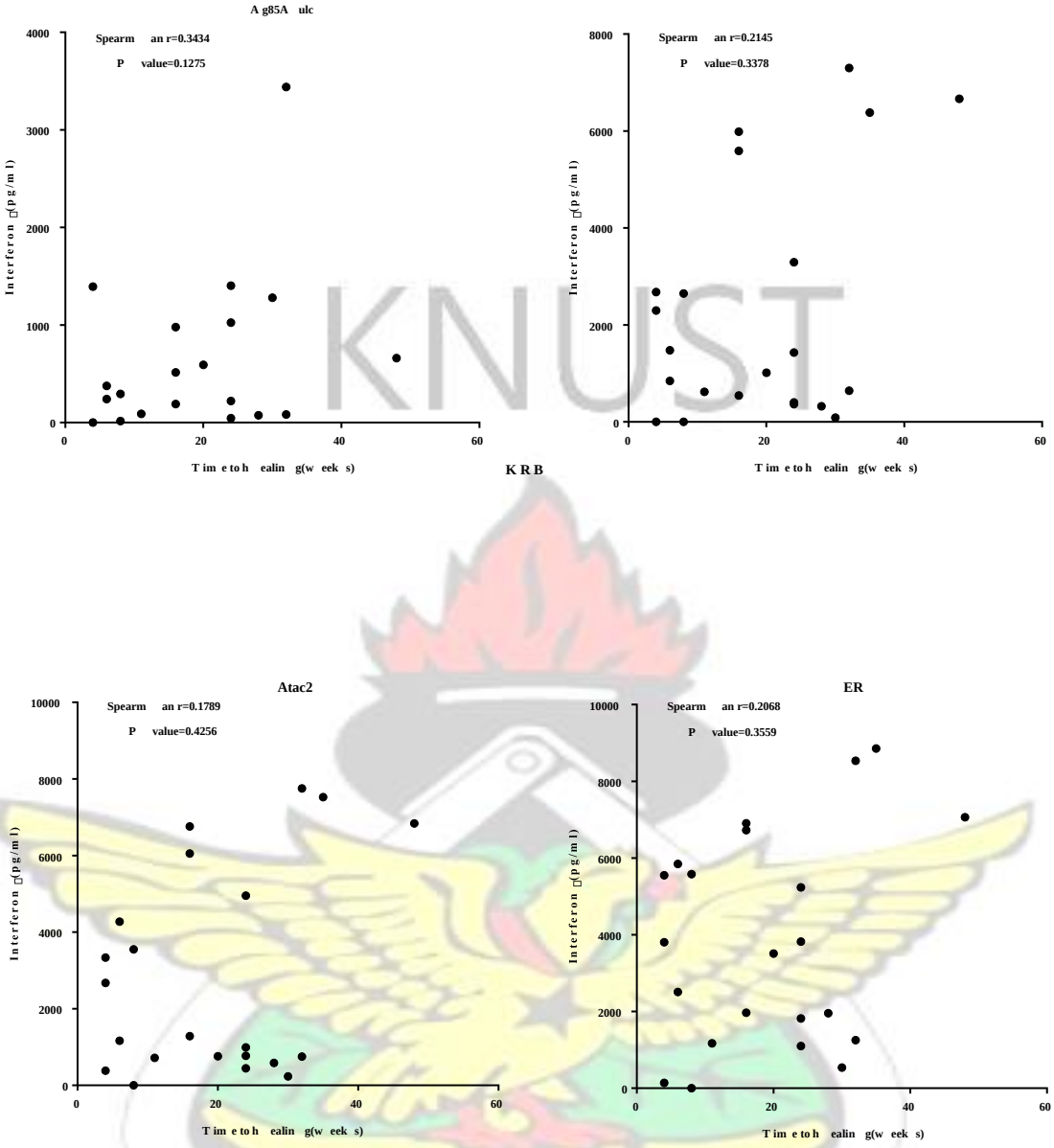


FIGURE 21.0(C) CORRELATION BETWEEN INTERFERON-γ RESPONSE AND TIME TO HEALING OF PARTICIPANTS WITH BURULI ULCER DISEASE.

KNUST

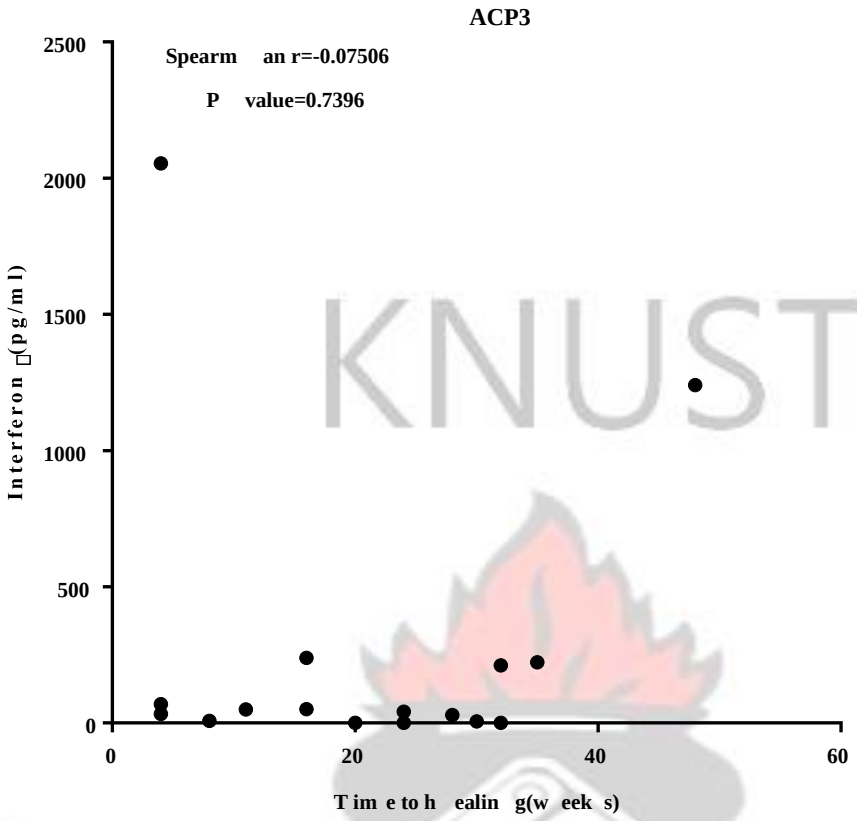


FIGURE 22.0(D) CORRELATION BETWEEN INTERFERON-Ɣ RESPONSE AND TIME TO HEALING OF PARTICIPANTS WITH BURULI ULCER DISEASE.

KNUST
NYANSAPƆ WƆSANE NO BADWENMA

4.3 Characteristics of participants in the paradoxical reactions study.

Paradoxical reaction is defined as an increase in inflammatory changes with increase in lesion size of greater than 100%, after initial improvement and decrease in size; and/or the appearance of new lesions following or during antimycobacterial treatment. These reactions are thought to be immune mediated reactions. The immune response in patients that develop such reactions have not been studied.

To study these reactions, immune responses of 38 patients with all forms of active Buruli ulcer were studied. Nineteen developed paradoxical reactions after treatment initiation and 19 age-matched patients had no paradoxical reactions after antibiotic treatment initiation.

Table 3 shows that for participants with Buruli ulcer who developed paradoxical reaction after treatment initiation the median age (range) was 14(5-58) years; there were 10(38%) males and 9(29%) females. Lesions comprised 8(42%) nodular forms, 4(21%) plaques and 7(37%) ulcers. Thirteen (68%) of the patients had paradoxical reactions 28 weeks after antibiotic treatment initiation and the remaining had it 1-35 weeks after 8 weeks of antibiotic therapy.

Similarly, for 19 patients with no paradoxical reactions their median age (range) was 16(6-51); 7(27%) were males and 12(39%) females. Lesions comprised 3(16%) nodules, 7(37%) plaques and 9(47%) ulcers.

There was no statistically significant differences in age or gender among, the study participants.

TABLE 3.0 CHARACTERISTICS OF PARTICIPANTS WITH BURULI ULCER WITH OR WITHOUT PARADOXICAL REACTIONS AFTER ANTIBIOTIC INITIATION AND CONTACTS.

Parameter	Patients with Buruli ulcer and:			
	Paradoxical reaction n=19	no Paradoxical reaction n=19	Total	p value
Age(years)				
Median (range)	14(5-58)	16(6-51)	16(5-58)	0.9994
Sex (%)				
Male	10(38)	7(27)	26(100)	
Female	9(29)	12(39)	31(100)	0.6095
Lesion form n(%)				
Nodule	8(42)	3(16)	11(58)	
Plaque	4(21)	7(37)	11(58)	
Ulcer	7(37)	9(47)	16(84)	
Time of paradoxical reaction				
week(%)				
2 to 8	13(68)	NA	13(68)	
9 to 15	3(16)	NA	3(16)	
16 to 22	0(0)	NA	0(0)	
23 to 29	2(11)	NA	2(11)	
30 to 36	0(0)	NA	0(0)	
37 to 43	1(5)	NA	1(5)	

4.3.1 Comparison of baseline Th1 and Th2 responses of patients with Buruli ulcer disease who developed or did not develop paradoxical reactions after antibiotic initiation

To determine if there are differences in baseline Th1 and Th2 responses of patients with Buruli ulcer disease that developed or did not have paradoxical reactions after treatment initiation, patients and controls were compared. Antibiotic therapy was initiated for all patients followed up and monitored for development of paradoxical reaction. Immune responses at baseline were compared by obtaining venous blood and whole blood stimulated for baseline samples with *Mu* sonicate antigens and incubated overnight at 37^{o}C for 24 hours. Plasma supernatants were quantified for interferon-γ for Th1 and IL-5 for Th2 responses as described in section 3.7 of methods (See Page 54).

Table 4.0 shows that patients who later developed paradoxical reactions mounted a lower interferon- γ response [median (range) 754.32 (50.93-4190.4) pg/ml] at baseline compared to patients who had no paradoxical reaction [1246.93 (81.11-6969) pg/ml)] but the difference did not reach statistical significance. By contrast patients elicited comparable interleukin-5 (IL-5) response levels when those that later developed paradoxical reactions 35.61 (24.79-67.83) pg/ml and those who had no paradoxical reaction 35.52 (11.82-993.90) pg/ml) were compared albeit the compared responses were not statistically significant.

These results suggest that later development of a paradoxical reaction may be associated with initial low Th1 cytokine response indicative of poor inflammatory response.

TABLE 4.0 COMPARISON OF BASELINE INTERFERON-ɣ AND INTERLEUKIN 5 RESPONSES OF PARTICIPANTS WITH BURULI ULCER WITH OR WITHOUT PARADOXICAL REACTIONS AFTER ANTIBIOTIC INITIATION.

KNUST

Cytokine measured	**Patients with Buruli ulcer and:**		Mann whitney
	Paradoxical reaction n=19	no Paradoxical reaction n=19	P value
Interferon- γ(pg/ml)			
Median	747.6	1158	0.8
Range	50.93-4190.49	81.11-6969.29	
Interleukin 5 (pg/ml)			
Median	35.61	35.52	0.4
Range	24.79-67.83	11.82-993.9	

4.4 Development of the Th1 and Th2 immune response to Mu sonicate antigens with treatment, in Buruli ulcer patients with or without associated paradoxical reaction

To establish the trend of the Th1 and Th2 immune response of patients with Buruli ulcer disease that developed or did not have paradoxical reactions after treatment initiation, patient immune responses were monitored with treatment. Antibiotic therapy was initiated and patients followed up for development of paradoxical reaction. Immune responses at baseline were compared by obtaining venous blood from *M. ulcerans* infected patients at baseline, week 8 and 8 weeks after completion of antibiotic therapy (week 16). Whole blood was stimulated with *Mu* sonicate antigens and incubated overnight at 37^{o}C for 24 hours. Plasma supernatants were quantified for interferon-γ for Th1 and IL-5 for Th2 responses.

Figure 23, shows a general trend of higher interferon-γ with treatment in all patients with Buruli ulcer. However, interferon-γ responses [median (range) pg/ml] at baseline [1246.93 (81.11-6969.29) pg/ml], week 8 [1406.00 (278.12-7628.00) pg/ml] and week 16 [2336.65 (650.42-8688.00) pg/ml] in patients without paradoxical reactions were higher than those with paradoxical reactions at baseline [754.320 (50.93-4190.49) pg/ml], week 8 [634.31(54.32-5148.25) pg/ml)] and week 16 [1452.15 (134.535143.61) pg/ml]. The difference however was not statistically significant.

By contrast, figure 24 shows that IL-5 responses were generally low in all patients compared to interferon-γ responses at all time points. Although figure 19.0, shows comparable IL-5 levels at baseline there was a trend of higher and increasing responses with treatment at week 8 [44.79(15.96-566.29) pg/ml] and week 16

[62.50(10.09410.83) pg/ml] in patients that did not develop paradoxical reactions. Comparatively, there were relatively decreasing and lower IL-5 responses for those that developed paradoxical reaction at week 8 [24.89pg/ml (17.62-566.3) pg/ml] and week 16 [24.89(17.96-132.6) pg/ml]. The IL-5 responses were slightly higher at the time of the paradoxical reaction [41.78 (24.69-394.08) pg/ml] compared to those at baseline, week 8 and week 16.

In conclusion these results show that although there were higher Th1 (interferon-γ) responses there were correspondingly increasing Th2 (IL-5) responses with treatment in patients who did not develop paradoxical reaction. In patients that had paradoxical reactions, increasing trend in inflammatory Th1 (interferon-γ) responses were associated with decreasing anti inflammatory Th2 (IL-5) responses.

KNUST

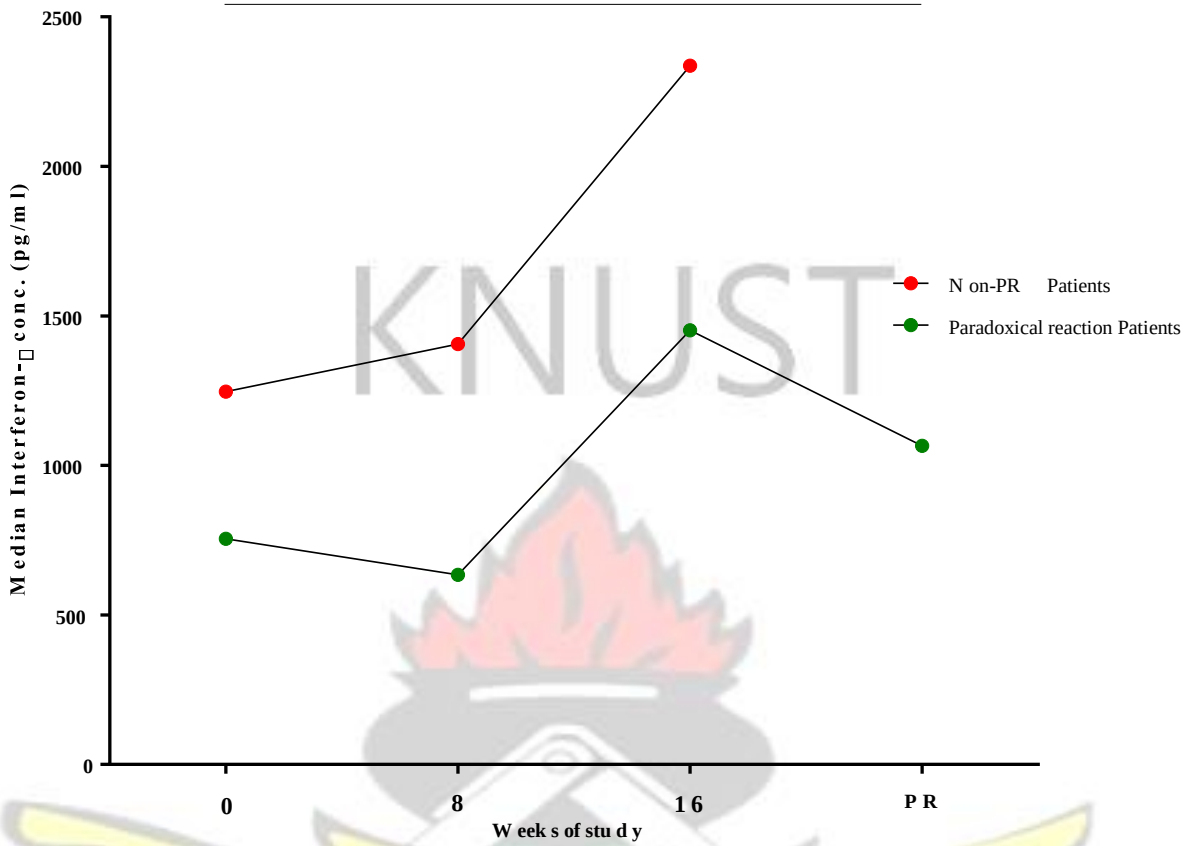


FIGURE 23.0 COMPARISON IN DEVELOPMENT OF INTERFERON-Γ RESPONSES AMONG PATIENTS WITH BURULI ULCER WITH OR WITHOUT PARADOXICAL REACTIONS AFTER ANTIBIOTIC INITIATION.

PR-INDICATES INTERFERON- LEVELS DURING THE PARADOXICAL EPISODE.

KNUST
NYANSAPƆ WƆSANE NO BADWENMA

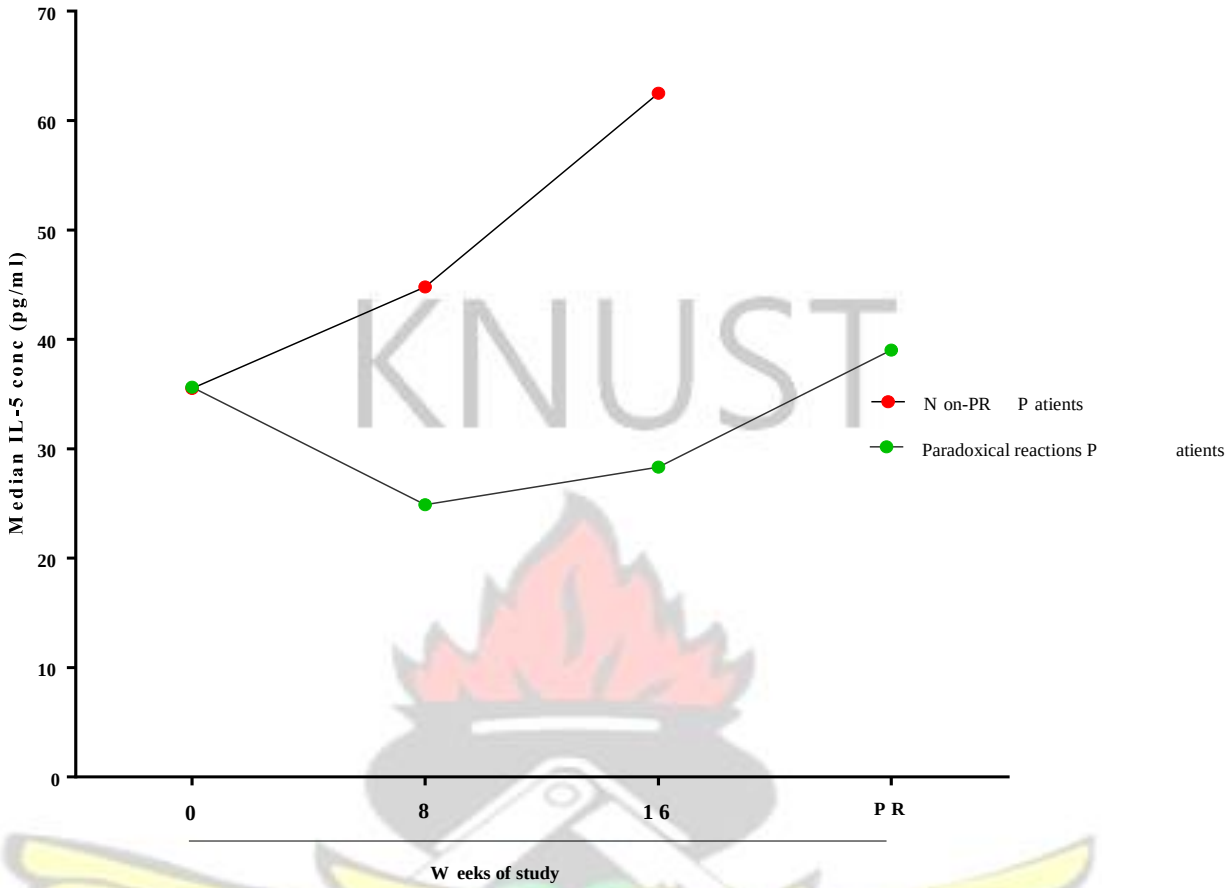


FIGURE 24.0 COMPARISON IN DEVELOPMENT OF IL-5 RESPONSES AMONG PATIENTS WITH BURULI ULCER WITH OR WITHOUT PARADOXICAL REACTIONS AFTER ANTIBIOTIC INITIATION. PATIENTS WITH PARADOXICAL REACTION SHOWED DECREASING TREND IN IL-5 LEVELS WITH TREATMENT WHILST THOSE WITHOUT SHOWED INCREASING LEVELS.

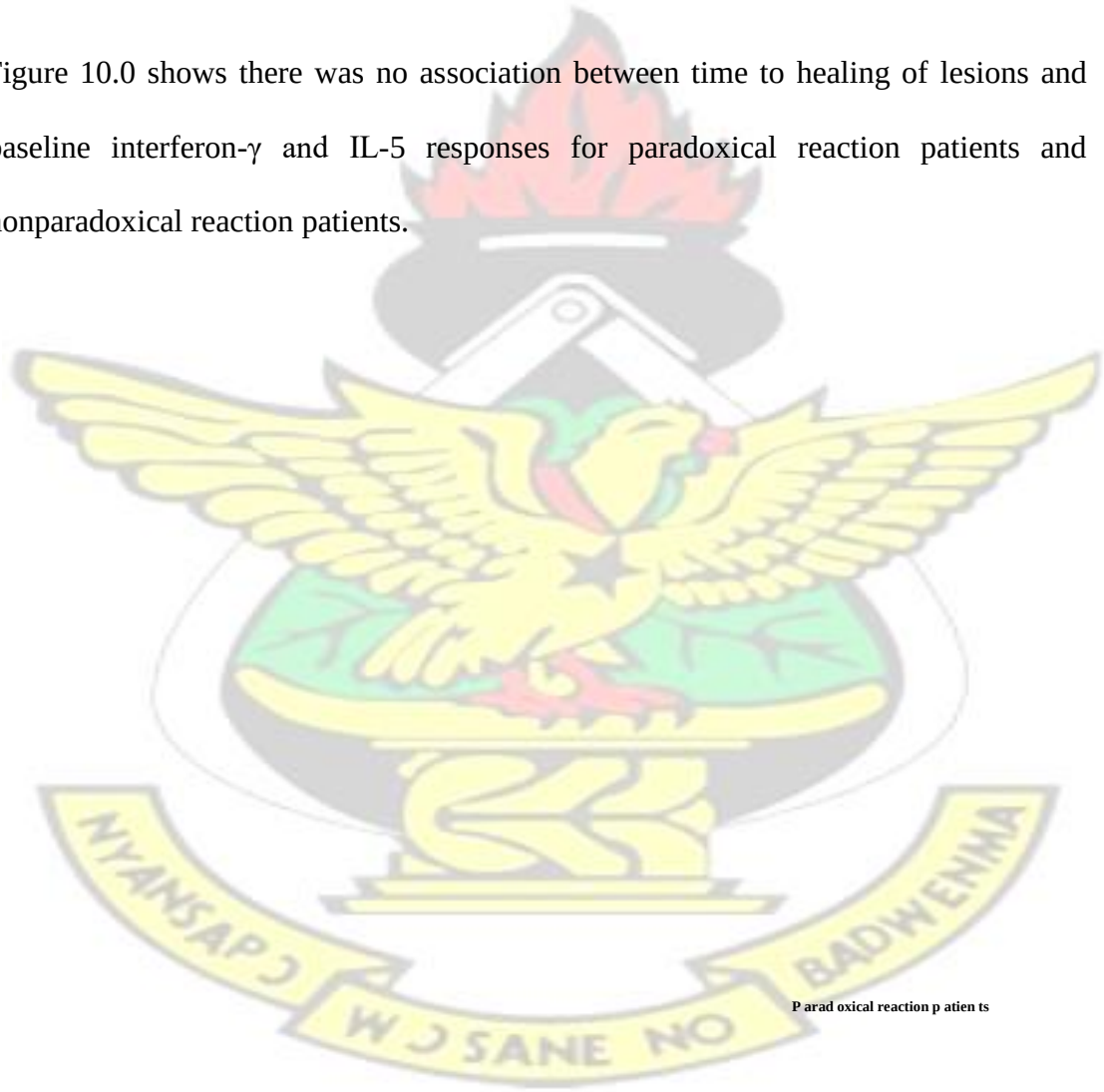
PR-INDICATES IL-5 LEVELS DURING THE PARADOXICAL REACTION EPISODE.

KNUST

4.5 Correlation of immune response with time to healing

It was further determined if cytokine responses of Buruli ulcer disease patients with or without paradoxical reaction had an association with time to healing of lesions. Whole blood obtained from such patients at baseline, were stimulated using the Mu sonicate antigen and culture supernatants collected after overnight incubation. Culture supernatants were used to quantify interferon-γ and IL-5 responses. Lesions were monitored for healing 2 weekly until complete healing.

Figure 10.0 shows there was no association between time to healing of lesions and baseline interferon-γ and IL-5 responses for paradoxical reaction patients and nonparadoxical reaction patients.



P arad oxical reaction p atien ts

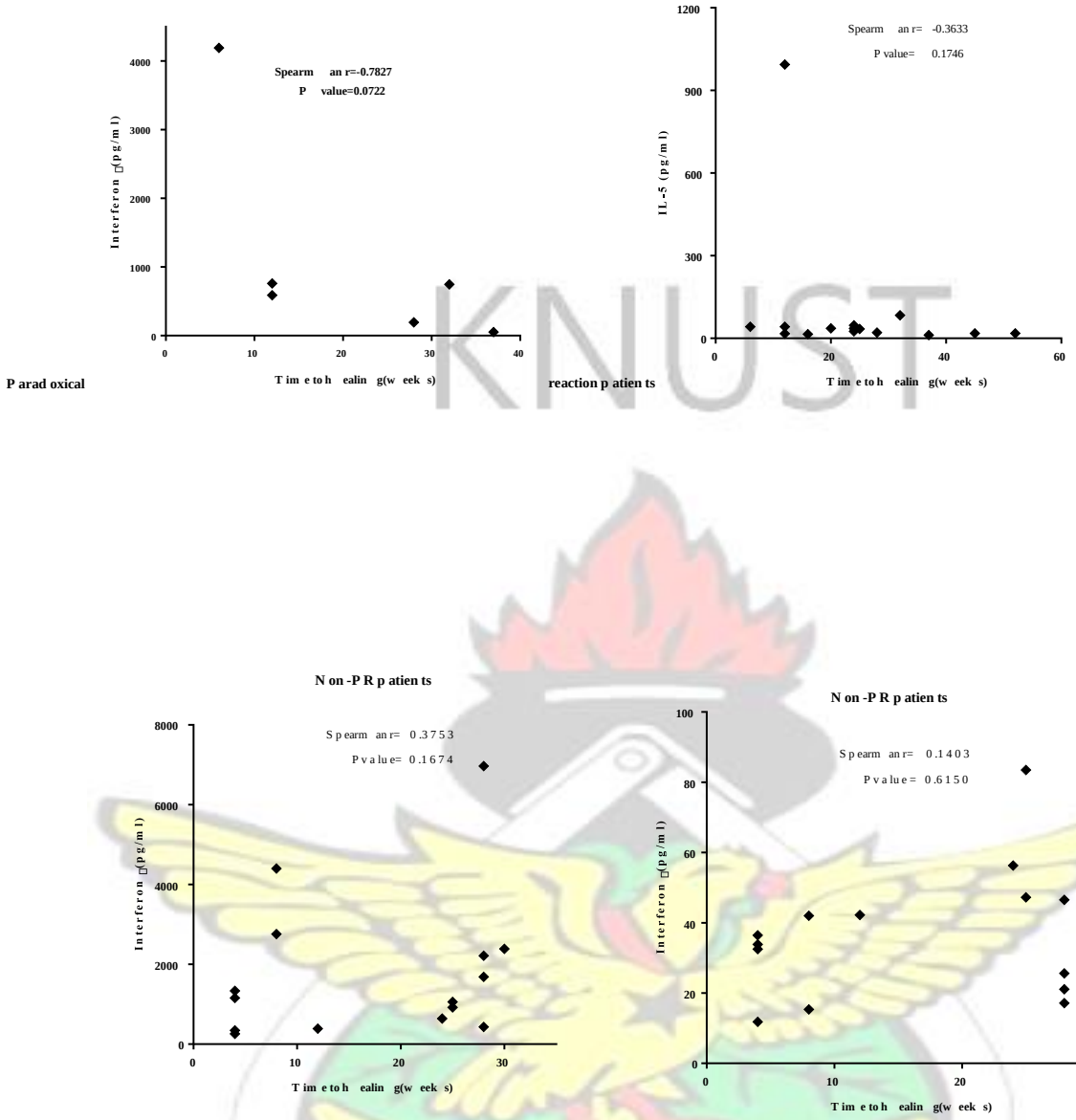


FIGURE 25.0 CORRELATION OF TIME WITH HEALING WITH EXPRESSION OF CYTOKINE FROM PATIENTS WHO DEVELOPED PARADOXICAL REACTION AND THOSE WHO DID NOT.

CHAPTER 5

DISCUSSION

5.1 Immune response to antigens encoded by mycolactone polyketide synathase domains(PKS) in patients with *Mycobacterium ulcerans* infection.

Current control efforts aim at outreach education and early detection activities with a network of trained community based volunteers so cases are referred early to hospital for treatment (Demangel *et al.*, 2009; WHO, 2014). The late reporting of cases to health facilities, long duration of antibiotic therapy, and eventual scarring and permanent disability make a vaccine against Mu *(M. ulcerans)* highly desirable (Bolz *et al*., 2015). We have demonstrated for the first time the immunogenicity of 12 PKS antigens and mycolyltransferase Ag85A of *M. ulcerans.* The participants induced stronger interferon-γ responses to PKS antigens. ER and Ksalt were the most immunogenic antigens as they induced 100% responses from the patients and their contacts. This confirms results of a recent study using a plasmid DNA prime / recombinant protein boost protocol, where the similar domain antigens in mouse model were immunogenic and also elicited strong antigen specific antibodies and Th1 type cytokine response (Roupie *et al.*, 2014). Elsewhere Polyketide synthase domains were also capable of inducing antibodies in Mu disease patients and healthy endemic controls (Pidot *et al*., 2010).

Interestingly, endemic controls produced high interferon-γ responses to PKS antigens but not in non-endemic controls with the exception of one. The only exception was a European who had worked at a hospital close to an endemic area for several months but there had not been any known exposure to Mu. These data strongly suggest the likelihood of previous exposure to Mu in the environment and the possibility of immune protection in selected individuals. This may be indicative of the absence of immunological memory in non-endemic controls and hence an inability to recognize

antigens to Mu. Prolonged follow-up for at least a year will be needed to confirm protection in such individuals. It was earlier hypothesized that a significant proportion of the population residing in an area of Mu endemicity may have been exposed to the organism but had not developed disease (Gooding *et al*., 2002). Exposure to BCG is not likely to be responsible for this observation and there was no difference in BCG exposure between endemic controls and non-endemic controls (data not shown).

Endemic controls also produced a significantly higher response to most of the PKS antigens except Atac1, ACP3 and KRA compared to the patients. The low responses of BUD patients to the antigens may be due to the immunosuppressive properties of mycolactone. At non-cytotoxic concentrations, mycolactone displays immunomodulatory properties on human primary monocytes and dendritic cells, indicating that it may limit the initiation of innate immune responses in vivo (Fraga *et al.*, 2011). By blocking the capacity of primary T cells to produce multiple cytokines upon activation and by impairing T-cell migration and homing into lymph nodes, mycolactone significantly inhibit the development of adaptive immune responses to *M. ulcerans* (Yotsu *et al*., 2015). More recent evidence has also revealed that mycolactone exerts a profound effect on protein secretion by blocking the co-translational translation of a plethora of proteins that pass through the endoplasmic reticulum for secretion or placement in cell membranes (Yotsu *et al.*, 2015).

In mice when interleukin-2 and interferon-γ levels to the same plasmid antigens were compared in naïve and vaccinated mice, naïve mice elicited low responses similar to what was seen in non-endemic controls of Buruli ulcer patients whereas vaccinated mice elicited high responses (Roupie *et al*., 2014) similar to those produced by endemic controls in this study. There were variations in the intensity when responses in this

study were compared to those in mice. The highest responses were to ER followed by KRA in humans by contrast to Ag85A followed by ATp in mice. After prime/boost with PKS protein and exposure to infection in mice those exposed to Ag85A, ER, Atac1 showed significantly lower numbers of AFB after being sacrificed compared to naïve mice and mice survival was better for those exposed to Ag85A, ATp and KR A, ACP2. The differences in response intensity may be due to difference in incubation period in humans (5days) and mice (3 days).

In this study there was a trend to increasing interferon gamma responses after antibiotic therapy but only responses to ACP2 and ATac2 reached significant levels compared to baseline suggestive of a slow recovery in interferon-γ levels as seen in previous human studies (Sarfo *et al*., 2009; Torrado *et al*., 2010). Already at week 8 following antibiotic initiation, plasmid antigens ATp, Ksalt, and ER induced significantly higher interferonγ responses compared to baseline and at completion of antibiotic therapy. Recovery of interferon-γ after antibiotic therapy could be associated with killing of Mu permitting phagocytosis of organisms, processing by macrophages and dendritic cells and presenting of antigens to T lymphocytes (Sarfo *et al*., 2009). Recent evidence of positive cultures after therapy suggesting persisting mycolactone (Sarfo *et al*., 2014) resulting in immune cell death and halting the recruitment of cells.

The pattern of secretion of interferon-γ responses of patients with non-ulcerative forms were markedly higher compared to ulcerative forms. ACP3 was the only antigen that induced a similar response from the non-ulcerative forms compared to the ulcerative forms. Even though ACP3 was the least immunogenic antigen, the best responses were from patients who presented with non-ulcerative. As previous studies have shown that severity of disease have an effect on immune responses of Mu patients, probably the

concentration of mycolactone in the tissues could give an indication on the extent of immunosuppression caused by mycolactone. This result was inconsistent with a study which used Mu sonicate as stimulus, incubated overnight and demonstrated that there were similar median interferon-γ responses between patients with ulcers and those with healed Buruli ulcers. But those with nodular forms showed lower responses (Phillips *et al*., 2006). Perhaps, this study provided different result due to the fact that overnight culture supernatants were used. In this present study 5 days culture supernatants were used and the antigens were produced differently. The overnight culture (24 hours) supernatant are used to measure prevailing effector responses whiles the long incubation assay (5 days) focus on insight into memory and regulatory T-cell responses.

There were limitations to this aspect of the study. We did not account for activated T cell and other immune cells capable of secreting the interferon-γ. This would have been important to finding out whether there is a correlation between proportions of activated T cells and interferon-γ levels measured. However, we used sandwich ELISA to measure the concentration of interferon-γ secreted. The present study did not investigate into whether there is an association between levels of interferon-γ secreted and mycolactone concentration.

5. 2 Effect of paradoxical reaction on the immune profile of Buruli ulcer patients

The key mechanism of the paradoxical reaction have been attributed to an immune reconstitution inflammatory syndrome (IRIS) (Beissner *et al.*, 2010). A previous study attributed this phenomenon to increased exposure to mycobacterial antigens, decrease in suppressor mechanisms or improved host cell-mediated immunity following

antibiotic therapy (Nienhuis *et al.*, 2012). During treatment of Buruli ulcer, the timing of paradoxical reactions is unpredictable, as this may occur from a few days to many months after the start of antibiotic treatment. The extent of severity and duration of the reactions are unpredictable as well (Beissner *et al*., 2010).

The present study demonstrated the effect of paradoxical reaction on the immune profile of Buruli ulcer disease patients. Overall, patients who developed paradoxical reactions (PR patients) mounted lower Th1 (interferon-γ) and Th2 (IL-5) responses compared to BUD patients who did not develop paradoxical reaction (non-PR patients). Our findings revealed that at baseline, non-PR patients mounted a markedly higher interferon-γ and IL-5 responses compared to the PR patients. Although there was a lower Th1 cytokine response trend in PR patients compared with non-PR patients, increasing interferon-γ concentrations were associated with decreasing concentrations of IL-5. This findings support the idea from a previous tuberculosis study which reported that the release of numerous cytokines, including interferons and TNF-α and elevated T-helper cell-1(Th1) and inflammatory cytokine and chemokine levels, but not

Th2 cytokines, have been associated with tuberculosis-IRIS (TB-IRIS) (Lagier *et al*., 2014). Lower cytokine responses in PR patients is likely to be caused by presence of mycolactone. On the other hand, *M. ulcerans* infected patients who developed no paradoxical reaction mounted a higher Th1 response and a lower Th2 response. This strongly suggest that a bacterial factor impedes cellular responses (Phillips *et al*., 2006). Our findings suggest that patients with lower interferon-γ response at baseline may be linked with development of paradoxical reaction after treatment is initiated.

This study identified a general trend of improving Th1 and Th2 response of patients with treatment. Although, Buruli ulcer patients are known to have a slowly improving interferon-γ response with treatment, it was shown that at week 8, interferon-γ and IL5 responses of patients who developed paradoxical reactions decreased. The decrease could be as a result of the episode of paradoxical reaction after the initiation of treatment. This finding supports the idea of a case series which reported of significant deterioration of lesions 1-2 months after antibiotic therapy began in patients who developed paradoxical reactions (Friedman *et al.*, 2012). In addition, this may be interpreted that at week 8, immune recovery may not have commenced and the effect of mycolactone on the immune system may still be prevailing. Interferon-γ responses was at a higher concentration in non-PR patients and at a consistently lower level in PR patients. IL-5 concentrations were partly comparable for both study groups especially at baseline.

However, at week 16, there was an improvement in Th1 and Th2 responses of Non-PR and PR patients. This could be attributable to a restoration of active humoral and cellular immune responses. In addition, it was suggested that a local cellular immune response develop in these situations as production of mycolactone is reduced presumably in response to persisting mycobacterial antigens (Friedman *et al.*, 2012). A previous study also reported of an increase in interferon-γ secretion after 4 and 8 weeks' antibiotic therapy, in contrast to our findings in PR patients. But an improved interferon-γ response was observed in non-PR patients after treatment. This variation could be attributed to the fact that this study was focused on BUD patients who developed paradoxical reaction while the previous study looked into BUD patients (Sarfo *et al.*, 2009).

We showed that there was no association between cytokine responses and time to healing of the lesions for non-PR patient and PR patients. This finding could be attributed to the variation in duration of lesions before presentation for treatment.

KNUST

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

Currently, there is no vaccine for Buruli ulcer disease. The main goal of this study was to determine the systemic immune response of *M. ulcerans* disease patients to the plasmid DNA encoding mycolactone polyketide synthase domains. To answer that question, interferon-γ responses of BUD patients were compared with endemic and non-endemic controls. Our findings showed that the PKS antigens are immunogenic in humans. Overall responses of the patients and endemic controls were estimated to be more than 80%. Interferon-γ responses of the patients were consistent to most of the 13 antigens. Endemic controls mounted significantly higher responses compared to the patient while the patients mounted significantly higher responses when compared to the non-endemic controls. This finding represent significant advance in the field of Buruli ulcer research since this study provides first data on the responses of humans to the PKS antigens. We have confirmed the immunogenicity of the plasmid antigens and discovered that immune response of patients to PKS domains is varied from that of the contacts, which could be suggestive to the immunosuppressive effect of mycolactone on immune response. ER and Ksalt were the most immunogenic antigens. A vaccine made up of the most immunogenic plasmid DNA encoding mycolactone polyketide synthase domains and Ag85A is an interesting possibility that require further study.

Paradoxical reaction has been shown to affect Buruli ulcer clinical outcome (O'Brien *et al*. 2009; Beissner *et al*., 2010; Nienhuis *et al*., 2012; Friedman *et al*., 2012; Lagier *et al*., 2014; O'Brien *et al*., 2014b). This study demonstrated the effect of paradoxical reaction on the immune response of BUD patients. Th1 and Th2 response of patients

who developed PR was compared to those who did not. Our results showed that patients who developed PR had a consistently lower Th1 response to the Mu sonicate antigen. Th2 responses were partly comparable to those who did not develop PR. These findings showed that BUD patients with much lower interferon-γ responses are most likely to develop PR. Furthermore, Th1 and Th2 responses of the patients at the week of occurrence of PR was comparable to their week 16 responses. This could be attributed to the role of the pro-inflammatory cytokine interferon-γ. This novel study is an important advancement in understanding PR in BUD. We have confirmed that paradoxical reaction have an effect on the immune response of patients. This could be caused by susceptibility gene as PR patients seem to have a distinctly lower interferonγ response at baseline. They mounted a markedly low Th1 and Th2 responses when compared with patients who did not develop paradoxical reactions.

6.2 Recommendation

1. The development of a vaccine made up of the most immunogenic PKS antigens and Ag85Aulc is most desirable for further research.
2. A future study to explore possible genetic markers that play a role in responses of endemic controls should be explored.
3. A further study into the specificity of the Plasmid antigens to Mu by testing in patients with other mycobacteria diseases (*M. tuberculosis, M. leprae)* would provide further understanding.
4. Further testing of the PKS antigens in other endemic countries will improve our understanding of responses to these plasmid antigens.

5. A future study exploring the role susceptibility genes play in the development of Paradoxical reaction could be explored.

REFERENCES

Ablordey, Anthony S, Koen Vandelannoote, Isaac A Frimpong, Evans K Ahortor, Nana Ama Amissah, Miriam Eddyani, Lies Durnez. (2015). "Whole Genome Comparisons Suggest Random Distribution of *Mycobacterium ulcerans* Genotypes in a Buruli Ulcer Endemic Region of Ghana." *PLoS Neglected Tropical Diseases* 9 (3). Public Library of Science: e0003681.

Adjei, O, MR Evans, and A. Asiedu. (1998). "Phenytoin Ulcer in the Treatment of Buruli." *Trans R Soc Med Hyg* 92: 108–9.

Adu, Emmanuel J K, and Edwin Ampadu. (2015). "*Mycobacterium ulcerans* Disease in the Middle Belt of Ghana : An Eight-Year Review from Six Endemic Districts." *International Journal of Mycobacteriology* 4 (2). 2014 Asian African Society for Mycobacteriology. Production and hosting by Elsevier Ltd. All rights reserved.: 138–42.

Adusumilli, Sarojini, Armand Mve-Obiang, Tim Sparer, Wayne Meyers, John Hayman, and Pamela Long Claus Small. (2005). "*Mycobacterium ulcerans* Toxic Macrolide, Mycolactone Modulates the Host Immune Response and Cellular Location of M. Ulcerans in Vitro and in Vivo." *Cellular Microbiology* 7 (9): 1295–1304.

Agogo Prebyterian Hospital. (2015). "Agogo Prebyterian Hospital." http://www.agogopresbyhospital.org/main/.

Amofah, George, Frank Bonsu, Christopher Tetteh, Jane Okrah, Kwame Asamoa, Kingsley Asiedu, and Jonathan Addy. (2002). "Buruli Ulcer in Ghana: Results of a National Case Search." *Emerging Infectious Diseases* 8 (2): 167–70.

Ashworth, T. G., E. M. Andersen, R. C. Ballard, M. Barral-Netto, A. L. Bittencourt, V. Boonpucknavig, H. J. Diesfeld. (1995). *Tropical Pathology*. Vol. 8. Spezielle Pathologische Anatomie. Berlin, Heidelberg: Springer Berlin Heidelberg.

Asiedu, Kingsley, and Samuel Etuaful. (1998). "Socioeconomic Implications of Buruli Ulcer in Ghana: A Three-Year Review." *American Journal of Tropical Medicine and Hygiene* 59 (6): 1015–22.

Atwima Nwabiagya. (2015). "Ghana » Ashanti Region » Atwima Nwabiagya." http://atwimanwabiagya.ghanadistricts.gov.gh/?arrow=nws&read=3157.

Beissner, Marcus, Karl-Heinz Herbinger, and Gisela Bretzel. (2010a). "Laboratory Diagnosis of Buruli Ulcer Disease." *Future Microbiology* 5 (3): 363–70.

Bessis, Didier, Marie Kempf, and Laurent Marsollier. (2014). "*Mycobacterium ulcerans* Disease (Buruli Ulcer) in Mali : A New Potential African Endemic Country," 6–7.

Bieri, Raphael, Miriam Bolz, Marie Thérèse Ruf, and Gerd Pluschke. (2016). "Interferon-gamma Is a Crucial Activator of Early Host Immune Defense against *Mycobacterium ulcerans* Infection in Mice." *PLoS Neglected Tropical Diseases* 10 (2): 1–13.

Bolz, Miriam, Sarah Kerber, Gert Zimmer, and Gerd Pluschke. (2015). "Use of Recombinant Virus Replicon Particles for Vaccination against *Mycobacterium ulcerans* Disease," 1–18.

Bourreau, Eliane, Herve Pascalis, Roger Pradinaud, Audrey Tanghe, Kris Huygen, and Pascal Launois. (2004). "Differential Production of Systemic and Intralesional Gamma Interferon and Interleukin-10 in Nodular and Ulcerative Forms of Buruli Disease" 72 (2): 958–65.

Boyd, Sarah C, Eugene Athan, N Deborah Friedman, Andrew Hughes, Aaron Walton, Peter Callan, Anthony McDonald, and Daniel P O'Brien. (2012). "Epidemiology, Clinical Features and Diagnosis of *Mycobacterium ulcerans* in an Australian Population." *The Medical Journal of Australia* 196 (5): 341–44.

Bratschi, Martin W., Marie Thérèse Ruf, Arianna Andreoli, Jacques C. Minyem, Sarah Kerber, Fidèle G. Wantong, James Pritchard. (2014). "*Mycobacterium ulcerans* Persistence at a Village Water Source of Buruli Ulcer Patients." *PLoS Neglected Tropical Diseases* 8 (3).

Chauty, Annick, Marie-Françoise Ardant, Ambroise Adeye, Hélène Euverte, Augustin Guédénon, Christian Johnson, Jacques Aubry, Eric Nuermberger, and Jacques Grosset. (2007). "Promising Clinical Efficacy of StreptomycinRifampin Combination for Treatment of Buruli Ulcer (Mycobacterium Ulcerans Disease)." *Antimicrobial Agents and Chemotherapy* 51 (11): 4029–35.

Coleman, Susan. (2004). "Assessing Water-Related Risk Factors for Buruli Ulcer: A Case-Control Study in Ghana" 71 (4): 387–92.

Converse, Paul J, Deepak V Almeida, Eric L Nuermberger, and Jacques H Grosset. (2011). "BCG-Mediated Protection against *Mycobacterium ulcerans* Infection in the Mouse." *PLoS Neglected Tropical Diseases* 5 (3): e985.

Coutanceau, Emmanuelle, Pierre Legras, Laurent Marsollier, Gilles Reysset, Stewart T. Cole, and Caroline Demangel. (2006). "Immunogenicity of *Mycobacterium ulcerans* Hsp65 and Protective Efficacy of a *Mycobacterium leprae* Hsp65-Based DNA Vaccine against Buruli Ulcer." *Microbes and Infection* 8 (8): 2075–81.

Coutanceau, Emmanuelle, Laurent Marsollier, Roland Brosch, Emmanuelle Perret, Pierre Goossens, Myriam Tanguy, Stewart T Cole, Pamela L C Small, and Caroline Demangel. (2005). "Modulation of the Host Immune Response by a Transient Intracellular Stage of *Mycobacterium ulcerans*: The Contribution of Endogenous Mycolactone Toxin." *Cellular Microbiology* 7 (8): 1187–96.

Demangel, Caroline, Timothy P Stinear, and Stewart T Cole. (2009). "Buruli Ulcer: Reductive Evolution Enhances Pathogenicity of Mycobacterium Ulcerans." *Nature Reviews. Microbiology* 7 (1): 50–60.

Dobos, K M, E a Spotts, B J Marston, C R Horsburgh, and C H King. (2000). "Serologic Response to Culture Filtrate Antigens of *Mycobacterium ulcerans* during Buruli Ulcer Disease." *Emerging Infectious Diseases* 6 (2): 158–64.

Ebong, Solange Meyin A, Sara Eyangoh, Estelle Marion, Jordi Landier, Laurent Marsollier, Jean François Guégan, and Philippe Legall. (2012). "Survey of

Water Bugs in Bankim, a New Buruli Ulcer Endemic Area in Cameroon." *Journal of Tropical Medicine* 2012: 1–9.

Eddyani, Miriam, David Ofori-Adjei, Guy Teugels, David De, Daniel Boakye, Wayne M Meyers, and David De Weirdt. (2004). "Potential Role for Fish in Transmission of Mycobacterium Ulcerans Disease (Buruli Ulcer): An Environmental Study Potential Role for Fish in Transmission of *Mycobacterium ulcerans* Disease (Buruli Ulcer): An Environmental Study." *Society* 70 (9): 5679–81.

Etuaful, S., B. Carbonnelle, J. Grosset, S. Lucas, C. Horsfield, R. Phillips, M. Evans. (2005). "Efficacy of the Combination Rifampin-Streptomycin in Preventing Growth of *Mycobacterium ulcerans* in Early Lesions of Buruli Ulcer in Humans." *Antimicrobial Agents and Chemotherapy* 49 (8): 3182–86.

Fakult, Philosophisch-naturwissenschaftlichen. (2012). "Development and Application of New Approaches to Study the Epidemiology of *Mycobacterium ulcerans* Disease (Buruli Ulcer) in Ghana."

Fakult, Philosophisch-Naturwissenschaftlichen, and Daniela Sch. (2009). "Approaches to Improve Treatment and Early Diagnosis of Buruli Ulcer : The Role of Local and Systemic Immune Responses."

Finch, Christopher K., Cary R. Chrisman, Anne M. Baciewicz, and Timothy H. Self. (2002). "Rifampin and Rifabutin Drug Interactions." *Archives of Internal Medicine* 162 (9). American Medical Association: 985.

Fraga, Alexandra G., Teresa G. Martins, Egídio Torrado, Kris Huygen, Françoise Portaels, Manuel T. Silva, António G. Castro, and Jorge Pedrosa.

(2012). "Cellular Immunity Confers Transient Protection in Experimental Buruli Ulcer Following BCG or Mycolactone-Negative *Mycobacterium ulcerans* Vaccination." *PLoS ONE* 7 (3): 1–10.

Fraga, Alexandra G, Andrea Cruz, Teresa G Martins, Margarida Saraiva, Daniela R Pereira, M Wayne, Françoise Portaels. (2011). "*Mycobacterium ulcerans* Triggers T-Cell Immunity Followed by Local and Regional but Not Systemic Immunosuppression □."

Friedman, N Deborah, Anthony H McDonald, Michael E Robson, and Daniel P O'Brien. (2012a). "Corticosteroid Use for Paradoxical Reactions during Antibiotic Treatment for *Mycobacterium ulcerans*." *PLoS Neglected Tropical Diseases* 6 (9):

Garchitorena, Andrés, Benjamin Roche, Roger Kamgang, Joachim Ossomba, Jérémie Babonneau, Jordi Landier, Arnaud Fontanet. (2014). "*Mycobacterium ulcerans* Ecological Dynamics and Its Association with Freshwater Ecosystems and Aquatic Communities: Results from a 12-Month Environmental Survey in Cameroon." *PLoS Neglected Tropical Diseases* 8 (5).

George, K. M. (1999). "Mycolactone: A Polyketide Toxin from *Mycobacterium ulcerans* Required for Virulence." *Science* 283 (5403). American Association for the Advancement of Science: 854–57.

Ghana Districts. (2015). "Ghana Districts - A Repository of All Districts in the Republic of Ghana." http://www.ghanadistricts.com/districts/?news&r=3&_=162.

Gooding, Travis M, Paul D R Johnson, Dianne E Campbell, John A Hayman,

Elizabeth L Hartland, Andrew S Kemp, Roy M Robins-browne, and R O Y M Robins-browne. (2001). "Immune Response to Infection with *Mycobacterium ulcerans* Immune." *Society* 69 (3): 3–7.

Gooding, Travis M, Paul D R Johnson, May Smith, Andrew S Kemp, and Roy M Robins-browne. (2002). "Cytokine Profiles of Patients Infected with *Mycobacterium ulcerans* and Unaffected Household Contacts" 70 (10): 5562–67.

Gordon, Claire L., John a. Buntine, John a. Hayman, Caroline J. Lavender, Janet a M Fyfe, Patrick Hosking, Mike Starr, and Paul D R Johnson. (2010). "All-Oral Antibiotic Treatment for Buruli Ulcer: A Report of Four Patients." *PLoS Neglected Tropical Diseases* 4 (11): 1–7.

Hart, Bryan E, Laura P Hale, and Sunhee Lee. (2015). "Recombinant BCG Expressing *Mycobacterium ulcerans* Ag85A Imparts Enhanced Protection against Experimental Buruli Ulcer." *PLoS Neglected Tropical Diseases* 9 (9): e0004046.

Huber, Charlotte a., Marie Thérèse Ruf, Gerd Pluschke, and Michael Käser. (2008). "Independent Loss of Immunogenic Proteins in *Mycobacterium ulcerans* Suggests Immune Evasion." *Clinical and Vaccine Immunology* 15 (4): 598–606.

Huygen, Kris. (2003). "On the Use of DNA Vaccines for the Prophylaxis of Mycobacterial Diseases minireview On the Use of DNA Vaccines for the Prophylaxis of Mycobacterial Diseases" 71 (4): 1613–21.

Huygen, Kris, Ohene Adjei, Dissou Affolabi, Gisela Bretzel, Caroline Demangel, Bernhard Fleischer, Roch Christian Johnson. (2009). "Buruli Ulcer Disease: Prospects for a Vaccine." *Medical Microbiology and Immunology* 198: 69–77.

Huygen, Kris, Ohene Adjei, A V Dissou, Gisela Bretzel, Caroline Demangel, Bernhard Fleischer, Roch Christian. (2009). "Buruli Ulcer Disease : Prospects for a Vaccine," 69–77.

Jenke-kodama, Holger, Axel Sandmann, Rolf Mu, and Elke Dittmann. (2003). "Evolutionary Implications of Bacterial Polyketide Synthases."

Johnson, Paul D R, Joseph Azuolas, Caroline J Lavender, Elwyn Wishart, Timothy P Stinear, John A Hayman, Lynne Brown, Grant A Jenkin, and Janet A M Fyfe. (2007). "Mycobacterium Ulcerans in Mosquitoes Captured during Outbreak of Buruli Ulcer , Southeastern Australia" 13 (11): 1653–60.

Kindt, Thomas J, Richard A Goldsby, and Barbara A Osborne. (2007). *Kuby Immunology*. *Kuby Immunology*. Vol. 6.

Kotlowski, Roman, Anandi Martin, Anthony Ablordey, Karim Chemlal, Pierre Alain Fonteyne, and Françoise Portaels. (2004). "One-Tube Cell Lysis and DNA Extraction Procedure for PCR-Based Detection of *Mycobacterium ulcerans* in Aquatic Insects, Molluscs and Fish." *Journal of Medical Microbiology* 53 (9): 927–33.

Kumar, S., S. Basu, S. K. Bhartiya, and V. K. Shukla. (2015). "The Buruli Ulcer." *The International Journal of Lower Extremity Wounds* 14 (3): 217–23.

Lagier, J C, and Didier Raoult. (2014). "Immune Reconstitution Inflammatory Syndrome Associated with Bacterial Infections." *Expert Opin Drug Saf* 13 (3): 341–50.

Maccullum, P. (1948). "A New Mycobacterial Infection in Man; Clinical Aspects." *The Journal of Pathology and Bacteriology* 60 (1): 93–102.

Marsollier, Laurent, Raymond Robert, Jacques Aubry, Jean-Paul Saint André, Henri Kouakou, Pierre Legras, Anne-Lise Manceau, Chetaou Mahaza, and Bernard Carbonnelle. (2002). "Aquatic Insects as a Vector for *Mycobacterium ulcerans*." *Applied and Environmental Microbiology* 68 (9): 4623–28.

Marston, B J, M O Diallo, C R Horsburgh, I Diomande, M Z Saki, J M Kanga, G Patrice, H B Lipman, S M Ostroff, and R C Good. (1995). "Emergence of Buruli Ulcer Disease in the Daloa Region of Cote d'Ivoire." *The American Journal of Tropical Medicine and Hygiene* 52 (3): 219–24.

McAllister, W A, P J Thompson, S M Al-Habet, and H J Rogers. (1983). "Rifampicin Reduces Effectiveness and Bioavailability of Prednisolone." *BMJ* 286 (6369): 923–25.

Merritt, Richard W., Edward D. Walker, Pamela L C Small, John R. Wallace, Paul D R Johnson, M. Eric Benbow, and Daniel A. Boakye. (2010). "Ecology and Transmission of Buruli Ulcer Disease: A Systematic Review." *PLoS Neglected Tropical Diseases* 4 (12): 1–15.

Nackers, Fabienne, Michèle Dramaix, Roch Christian Johnson, Claude Zinsou, Annie Robert, Elisa DE Biurrun Bakedano, Judith R Glynn, Françoise Portaels, and René Tonglet. (2006). "BCG Vaccine Effectiveness against Buruli Ulcer: A Case-Control Study in Benin." *The American Journal of Tropical Medicine and Hygiene* 75 (4): 768–74.

Nielsen, Suzanne, Raimondo Bruno, Louisa Degenhardt, Mark a. Stoove, Jane a. Fischer, Susan J. Carruthers, and Nicholas Lintzeris. (2013). "Treatment and Prevention of *Mycobacterium ulcerans* Infection (Buruli Ulcer) in Australia:

Guideline Update." *Medical Journal of Australia* 199 (10): 696–99.

Nienhuis, Willemien a., Ymkje Stienstra, K. Mohammed Abass, Wilson Tuah, William A. Thompson, Peter C. Awuah, Nana Yaa Awuah-Boateng. (2012a). "Paradoxical Responses after Start of Antimicrobial Treatment in *Mycobacterium ulcerans* Infection." *Clinical Infectious Diseases* 54 (4): 519–26.

O'Brien, D. P., N. Ford, M. Vitoria, V. Christinet, E. Comte, A. Calmy, Y. Stienstra, S. Eholie, and K. Asiedu. (2014). "Management of BU-HIV CoInfection." *Tropical Medicine & International Health* 19 (9): 1040–47.

O'Brien, Daniel P, Grant Jenkin, John Buntine, Christina M Steffen, Anthony McDonald, Simon Horne, N Deborah Friedman. (2014). "Treatment and Prevention of *Mycobacterium ulcerans* Infection (Buruli Ulcer) in Australia: Guideline Update." *The Medical Journal of Australia* 200 (5): 267–70.

O'Brien, Daniel P, Michael E Robson, Peter P Callan, and Anthony H McDonald. (2009). "'Paradoxical' immune-Mediated Reactions to *Mycobacterium ulcerans* during Antibiotic Treatment: A Result of Treatment Success, Not Failure." *The Medical Journal of Australia* 191 (10): 564–66.

Owusu-Sekyere, Ebenezer, and Daniel A. Bagah. (2014). "Where Are the *Mycobacterium ulcerans*? Mapping the Risk and Vulnerable Areas of Mycobacterium Infection in the Amansie West District of Ghana." *Journal of Applied & Environmental Microbiology* 2 (6). Science and Education Publishing: 273–80.

Phillips, R, C Horsfield, S Kuijper, A Lartey, I Tetteh, S Etuaful, B Nyamekye. (2005). "Sensitivity of PCR Targeting the IS2404 Insertion Sequence of

Mycobacterium ulcerans in an Assay Using Punch Biopsy Specimens for Diagnosis of Buruli Ulcer." *Journal of Clinical Microbiology* 43 (8): 3650–56.

Phillips, R, C Horsfield, S Kuijper, S F Sarfo, S Etuaful, B Nyamekye, P Awuah, S Lucas. (2006a). "Cytokine Response to Antigen Stimulation of Whole Blood from Patients with *Mycobacterium ulcerans* Disease Compared to That from Patients with Tuberculosis" 13 (2): 253–57.

Phillips, R, C Horsfield, J Mangan, K Laing, S Etuaful, P Awuah, K Nyarko, P Butcher, and S Lucas. (2006). "Cytokine mRNA Expression in *Mycobacteriam ulcerans* -Infected Human Skin and Correlation with Local Inflammatory Response" 74 (5): 2917–24.

Phillips, R O, F S Sarfo, F Osei-Sarpong, A Boateng, I Tetteh, A Lartey, E Adentwe, W Opare, K B Asiedu, and M Wansbrough-Jones. (2009). "Sensitivity of PCR Targeting *Mycobacterium ulcerans* by Use of Fine-Needle Aspirates for Diagnosis of Buruli Ulcer." *Journal of Clinical Microbiology* 47 (4): 924–26.

Phillips, Richard O, Fred S Sarfo, Mohammed K Abass, Justice Abotsi, Tuah Wilson, Mark Forson, Yaw A Amoako, William Thompson, Kingsley Asiedu, and Mark Wansbrough-Jones. (2014). "Clinical and Bacteriological Efficacy of Rifampin-Streptomycin Combination for Two Weeks Followed by Rifampin and Clarithromycin for Six Weeks for Treatment of Mycobacterium Ulcerans Disease." *Antimicrobial Agents and Chemotherapy* 58 (2): 1161–66.

Phillips, Richard Odame, Delphin Mavinga Phanzu, Marcus Beissner, Kossi Badziklou, Elysée Kalundieko Luzolo, Fred Stephen Sarfo, Wemboo Afiwa

Halatoko. (2015). "Effectiveness of Routine BCG Vaccination on Buruli Ulcer Disease: A Case-Control Study in the Democratic Republic of Congo, Ghana and Togo." *PLoS Neglected Tropical Diseases* 9 (1): e3457.

Phillips, Richard, Fred S Sarfo, Laure Guenin-Macé, Jérémie Decalf, Mark Wansbrough-Jones, Matthew L Albert, and Caroline Demangel. (2009). "Immunosuppressive Signature of Cutaneous *Mycobacterium ulcerans* Infection in the Peripheral Blood of Patients with Buruli Ulcer Disease." *The Journal of Infectious Diseases* 200 (11): 1675–84.

Pidot, Sacha J, Jessica L Porter, Laurent Marsollier, Annick Chauty, Florence Migot-Nabias, Cyril Badaut, Angèle Bénard. (2010). "Serological Evaluation of *Mycobacterium ulcerans* Antigens Identified by Comparative Genomics." *PLoS Neglected Tropical Diseases* 4 (11): e872.

Portaels, F, J Aguiar, M Debacker, A Gue, and W M Meyers. (2004). "*Mycobacterium bovis* BCG Vaccination as Prophylaxis against Mycobacterium Ulcerans Osteomyelitis in Buruli Ulcer Disease" 72 (1): 62–65.

Portaels, F, J Aguiar, M Debacker, C Steunou, C Zinsou, A Guédénon, and W M Meyers. (2002). "Prophylactic Effect of *Mycobacterium bovis* BCG Vaccination against Osteomyelitis in Children with *Mycobacterium ulcerans* Disease (Buruli Ulcer)." *Clinical and Diagnostic Laboratory Immunology* 9 (6): 1389–91.

Portaels, Françoise, Wayne M Meyers, Anthony Ablordey, António G Castro, Karim Chemlal, Pim de Rijk, Pierre Elsen. (2008). "First Cultivation and Characterization of Mycobacterium Ulcerans from the Environment." *PLoS Neglected Tropical Diseases* 2 (3): e178.

Röltgen, Katharina, and Gerd Pluschke. (2015). "*Mycobacterium ulcerans* Disease (Buruli Ulcer): Potential Reservoirs and Vectors." *Current Clinical Microbiology Reports* 2 (1): 35–43.

Roupie, Virginie, Sacha J Pidot, Tobba Einarsdottir, Christophe Van Den Poel, Fabienne Jurion, Timothy P Stinear, and Kris Huygen. (2014). "Analysis of the Vaccine Potential of Plasmid DNA Encoding Nine Mycolactone Polyketide Synthase Domains in *Mycobacterium ulcerans* Infected Mice." *PLoS Neglected Tropical Diseases* 8 (1): 47.

Sarfo, F. S., R. O. Phillips, E. Ampadu, F. Sarpong, E. Adentwe, and M. Wansbrough-Jones. (2009). "Dynamics of the Cytokine Response to *Mycobacterium ulcerans* during Antibiotic Treatment for M. Ulcerans Disease (Buruli Ulcer) in Humans." *Clinical and Vaccine Immunology* 16 (1): 61–65.

Sarfo, Fred S, Richard O Phillips, Jihui Zhang, Mohammed K Abass, Justice Abotsi, Yaw Amoako, Yaw Adu-Sarkodie, Clive Robinson, and Mark H Wansbrough-Jones. (2014). "Kinetics of Mycolactone in Human Subcutaneous Tissue during Antibiotic Therapy for *Mycobacterium ulcerans* Disease." *BMC Infectious Diseases* 14 (1). BMC Infectious Diseases: 202.

Sarfo, Fred Stephen. (2014). "The Kinetics of Mycolactone in Relation to the Microbiological, Clinical and Immunological Responses to Antibiotic Therapy for *Mycobacterium ulcerans* Disease."

Sarfo, Fred Stephen, Richard Phillips, Kingsley Asiedu, Edwin Ampadu, Nana Bobi, E Adentwe, Awuli Lartey, Ishmael Tetteh, and M Wansbrough-Jones. (2010). "Clinical Efficacy of Combination of Rifampin and Streptomycin for

Treatment of *Mycobacterium ulcerans* Disease." *Antimicrobial Agents and Chemotherapy* 54 (9): 3678–85.

Schu, Daniela, Elisabetta Peduzzi, Paul Zajac, Simona Rondini, Ernestina Mensah-quainoo, Giulio Cesare Spagnoli, Gerd Pluschke, and Claudia Andrea Daubenberger. (2007). "Local Activation of the Innate Immune System in Buruli Ulcer Lesions" 127.

Silva, Manuel T, Françoise Portaels, and Jorge Pedrosa. (2009). "Pathogenetic Mechanisms of the Intracellular Parasite *Mycobacterium ulcerans* Leading to Buruli Ulcer." *The Lancet Infectious Diseases* 9 (11): 699–710.

Smith, P G, W D Revill, E Lukwago, and Y P Rykushin. (1976). "The Protective Effect of BCG against *Mycobacterium ulcerans* Disease: A Controlled Trial in an Endemic Area of Uganda." *Transactions of the Royal Society of Tropical Medicine and Hygiene* 70 (5–6): 449–57.

Sopoh, Ghislain Emmanuel, Yves Thierry Barogui, Roch Christian Johnson, Ange Dodji Dossou, and Michel Makoutode. (2010). "Family Relationship , Water Contact and Occurrence of Buruli Ulcer in Benin" 4 (7).

Stanford, J L, W D Revill, W J Gunthorpe, and J M Grange. (1975). "The Production and Preliminary Investigation of Burulin, a New Skin Test Reagent for *Mycobacterium ulcerans* Infection." *The Journal of Hygiene* 74 (1): 7–16.

Stienstra, Ymkje, Winette T A V A N D E R Graaf, Kwame Asamoa, and Tjip S V A N D E R Werf. (2002). "Beliefs and attitudes toward Buruli ulcer in Ghana" 67 (2): 207–13.

Stinear, Timothy P, Armand Mve-Obiang, Pamela L C Small, Wafa Frigui,

Melinda J Pryor, Roland Brosch, Grant a Jenkin. (2004). "Giant PlasmidEncoded Polyketide Synthases Produce the Macrolide Toxin of *Mycobacterium ulcerans.*" *Proceedings of the National Academy of Sciences of the United States of America* 101 (5): 1345–49.

Sumithra, T G, V K Chaturvedi, A K Rai, S S Chougule, L S Rajan, S S Jacob, and Cherian Susan. (2013). "Progress in DNA Vaccinology against Bacterial Diseases - an Update." *Advances in Animal and Veterinary Sciences* 1 (6): 164–77.

Tanghe, Audrey, Pierre-yves Adnet, Tatiana Gartner, and Kris Huygen. (2007). "A Booster Vaccination with *Mycobacterium bovis* BCG Does Not Increase the Protective Effect of the Vaccine against Experimental *Mycobacterium ulcerans* Infection in Mice □" 75 (5): 2642–44.

Tanghe, Audrey, Jean Content, Jean-paul Van Vooren, Françoise Portaels, Kris Huygen, and Jean-paul V A N Vooren.(2001). "Protective Efficacy of a DNA Vaccine Encoding Antigen 85A from *Mycobacterium bovis* BCG against Buruli Ulcer." *Infection and Immunity* 69 (9): 5403–11.

Tanghe, Audrey, Jean-pierre Dangy, Gerd Pluschke, and Kris Huygen. (2008). "Improved Protective Efficacy of a Species-Specific DNA Vaccine Encoding Mycolyl-Transferase Ag85A from *Mycobacterium ulcerans* by Homologous Protein Boosting" 2 (3): 1–10.

Thangaraj, Harry S., Mark R W Evans, and Mark H. Wansbrough-Jones. (1999). "*Mycobacterium ulcerans* Disease; Buruli Ulcer." *Transactions of the Royal Society of Tropical Medicine and Hygiene* 93 (4): 337–40.

Torrado, Egídio, Alexandra G Fraga, Elsa Logarinho, Teresa G Martins, Jenny a Carmona, José B Gama, Maria a Carvalho, Fernanda Proença, Antonio G Castro, and Jorge Pedrosa. (2010). "IFN-Gamma-Dependent Activation of Macrophages during Experimental Infections by *Mycobacterium ulcerans* Is Impaired by the Toxin Mycolactone." *Journal of Immunology (Baltimore, Md. : 1950)* 184 (2): 947–55.

Uganda Buruli Group. (1970). "Clinical Features and Treatment of Pre-Ulcerative Buruli Lesions (*Mycobacterium ulcerans* Infection). Report II of the Uganda Buruli Group." *British Medical Journal* 2 (5706): 390–93.

Walsh, Douglas S., Françoise Portaels, and Wayne M. Meyers. (2008). "Buruli Ulcer (*Mycobacterium ulcerans* Infection)." *Transactions of the Royal Society of Tropical Medicine and Hygiene* 102 (10): 969–78.

Wanda, Franck, Patrick Nkemenang, Genevieve Ehounou, Marie Tchaton, Eric Comte, Laurence Toutous Trellu, Isabelle Masouyé, Vanessa Christinet, and Daniel P O'Brien. (2014). "Clinical Features and Management of a Severe Paradoxical Reaction Associated with Combined Treatment of Buruli Ulcer and HIV Co-Infection." *BMC Infectious Diseases* 14 (1): 423.

Wansbrough-Jones, Mark, and Richard Phillips. (2006). "Buruli Ulcer: Emerging from Obscurity." *Lancet* 367 (9525): 1849–58.

WHO. (2014). "WHO | Buruli Ulcer." *WHO Website*. World Health Organization. http://www.who.int/mediacentre/factsheets/fs199/en/.

Wiker, H G, and M Harboe. (1992). "The Antigen 85 Complex: A Major Secretion Product of *Mycobacterium tuberculosis*." *Microbiological Reviews* 56 (4): 648–

61.

Willson, Sarah J, Michael G Kaufman, Richard W Merritt, Heather R Williamson, David M Malakauskas, and Mark Eric Benbow. (2013). "Fish and Amphibians as Potential Reservoirs of *Mycobacterium ulcerans*, the Causative Agent of Buruli Ulcer Disease." *Infection Ecology & Epidemiology* 3 (January).

World Health Organization. (2014). "WHO | *Mycobacterium ulcerans* (Buruli Ulcer)." World Health Organization. http://www.who.int/topics/mycobacterium_ulcerans/en/.

Yeboah-Manu, Dorothy, Grace S. Kpeli, Marie Thérèse Ruf, Kobina AsanAmpah, Kwabena Quenin-Fosu, Evelyn Owusu-Mireku, Albert Paintsil. (2013). "Secondary Bacterial Infections of Buruli Ulcer Lesions Before and After Chemotherapy with Streptomycin and Rifampicin." *PLoS Neglected Tropical Diseases* 7 (5).

Yotsu, Rie R., Chiaki Murase, Mariko Sugawara, Koichi Suzuki, Kazue Nakanaga, Norihisa Ishii, and Kingsley Asiedu. (2015). "Revisiting Buruli Ulcer." *The Journal of Dermatology*, no. June: n/a-n/a.

Yotsu, Rie R, Kazue Nakanaga, Yoshihiko Hoshino, Koichi Suzuki, and Norihisa Ishii. (2012). "Buruli Ulcer and Current Situation in Japan: A New Emerging Cutaneous Mycobacterium Infection." *The Journal of Dermatology* 39 (7): 587–93.

APPENDIX

APPENDIX I

PROCEDURE FOR OBTAINING SWAB SAMPLES

KNUST

Materials for swab samples

1. Sterile cotton-wool on an applicator(swab)
2. Disinfectant (70% ethanol)
3. Disposable gloves
4. Labelled tubes with appropriate media
5. Cotton-wool pads

Procedure

After disinfecting the lesion site, a sterile swab was gently inserted underneath the undermined edges of the ulcer. The swab was used to twirl the tissue beneath the edges of the ulcer in a clockwise manner. The swabs are then placed in well labelled tubes containing appropriate media.

Procedure for Obtaining Fine-Needle Aspiration(FNA)

Materials for FNA

1. 10 ml syringe and 12 or 22- gauge needle
2. Labelled tubes with transport media (CLS for PCR)
3. Labelled microscope slides
4. Cotton-wool pads and disinfectant (70%)

5. Dressing materials
6. Disposable gloves

Procedure

Prior to taking samples, patients were assured that the samples are required to find out what is causing the lesion (nodule, plaque or edema), so that appropriate treatment can be given. The procedure is briefly explained to the patient.

KNUST

APPENDIX II

BUFFERS AND REAGENTS

ELISA ASSAYS

Note: Do not use sodium azide in these preparations. Sodium azide inactivates the horseradish peroxidase enzyme.

Coating Buffer - 0.1 M Sodium Carbonate, pH 9.5

7.13 g $NaHCO_3$, 1.59 g Na_2CO_3; q.s. to 1.0 L; pH to 9.5 with 10N NaOH. Freshly prepare or use within 7 days of preparation, stored at 2-8°C.

Assay Diluent

PBS* with 10% FBS#, pH 7.0. *Phosphate-Buffered Saline: 80.0 g NaCl, 11.6 g Na_2HPO_4, 2.0 g

KH_2PO_4, 2.0 g KCl, q.s. to 10 L; pH to 7.0. Freshly prepare or use within 3 days of preparation, with 2-8°C storage.

Wash Buffer

PBS* with 0.05% Tween-20. Freshly prepare or use within 3 days of preparation, stored at 2-8°C.

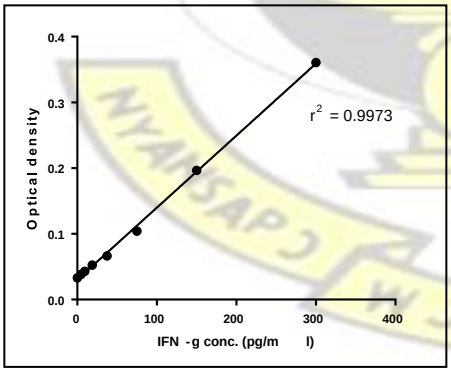
Substrate Solution

Tetramethylbenzidine (TMB) and Hydrogen Peroxide.

Stop Solution - 1 M H_3PO_4 or $2NH_2SO_4$

ELISA STANDARDIZATION

Examples of interferon-γ standard curve



APPENDIX III

BU01 FORM

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 _____ ID#: _____

Address (village or town): _____ District: _____

Province/Region/State_: _____ Country: _____

Age (yrs): _____ Sex : ☐Male ☐Female

Weight (Kg): _____ Profession: _____

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) ☐ Lower limb (LL)

☐Abdomen (AB) ☐Back (BK)

☐Buttocks and Perineum (BP) ☐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
1b: Antibiotic treatment not completed
2a: Healed without surgery
2b: Healed with surgery
3a: Healed without limitation of movement at any joint
3b: Healed with limitation of movement at any joint
4: Referred for further treatment
5: Lost to follow up
Died