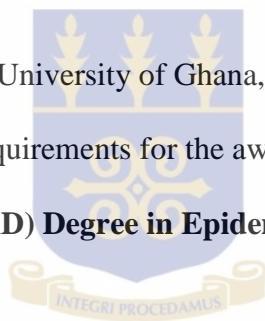


EVALUATING AND IMPROVING MICROBIOLOGICAL METHODS FOR THE DIAGNOSIS OF BURULI ULCER DISEASE

Enid Owusu MPhil., B Sc.

(ID # 10297443)

This thesis is submitted to the University of Ghana, Legon, in partial fulfilment of the requirements for the award of
Doctor of Philosophy (PhD) Degree in Epidemiology and Disease control.



Department of Epidemiology and Disease control

School of Public Health. College of Health Sciences. University of Ghana

MARCH, 2013

DECLARATION

This thesis is the result of research work undertaken by me at the Microbiology Department of the University of Ghana Medical School (UGMS), College of Health Sciences, the Public Health Reference Laboratories (PHRL) of the Korle-Bu and the Noguchi Memorial Institute for Medical Research (NMIMR). I hereby declare that with the exception of the references cited to other people's work which has been duly acknowledged, this work is the result of my own research work done under supervision and has neither in part or whole been presented elsewhere for another degree

Sign

Date

Student: **Mrs. Enid Owusu**



Sign

Date

Supervisor: **Professor Mercy J. Newman**

Sign

Supervisor: **Professor Gerd Pluschke**

Date

DEDICATION

This work is dedicated to God Almighty through whom all things are possible.

To my husband, Kwame and my daughters Nana Abena Asoh and Maame Yaa Gyekyawaa.

To the memory of my wonderful parents Mr. and Mrs. Appiah-Kyeremeh whose true love kept me going in a world of challenges. To my wonderful and dedicated siblings whose constant care and laughter helped me focus only on the brighter side of life.

To a great and wonderful “mother” Professor Mercy J. Newman for being there for me.



ACKNOWLEDGEMENTS

I am grateful to the Almighty God, who has made this wonderful period in my life possible in his chosen time.

I really appreciate you, Professor Mercy J. Newman for allowing yourself to be the facilitator of God's mighty deed, God richly bless you. I thank Professor Fred Binka for his efforts at manpower development in the University of which I am a grateful beneficiary. I also thank Professor Edwin Wiredu, former Dean of the School of Allied Health Sciences for allowing me to enrol on this PhD programme. A special gratitude to Professor Patrick Ayeh-Kumi, Dean of the School of Allied Health Sciences who took a divine decision to allow me time off teaching to enable me to complete this programme. God bless you, Prof.

I thank my supervisors Professor Mercy J. Newman, Professor Gerd Pluschke and the supervisory team for their diligent guidance throughout this study period. I am also grateful to heads and faculty of these institutions and departments under the college, Epidemiology and disease Control, SPH, Microbiology Department, UGMS, Bacteriology, NMIMR and Medical Laboratory Sciences, SAHS for the various roles they played during this study.

I greatly appreciate DAAD for the financial support and the team of experts and students from the North-South partnership.

I am particularly grateful to these special individuals for the unique roles they played in this programme : - Mr.Amos Akumwena, Mr. Michael Olu-Taiwo, Mr. Honesty Ganu, Mr. Michael Omari, Mr. Emmanuel Osiogwu, Mr. Lawrence Ofosu- Appiah ,Mr. William Ansah and Mr. Christian Mahu of Paakro health Centre for excellent technical support. I also thank Mr. Martin Adjuick and Rev. Richard Ndanu for excellent advice with the statistical analysis. Special thanks

to Dr. Elizabeth Bannerman, Dr. Henry J. Lawson of the Family Medicine Unit and Dr. Fenna Veltmann for moral support.

I wish to thank my entire family especially my dear husband Kwame Owusu, my sweet daughters Nana Abena Asoh Owusu and Maame Yaa Gyekyawaa Owusu for your patience and encouraging words amidst periods of great laughter even under challenging moments. To my wonderful siblings, Patricia, Michael, Kenneth and Harriet, I say thank you for your support at all times.

Lastly to the memory of my wonderful parents, even-though you left me when I needed you most, I still thank you and I appreciate you more after each day's encounter with life. God bless you for your efforts at leaving a wonderful legacy.

Thank you all for your support overtly and covertly



TABLE OF CONTENT

DECLARATION.....	i
DEDICATION.....	ii
ACKNOWLEDGEMENTS	iii
TABLE OF CONTENT.....	v
LIST OF FIGURES	xii
LIST OF TABLES	xiv
LIST OF ABBREVIATIONS	xv
ABSTRACT.....	xvi
CHAPTER ONE	1
INTRODUCTION.....	1
1.1 General Introduction	1
1.2 Rationale	3
1.2.1 Problem statement.....	3
1.2.2 Study justification	7
1.3 Study Hypothesis.....	8
1.4 Objectives.....	8
1.4.1 Main objective	8
1.4.2 Specific objectives	8

CHAPTER TWO	10
LITERATURE REVIEW	10
2.1 Buruli ulcer disease.....	10
2.1.1 Epidemiology and Historical perspective of Buruli ulcer disease	10
2.1.2 Burden of Buruli ulcer disease.....	11
2.1.2.1. World burden	11
2.1.2.2 Buruli ulcer-disease burden in Ghana.....	133
2.1.3 Buruli ulcer disease transmission and risk factors	155
2.1.4 <i>Mycobacterium ulcerans</i> –Etiological Agent of Buruli ulcer.....	17
2.1.5 Histo-pathogenesis of <i>Mycobacterium ulcerans</i> infection.....	199
2.1.6 Clinical features of Buruli ulcer disease	21
2.1.7 Diagnosis of Buruli ulcer disease	244
2.1.7.1 The Laboratory diagnosis of Buruli ulcer disease by microscopy as a diagnostic tool.....	25
2.1.7.2 Laboratory diagnosis of Buruli ulcer disease by culture	32
2.1.7.3 Detection of <i>M. ulcerans</i> DNA by PCR Technique.....	39
2.1.7.4 Histopathological Analysis for BU diagnosis.....	40
2.1.8 Hosts' Immune responses to Buruli ulcer disease.....	41
2.1.9 Management of Buruli ulcer disease.....	42
2.1.9.1 BU disease prevention strategies	43
2.1.9.2 Drug Resistance in <i>Mycobacterium</i>	43
2.1.9.3 Antimicrobial Susceptibility Testing for <i>Mycobacterium ulcerans</i> isolates.....	48
CHAPTER THREE.....	51

MATERIALS AND METHODS	51
3.1 Materials	51
3.1.1 Study area.....	51
3.1.1.1 Akuapem South District	53
3.1.1.2 Suhum-Kraboia-Coaltar district	54
3.1.2 Study design	56
3.1.3 Sample size estimation.....	56
3.1.4 Eligibility criteria.....	57
3.1.4.1 Inclusion Criteria	57
3.1.4.2 Exclusion Criteria	57
3.2 Methods.....	60
3.2.1 BU specimen collection and storage	60
3.2.2 Laboratory Procedures for the Diagnosis of Buruli ulcer by Conventional Methods.....	61
3.2.2.1 Detection of Acid Fast Bacilli (AFB) from clinical BU specimens by conventional methods.....	63
3.2.2.2 Culture of <i>Mycobacterium ulcerans</i> from clinical BU Specimens by Conventional methods.....	67
3.2.2.3 Detection of <i>M. ulcerans</i> DNA from BU specimens by conventional IS2404 specific PCR.....	69
3.2.3 Diagnosis of Buruli ulcer Disease Using Modified Laboratory Procedures.....	76
3.2.3.1 Identification and Selection of Appropriate Modification Protocols for the Detection of AFB from BU specimens.....	76
3.2.3.2 Detection of Acid fast bacilli (AFB) from Clinical BU Specimens by Modified Procedures.....	76

3.2.4 Culture of Mycobacterium ulcerans from Clinical BU Specimens by	80
3.2.4.1 Investigating inhibitory effect of selected chemical agents on clinical isolates of potential skin contaminants.....	80
3.2.4.2 Assessing Inhibitory Activities of Selected Chemical Agents on Microbial Contaminants in BU samples.....	81
3.2.4.3 The isolation of <i>M. ulcerans</i> by Modified Chemical Decontamination Protocols	82
3.2.4.4 Investigating the susceptibility of <i>M. ulcerans</i> isolates to rifampicin and streptomycin.....	83
3.2.5 Quality Control Checks.....	87
3.2.6 Ethical considerations	87
3.2.7 Data Analyses	87
CHAPTER FOUR.....	90
RESULTS	90
4.1 Baseline demographic characteristics of study population.....	90
4.1.1 Baseline data on the demographic features of study population.....	90
4.1.2 Clinical Epidemiology of Study Population	92
4.2 BUD Case Confirmation by Conventional Laboratory Procedures.....	94
4.3 Modified Laboratory Procedures for the Confirmation of BUD	100
4.3.1 Modified Laboratory Procedures for the Confirmation of BUD	100
4.3.1.1 AFB Detection by Modified Smear Preparation Methods.....	100
4.3.1.2 Analysis of Smear Preparation Protocols	102
4.3.2 <i>M. ulcerans</i> Culture by Modified Methods.....	107
4.3.2.1 Inhibition of Potential Microbial Skin Contaminants by Decontamination Protocols	107

4. 3.2.2 Inhibitory Activities of Chemical Agents on Microbial Contaminants in BU	
Samples.....	110
4.3.2.3 Performances of selected decontamination protocols.....	112
4.3.2.4 Analysis of microbial contamination after decontamination protocols.....	114
4.3.2.5 Performances of protocols in comparison to conventional methods.....	116
4.3.2.6 Drug susceptibility profiles of <i>M. ulcerans</i> isolates.....	118
CHAPTER FIVE.....	120
DISCUSSION.....	120
5.1 Confirmation of BUD cases.....	120
5.2 Detection of AFBs by microscopy.....	122
5.3 Decontamination protocols in <i>M. ulcerans</i> culture.....	127
5.4 Culture of <i>M. ulcerans</i> after decontamination.....	129
5.5 Drug susceptibility profile of <i>M. ulcerans</i> isolates.....	131
CHAPTER SIX.....	135
CONCLUSIONS AND RECOMMENDATIONS AND FUTURE PROJECTIONS.....	135
6.1 Conclusions.....	135
6.2. Recommendations.....	137
6.3 Future Projections.....	138
REFERENCES.....	139
APPENDICES.....	164

APPENDIX A	164
A.1. Questionnaires administered to suspected Buruli ulcer cases in the Akuapem south and the Suhum- Kraboa-Coaltar districtdistricts of the eastern region of Ghana.....	164
A.2. A copy of the consent form used in this study	168
APPENDIX B:	171
B.1 Media, reagents and equipment used in this study.....	171
B.1.1 Preparation of agar media and broths for culture, bacteria identification:.....	171
B.1.2 Preparation of Löwenstein-Jensen medium	172
B.1.3 Preparation of Mueller-Hinton (MH) agar (per litre).....	174
B.2 Chemicals & Reagents	175
B.2.1. Chemical agents 1: microscopy.....	175
B.2.2 Chemical Agents 2: Culture	176
B.3. Antibiotics used in this study:	177
B.4. The incorporation of antibiotics into Lowenstein-Jensen (L-J) medium.	178
APPENDIX C	179
C.1. Equipments used in this study	179
APPENDIX D: Microbiological Procedures	180
D.1.Ziehl-Neelsen (ZN) staining procedure for AFB (acid fast bacilli).....	180
D.2. Evaluation criteria for AFB.....	181
D.3 Evaluation criteria for modified protocols for the detection of acid fast bacilli (AFB) by microscopy.....	181
D.4. Decontamination protocols in culture	182

D.4.1 Conventional Procedure using 5% oxalic acid	1822
D.4.2 Modified Procedures with selected decontamination protocols	1822
D.4.3. Evaluation criteria for <i>M. ulcerans</i> culture: Colonies appearing after 8 weeks on L-J media.....	18382
APPENDIX E:	183
E.1 List of microbes used in this study.....	183
APPENDIX F:	184
F.1. Microscopy for the detection of AFB.....	184
F.2. Tables and figures for results	185
<i>F. 2.1 Tables</i>	<i>185</i>
<i>F2.2. Figures</i>	<i>187</i>

LIST OF FIGURES

Figure 2.1 Global distribution of Buruli ulcer disease.....	12
Figure 2.2: Ziehl-Neelsen stained smear showing <i>M.ulcerans</i> as acid fast bacilli (AFB)	18
Figure 2.4 showing palpable nodular lesion	23
Figure 2.3 showing typical Buruli ulcer lesion	23
Figure 2.6 showing a plaque (BU lesion)	23
Figure 2.5 showing an oedematous lesion	23
Figure 2.7 Chemical structure of Sodium hypochlorite.....	27
Figure 2.8 Chemical Structure of Ammonium Sulphate.....	29
Figure 2.10 Chemical Structure of Cetyl pyridinium chloride	37
Figure 2.11 Chemical Structure of Streptomycin	45
Figure 2.12 Chemical structure of Rifampicin.....	47
Figure 3.1 Map of Ghana showing administrative regions including Eastern Region where study sites are located.....	52
Figure 3.2 Map showing geographical location of Suhum-Krabo-Coaltar and Akuapim –South districts of the Eastern region of Ghana.....	55
Figure 3.3 Schematic presentation of Summary of Study Protocol	59
Figure 3.4 Study flow chart: Laboratory standards for reporting on confirmation of BUD.....	62
Figure 3.5 Detection of AFB from BUD specimens by conventional procedures	64

Figure 3.6 Flow chart showing procedures on <i>M. ulcerans</i> culture from BUD samples.....	68
Figure 3.7 Flowchart on Procedures for the extraction of <i>M. ulcerans</i> DNA from BU samples	71
Figure 3.8 Procedures for the detection of <i>M. ulcerans</i> DNA from BU samples by IS2404 PCR using QIAmp PCR kit.....	73
Figure 3.9 Electrophoretic patterns of amplified PCR products on 2% agarose gel incorporated with ethidium bromide.....	75
Figure 3.10 Schematic diagrams of modified laboratory procedures for the detection of AFB from BUD specimens.....	79
Figure 3.11 Procedures for determining drug susceptibility patterns of <i>M. ulcerans</i> isolates.....	86
Figure 4.1 Clinical presentation of lesions on different parts of the body of patients	93
Figure 4.2 ZN stained smears of modified procedures on AFB detection.....	106

LIST OF TABLES

Table 2.1 Outcome of some works aimed at improving sensitivity of BU diagnosis.....	31
Table 3.1 Modified protocols for microscopy-evaluation criteria	66
Table 4.1 Age Range and Sex distribution of BU Cases (n=135).....	91
Table 4.2 Confirmation of Buruli ulcer by Conventional Laboratory Procedures.....	95
Table 4.3 <i>M. ulcerans</i> detection from Swabs by Conventional Laboratory Procedures.....	97
Table 4.4 <i>M. ulcerans</i> detection from FNA by Conventional Laboratory Procedures	99
Table 4.5 Performance of smear preparation protocols based on evaluation criteria(AFB).....	101
Table 4.6 Comparative Analysis of Smear Preparation Protocols (conventional & modified) .	104
Table 4.7 Inhibitory effect of selected chemical agents on clinical isolates of Potential skin contaminants	106
Table 4.8 Effect of chemical agents on microbial contaminants in BU samples.....	111
Table 4.9 Comparative Analysis of <i>M. ulcerans</i> Recovery after Treatment with Selected Decontamination Protocols (conventional & modified)	113
Table 4.9.1 Comparative Analysis of Levels of Microbial Contamination after Treatment with Decontamination Protocols (conventional & modified)	115
Table 4.10 Performances of protocols in comparison to conventional procedure for a particular laboratory diagnostic method.....	117
Table 4.11 <i>In-vitro</i> Susceptibility of Clinical Isolates of 34 <i>M. ulcerans</i> to Rifampicin And Streptomycin (n=34)	119

LIST OF ABBREVIATIONS

<i>et al</i>	and others
BU	Buruli Ulcer
BUD	Buruli Ulcer Disease
e.g.	For example
G	gramme
L-J	Lowenstein-Jensen
mg	milligramme
ml	millilitre
µg	microgramme
MU	<i>Mycobacterium ulcerans</i>
<i>M. ulcerans</i>	<i>Mycobacterium ulcerans</i>
MTB	<i>Mycobacterium tuberculosis</i>
Mg /ml	milligramme per milliliter
NMIMR	Noguchi Memorial Institute for Medical Research
#	Number
%	percentage
PHRL	Public Health Reference Laboratory
i.e.	that is
UGMS	University of Ghana Medical School
vol /vol	volume per volume
WHO	World Health Organisation
Z-N	Zeihl-Neelsen

ABSTRACT

Background

Challenges associated with early diagnosis of Buruli ulcer disease (BUD), an infection caused by *Mycobacterium ulcerans* (*M. ulcerans*) is a major setback in public health and disease control. Lack of simple, convenient, rapid and sensitive diagnostic procedures, readily available to rural endemic communities has hampered control efforts. Improving the sensitivity of simple diagnostic methods such as microscopy for AFB detection constitutes a crucial effort in this direction. *M. ulcerans* isolation by culture though slow, provides isolates critical to performing important investigations to provide information on drug susceptibility profiles of *M. ulcerans* isolates and molecular epidemiology of the disease. Techniques aimed at improving the sensitivities of the two diagnostic methods will facilitate early disease diagnosis and subsequently improve disease control.

Objective

This study assessed procedures aimed at improving the sensitivities of methods of AFB detection by microscopy and the isolation of *M. ulcerans* in culture.

Methodology

The performances of eight smear preparation protocols were assessed for the effective detection of acid fast bacilli (AFB). Swabbed samples from ulcer lesions of Buruli ulcer (BU) patients in two BU endemic districts of the Eastern region of Ghana were used. Smear preparation was based on physical and chemical modifications, of the conventional methods for Zeihl-Neelsen (ZN) staining protocols, for the detection of acid fast bacilli (AFB) from BU cases.

Additionally, two dilutions of 5 selected chemical agents were investigated for their potential use as decontamination agents in *M. ulcerans* in culture. The activities of the chemical agents were preliminarily assessed against clinical isolates of potential skin contaminants. Effective chemical agents were simultaneously evaluated for (i) their decontamination activity against contaminants in BU samples and (ii) their effect on the growth of *M. ulcerans* in culture. Broth dilution methods were applied in these investigations. *M. ulcerans* from the study isolates were tested on rifampicin and streptomycin by the agar proportion method described by Canetti to assess the drug susceptibility.

Results

A total number of 135 clinically diagnosed BUD patients were recruited for the study. The age of the patients ranged between 1 to 92 years, with a mean age of 39 years. Swabs were taken from 123 cases while fine needle aspirates (FNAs) were taken from the 12 remaining BUD cases. Out of 123 swabs taken, PCR detected 111 positive cases, followed by microscopy with 62 cases and then culture with 52 cases. The observed differences between the three methods was statistically significant ($p < 0.001$). Out of 12 FNA samples collected, PCR detected 10 positive cases, followed by microscopy with 6 cases, while laboratory diagnosis from culture detected 5 cases. The difference between the three methods for the detection of *M. ulcerans* in FNA samples was also statistically significant ($p < 0.001$).

Eight smear preparation protocols were investigated. Among the protocols, Ziehl-Neelsen (Z-N) stained smears from samples processed with 5% phenol in 4% ammonium sulphate (5%/4% phenol ammonium sulphate) and concentrated by gravitational sedimentation had the best outcome at a score rating of 686, whilst the least effective protocol was 3.5% sodium hypochlorite concentrated by gravitational sedimentation at a score rating of 360. Four (4) out of five (5) chemical agents at 2 dilutions each, tested on potential skin contaminants had activities

against all. Povidone iodine at 0.5% and 1%, 2% CPC/4% NaCl, 1% virkon and 10% oxalic acid inhibited the growth of all microbes tested. Forty percent (40%) benzalkonium chloride, 1% CPC/2% NaCl, 5% oxalic acid could not inhibit the growth of two of the microbes. The least effective was twenty percent (20%) benzalkonium chloride (BC), inhibiting the growth of 6 out of 9 microbes tested. Of the 35 BU samples tested in duplicates against the selected chemical agents, 1% virkon and 1% povidone iodine (PI) recorded the highest activity of 99%. Whilst the least activity was recorded by 20% BC at 86%.

Outcomes for assessing the usefulness of the selected chemical agents as decontamination agents in *M. ulcerans* culture indicated that, 2% CPC / 4% NaCl and 0.5% virkon were the most effective at 52.8%, and 51.4%, *M. ulcerans* recovery rates respectively. *M. ulcerans* recoveries were arithmetically higher than that of the conventional method (5% oxalic acid) at a yield of 44%. The observed differences were however not statistically significant (2% CPC / 4% NaCl, $p=0.7608$ and 0.5% virkon, $p=0.4070$). The highest contamination rate of 29% was exhibited by 5% oxalic acid and 1% CPC/2% NaCl of 21% in culture.

Six (6) *M. ulcerans* isolates showed resistance to rifampicin, whilst 1 was resistant to streptomycin *in-vitro*. Resistance to both drugs was not observed among any of the isolates.

Conclusions and recommendations

In conclusion, BU specimens processed with 5% phenol /4% ammonium sulphate and concentration by gravitational sedimentation will improve AFB detection. In the peripheral centres where equipments like centrifuge may not be readily available, this sedimentation procedure can be used with adequate training. 2% CPC / 4% NaCl and 0.5% virkon are effective decontamination agents for the isolation *M. ulcerans*. Potential resistance to drugs (rifampicin and streptomycin), could also be a concern in the study area. In view of this, regular

susceptibility testing of isolates and surveillance of susceptibility results (as is being done for *Mycobacterium tuberculosis*), is recommended.

Key words: Ghana, Buruli ulcer, *Mycobacterium ulcerans*, diagnostics, acid fast bacilli, chemical agents, decontamination, drug susceptibility testing, culture, microscopy, povidone iodine, cetyl pyridinium chloride.

CHAPTER ONE

INTRODUCTION

1.1 General Introduction

Buruli ulcer disease (BUD) is a chronic, necrotic, infectious skin condition caused by an environmental organism, *Mycobacterium ulcerans* (*M. ulcerans*) (Thangaranj *et al*, 1999). It is the third most widespread *Mycobacterium* infection after tuberculosis and leprosy causing morbidity in immune-competent humans worldwide (WHO, 2001). Though all people are susceptible, the disease has been reported in localized rural, remote, riverine communities, with limited access to health care centres. More cases have been reported among children of fifteen years and below (Marsollier *et al.*, 2003; Aiga *et al*, 2004).

Sir Robert Cook described the disease among patients in the Mengo hospital of Uganda in 1897 (Cook, 1897). The aetiological agent *M. ulcerans*, was subsequently isolated and identified by Professor Peter McCallum in Bairnsdale, Australia in 1948 (MacCallum *et al.*, 1948). The disease has since been reported from over thirty-two tropical and subtropical locations globally. This includes Ghana (Johnson *et al*, 2005).

Since the 1980s, there has been a sharp rise in disease incidences, with most of the cases reported from countries in Sub-Saharan Africa including Benin (Muelder *et al*, 1990), Cote d'Ivoire (Marston *et al* 1995), Togo (Meyers *et al*, 1996), Nigeria (Chukwuekezie *et al* 2007) and Ghana (Bayley, 1971). In Ghana, an estimated 6,000 cases were reported in a countrywide survey by Amofah's group in 2002 (Amofah *et al*, 2002). They also showed a prevalence of 130 per 100,000 cases some in communities, and a up to twenty-two percent (22%) of inhabitants of some communities were also infected, replacing tuberculosis and leprosy as the most prevalent Mycobacteriosis in the community (Amofah *et al*, 2002). The disease has also been reported in points of topographical disturbances such as earthquakes, floods and mining activities (Clancy *et*

al 1961). In Ghana, Aiga *et al* reported on water related risk factors for the disease (Aiga *et al* 2004). Endemic communities were cited in areas including tropical rain forests in Amansie West district of the Ashanti region (Asiedu *et al*, 1998; van der Werf *et al* 1999). In addition, cases were also reported in swampy grasslands around the banks of the Densu river in Nsawam and Amasaman (Bayley *et al*, 1971). It has also been reported in areas north of the Afram River among others (van der Werf *et al*,1999).

The reservoir of *M. ulcerans* and its route of transmission is unclear (Hayman *et al* 1985), but an antecedent break in host skin has been reported as possible routes, and some aquatic fauna and flora have been implicated as potential reservoirs for the organism (Marsollier *et al*, 2002 ; 2003).

Mycobacterium ulcerans proliferates in the host after inoculation. It subsequently produces a toxin referred to as mycolactone. This is a polyketide derived compound with immunosuppressive and cytotoxic properties. The toxin is the major virulence determinant in the mediation of BUD pathogenesis. This results in typical clinical features of BUD (George *et al*, 1999). The immune response of the host at the early stages of infection is lethargic or non-existent. However, during the later stages of the disease, there is some degree of immune response. The disease is resolved and the patient is left with deformities. This leads to the morbid state associated with BU disease (WHO, 2001).

The clinical manifestations of a patient form the basis for the clinical diagnosis of BUD, particularly in an endemic area. Two forms of the disease have been recognized by the World Health Organization (WHO); the active and inactive forms. The active form is characterized by non-ulcerative papules, nodules, plaques, and oedema and ulcerative forms. The inactive form is characterized by evidence of previous infection with a depressed stellate scar with or without sequelae in the form of deformities (Asiedu *et al*, 2000; WHO, 2003). Clinically diagnosed

cases can be confirmed by laboratory analyses. These include (i) the detection of acid fast bacilli (AFB) from Zeihl-Neelsen (ZN) stained smears. (ii) The culture of *M. ulcerans* on *Mycobacterium* media including the Löwenstein-Jensen and the Middle brook series and (iii) the detection of *M. ulcerans* DNA from BU specimens using the IS2404 polymerase chain reaction (PCR) technique (Stinear *et al*, 2004; Siegmund *et al*, 2005) and (iv) histopathological examination of biopsied tissue specimens from infected patients, showing necrotic cells and remnants of *M. ulcerans* (Hayman, 1993 ; Asiedu *et al*, 2000).

Currently the treatment of BUD involves the administration of rifampicin and streptomycin for an eight week period. This is a treatment regimen recommended by the world health organization (WHO, 2004). It has been found to be particularly useful at the pre-ulcerative and the early ulcerative stages (WHO, 2004; Chauty *et al*, 2007). Wide surgical excision to repair and replace damaged skin surface is recommended for very large complicated stages of the disease (WHO, 2001). Other treatment options at the clinical trial stage include the use of heat for treatment and hyperbaric oxygen. In addition, topical treatments including nitrite and phenytoin have been applied (Krieg *et al*, 1974 ; Junghanss *et al*, 2009).

1.2 Rationale

1.2.1 Problem statement

The diagnosis of Buruli ulcer disease is based on presenting clinical features of a suspected case. However, clinical diagnosis is error prone; this is because other skin infections can manifest with similar features. In a study conducted by Etuaful and co-workers, one third of cases on antibiotic treatment turned out to be conditions other than BUD (van der Werf *et al*, 1999 ; Etuaful *et al*, 2003). The World Health Organization (WHO) therefore recommends that all clinically diagnosed cases be confirmed with any two of the four recommended laboratory

methods. Histopathological analysis and IS2404 PCR are the most sensitive (> 90%) but they require more specialized inputs. Inputs required includes maintaining a cold chain, stable electric supply and qualified laboratory technicians. These facilities may not be readily available in peripheral health facilities (Bretzel *et al*, 2007; Herbingner *et al*, 2009). Conventional smear microscopy involves examining Zeihl-Neelsen (ZN) stained smears at high power to detect AFB. This is a relatively easy, cheap, rapid and most readily available method which can easily be applied in most peripheral health facilities. The rate of detection of AFB by microscopy is however low. It is only able to detect AFB levels of $5-10 \times 10^3$ bacilli / ml in 100 microscopy fields. Reliance on this method can lead to the generation of false negative results due to its low specificity. This can lead to misdiagnosis, delay diagnosis and ultimately affect disease control. Improving the sensitivity of AFB detection by microscopy is therefore an important public health priority (Steingart *et al.*, 2006) .

Studies have shown that the sensitivity for the detection of acid-fast bacilli can be improved . This is when diagnostic specimens are concentrated into smaller volumes by either centrifugation or overnight gravitational sedimentation (Miörner *et al*, 1996 ; Gebre-Selassie ,2003). These procedures increase the quantum of AFB per volume per field; enhancing visualization by microscopy (Ängeby *et al*, 2000; Bruchfeld *et al*, 2000, Gebre-Selassie, 2003) . Furthermore, chemical agents can also be used to facilitate the release of AFB embedded in host cell matrix. This procedure can clear the viewing field and decrease the time taken to read the slides. Chemical agents such as 1% , 3.5 % sodium hypochlorite and 5% phenol (4%) ammonium sulphate have been successfully used to improve AFB detection in *Mycobacterium tuberculosis* (MTB) microscopy (Miörner *et al*, 1996; Selvakumar *et al* 2002). Outcomes from these studies have shown improved sensitivities especially in the area of MTB studies. This is in comparison with conventional smear-microscopy after bleach treatment (Van Deun *et al*, 2000 ; Ängeby *et al*, 2004; Cattamanchi *et al*, 2010).

Culturing *M. ulcerans* from specimens obtained from Buruli ulcer lesions is the only microbiological method that provides isolates. Subcultures of viable *M. ulcerans* isolates are useful because

- i) Mycolactone, the key virulent factor mediating Buruli ulcer disease can be extracted for further research.
- ii) Drug susceptibility testing can be conducted to obtain information on the resistance profiles of the obtained isolates to the current treatment regimen of streptomycin and rifampicin..
- iii) Information on the molecular epidemiology of the disease can be obtained.

In spite of its usefulness, the culture of *M. ulcerans* is a slow diagnostic process and takes over eight weeks to obtain results; furthermore, the recovery rate of *M. ulcerans* from swabs is particularly low (about 20-45%). This is because (i) a majority of clinical specimens collected for isolation of *M. ulcerans* are usually contaminated to varying degrees with more rapidly growing commensals. These are potentially capable of contaminating the *M. ulcerans* cultures (Palomino *et al*, 1998; McClean *et al*, 2011; Yeboah-Manu *et al*, 2004). Conventionally, collected specimens are processed with specialised chemical digestion/decontamination agents. These include zwitterionic compounds, weak alkalis and acids with selective antimicrobial properties. In the process of inhibiting / eliminating the fast growers, some of these chemical agents may have varying degrees of harsh effects on *M. ulcerans* viability in culture. In effect useful isolates are lost through the decontamination process.

This constitutes an important challenge for the diagnosis of BU by culture; a very useful laboratory method for *M. ulcerans* diagnosis. In this regard, various decontamination methods have been studied for *Mycobacterium tuberculosis* (MTB) and *M. ulcerans* isolation. These include 5% oxalic acid and 4% sodium hydroxide (Petroff method). Currently, 5% oxalic acid

decontamination method is the most widely used; particularly for the isolation of *M. ulcerans* from tissue (Yeboah-Manu *et al*, 2004).

BU specimens, particularly swabs from open ulcers also contain spores of contaminating organisms including fungal spores. These are difficult to remove by the present decontaminating method. They grow uninhibited even after decontamination, outgrowing *M. ulcerans* in culture.

This study will therefore investigate selected chemical agents with known antimicrobial activities including antiseptic and disinfecting properties. In addition, 5% oxalic acid (conventional method) will be included.

These include cetylpyridinium chloride–sodium chloride (CPC / NaCl) solution, a chemical agent with known antifungal properties (Smithwick *et al*, 1975) (Pardini *et al*, 2005). Povidone iodine is an effective antiseptic for dressing wounds. Virkon disinfectant is used in veterinary facilities for reducing levels of contamination. Benzalkonium chloride (alkyl dimethyl benzyl ammonium chloride-40-active) with the brand name Timsen. This is also used for dressing diabetic wounds

Currently, streptomycin and rifampicin have been recommended by WHO as the antibiotics for the treatment of Buruli ulcer. (WHO, 2004). This implies that biopsied tissue from surgical excision would be less available as diagnostic specimens. These will gradually be replaced with swabs from ulcers and fine needle aspirates (FNA) from pre-ulcerative lesions. (Stienstra *et al*, 2002, Aujoulat *et al*, 2003). FNA technique is an adaptation from a method used to obtain cellular material for cytological studies from tumours (Ersoz *et al*, 1998, Lester and Layfield, 2007). Other investigators have shown that this method looks promising for the diagnosis of pre-ulcerative BU cases and for follow-ups (Eddyani *et al*, 2009; Phillips *et al.*, 2009). *M. ulcerans* recovery from swabs and FNA in culture is however challenging especially from paucibacillary

lesions. This study will investigate the effective detection and recovery of *M. ulcerans* from swabs and fine needle aspirates.

The possibility of administering rifampicin and streptomycin to misdiagnosed (false positives) cases exists. This can lead to the generation/selection of resistant mutants as a result of inappropriate exposure to the drugs. This can occur in developing countries where the possibility of co-infection with Tuberculosis (TB) and Buruli ulcer exists. When such patients are treated first for BUD, they may develop a resistance to TB. Antimicrobial susceptibility testing of *M. ulcerans* isolates will provide useful information on resistance profile of circulating strains within the endemic communities. This information is crucial for public health and disease control.

1.2.2 Study justification

Buruli ulcer disease occurs predominantly in localized rural communities of developing countries with limited access to basic health care. This is an important public health challenge to the health sector. Improving the laboratory diagnosis of Buruli ulcer disease is crucial to the management of BUD especially in endemic communities. Relatively simple procedures such as microscopy for the detection of acid fast bacilli can be enhanced by investigating smear preparation methods that will improve the detection of acid fast bacilli by microscopy. The detrimental effects of decontaminating agents on *Mycobacterium* are well known. Investigating effective decontamination protocols will improve the isolation of *M. ulcerans* from culture. There is therefore the need to develop appropriate decontamination protocols that will preserve the viability of *M. ulcerans* and effectively eliminate contaminating organisms. There is also the need to investigate the susceptibility/resistant profiles of the isolates obtained. Information obtained from these investigations will help influence public health policies on the control of Buruli ulcer disease.

1.3 Study Hypothesis

Modifications of conventional laboratory diagnostic protocols will not improve Buruli ulcer disease diagnosis.

1.4 Objectives

1.4.1 Main objective

To assess laboratory procedures, for effective detection and isolation of *M. ulcerans*, from BU specimens.

1.4.2 Specific objectives

1. To identify and assess the effectiveness of various modifications of detecting AFB from BU specimens by smear microscopy
 - To identify useful smear preparation protocols for the effective detection of AFB from BU specimens
 - To assess the effectiveness of the smear preparation protocols on the detection of AFB from BU specimens
2. To determine the inhibitory activities of selected chemical agents and assess their effectiveness on decontamination of *M. ulcerans* culture
 - i) To determine the inhibitory activities of selected chemical agents on isolates of potential skin contaminants.
 - ii) To assess the inhibitory activities of the selected chemical agents on microbial contaminants in BU samples.

- iii) To investigate the effectiveness of the selected decontamination protocols on *M. ulcerans* isolation in culture.
- iv) To determine the drug susceptibility profiles of the *M. ulcerans* isolates.

CHAPTER TWO

LITERATURE REVIEW

2.1 Buruli ulcer disease

2.1.1 Epidemiology and Historical perspective of Buruli ulcer disease

Preventable mortality and morbidity resulting from infectious conditions have been a cause for global public health concern particularly in developing countries. Buruli ulcer disease (BUD) is one of such infectious conditions affecting the skin and soft tissue. The disease is caused by *Mycobacterium ulcerans* (*M. ulcerans*), a slow growing and toxin producing environmental pathogen. The condition is the third most widespread *Mycobacterium* disease after tuberculosis and leprosy. The disease has also been recognized by World Health Organization (WHO) as one of thirteen neglected infectious conditions affecting immune-competent humans (Thangaranj *et al* 1999; Portaels *et al*, 2001; Molyneux *et al*, 2005).

Professor Peter MacCallum and his team of researchers were the first to isolate the causative organism from the lesion of patients in Australia. They identified it as *Mycobacterium ulcerans*, an organism that could be cultured at a relatively lower temperature of 32°C (MacCallum *et al*, 1948). The condition had previously been described in 1897 by Sir Albert Cook, a British physician who was then working at the Mengo Hospital in Uganda (Cook, 1897) and also by Klein Schmidt in North-eastern Congo during the period of the 1920s (Meyers *et al*, 1996). The period preceding the early 80s saw a global reportage of increased disease incidence with most of the cases from countries including Congo (Smith *et al*, 1976), Uganda (Clancy *et al*, 1961), Nigeria (Oluwasanmi *et al*, 1976) and Ghana (Bayley, 1971). The name 'Buruli ulcer' was obtained from the area Buruli county in Uganda where several cases of the condition were reported among a group of refugees who were camped near Lake Kyoga of the Nile Delta

(Clancy *et al*, 1962). After this period and during the early 80s, remarkable increases in disease incidences were observed in many countries of West Africa including Ghana.

2.1.2 Burden of Buruli ulcer disease

2.1.2.1. World burden

Buruli ulcer disease has since been confirmed in over thirty-two countries worldwide, with a majority of the endemic areas geographically located in tropical and subtropical regions (Figure 2.1). These include countries in Africa, South America, South-East Asia (Pettit *et al*, 1966, Pradinaud, 2003) and Australia where new cases and new endemic foci have reportedly increased over the past two decades (Hayman, 2003).

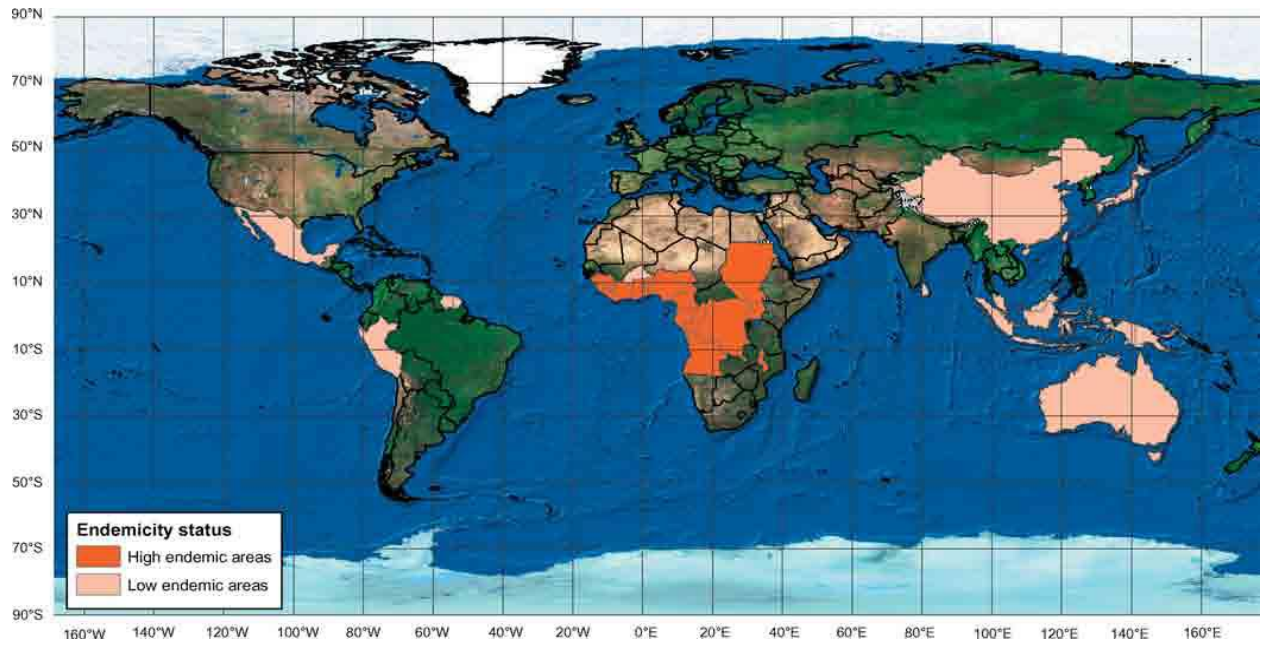


Figure 2.1 Global distribution of Buruli ulcer disease

In Africa, the most endemic areas are located in sub-Saharan region; with East and West Africa being the worst hit. The condition has been reported from countries in Uganda, Guinea, Zaire, Congo, Cameroon, Nigeria (Oluwasanmi *et al*, 1976) Benin (Debacker *et al*, 2003), Liberia (Monson *et al*, 1984) and the Ivory Coast (Marston *et al*, 1995) Ghana (Amofah *et al*, 2002) and the most recent addition Togo (Meyers *et al*, 1996).

Buruli ulcer disease has a tendency to concentrate in specific areas. This pattern of distribution occurs even in endemic regions (Johnson *et al*, 2005). Incidence rates of the disease reported over the past two decades in some of the endemic areas are approximately 15000 cases in Cote d'Ivoire with a 16% prevalence rate (Marston *et al*, 1995). Four thousand cases detected in southern Benin with an annual prevalence rate of 21.5 per 100,000, a rate much higher than that reported for tuberculosis and leprosy (Muelder and Nourou, 1990; Debacker *et al*, 2003). In Ghana, Amofah and his team estimated that the disease prevalence in some of the highly endemic areas was about 150.8 /100,000 individuals (Amofah *et al.*, 2002). In West Africa, an estimated 25% of the cases reported were predominantly children, who were usually left with permanent disabilities. Other areas outside Africa have reported many cases of the disease including Australia. Even though Buruli ulcer disease remains uncommon in Australia, there have been increases with both incidence and the number of endemic areas in the last twenty years (W H O, 2003; Johnson *et al*, 2005).

2.1.2.2 Buruli ulcer-disease burden in Ghana

Ghana is a BU endemic country in the West African sub-region and records indicate that the first case was a patient from an endemic community along the Densu River who had reported at the Korle-Bu Teaching Hospital for medical attention in 1971 (Bayley *et al*, 1971). Other cases

were reported from other endemic communities located particularly along River bodies (Aiga *et al.*, 2004). These included Agogo within the Afram valley where van der Werf and his team recorded over ninety cases (van der Werf *et al.*, 1989; van der Werf *et al.*, 1999). In 1993, Amofah and his team, working in the Amansie West district of the Ashanti region, alerted health authorities on a significant number of cases in the Ashanti region of Ghana (Amofah *et al.*, 1993). Subsequent to this, cases were reported from focalized endemic areas spread over the country. Presently, cases of the disease represent a substantial fraction of patients patronizing health facilities in the endemic communities. The disease has been reported in all ten regions of the country. In 2002, a survey conducted by Amofah and his team on the prevalence of Buruli ulcer disease, indicated that an approximated number of 5,619 cases with a total of number 6,332 clinical lesions at various stages was recorded (Amofah *et al.*, 2002). Amofah and his group further reported that, people with Buruli ulcer disease constituted 37.6% of patients registered in healthcare facilities, within endemic areas. Reports indicate that 8.4% of the population in Tontokrom of the Amansie West district of the Ashanti region had the active form of the disease, with the farming women recording the highest number. This report corroborated reports that women and children under the age of 15 years were mostly affected. Information from his study showed that, even though the disease was widespread, documented cases were relatively few, revealing inadequacies in the documentation process. This was generally attributable to resource constraints, particularly within rural endemic communities. The study reported a 22% prevalence rate for Buruli ulcer disease, clearly higher than that for tuberculosis and leprosy (Amofah *et al.*, 2002).

2.1.3 Buruli ulcer disease transmission and risk factors

Mycobacterium ulcerans, the aetiological agent of Buruli ulcer disease (BUD) is an environmental pathogen, which primarily affects humans (Barker, 1972). The organism has been reported to also infect animals such as pandas, armadillos and tree-living koalas, in remote endemic regions of Australia (McOrist *et al*, 1985). Though the reservoir of the pathogen, and the means by which it is transferred to its host is still not clear, studies have shown that *M. ulcerans* was first detected in the environment in the 1990s by Australian researchers using polymerase chain reaction (PCR)(Ross *et al*, 1997). Other researchers identified *M. ulcerans* in some aquatic insects from endemic regions in Africa by PCR (Portaels *et al*, 1997). This led to the proposition that, aquatic insects of the insect order Hemiptera (Naucoridae and Belostomatidae) among others could be reservoirs for the organism. In subsequent studies *M. ulcerans* was detected in other aquatic organisms. These included snails, fish and biofilm of aquatic plants (Marsollier *et al*, 2002; 2004). Even though the transmission pathway of the organism to the host remains an enigma, a possible access through a previous skin trauma has been conjectured (Hayman *et al*, 1993; Veitch *et al*,1997).The predominance of BUD lesions on the left upper limb and the right lower limbs, demonstrated in van der Werf's study, indicated a possible transmission through injury in the course of outdoor activities. Some of these activities included farming, an activity categorised as a BUD risk related activity (van der Werf *et al*, 1989; Raghunathan *et al* 2005). A case of person-to-person spread has been reported (Horsburgh *et al*, 1997; Debacker *et al*, 2003). The upsurge of BUD is usually associated with drastic changes in the environment coupled with topographical disturbances (Hayman *et al*, 1993; Marston *et al*, 1995) . These occurrences which include earthquakes; increased farming, mining activities and flooding have also been mentioned. Until recently, cultures of water samples from BU endemic areas were negative for the presence of *M. ulcerans*. Portaels and her team proved

the environmental nature of the organism by culturing it from the environment, providing a breakthrough for the confirmation of the organism as an environmental one (Ross *et al*, 1997, Portaels *et al*, 2008).

The occurrence of the disease has been associated with aquatic environments. The first cases reported in Australia occurred between two and three years after severe flooding and the disease was found first in koalas and then in humans (McOrist *et al*, 1985; MacCallum *et al*, 1948). In Uganda disease incidences were also reported between 1962 and 1964 on the Eastside of the Victoria Nile (Clancy *et al*, 1961; Connor and Lunn, 1966). The outbreak was presumably associated with severe flooding caused by heavy rains. The incidence pattern of the disease was the same for the two areas. Even though, *M. ulcerans* has not yet been isolated from the soil, it has been considered as a possible reservoir. Seasonality patterns with a higher incidence in the drier months have been reported (Barker *et al* 1972). In West Africa, Cote D'Ivoire reported cases from individuals living in close proximity to the main river where farming is their main occupation (Marston *et al*, 1995). Cases were also reported amongst children associated with seasonal flooding in a local village in Togo (Meyers *et al*, 1996). In Liberia, the disease was reported in an area where swamp rice was introduced to replace upland cultivation. The construction of dams for this purpose caused an artificial extension of wetlands (Monson *et al* 1984). Similar situations were reported from other endemic areas including Papua New Guinea and Phillips Island (Flood *et al*, 1994). In Ghana, endemic areas have been found in close proximity to river bodies. For instance, Agogo, an endemic area is close to the Afram River (van der Werf *et al*, 1989) Amasaman, Nsawam and Suhum are close to River Densu. The Amansie West district of the Ashanti Region is a very important endemic area close to the Offin River (Amofah *et al*, 1993).

Mycobacterium ulcerans infection can affect all people regardless of race, sex, and age. However, most studies have shown that the age range that is most affected are children under the age of fifteen years (Marston *et al*, 1995). Even though the disease has been reported in persons with human immunodeficiency virus (HIV/AIDS), no predilection for BU has been noted in HIV infected persons or other immune-deficient patients. This is in spite of the substantial rates of HIV in many BU endemic areas (Horsburgh *et al*, 1997).

2.1.4 Mycobacterium ulcerans –Etiological Agent of Buruli ulcer

Mycobacterium ulcerans (*M. ulcerans*) is the aetiological agent of Buruli ulcer disease (Figure 2.2). It is classified as a member of the phylum Actinobacteria, order Actinomycetales, in the suborder Corynebacteriaceae and the genus *Mycobacterium* (Connor and Lunn, 1965).

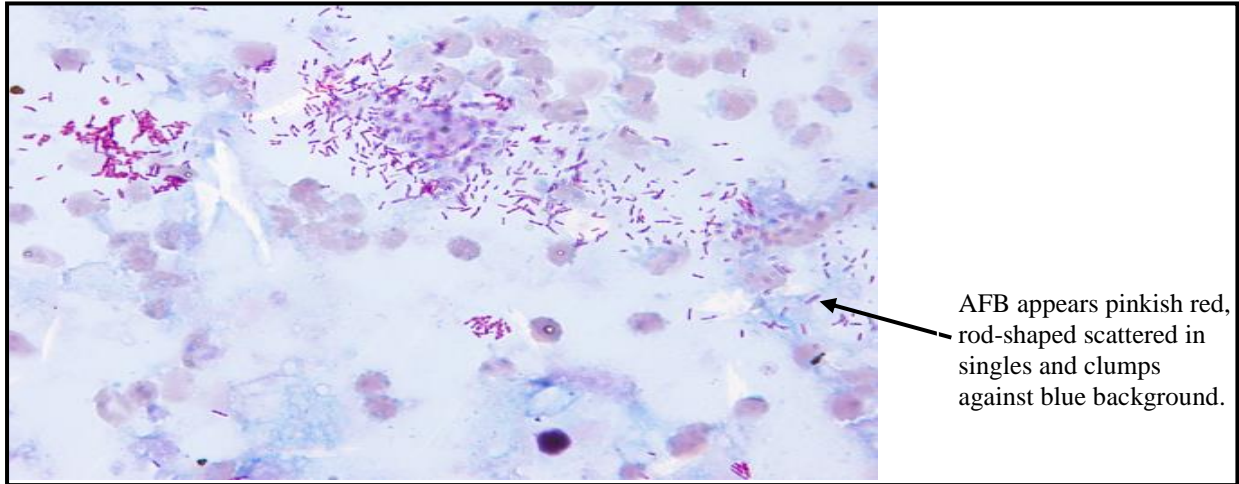


Figure 2.2: Ziehl-Neelsen stained smear showing *MU* as acid fast bacilli (AFB) *M. ulcerans* from clinical BU specimens observed under oil immersion (x1000).

Structurally, *M. ulcerans* has a typical Mycobacterium cell structure made up of a tightly wound nuclear structure with a high Guanine+Cytosine (G+C) DNA content of 65% (Barksdale and Kim, 1977; George *et al*, 1999 ; Siegmund *et al*, 2005,). A cell cytoplasm enclosed by the plasma membrane and the peptidoglycan layer which is bordered on the exterior by layers of complex fatty acids and lipids including mycolic acids from which the mycolactone, a toxin that mediates pathogenesis is derived (Collins *et al*, 1997 ; Adusumilli *et al*, 2005). The lipid rich layer of the cell wall makes it waxy. This makes it relatively impervious to hydrophilic compounds, including basic dyes used for other bacteria. It also makes it impermeable to most disinfectants except phenol compounds. *Mycobacterium* is generally termed 'acid fast' organisms because, they cannot be decolorized by acids or acid/alcohol decolorizes once stained. The waxy cell wall makes them clumpy in suspension and in culture. This contributes to the characteristically slow growth rate, with a generation period of 20 hours (Baron, 1994). *Mycobacterium ulcerans* will grow on typical *Mycobacterium* media including Lowenstein Jensen(L-J) medium at an optimum temperature range of 29°C to 33°C and a pH of 5.4 -7.4. It is an aerobic organism which grows best under micro-aerophilic conditions. It requires a minimum growth period of eight weeks. The colonies of *Mycobacterium ulcerans* range from colourless to pale yellow. The colonies have a matt texture and may be convex or flat, and not more than 3mm in diameter. Biochemically, *M. ulcerans* does not produce niacin, but has moderate catalase activity (Barksdale *et al*, 1977 ; Collins *et al*, 1997).

2.1.5 Histo-pathogenesis of *Mycobacterium ulcerans* infection

Mycobacterium ulcerans is a slow growing environmental organism, with an opportunistic existence. It can occasionally evolve into a pathogenic existence under different conditions such as trauma and immune-compromised state of host (Collins *et al*, 1997). Once it accesses the host, *M. ulcerans* proliferates within the subcutaneous tissues and the skin above it. It then

produces a family of toxic macrolides, referred to as mycolactone. These toxins have immunosuppressive and necrotizing properties and they mediate the virulence exhibited by *M. ulcerans* (Hayman, 1985, George *et al*, 1999). The production of mycolactone seems to be peculiar to *M. ulcerans* though other toxic lipid compounds can be produced by *Mycobacterium* in general (George *et al*, 1999). Mycolactone produced by *M. ulcerans* induces apoptosis or programmed cell destruction. This causes necrosis and suppresses the activities of the immunocytes. In effect, there is extensive necrosis of tissue with impaired local immune response.

The organism continues to replicate and spread to infect adjoining tissue (Rondini *et al*, 2005). This potentially increases the necrotic base and in extreme cases spread to affect the underlying bone, causing osteomyelitis associated with advanced cases of the condition (George *et al*, 1999 ; Gooding *et al*, 2001 ; Portaels and Hernandez, 2006).

The effect of mycolactone on the cells can be clearly observed by histo-pathological presentations of infected tissue under the microscope (Mve-Obiang *et al*, 2003). It reveals large clumps of extracellular acid-fast organisms and globular fatty cells surrounded by areas of necrosis with poor or absent inflammatory response (Dodge, 1964; Connor *et al*, 1965 ; 1966) . The major mediator of virulence exhibited by *M. ulcerans* is mycolactone, a polypeptide derived macrolide (Hockmeyer *et al*, 1978; George *et al*, 1999). Macrolides are chemical metabolites produced by some soil bacteria including *M. ulcerans* (Baron *et al*, 1994 ; Benowitz *et al*, 2001). Studies show that a single bacterium can produce a number of related macrolides and congeners. This was evidenced by a study from Cadapan; he showed that *M. ulcerans strain* 1615 from which mycolactone A/B had been isolated and also produced minor congener molecules (Cadapan *et al*, 2001).The synthesis of mycolactone was encoded by a giant circular plasmid with a molecular size of 174kb named pMUM001 encoded (Stinear *et al*, 2004).

Studies show that mycolactone has a cytotoxic effect in guinea pigs similar to that seen in human patients (Krieg *et al*, 1974; George *et al* 1999 ; Gooding *et al*, 2001; 2003). It also had selective *in-vitro* activity against some immune cells; particularly those responsible for immunity against *Mycobacterium* infection (Read *et al*, 1974).

The conclusion drawn from these observations were that, mycolactone produced from the invading bacteria were responsible for the extensive necrotic damage, to the host tissues. It was also responsible for local suppression of the immune system. Portions of the skin most affected were the dermis, panniculus and fascia (Hockmeyer *et al*, 1978).

The condition could however resolve after a variable period usually leaving the patient deformed. Studies on murine models suggested the systemic nature of Buruli ulcer disease and results from Addo and team indicated spread by the lymphatic pathway (Addo *et al*, 2005; Pszolla *et al*, 2003).

2.1.6 Clinical features of Buruli ulcer disease

The effect of the proliferating *M. ulcerans* on the host is eventually manifested as typical BU lesions (Figure 2.3 – 2.6). The period between infection and clinical manifestation is referred to as the incubation period. In *Mycobacterium ulcerans*, this may vary between two weeks and three months. This is dependent on factors including the infecting *M. ulcerans* strain and the host factors.

The clinical features may manifest at any part of the body but a predisposition to the extremities, particularly the limbs has been reported (Hospers *et al*, 2005; Horsburgh *et al*, 1997). Clinically the onset of the disease is characterized by a localized, firm and painless swelling in the skin at the entry point (WHO, 2003; Sizaire *et al*, 2006; WHO, 2008).

The swelling is described as a papule when it is less than one centimetre in diameter; and as a nodule (Figure 2.4), when it ranges between of one to two centimetres and attached to the skin (subcutaneous). If it appears flat and irregular at the edges and covers an area that is more than two centimetres then it is referred to as a plaque.

These are features that are ignored by the patient as it is assumed to be other conditions including yaws (Aujoulat *et al*, 2003). Without the necessary therapeutic intervention, the swellings develop into an ulcer (Figure 2.3). The clinical features of the disease have been clearly defined by the WHO (Hayman and McQueen, 1985; Asiedu *et al*, 2000) .



Figure 2.3 showing typical Buruli ulcer lesion



Figure 2.4 showing palpable nodular lesion



Figure 2.5 showing an oedematous lesion



Figure 2.6 showing a plaque (BU lesion)

Source: National Buruli Ulcer Control Programme (NBUCP)

Buruli ulcer patients studied by Meyers and colleagues, presented with clinical features of rapidly progressing diffuse oedema of a whole anatomical site, without an obvious initial focal lesion (Meyers *et al*, 1996). The whole affected anatomical site or sections of it, subsequently ulcerates to form the typical Buruli ulcer lesion. Studies have shown that timely therapeutic intervention can substantially reduce the necrotic outcome.

The classical Buruli ulcer lesion presents as a necrotic skin ulcer with deeply undermined edges. This is accompanied with a basal slough with a cotton-wool-like appearance. In general, the ulcers are painless unless secondarily infected. The affected individual appears systemically well. This explains why most patients report late for medical attention. Contractures and gross deformities seen in Buruli ulcer disease are as a result of bone destruction (osteomyelitis) from the *M. ulcerans* infection (Portaels *et al*, 2002).

2.1.7 Diagnosis of Buruli ulcer disease

The diagnosis of Buruli ulcer disease is based on the clinical and laboratory assessment. In an endemic area, an assessment of presenting features by an experienced physician forms the basis for the clinical diagnosis of BUD. Other considerations include the patient's transient or permanent exposure to an endemic area, and the location of the presenting lesion among others. Since other infectious conditions may have clinical signs similar to BUD, WHO strongly recommends that all clinically diagnosed BU cases should be confirmed by the recommended laboratory procedures (Portaels *et al*, 2001). The confirmatory tests include the detection of acid fast bacilli (AFB), from Zeihl-Neelsen stained smears prepared from BU specimens. Positive *M. ulcerans* cultures on *Mycobacterium* media, including Lowenstein Jensen (LJ). The detection of *M. ulcerans* DNA using the IS2404 PCR method and histopathological analysis of tissue biopsies (Guarner *et al*, 2003; Phillips *et al*, 2005).

Diagnostic specimens for these tests include fine needle aspirates (FNA), tissue from biopsy and swabs from BU lesions (Ersoz *et al*, 1998; Eddyani *et al*, 2009; Phillips *et al*, 2009; Cassisa *et al*, 2010) . The World Health Organization further recommends that the disease can be confirmed by at least any two of the four procedures (WHO, 2001). These laboratory methods are unique in sensitivity, specificity, rapidity and expense. Some publications have however pointed out the difficulties associated with the above criteria (Bretzel *et al*, 2007; Herbingner *et al*, 2009).

2.1.7.1 The Laboratory diagnosis of Buruli ulcer disease by microscopy as a diagnostic tool

Microscopy for the detection of acid fast bacilli (AFB) provides a useful tool, for the preliminary diagnosis of the disease. It is a relatively simple method which requires the use of a microscope and staining reagents. This method can easily be used in less sophisticated laboratories especially in rural endemic communities (Wilkinson and Sturm, 1997). Conventionally, smears prepared from diagnostic specimens are stained with the specialized Zeihl-Neelsen (ZN) staining technique for acid fast bacilli (AFB) (Allen and Mitchison, 1992; Collins *et al*, 1997). The presence of *M. ulcerans* can be observed as pink bacilli (rods) against a bluish background. Other staining methods include the Kinyuon (cold stain), the auramine / rhodamine fluorescent methods (American Thoracic Society, 1981).

Microscopy for the detection of AFB is able to detect levels of 5×10^3 - 10^4 bacteria per millilitre (bact./ml). This makes it relatively low in sensitivity in comparison to culture. Culture can detect AFB levels of 10-100 viable bacteria per millilitre of sample. Microscopy as a diagnostic method lacks specificity as it is limited in differentiating AFB from different *Mycobacterium* species. For example it lacks the capacity to differentiate AFB from *M. ulcerans* from *M.*

marinum (Zumla and Grange 2002). In spite of these challenges, microscopy for the detection of AFB remains very useful for these reasons:-

- i) It provides a presumptive diagnosis of *Mycobacterium* disease
- ii) It enables the rapid identification of most infections i.e. smear positive cases
- iii) It may be used to follow the progress of patients with *Mycobacterium* infections on chemotherapy
- iv) It is of vital importance with regard to the patient's discharge from the hospital, or return to gainful employment
- v) It can confirm that cultures growing on media are indeed acid fast bacilli.
- vi) It is also useful in determining appropriate dilutions of sediments for direct drug susceptibility tests.

Outcomes from studies indicate that the presence of debris and host protein material, found in diagnostic specimens, hampers the effective detection of AFB by microscopy. Lysing or digestion procedures have been adapted, by several studies to overcome this challenge (Ängeby *et al*, 2000; Daley *et al*,2009). Sodium hypochlorite (NaOCl) and phenol ammonium sulphate (PhAS) are some of the chemical agents widely used for such procedures by some investigators, particularly in tuberculosis research (Ängeby *et al*, 2004).

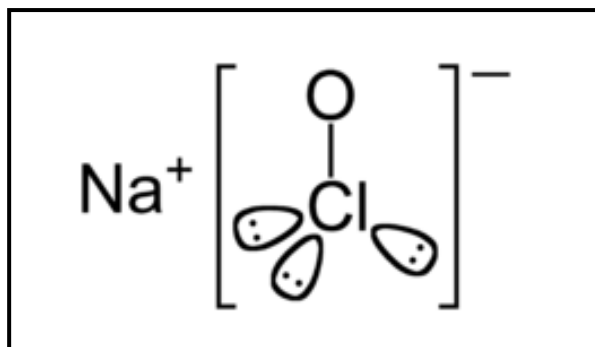


Figure 2.7 Chemical structure of sodium hypochlorite

Sodium hypochlorite or household bleach is a chemical compound with the formula NaOCl (Figure 2.7). It is a disinfectant with cleaning, bleaching properties. It was first produced in France by Claude Louis Berthollet in 1789. Its cleaning / bleaching activities is based on its oxidizing property, even though it can be corrosive to metallic surfaces. This inherent property has been exploited, in the area of Mycobacteriology as digestants/disinfectant for processing of diagnostic specimens (Van Deun *et al*, 2000; Cattamanchi *et al*, 2010).

Phenol ammonium sulphate (PhAS) is a chemical compound with similar properties. It is being used for the processing of diagnostic specimens for the detection of AFB, especially in the area of MTB microscopy. Phenol ammonium sulphate is a solution made from phenol and ammonium sulphate compounds (Karl-Heinz, 2012).

Phenolics generally have antimicrobial properties, and are useful for formulating disinfectants, especially in Mycobacteriology laboratories. Phenols can be synthesized industrially, or obtained from natural sources.

Ammonium sulphate with the molecular formula $(\text{NH}_4)_2\text{SO}_4$ is a fine grained crystal inorganic salt soluble in water. It is made up of 21% nitrogen as ammonium cations and 24% sulphur as sulphate anions (Figure 2.8).

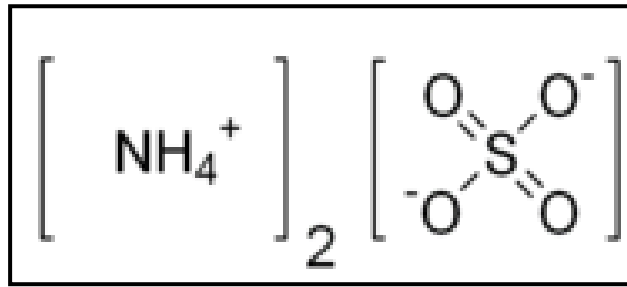


Figure 2.8 Chemical Structure of Ammonium Sulphate

Ammonium sulphate can be prepared by various methods, including the treatment of ammonium with sulphuric acid. It can also be obtained naturally from volcanic fumaroles and from coal fires on dumps. It is a salt of a strong acid and a weak base. Ammonium sulphate salt is acidic with a pH of 5.5. It has a property that makes it useful for purifying proteins the area of biochemistry ([http://www.encorbio.com/protocols/AM-SO₄.htm](http://www.encorbio.com/protocols/AM-SO4.htm)). It is highly soluble in water, and can make concentrated solutions that can precipitate protein, in a 'salting out' procedure. This property, in addition to its acidic property, makes it very useful for processing diagnostic specimens from *Mycobacterium* infections.

Other studies have also attempted to improve the detection rate by concentrating the diagnostic samples. This is done by reducing the volume of sample and increasing observable amount of available AFB. This procedure was found to be effective by some researchers (Bruchfeld *et al*, 2000, Gebre-Selassie, 2003). These are all procedures that facilitate the release of the AFB from the diagnostic sample. Results have shown improved reading of AFB by microscopy using these procedures. These methods could easily be applied in rural communities, where *Mycobacterium* infections such as tuberculosis, leprosy and Buruli ulcer are endemic.

Table 2.1 Outcome of some works aimed at improving sensitivity of AFB detection

References	Methodology adapted	Outcome
Selvakumar <i>et al</i> , 2002	Used phenol ammonium sulphate in smear preparation	Increased sensitivity (>85%), when compared to conventional method It is more acceptable to laboratory workers

Frimpong <i>et al</i>, 2005	Used sodium hypochlorite	Increased sensitivity (>85%) compared to conventional AFB detection method (>77.2%) for MTB
Chew <i>et al</i>, 2011	Bleach (15%, 6% & 3%) sedimentation for smear preparation	Outcome for bleach sedimentation depends on the characteristic of the sample Gives a relatively low inter observer rate
Gebre-Selassie, 2003	Compared and evaluated sample concentration procedures; direct and after mechanical sedimentation and centrifugation methods followed by treatment with NaCl	Sedimentation and centrifugation methods followed by treatment with NaCl increased sensitivity to 77.9%.
Yeboah-Manu <i>et al</i>, 2011	Evaluated decontamination methods	Improved sensitivity of AFB detection by microscopy with concentration method

Possible limitations associated with this diagnostic method include the presence of other AFB that may be contaminants in water being used for the procedure e.g. *M. gordonae* a non-pathogenic contaminant in water can affect results. Cross contamination can also affect specificity with the generation of false positives (Miörner *et al*, 1996; Steingart *et al*, 2006).

Specimen types, age, conditions of culture and staining procedures can influence the outcomes for the sensitivity of AFB detection (Table 2.1). The overall sensitivity of microscopy for the detection of AFB could be as low as 20%, therefore in order to increase the sensitivity some researchers have adopted and investigated various modified methodologies with some yielding promising outcomes (Table 2.1). It is due to these limitations associated with microscopy for the detection of AFB that a positive presumptuous result from microscopy had to be confirmed with culture.

2.1.7.2 Laboratory diagnosis of Buruli ulcer disease by culture

The culture of *Mycobacterium ulcerans* from BU specimens on *Mycobacterium* media including Lowenstein-Jensen (L-J) is crucial to the laboratory diagnosis of Buruli ulcer disease (Figure 2.9). Culture remains an important diagnostic tool because it is able to detect very small amounts of AFB and provide information on viability of the organism (Kent and Kubica 1985; Lennette *et al*, 1985).

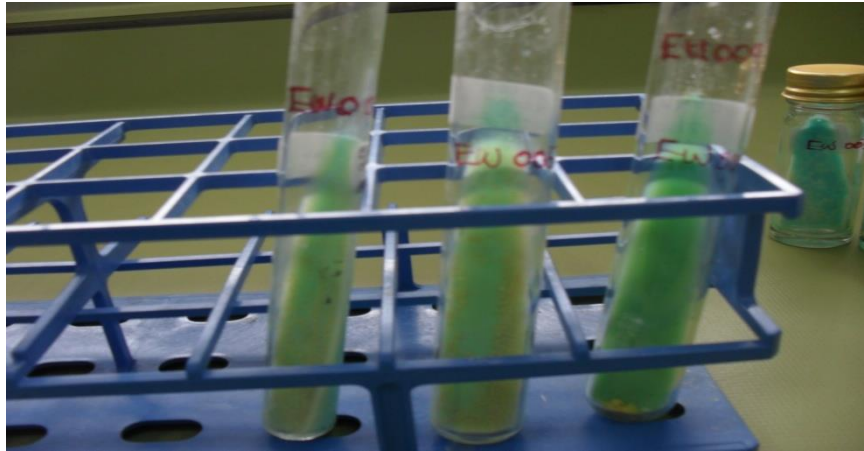


Figure 2.9 *M. ulcerans* colonies on Lowenstein-Jensen media

Conventionally, homogenate suspensions and aspirates from diagnostic specimens are processed with standard 5% oxalic acid or 4% sodium hydroxide (Petroff) decontamination methods. They are then inoculated onto slopes of *Mycobacterium* media (e.g. Lowenstein-Jensen media) and incubated at 29°C-32°C. It is expected to show colonies of *M. ulcerans* after eight week incubation if AFB is present in diagnostic specimens. *M. ulcerans* isolates obtained by this procedure do not only confirm the presence of *M. ulcerans* but can also be the source of isolates for further investigations of public health benefit (Portaels *et al*, 2001, Yeboah-Manu *et al*, 2004). In spite of its relatively high sensitivity as compared to microscopy, it is a rather slow procedure with other associated limitations that influence outcomes, especially from specimens such as swabs.

Essentially, the presence of organic debris such as mucin, tissue, serum, other proteinaceous material and other faster growing organisms accompanying the mycobacterium in diagnostic specimens can affect culture (McClellan *et al*, 2011). The faster growing contaminants outgrow the *Mycobacterium* in culture. Processing diagnostic specimens with chemical agents usually minimizes this difficulty. These chemical agents have antimicrobial / cleaning properties that will not only digest the debris but will inhibit or eliminate the contaminants in a process referred to as decontamination (Baron *et al*, 1994).

Decontamination methods take advantage of the relative resistance of *Mycobacterium* to chemical agents with selective antimicrobial activities and these include acids, bases, antiseptics and disinfectants. Over the years these chemical agents have been used for the culture of *Mycobacterium* (Palomino and Portaels, 1998). All these decontamination protocols have varying degrees of inherent limitations. This is as a result of the varying selective susceptibilities of the target organisms (contaminants) or the *Mycobacterium* species to be isolated in culture (Baron, 1994; Thornton *et al*, 1998). Despite these limitations, Mycobacteriologists are still

required to maximize the survival and detection of *Mycobacterium* from diagnostic specimens on the one hand whilst increasing the elimination rate of the contaminating microbes. Ideally, an effective decontamination agent is one with the potential to eliminate contaminants with minimal detrimental effect on the *Mycobacterium* (Baron *et al*, 1994).

The decontamination step is therefore crucial to *Mycobacterium* culture. Outcomes from effective and comparative decontamination methods have been the basis for which other tests have been conducted (Yajko *et al*, 1993). Historically, Petroff's decontamination method utilizing sodium hydroxide and hydrochloric acid was one of the earliest decontamination methods applied for the isolation of MTB (Kent *et al*, 1985). Subsequently, tri-sodium phosphate was also used in 1946 (Corper and Stoner, 1946), followed by pancreatic desogen in 1955. Other studies sought to decontaminate diagnostic specimens by combining various chemical agents, e.g. N-acetyl-L-cysteine (NALC)–sodium hydroxide (NaOH) which was used in 1963. The N-acetyl-L-Cysteine-NaOH method is the most frequently used for MTB due to its rapidity. Sodium hydroxide is also useful with samples that are mucolytic especially sputum samples. Though several chemical agents are capable of liquefying specimens for the release of AFB they cannot on their own eliminate the faster growing microorganisms. These digestants are sometimes combined with decontaminating agents for effectiveness. A typical example is the use of NaOH in combination with N-acetyl-L-cysteine (NALC). Specimen decontamination is facilitated by the treatment of the specimens with liquefiers and digestants or mucolytic agents (sputum) such as tri-sodium phosphate, and NALC (Kent and Kubica, 1985). Most of these decontamination digestion methods have been applied in the processing of sputum for the isolation of MTB (Riemenschneider and Tanifuji, 2002). Chemical decontaminating agents generally have antimicrobial activities and have properties similar to sodium hypochlorite, phenol, benzalkonium chloride (Timsen), cetrimide, povidone iodine, cetylpyridinium chloride, boric acid, methyl salicylate and virkon among others (Smithwick *et al*, 1975; Reimer and

Fleischer, 1997). These properties exhibited by some of these chemical agents would be utilized in this study (Fletcher *et al*, 1982). Benzalkonium chloride (BC) (*brand name*: ‘Timsen’) is a cationic quaternary ammonium surfactant, phase transfer compound with antimicrobial properties. It is a useful, fast acting antiseptic that works by destroying microbial cellular membrane, resulting in leakage of metabolic enzymes. It is an effective non alcoholic sanitizer and widely used in health institutions. It is deactivated in the presence of other anionic surfactants. The use of Benzalkonium chloride (BC) in addition to Zephiran, -trisodium phosphate have also been described (Wells, 1984).

Cetylpyridinium chloride (CPC) chemical is a cationic quaternary ammonium compound with a chemical formula of $C_{21}H_{38}NCl$ (Figure 2.10). It has antimicrobial properties that have been extensively and effectively used in commercial dental health products. It is soluble in inorganic solvents and is effective even at relatively low concentrations.

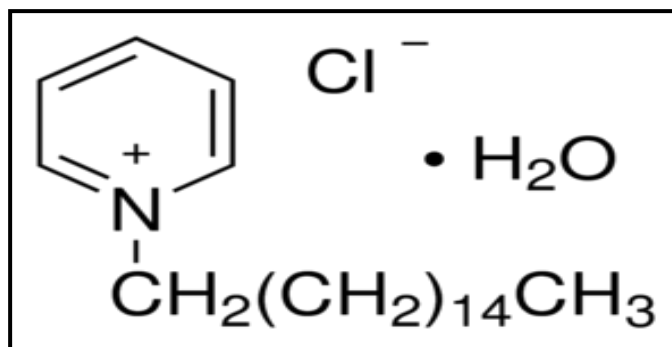


Figure 2.10 chemical structure of cetyl pyridinium chloride

CPC has been used in addition to sodium chloride as a decontaminating agent for the isolation of MTB from sputum specimens. A study conducted by Smithwick in 1975 demonstrated that a solution of 1% CPC / 2% NaCl was very useful for sputum samples in transit and also as decontaminating agent (Smithwick *et al*, 2002; Lumb *et al*, 2006). Another study similar to this conducted by Pardini also compared the recovery of MTB by the CPC / NaCl and NALC-NaOH methods and concluded that recovery from CPC / NaCl was more effective (Pardini *et al*, 2005). Selvakumar and his team however observed that sputum samples transported in CPC / NaCl had relatively lowered AFB detection rates by microscopy (Selvakumar *et al*, 2002). A solution of cetyl pyridinium chloride and sodium chloride has antifungal properties

Oxalic acid is a generally strong acid with reducing properties that confers on it antimicrobial properties (Wells, 1984; Riemenschneider and Tanifuji, 2002,). In 1949 a study by Corper and Nelsen recommended the use of oxalic acid for the processing of heavily contaminated specimens from *Mycobacterium tuberculosis* (MTB) (Corper and Nelsen, 1949). In 2004, 5% oxalic acid was effectively used as a decontaminant for the isolation of *M. ulcerans* from tissue specimens from Buruli ulcer lesions (Yeboah-Manu *et al*, 2004). In a previous study however, Palomino working with Portaels reported in their study that oxalic acid markedly reduced the growth rate of the *M. ulcerans* in the BACTEC system (Palomino and Portaels, 1998), whereas Yajko demonstrated the effectiveness of oxalic acid for the recovery of *M. avium* from stool (Yajko *et al*, 1993).

Povidone iodine is a chemical complex of polyvinylpyrrolidone (PVP) and elemental iodine (9-12% dry weight). HA Shelanski and MV Shelanski first found out about this compound and subsequently demonstrated its antimicrobial activity and its superiority to other iodine compounds in terms of toxicity and antiseptics it was first formulated in 1955 (Reimer and Fleischer, 1997). It is a very good antiseptic and is used effectively on the skin particularly in

health facilities. Currently no study has as yet reported of its use as a decontaminating agent for the isolation of *Mycobacterium* species though it has a potential for this purpose.

Virkon disinfectant is a known chemical compound made of oxone (potassium peroxymonosulphate) sodium dodecyl benzenesulfonate, sulphamic acid and inorganic buffers readily soluble in water. It is a multi-purpose biocide with a wide spectrum of activity (active against bacteria, viruses and fungi). It is useful for cleaning up hazardous spills, disinfecting surfaces and decontaminating equipment. It is also used in health facilities (hospitals and veterinaries) and places where microbial control is a requirement. It is effective at concentrations of 1-3% (Gasparini *et al*, 1995). A solution of virkon disinfectant has a pink shade which is affected by concentration and age (It is stable for a maximum of 7 days).It is effective as an antiseptic at low concentrations between 0.5-15% (Hernandez *et al*, 2000).Virkon was selected for this study because of its usefulness as a disinfectant.

2.1.7.3 Detection of *M. ulcerans* DNA by PCR Technique

The detection of *M. ulcerans* deoxyribonucleic acid (DNA) from BU samples is based on a procedure utilizing insertion sequence (IS) Polymerase Chain Reaction (PCR) technique. This is a technique that is dependent on the unique DNA constitution of *M. ulcerans*. It is useful for its speed and sensitivity (Ross *et al*, 1997; Portaels *et al*,1997). Relatively small quantity of sample is required for this method. It however cannot be easily applied routinely in rural health facilities where BU is endemic because it requires expensive input to prevent false results and is beyond the means of most endemic communities in developing countries. It is however useful and can be utilized in reference facilities of endemic countries, especially for the rapid differential diagnosis of cases that prove to be difficult on clinical grounds alone. Several PCR-based assays for detecting different genomic targets have been developed (Fyfe *et al*, 2007; Kaser *et al*,

2009). Some of the *M. ulcerans* specific DNA targets are the 16s rRNA gene, the 65-kDa heat shock protein gene and the repetitive DNA sequence *IS2404* (Phillips *et al*, 2005).

Presently, the recommended target for the diagnosis of BU is *M. ulcerans* specific insertion sequence *IS2404*, which is present in at least 50 copies in the mycobacterium genome, therefore improving the sensitivity of the assay. It has also been reported to be more specific than other targets (Ross *et al*, 1997). In Ghana, a recent report has described a new dry-reagent PCR for Buruli ulcer that could be used in small regional centres. The report indicates a 95% detection rate (Siegmund *et al*, 2005).

2.1.7.4 Histopathological Analysis for BU diagnosis

Histopathological examination of processed biopsy from excised *M. ulcerans* infected tissue can reveal histopathologic changes that are characteristic of Buruli ulcer disease. The observed characteristics are however nonspecific and the changes are usually evident with the development from the nodular to the ulcerative stage. Different histopathological changes have been described by several authors at the various clinically progressive stages of the disease (Hayman *et al*, 1985 ; Hayman *et al*,1993). Histopathological examination of early lesions reveals extensive necrosis of cutaneous tissue, numerous extracellular acid fast bacilli distributed in clumps and very few inflammatory cells. This is probably as a result of the immunosuppressive effect of the toxin. Examination of infected tissue also shows centrally necrotized subcutaneous fat surrounded by granulation tissue with giant cells, which lacks the typical caseation or tubercles seen in tuberculosis (Guarner *et al*, 2003). Typically reliable histopathological features are observed necrosis of subcutaneous tissues and dermal collagen with minimal inflammation and acid-fast bacilli (AFB) are considered for the diagnosis of Buruli ulcer disease. This means that the specimen quality and the collection method are essential for *M. ulcerans* detection at various stages of the infection. Tissue from the necrotic

subcutaneous and undermined edges of an ulcerative lesion is good for diagnosis from ulcers and specimens from skin and subcutaneous tissue are good for pre-ulcerative cases (Asiedu *et al*, 2000).

2.1.8 Hosts' Immune responses to Buruli ulcer disease

The immune mechanisms involved in the Buruli ulcer disease is not well understood (Gooding, 2001). Reports from studies conducted however indicate that peripheral blood mononuclear cells obtained from people with a history of the disease and those with current *M. ulcerans* infection show a strong T-helper (Th)-2 cytokine response when exposed to *M. ulcerans in vitro* (Gooding *et al*, 2002, Adusumilli *et al*, 2005). Their household contacts (healthy controls) on the other hand exhibit a T-helper-(Th)-1 response. This implies that natural resistance could be determined by cell-mediated immune mechanisms directed against intracellular organisms. Another study showed that the development of ulcers involves an immune shift from T-helper-1 to the T helper-2 phenotype. Prevot and his team demonstrated that the cytokine interleukin-10 must be the key mediator in the shifts with the T-helper phenotypes in nodules and ulcers (Prevot *et al*, 2004). Humoral immunity involving antibodies could be involved with protection against *M. ulcerans* infection, since the organism is usually located extracellular during active disease (Krieg *et al*, 1979). The immune mechanisms can further be understood if investigations are conducted on the immune cell groups including B lymphocytes, T helper cells, and cytolytic T lymphocytes, cytokines, and monokines using murine models (Pahlevan *et al*, 1999). BUD, caused by *Mycobacterium ulcerans*, follows an indolent course of initial progression to ulceration followed by extensive tissue damage. It has been suggested that healing disease stages come with a protective immune response. The resolution of the disease has been hypothesized to be associated with a down regulation of T-helper 1, induced by the cytokines interleukin-4 (IL-4) - or IL-10. It has also been conjectured to play an important role in disease progression and

resolution. The role of gamma interferon (IFN- γ), IL-4, and IL-10 responses have also been documented (Gooding *et al*, 2002).

2.1.9 Management of Buruli ulcer disease

Buruli ulcer disease accounts for a substantial proportion of morbidity among individuals within the endemic communities. This is despite extensive public health efforts at disease control. The current treatment option available involves the administration of streptomycin and rifampicin for an eight week period. This has been found to be very effective particularly at the pre-ulcerative stages. This was subsequent to a recommendation by WHO in 2004, based on a successful clinical trial by Etuaful and his team in Agroyesum (Etuaful *et al*, 2003; WHO, 2004). Previous studies had shown that though *M. ulcerans* was susceptible to some antibiotics *in-vitro*, its use for the treatment of the disease was generally disappointing (Thangaranj *et al*, 2000 ; Dega *et al*, 2002)

Until recently however, surgical excision aimed at removing and repairing infectious/damaged tissue by skin grafting was the definitive treatment for the disease (Cornet *et al*, 1992). Efforts at treatment have been particularly frustrating as most patients report late with advanced stages of the disease. These resulted in post-surgical loss of large portions of infected tissue leading to contractures and deformities and long hospitalization periods, the cost of which may be beyond the means of the affected people especially in developing countries. Even in developed countries like Australia, the economical effect can be substantial. This situation has a socio-economic impact on the affected people (Asiedu *et al*, 1998). Estimated 18%–47% post-surgical cases recurrences have been reported. Other treatment options that have been found to be helpful include heat treatment (Krieg *et al*, 1979). This treatment had been previously used by some

workers and in recent times by Junghanns and his team of researchers in the Cameroon (Junghanns *et al*, 2009). Topical applications including phenytoin, acidified nitrite creams, and hyperbaric oxygen have also been used effectively (Grange, 1988), with varying degrees of success (BCG, 1969; Meyers *et al*, 1974; Adjei and Asiedu, 1998).

2.1.9.1 BU disease prevention strategies

Even though there are currently no vaccines available for the specific prevention of the disease, Bacilli Calmette Guerin (BCG) vaccines are being administered to boost immune system to *Mycobacterium ulcerans* infection. This is despite the limited effectiveness (Huygen, 2003; Portaels *et al*, 2004). In spite of its limited effect, it is currently being administered routinely as part of the World Health Organization's Expanded Programme on Immunization for Tuberculosis. Though it is not being given routinely, double doses (booster doses) are being encouraged in infants in highly endemic communities, because the protective effect of the vaccine is generally transient. Currently, there are attempts to improve the present vaccine using attenuated or live *M. ulcerans* isolate or sub unit vaccines made up of protein components or by the mycolactone (Huygen *et al*, 2003). Other preventive measures include educating people within endemic communities on disease preventive measures (Thangaranj *et al*, 1999; Asiedu *et al*, 2000; Evans *et al*, 2003).

2.1.9.2 Drug Resistance in *Mycobacterium*

Despite efforts at disease control with antibiotics, some organisms manage to survive therapeutic levels of antimicrobial agents. These strains are categorized as resistant strains. These strains have inherent (genetic) or acquired properties (phenotypic) that enable them survive.

Drug resistance properties exhibited by *Mycobacterium* are usually attributed to the characteristically impervious lipid-waxy cell wall. The impervious nature of the cell wall prevents easy access of most antimicrobials. This property necessitates use of at least two drugs for the treatment of *Mycobacterium* infections. This is because currently, rifampicin and streptomycin are the two antibiotics being used for the treatment of *M. ulcerans* infection (Sensi *et al*, 1959; Satoskar 2001).

Streptomycin, an important aminoglycoside has been extensively used for the treatment of *Mycobacterium* infections especially tuberculosis and it is presently being used for the treatment of Buruli ulcer.

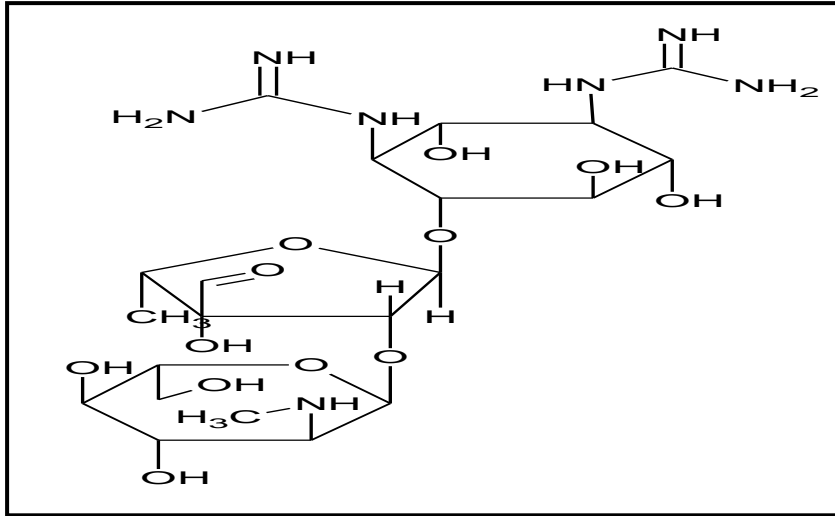


Figure 2.11 Chemical structure of streptomycin

It is a polycationic compound made up of an amino sugar in a glycosidic linkage (Figure 2.11) and its activity is enhanced in an alkaline environment. It is produced by *Streptomyces griseus*. It is administered intramuscularly. It is bactericidal at high concentrations and bacteriostatic at low levels and bacteria easily develop resistance to it exhibiting cross-resistance to other aminoglycosides. Streptomycin penetrates bacterial cell wall and targets the DNA. It further inhibits the protein synthesis machinery of the bacteria by binding to the 23S rRNA molecule of the bacterial ribosome, thus preventing the release of the growing protein. Streptomycin induced ototoxicity has been reported (Singh and Mitchison, 1954).

Rifampicin is one of the antibiotics recommended for the treatment of Buruli ulcer. It is a semi-synthetic derivative of rifamycin B (Figure 2.12), isolated from *Streptomyces spp.* (Satoskar *et al* 2001).

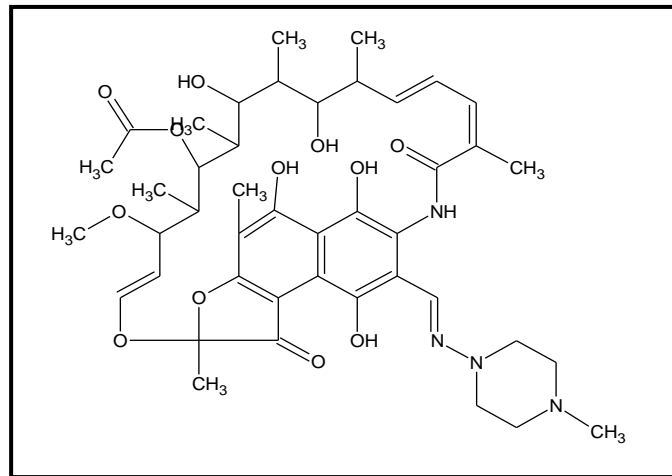


Figure 2.12 Chemical structure of Rifampicin

The 4-methyl-1-piperazinaminy derivative is the most effective. It stains body fluids with its intense red color. When taken with food the concentration absorbed by the body is reduced. Rifampicin is useful for the treatment of tuberculosis, leprosy and Buruli ulcer. It is also effective for the control of other challenging bacterial infections. Due to the high levels of resistance exhibited by bacteria to rifampicin, its use in combination with other drugs is highly recommended (Atlas, 1995; Satoskar *et al*, 2001). It works by targeting the bacteria RNA – dependent DNA synthesis. It is the only drug that acts against the ‘persisters’ (Havel and Pattyn, 1975). Extensive studies have been conducted on the use of rifampicin for the management of Buruli ulcer disease (Thangaranj *et al*, 2000; Etuaful *et al*, 2003; Chauty *et al*, 2005).

2.1.9.3 Antimicrobial Susceptibility Testing for *Mycobacterium ulcerans* isolates

Antimicrobial agents are used for the treatment of infectious conditions. Since resistance patterns of microbes are constantly changing, it is important to determine the drug susceptibility patterns of bacteria isolated from an infectious condition. The method widely used for this determination is referred to as the drug susceptibility test (DST). It is an *in-vitro* system usually conducted in the laboratory. Information on the potency levels of specific drugs against specific pathogens can be obtained (Baron *et al*, 1994; Kent and Kubica, 1985). Even though outcomes from *in-vitro* system may not necessarily translate *in-vivo*, it can inform decision on the appropriate selection of a drug for treatment.

The determination of drug susceptibility testing for *Mycobacterium* though based on the basic principle is a more specialised method. This is because most pathogenic *Mycobacterium* has a relatively slower growth rate. The resistant ratio, the absolute concentration and the proportion methods are the three major modifications of indirect agar dilution procedures used. The most

widely used however is the proportion method as described by Canetti's team (Canetti *et al*, 1963).

The proportion method: This method is fundamentally based on the premise that all wild strains of the tubercle bacilli would have some mutant strains that will exhibit some level of resistance to the antimicrobial treatment regimen. The apparent difference between a susceptible and a resistant strain is the total number of resistant bacteria population within a strain. The observed phenomenon can be calculated by conducting the drug susceptibility test. By seeding prepared standardized inocula onto dilutions of drug-incorporated media and drug –free media in appropriate *Mycobacterium* media such as Lowenstein-Jensen media ,the proportion of resistant strains can easily be deduced by comparing counts from the controls and the tests (Canetti *et al*, 1963). Susceptibility of a test organism is graded by the absence of colonies on therapeutic levels of drug incorporated medium or that the proportion of growth will be less than 1% of the proportion of growth on the drug-free medium. In a given test where the organisms exhibits susceptibility, it is expected that there would be no growth on the medium or that the proportion of growth exhibited would be less than 1% of the proportion exhibited on the drug free media (Grange , 1988, Collins *et al*, 1997).

In general, all the three drug susceptibility methods ; the resistant ratio method, the absolute concentration method and the proportion method basically produce similar results, however the proportion method is more simple and convenient method that can easily be applied and modified when necessary. Presently there are more improved methods using radiometry, its use is however limited because of its expense and cannot be easily applied in resource-constrained laboratories (Wilkinson and Sturn ,1997).

Other methods include:

The resistance ratio is determined by inoculating standardized suspension of the test strain and a number of known sensitive strains onto media containing doubling dilutions of the drug. After incubation, the end point for each strain (slope with 20 colonies or less) is determined. Test strains are then compared with the average or the modal resistance on the set of known sensitive strains. If the end point of tests and control is equal, the strain has a resistance ratio of 1. As doubling dilutions of the drugs are used 1, 2 or 3 tube differences in the end-points of test and control strain give resistance ratios of 2, 4 and 8 respectively. Strains with resistance ratios of 4 or more are reported as resistant.

The absolute concentration method is similar to the resistance ratio method, being based on the titration of the test strain along with adequate control strains on slopes of media containing known quantities of the drug in doubling dilutions. The difference is that the results are expressed in the actual endpoints concentration of the drug. In practice the activity of the drug may be less than its concentration in the medium owing to its denaturation during media preparation. Accordingly, this method has no real advantage over the resistance ratio method.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

3.1.1 *Study area*

This study was conducted in Asuboi in Suhum-Kraboia-Coaltar and Paakro in the Akuapem South, both districts of the Eastern Region of Ghana (Figure 3.1, right). Ghana is one of several West African countries lying along the Gulf of Guinea (Figure 3.1, left). It is bordered on the east by La Cote d'Ivoire, the west by Togo, the north by Burkina Faso and the south by the Atlantic Ocean. BU endemic communities are found in the countries lying along the Gulf of Guinea.

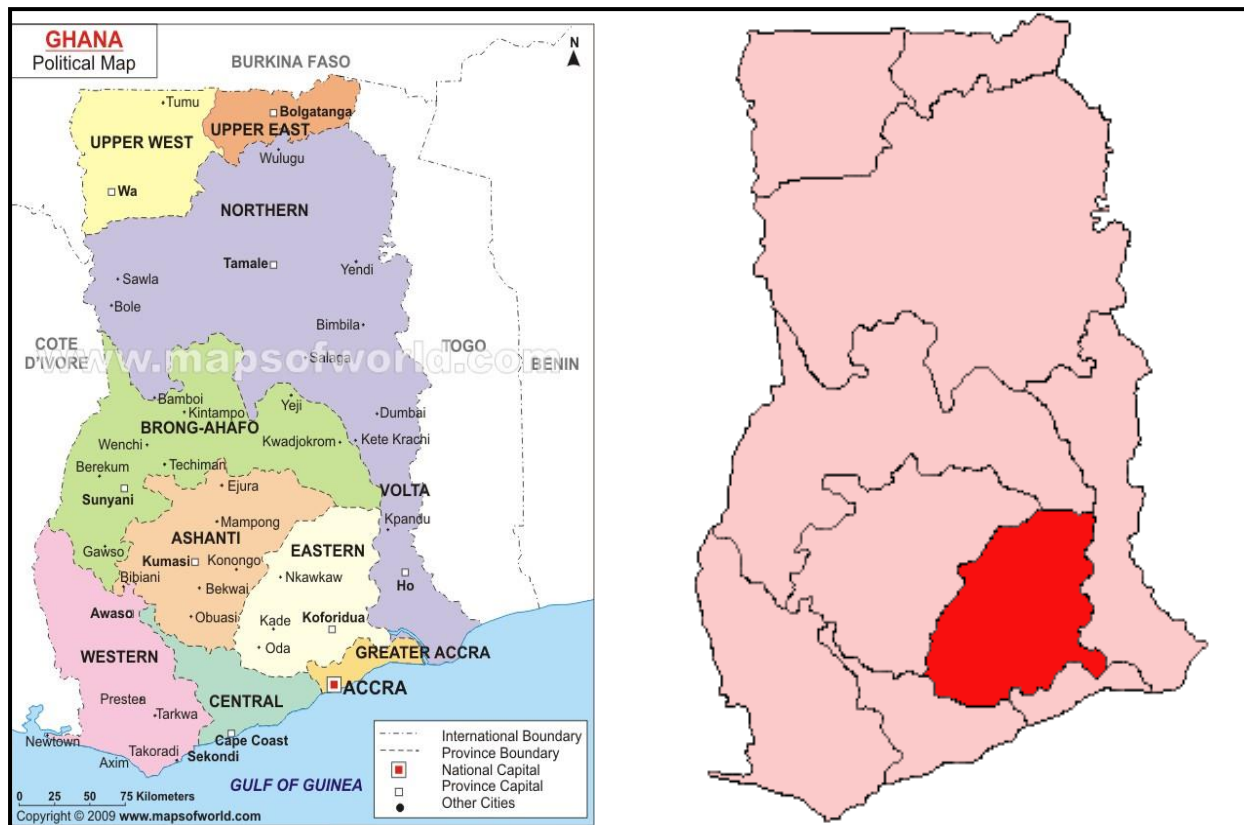


Figure 3.1 Map of Ghana showing administrative regions including Eastern Region where study sites are located. Left: Adapted from www.mapsofworld.com. Right: Adapted from <http://okwahumannyy.org>

Ghana is a tropical country located above the equator. It occupies an estimated 239,460 sq. km of land area. Data from the 2010 census indicates a population of approximately 24 million people. The rainfall is high within the periods of April to October, with variations in different areas over the entire country. The temperatures range between 21°C to 42°C with high humidity ranging between 50% and 80%. In most areas within the country, temperatures are highest in March and April and lowest within the periods of July and August. Very little differences have been noted between night and day temperatures.

The sites selected for this study were the health centres of Paakro in the Akuapem South district, and the Asuboi of the Suhum- Kraboa- Coaltar districts of the Eastern Region of Ghana. The region occupies an estimated 19,323 square kilometres, accounting for about 8.1% of Ghana's total land coverage. The region is divided into twenty one administrative districts, including municipalities. It is bordered on the north by the Ashanti and the Brong-Ahafo regions and on the east by the Volta region. The southern border areas include the Central and Greater Accra regions. It is the sixth largest region in the country. Parts of the Eastern Region have been identified by the Ghana Health Service, as areas endemic for Buruli ulcer disease. Endemic communities can be found in the environs of the two selected districts namely Suhum-Kraboa-Coaltar and the Akuapem-South Districts. (http://en.wikipedia.org/wiki/File:Eastern_Ghana_districtdistricts.png)

3.1.1.1 Akuapem South District

Akuapem South district is one of twenty one district and municipalities in the Eastern region of Ghana. It occupies about 403 square kilometres of land with total arable, cultivable land of approximately 20,000 hectares and an additional 600 hectares of land under cultivation along the

Densu Basin. It is surrounded by Ga West Municipal and Tema Metropolis to the south, Suhum-Kraboa-Coaltar district, Akwapim North and West Akyem Municipal to the north-west respectively. It has a bimodal pattern of rainfall of 1700mm per annum with mean annual rainfall of 1250 millimetres per annum. The soil type is sandy loam and clayey in valley bottoms.

Commercial and subsistence farming are the main occupation of the people, and about 60% of its population are engaged in farming for their livelihood. They mainly crop cassava, maize, palm and citrus in addition to vegetables and cocoa to a small extent. Some families keep livestock and poultry. The district has a lot of water bodies in the form of dams and dugout wells and rivers. The main rivers are the Densu, Ponpon, Dobro and Nsakyi. Crops cultivation is concentrated near the Densu River. About 80% of farmers in the municipality are crop farmers who practice subsistence farming with about 60%-70% as small scale holders and 20%-30% as large scale holders (http://en.wikipedia.org/wiki/File:Eastern_Ghana_districts.png).

3.1.1.2 Suhum-Kraboa-Coaltar district

Suhum-Kraboa Coaltar district is one of the 21 districts in the Eastern Region. The district is characteristically rural, with Suhum the district capital as the only town with urban characteristics. Suhum-Kraboa-Coaltar district is bounded by East Akim Municipal district to the North, Akuapem south Municipal district to the South, New Juaben and Akuapem North to the east and west Akim and Kwaebibrim to the west (Figure 3.2).

The district occupies an area of 1,018 km². About 70% of the economically active population engage in Agriculture for their livelihood. The population in the district depend on pipe-borne water, hand-dug wells with pumps and boreholes for their source of water supply.

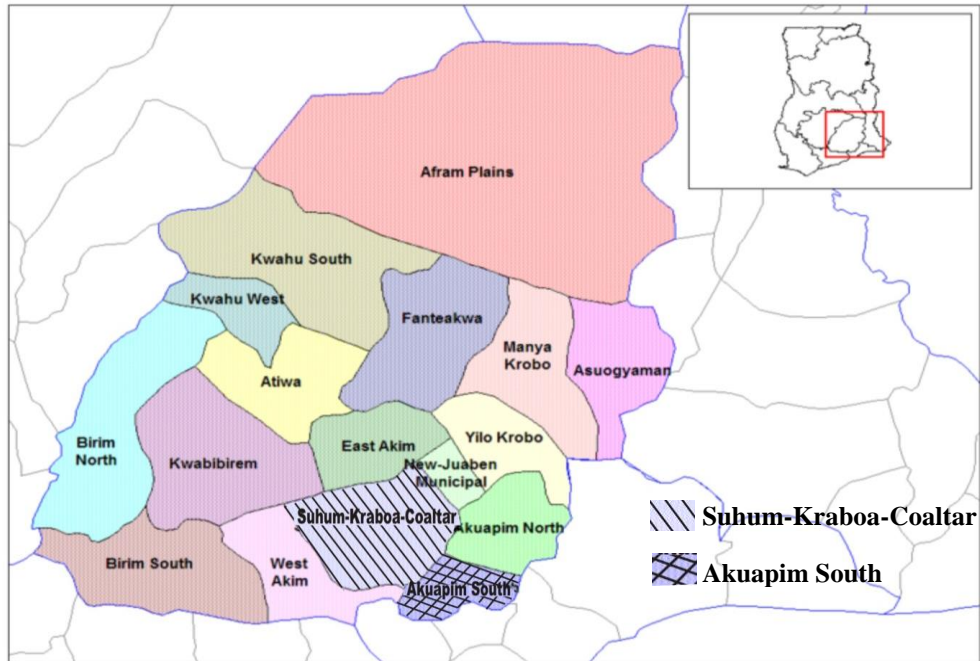


Figure 3.2 Map showing geographical location of Suhum-Krabo-Coaltar and Akuapim –South districts of the Eastern region of Ghana.

Some communities depend on streams and springs. Only 50% of the households have access to potable water from mechanized boreholes. The largest water body in the district is the Densu River flowing from the northern portion of the district to the south (http://en.wikipedia.org/wiki/File:Eastern_Ghana_districts.png)

3.1.2 *Study design*

This was an experimentally designed study, enrolling a cross section of one hundred and thirty five suspected Buruli ulcer patients between the periods of June 2010 to April 2012. The target study population were individuals who were clinically diagnosed with BU residing within the environs of Paakro in the Akuapem South district and Asuboi in the Suhum- Kraboa–Coaltar districts of the Eastern region of Ghana.

3.1.3 *Sample size estimation*

Sample size for this study had been earlier determined based on achieving a 95% p - value of detecting major causes or sources of Buruli ulcer with significant level set at $d=0.05$ using Eq.

(3.1)

$$n = \frac{z^2 pq}{d^2} = \left(\frac{z}{d}\right)^2 pq$$

Where n = sample size

z = confidence interval

d = significant level

p = proportion of population with BU

q = $(1-p)$ proportion without BU

No reliable record of p was known but based on an earlier study in Amasaman (DHMT data); p was not expected to exceed 23%. Substituting this into the Equation (3.1) a minimum sample size of 150 was determined:

$$n = \frac{(1.96)^2 (0.23)(0.77)}{0.05^2} = 150$$

3.1.4 Eligibility criteria

Individuals manifesting with skin conditions at the designated health facilities with clinical suspicion of Buruli ulcer disease (BUD) were eligible to enter into the study.

3.1.4.1 Inclusion Criteria

- Individuals with suspected Buruli ulcer lesions and were clinically diagnosed by an experienced clinician.
- Individuals who consented to be enrolled on the study after obtaining detailed information on their involvement and role in the study.
- Suspected BU cases who had agreed to provide information as required by the questionnaires administered.

3.1.4.2 Exclusion Criteria

- Suspected BU cases with lesions suggestive of BU disease but not clinically confirmed as such.
- Clinically diagnosed BU patients who were not in agreement with the terms and conditions associated with enrolment.

A structured questionnaire was administered, to obtain baseline demographic information, from persons enrolled in the study. Information sought from persons within this questionnaire included age, sex, residential address etc. Data was also obtained on disease presentations including lesion type, lesion location, lesion size and others provided in the questionnaire (See Appendix A for details)

A suspected Buruli ulcer patient was clinically confirmed by an experienced clinician based on defined WHO guidelines on the clinical diagnosis of Buruli ulcer. A case was defined as persons manifesting with any of the following clinical signs of Buruli ulcer disease as described:

- (i) A nodule as a firm, painless subcutaneous swelling less than 2cm in size and attached to the skin
- (ii) A plaque as a firm, raised lesion irregular at the edges, with a size usually greater than 10cm
- (iii) An edema as a characteristically painless, non-pitting swelling of an anatomical site and
- (iv) An ulcer as an open wound with a typical cotton-wool like basal slough with undermined edges. (W. H. O., 2001).

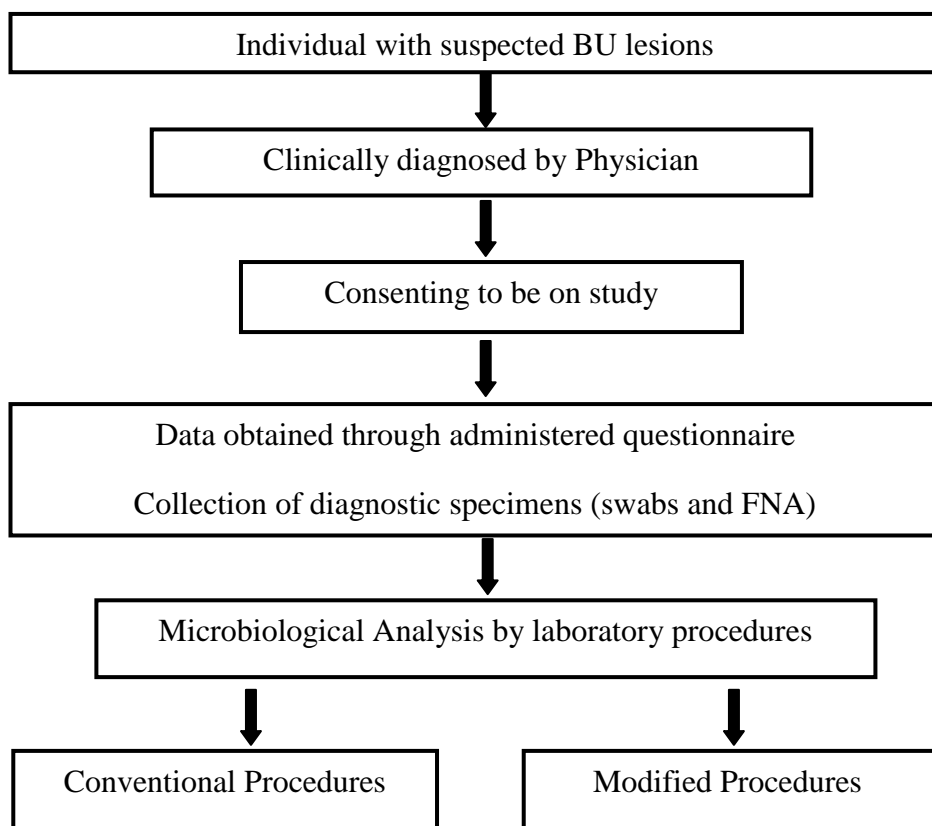


Figure 3.3 Schematic presentation of Summary of Study Protocol

3.2 Methods

3.2.1 *BU specimen collection and storage*

Specimens were collected from clinically diagnosed Buruli ulcer patients between June 2010 and April 2012 from the Paakro and Asuboi Health Centres, the designated health facilities.

Study participants with active Buruli ulcer lesions were categorized into two groups

(i) Those with pre-ulcerative lesions

(ii) Those with ulcerative lesions.

Swab specimens were collected from those with ulcers, whilst aspirates were collected from those with pre-ulcerative lesions.

Swab specimens were collected by circling the entire undermined edges of the lesion, and also crevices within the lesion. The tips of the swab sticks were aseptically transferred into a 15-ml conical tube (BD Falcon) containing 5ml of semi-solid transport medium incorporated with 2% PANTA (polymixin B, amphotericin B, nalidixic acid, trimethoprim and ampicillin) with the tip completely immersed in the medium (Eddyani *et al*,2008; Yeboah-Manu *et al*,2004) . Aspirates from pre-ulcerative lesions were collected using the fine needle aspiration technique as described (Phillips *et al*, 2009). They were aseptically transferred by flushing into a 1.5 ml sterile screw-capped tube, containing 100 µl of liquid transport medium (sterile phosphate buffered saline -PBS) (Portaels *et al*, 2001).

The samples were kept in an insulated container with ice packs, and transported to the laboratory. The analysis of the samples took place at the Bacteriology Department of the Noguchi Memorial Institute for Medical Research (N.M.I.M.R.), Legon, and the Tuberculosis

Laboratory of the Public Health Reference Laboratory (P.H.R.L.) and the chest clinic of the Korle-Bu Teaching Hospital (K.B.T.H.), Korle-Bu.

Most of the samples were processed for analysis within forty eight hours. Swab specimens were processed as suspensions, after being transferred into sterile screw-capped test tubes with PBS, and vortexed with 10-15 glass beads (3mm diameter; Merck, Germany). Each swab was eluted into two millilitres (ml) of PBS. This was to enhance the release of the lesion exudates from the tip of the swab into suspension. The sample suspensions obtained were appropriately aliquoted for the various microbiological procedures.

3.2.2 Laboratory Procedures for the Diagnosis of Buruli ulcer by Conventional Methods

Mycobacterium ulcerans infection was confirmed by the following microbiological methods: -

- (i) Zeihl-Neelsen (ZN) staining of smears prepared directly from BU specimens (swabs and fine needle aspirates) for the detection of acid fast bacilli (AFB) (Portaels *et al*, 2001).
- (ii) Culture for the isolation of *M. ulcerans*
- (iii) Insertion sequence (IS) 2404 polymerase chain reaction PCR for the detection of *M. ulcerans* DNA (deoxyribonucleic acid (Phillips *et al*, 2005)

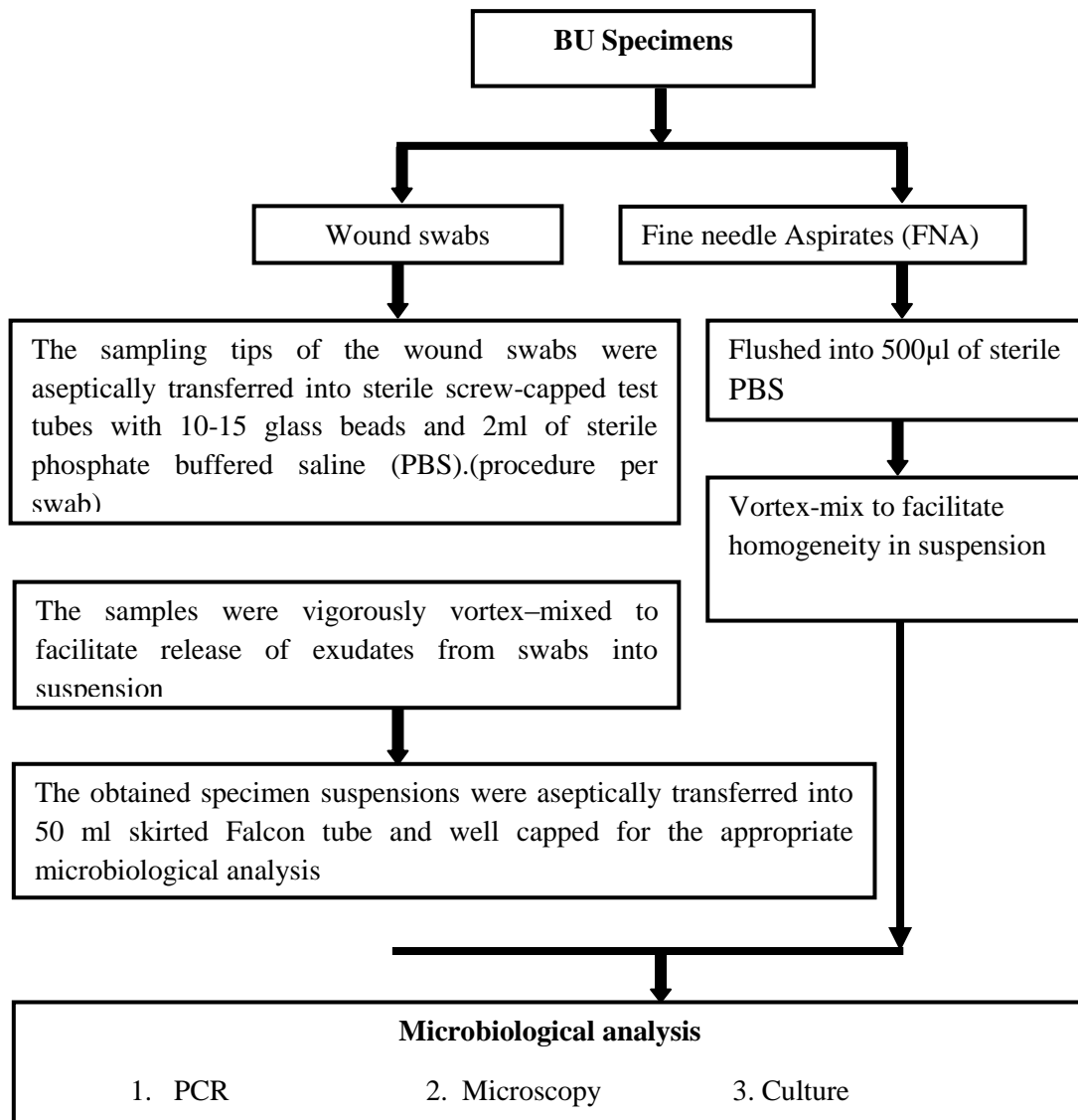


Figure 3.4 Study flow chart: Laboratory standards for reporting on confirmation of BU cases

3.2.2.1 Detection of Acid Fast Bacilli (AFB) from clinical BU specimens by conventional methods

Smears prepared from 20µl of BU specimen suspensions were air-dried, heat fixed and stained by the Ziehl-Neelsen (ZN) staining procedure for the detection of acid fast bacilli (AFB) as described (Grandjean L, 2008, American Thoracic Society , 1981). The smears were scanned at 1000X magnification under oil immersion with a light microscope as described (details in Figure 2.2 and Appendix D.1). Typical acid fast bacilli were revealed as red slender rods in singles or clumps against a blue background.

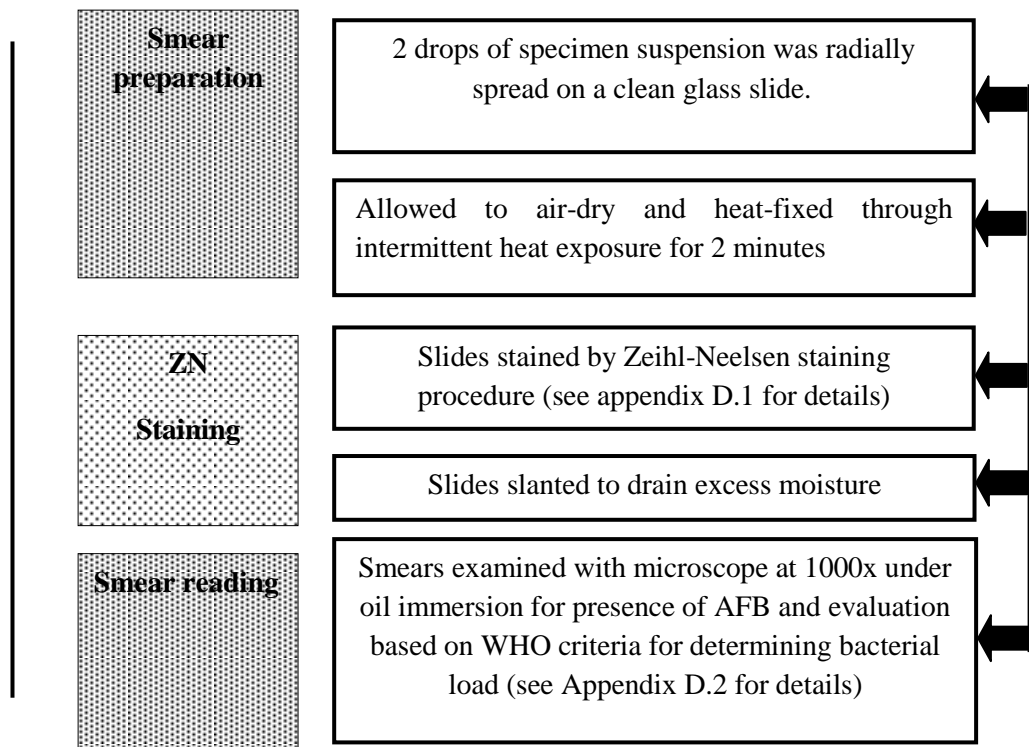


Figure 3.5 Detection of AFB from BU specimens by conventional procedures

Evaluation criteria for AFB detection: The smears were graded based on evaluation standards of the American Thoracic Society and the results were quantified in accordance with published standards (American Thoracic Society,1981 ; Kent and Kubica,1985 ; Tuberculosis Division and Disease,2005)

Table 3.1 Modified protocols for microscopy-evaluation criteria

AFB POSITIVITY RATING (P)			
	Observations (Readings)	Estimation (grading)	Score
P0	No AFBs per 100 fields	Negative	0
P1	1-9 AFBs per 100 oil immersion fields	Scanty	1
P2	10-99 AFBs per in 100 oil immersion fields	+1	2
P3	1-10 AFBs per oil immersion field	+2	3
P4	More than 10 AFBs per oil immersion field in at least 20 fields	+3	4
CLARITY AND CONTRAST (C)			SCORE
C0	Dark and AFBs indistinguishable		0
C1	Slight illumination of slide and relatively improved contrast		1
C2	brighter field and AFBs distinguishable		2
C3	Bright field, good contrast and AFBs clearly visible		3
C4	Very bright clear background, AFBs' visibility enhanced		4
R (RELEASE OF AFB EMBEDDED IN MATRIX)			SCORE
R0	AFB totally embedded in matrix material		0
R1	-AFB partially detached from matrix material		1
R2	AFB detached from matrix		2
R3	AFB distinguishably detached from matrix		3
R4	AFB totally detached and aids visible detection		4

Key

Smear from BU samples were evaluated based on these criteria (Table 3.1):

- i. Positivity (P)
- ii. Clarity & contrast (C)
- iii. Release of AFB from host material (R). Scores / criteria ranges from 0 - 4.
 - Maximum score / slide = $12 - P(4) + C(4) + R(4)$
 - Minimum score / slide = $0 - P(0) + C(0) + R(0)$
 - Sample smears prepared in duplicates
 - Score per sample smears was obtained based on cumulative values from assessment.

3.2.2.2 Culture of *Mycobacterium ulcerans* from clinical BU Specimens by Conventional methods

Primary isolation of *M. ulcerans* from clinical specimens was done using the following procedure. After decontamination of the specimen suspension by the standard 5% oxalic acid method as described (Yeboah-Manu *et al*, 2004). See Appendix D.4.1 for details, each specimen was inoculated onto two media

- i) Lowenstein-Jensen (L-J) media and
- ii) Lowenstein-Jensen media incorporated with antibiotics (PANTA).

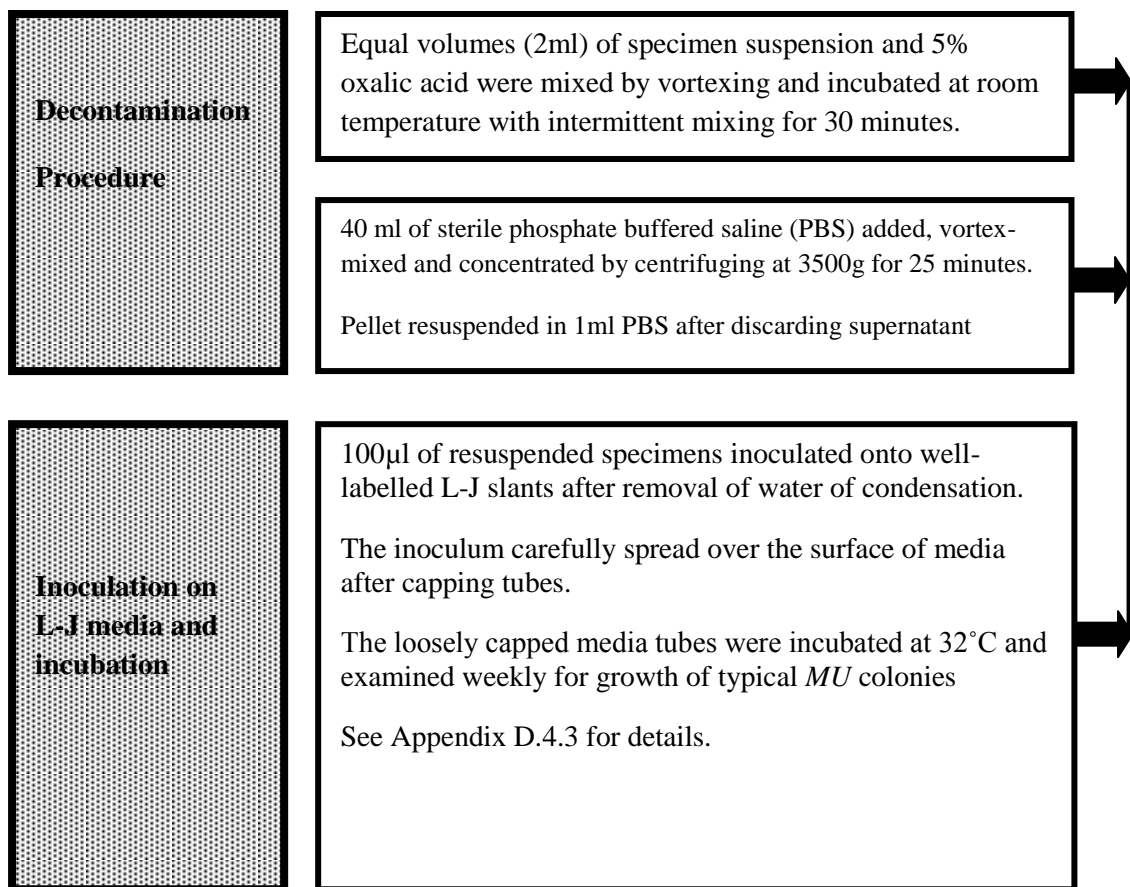


Figure 3.6 Flow chart showing procedures on *M. ulcerans* culture from BU samples

All L-J slants were incubated at 32°C and examined weekly for growth (Portaels *et al*, 1997) for a maximum period of 12 weeks (cultures that did not achieve growth after 12 weeks were left to incubate for six months).

Evaluation criteria for the culture of M. ulcerans

M. ulcerans cultures that showed growth were authenticated by standard phenotypic identification tests. They included the growth rate, pigmentation, photo-reactivity and morphology. ZN staining of smears was done for colonies appearing on the L-J media. Colonies that were matt, yellowish to buff, waxy with rough margins and grew after 8 weeks were presumptively identified as typical *M. ulcerans* colonies (W.H. O., 2001).

Cultures on Lowenstein- Jensen (LJ) media that did not show growth after 12 weeks were considered negative. A tube was considered contaminated when over 50% of the surface of the solid media was overgrown with other faster growing bacteria and also when the medium was liquefied. The number of weeks taken to obtain positive culture as well as the number of colonies observed on the L-J media was recorded.

3.2.2.3 Detection of M. ulcerans DNA from BU specimens by conventional IS2404 specific PCR

Extraction of M. ulcerans DNA from clinical BU specimen

Mycobacterium DNA was extracted from 500µl of BU sample by the QIAamp DNA extraction minikit (Qiagen, Hilden, Germany) as per instructions of manufacturer. In brief, 20µl of proteinase K was added to 400µl aliquot of BU sample and 400µl of lysis

buffer. This was mixed by vortexing and incubated at 56° C for 30 minutes after which 400µl of absolute ethanol was added to facilitate *Mycobacterium* DNA extraction. 700 µl of mixture was transferred to spin column attached with a collection tube and centrifuged at 6000g. The collection tube was discarded with the filtrate. The procedure was repeated for the remaining mixture. The filtrate was washed twice with two different washing buffers. The extracted *Mycobacterium* DNA was eluted in 150µl of the elution buffer provided in kit. Extracted DNA was stored at -20°C (Details in Figure 3.7)

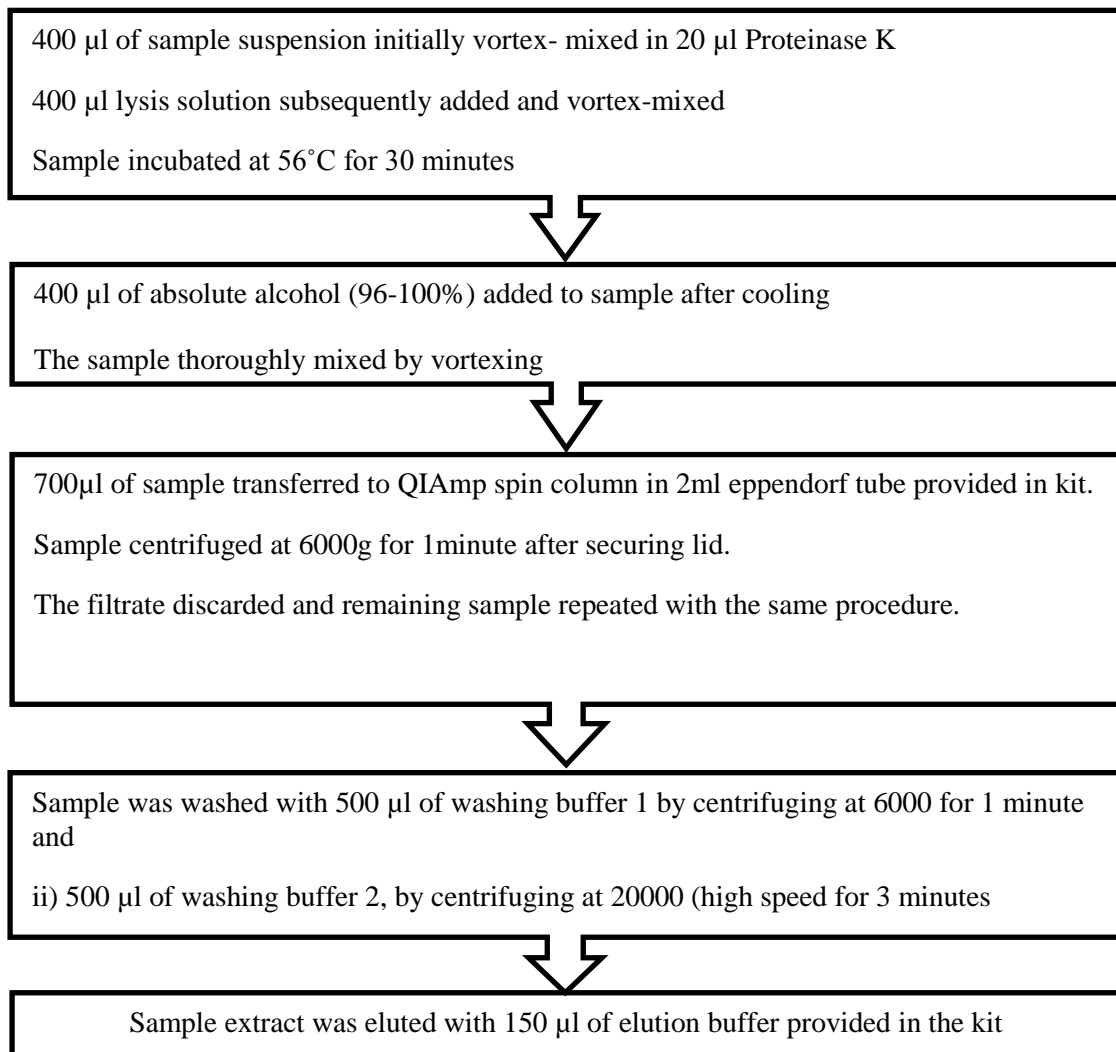


Figure 3.7 Flowchart on Procedures for the extraction of *M. ulcerans* DNA from BU samples

Amplification of extracted *M. ulcerans* DNA by thermo-cycling

Amplification of extracted *Mycobacterium* DNA was done with the QIAGEN PCR kit as per instruction provided by the manufacturer. Briefly 4µl of extracted *Mycobacterium* DNA was amplified in a final 20-µl volume PCR reaction mix (1_ *Taq* PCR buffer, deoxynucleoside triphosphates (0.2 mM each), 1 U of *AmpliTaq* Gold DNA polymerase (Perkin-Elmer Applied Biosystems), each primer at a concentration of 0.5 µM (represented in the PCR QIAGEN kit as master mix and Q solution in addition to 1 µl of forward Primer pMU1 and and 1µl of reverse pMU2 in addition to 10µl of Master Mix solution. The reaction was carried out using an Applied Biosystems thermal cycler starting with de-naturation for 5 min at 95°C. PCR was performed for 40 cycles of 60 seconds at 94°C, 60 seconds at 66°C, 72°C for 60 seconds .The reactions were terminated by incubation at 72°C for 10 minutes.

Visualization of PCR products

The products or amplicons from the polymerase chain reaction (PCR) were analyzed by agarose gel electrophoresis using 2% agarose incorporated with ethidium bromide and visualized with a ultra-violet trans-illuminator and estimated by comparing the bands to 1-kb size markers. The samples were run concurrently with negative and positive controls.

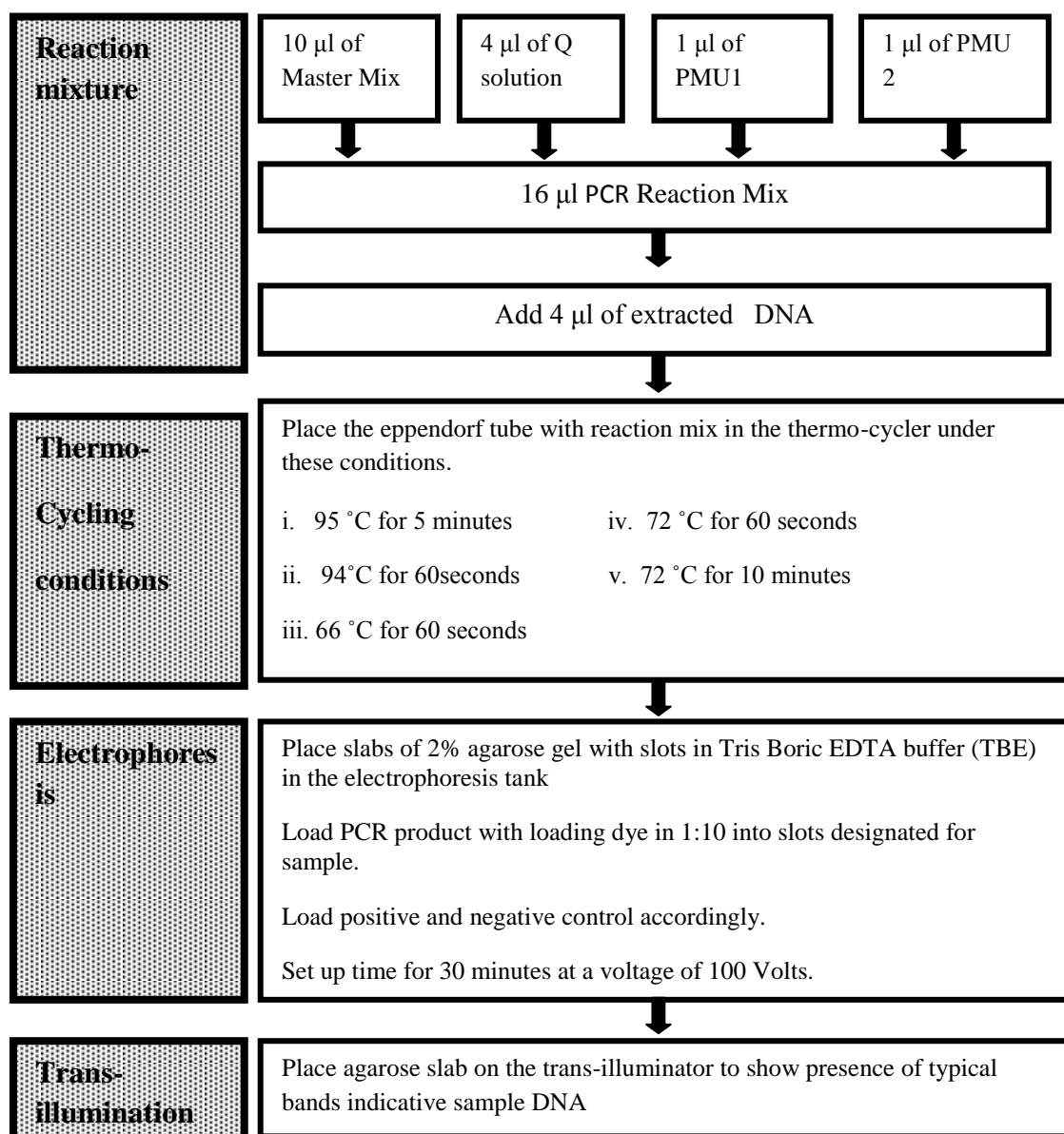


Figure 3.8 Procedures for the detection of *M. ulcerans* DNA from BU samples by IS2404 PCR using QIAmp PCR kit

Evaluation criteria for positive IS2404 sample

A sample positive for IS2404 was indicated by the alignment of its band with the band produced by the positive control sample as shown in the figure 3.9.

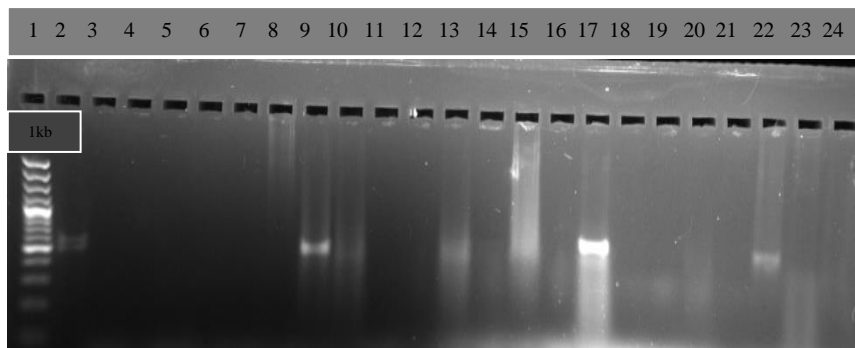


Figure 3.9 Electrophoretic patterns of amplified PCR products on 2% agarose gel incorporated with ethidium bromide.

Lane 1=1 kb base pair markers

Lane 2= positive control

Lane 3=negative control

Lanes 9 & 17=Clinical samples strongly positive for IS2404PCR

Lanes 10, 13, 15, 22=Clinical samples weakly positive for IS2404PCR

3.2.3 Diagnosis of Buruli ulcer Disease Using Modified Laboratory Procedures.

These investigations were part of the second phase, where seven modifications of specimen processing methods for the detection of AFB from smears were assessed in addition to the conventional method. This was aimed at improving the sensitivity of smear microscopy, to facilitate the early diagnosis of Buruli ulcer disease and ultimately, disease control.

3.2.3.1 Identification and Selection of Appropriate Modification Protocols for the Detection of AFB from BU specimens

The method applied for the protocol modifications for effective detection of acid fast bacilli was based on a review of methods for AFB detection from articles in the field of Mycobacteriology (Kubica, 1980; Miörner *et al*, 1996; Habeenzu *et al*, 1998; Ängeby *et al*, 2000; Van Deun *et al*, 2000; Selvakumar *et al*, 2002; Cattamanchi *et al*, 2009 ; 2010) . These methods were selected on the basis of the effectiveness in addition to the availability and accessibility of the described method. Most of the studies were based on smear preparation procedures aimed at improving the sensitivity of microscopy for the detection of AFB.

3.2.3.2 Detection of Acid fast bacilli (AFB) from Clinical BU Specimens by Modified Procedures.

Specimens positive for IS2404 PCR were selected for this study. Smears were prepared from BU samples by the following smear preparation protocols:-

(i) Direct smear preparation from swab

- (ii) Direct smear preparation from specimen suspension
- (iii) Smear preparation from sample concentrated by centrifugation
- (iv) Smear preparation from sample concentrated by overnight gravitational sedimentation.
- (v) Smears from gravitationally sedimented BU sample treated with 3.5% sodium hypochlorite.
- (vi) Smears from centrifuged BU sample treated with 3.5% sodium hypochlorite
- (vii) Smear from gravitationally sedimented BU sample treated with 5% phenol/ 4% ammonium sulphate
- (viii) Smear from centrifuged BU sample treated with 5% phenol /4% ammonium sulphate.

In brief, equal volumes (100 µl) of the various selected chemical agents and BU samples were vortex- mixed for 1 minute. They were then incubated at room temperature for a further nine minutes with intermittent mixing making up a total contact / exposure period of 10 minutes, after which 900 µl of sterile distilled water was added and mixed to neutralize effect of chemical agent used.

Centrifugation:-Samples were centrifuged at 2500 for 15 minutes, the supernatant carefully removed and a smear prepared from the re-suspended pellet.

Sedimentation: - Samples were allowed to stand overnight on a flat surface. The supernatants were removed and smears prepared from the sediment as per methods (Vasanthakumari, 1988) .

All smears were ZN stained as described (Appendix D.1 for details) after heat fixing. The slides were evaluated by scanning at 1000 x magnification with a light microscope. The AFB were quantified in accordance with published standards and further evaluated

based on other specified evaluation criteria (Kubica *et al*,1980a ; Collins *et al*,1997,in house evaluation criteria). See appendix D.2 and D.3 for details.

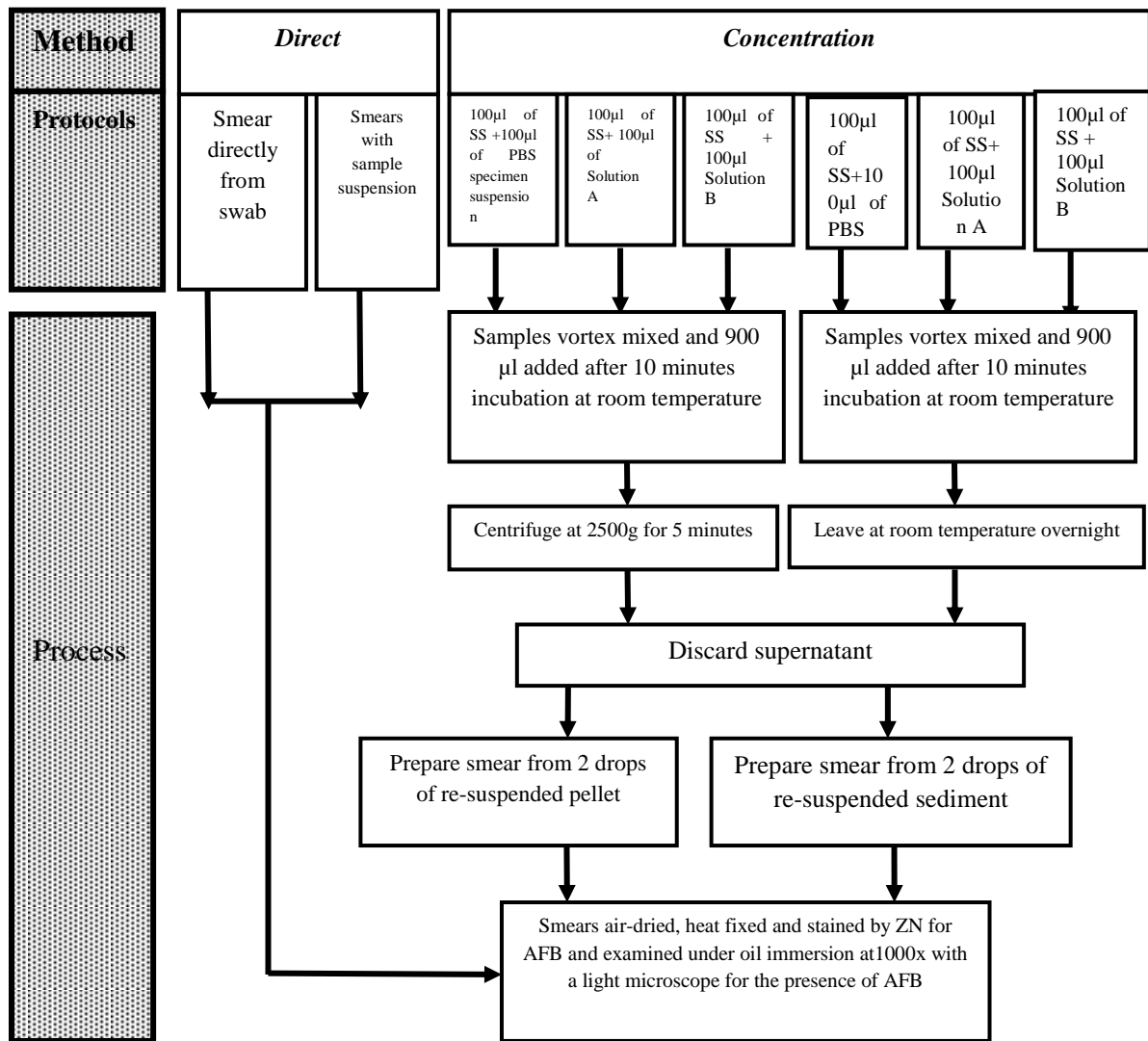


Figure 3.10 Schematic diagrams of modified laboratory procedures for the detection of AFB from BU specimens.

Solution A (HOCl) =hypochlorite

Solution B (PhAS) =phenol ammonium sulphate

ZN=Zeihl-Neelsen

SS=Sample suspension

3.2.4 *Culture of Mycobacterium ulcerans from Clinical BU Specimens by*

Modified Procedures

This aspect of the study investigated the usefulness of some selected chemical agents as potential decontamination agents for the effective isolation of *Mycobacterium ulcerans* from BU specimens.

3.2.4.1 Investigating inhibitory effect of selected chemical agents on clinical isolates of potential skin contaminants

The chemical agents were screened for their *in-vitro* inhibitory activity against the following potential microbial skin contaminants : *Klebsiella pneumoniae*, *Staphylococcus epidermidis*, *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Candida albicans* and *Aspergillus niger*. These were all clinical isolates obtained from the Microbiology Department of the University of Ghana Medical School.

Briefly, equal volumes (200µl) of microbial inocula with an equivalent turbidity of 0.5 McFarland's solution, were vortex-mixed with the selected chemical agents in 15 ml centrifuge tubes (BD Falcon). They were incubated at room temperature for thirty minutes after the effect of the chemical agents had been neutralized with 5ml of sterile distilled water. The effective inhibitory activities on the microbes was assessed by spreading 100µl of mixture on Mueller Hinton agar after gentle mixing. Microbial,

chemical agent, and media controls were set up alongside the tests. The plates were read after 24 hours of incubation at 37°C.

Chemical agents selected for these investigations were (i) 0.5% povidone iodine (PI) (ii) 1% povidone iodine (PI) (iii) 1% cetyl-pyridinium chloride / 2% sodium chloride (iv) 2% cetyl-pyridinium chloride / 4% sodium chloride (v) 5% oxalic acid (vi) 10% oxalic acid (vii) 0.5% virkon and (viii) 1% virkon.

3.2.4.2 Assessing Inhibitory Activities of Selected Chemical Agents on Microbial Contaminants in BU samples

The inhibitory effect of the selected chemical agents against the microbial contaminants in the BU samples were also assessed by the method described. Equal volumes (400µl) of BU samples and selected chemical agents were vortex mixed in 15 ml centrifuge tubes (Falcon BD). These were allowed to incubate at room temperature for 30 minutes with intermittent mixing. The reaction was stopped by neutralizing the effects of the chemical agents with 5ml of sterile distilled water. The effective inhibitory activities of chemical agents on the microbes was assessed by spreading 100µl of mixture on Mueller Hinton agar after gentle mixing. Microbial, chemical agent, and media controls were set up alongside the tests. The plates were read after 24 hours of incubation at 37°C.

Evaluation criteria: An effective decontamination agent was indicated by the absence of visible colonies on the Mueller-Hinton media after inoculation. An ineffective agent supported the growth of the test microbes, after 24 hours of incubation at 37°C.

3.2.4.3 The isolation of *M. ulcerans* by Modified Chemical Decontamination Protocols

The effects of the selected chemical agents on the isolation and yield of *M. ulcerans* from culture were evaluated. BU samples selected for these studies were positive for AFBs and IS2404 PCR. Briefly, equal volumes (500µl) of BU samples and selected chemical agents were mixed by vortexing in a 15 ml centrifuge tube (BD Falcon) to obtain final concentrations by w/v in 1ml of 0.25% PI (povidone iodine, 0.5% PI; 0.5% CPC (cetylpyridinium chloride/1% NaCl (sodium chloride), 1% CPC /2 % NaCl; 2.5% oxalic acid, 5% oxalic acid; 0.25% virkon tube and 0.5% virkon.

Ten milliliters (10 ml) of sterile distilled water was added to neutralize effect of chemical agents, after 30 minutes of incubation at room temperature, the mixture was vortexed and centrifuged at 3000g for 25 minutes. The supernatants were discarded into a discard jar with 5% phenol solution. The pellets were resuspended in 100 µl of sterile phosphate buffered saline (PBS), mixed by vortexing for 5 seconds and inoculated on labeled Lowenstein –Jensen media. The slants were incubated at 32°C and examined daily for 7 days and subsequently on a weekly basis for 12 weeks for the appearance of typical *M. ulcerans* colonies.

Controls: For each of the three investigative assays controls was set -up, where Buruli ulcer specimens were inoculated on media after decontamination with the chemical agents. They were incubated and read simultaneously with the tests at 32°C (Grandjean *et al*, 2008).

Control inoculums of authenticated *M. ulcerans* and MTB isolates were also set-up along-side the test BU specimen suspensions.

Evaluation criteria

The assessment of the chemical agents as a useful decontaminating agent was based on the following criteria:-

- i) Recovery of *M. ulcerans* colonies from culture and
- ii) The contamination rate per tests

A tested chemical agent was therefore classified as an effective decontaminating agent if higher yield of *M. ulcerans* was obtained in culture with reduced contamination rate.

3.2.4.4 Investigating the susceptibility of M. ulcerans isolates to rifampicin and streptomycin

The drug susceptibility pattern of thirty –five isolates of *Mycobacterium ulcerans* was determined for streptomycin and rifampicin using the indirect proportion method on Lowenstein-Jensen slants as described by Canetti (Canetti *et al*, 1963; Baron *et al*, 1994)

Preparation of M. ulcerans inoculums: (*M. ulcerans* inocula) were prepared from colonies of sub-cultured *M. ulcerans* isolates. Briefly, an inoculum of *M. ulcerans* was made by transferring 1-2 colonies of growing bacterial isolates into a sterile screw-capped test tube with 15 glass beads and 1 milliliter (ml) of sterile distilled water. This was vortexed gently, the clumps in suspension were allowed to settle and the supernatant aseptically transferred to a fresh sterile tube. The inoculums were made by adjusting the turbidity of the bacterial suspension to that of McFarland's standard 1 solution.

Preparation of drug incorporated L-J medium: Reagent grade streptomycin and rifampicin were purchased from a company called Sigma Chemicals were used for the test. Stocks of the two drugs were prepared by dissolving lyophilized powder in appropriate diluents-streptomycin was dissolved in sterile deionised water, whilst rifampicin was dissolved in absolute methanol. The prepared drug stocks were aliquoted and stored frozen at -70°C. The appropriate working dilutions were prepared for the two drugs at a final media incorporation dilution of 4ug/ml for streptomycin and 40ug/ml for rifampicin as per method described (See Appendix B.3 for details).

Inoculation onto Antibiotic-Incorporated L-J media: Tests:-The drug susceptibility test was done by inoculating 100ul of bacterial suspension onto well labelled drug incorporated L-J media. The tubes were gently rolled to ensure even spread of inoculums over media surface. All tests were set up in duplicates.

Controls: Serial dilutions (10^4 bacteria /ml and 10^2 bacteria /ml) were made of every isolate's inoculum (10^6) and inoculated onto drug free Lowenstein-Jensen media.

Un-inoculated drug-free LJ media and un-inoculated drug incorporated media were also set up. Tests and controls were set up in duplicates.

Incubation: All tubes were incubated at 32°C and examined daily for the first 7 days and subsequently observations and records were made on the twenty eighth and forty second days of incubation.

Evaluation criteria for the determination of DST: Drug resistance was expressed as the proportion of colonies growing on drug incorporated L-J medium to drug-free L-J medium with the critical proportion for resistance taken as 1%. A susceptible isolate exhibited colonial growth less than 1% of those that appeared on the control and resistant

if the colonies appearing had more than 10 % coverage as compared to that of the control. Isolates with colonies proportionally within 1-10 % of the growth on the controls was considered intermediately resistant (Canetti *et al*, 1963)

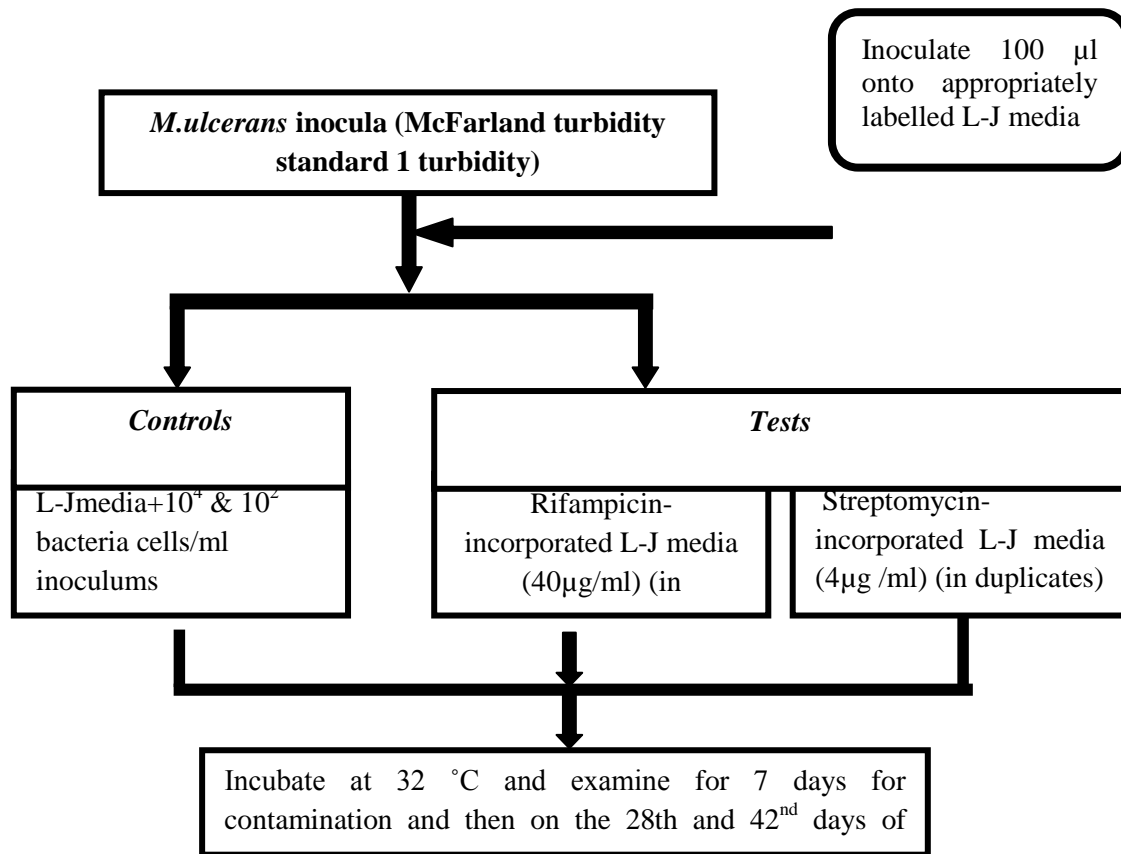


Figure 3.11 Procedures for determining drug susceptibility patterns of *M. ulcerans* isolates

3.2.5 Quality Control Checks

Information gathering with questionnaires was closely supervised. Samples were handled following standard procedures. Slide readings were done by the principal investigator and ten percent (10%) of slides were randomly selected for confirmation by a bacteriologist at the laboratory. Culture readings were done in consultation with other experts. All data were double -entered for consistency.

3.2.6 Ethical considerations

This study was reviewed and approved by the Ethical and Protocol Review Committee of the Noguchi Memorial Institute for Medical Research (NMIMR) and the Ghana Health Service (GHS), Ghana. Informed consent was sought from participating adults and from the parents or guardians of children. Participation was voluntary and enrolment was subject to individual's approval through signature or thumb-printing.

All procedures pertaining to the study alongside the study objectives were thoroughly explained to the participants. Individuals wishing to withdraw from the study were not coerced to stay, potential withdrawals were tolerated. Privacy and confidentiality were assured as patient information was kept under tight security. Individuals opting out of the study were still treated for their various conditions. This study was funded by the DAAD (German Academic Exchange Programme).

.

3.2.7 Data Analyses

The data collected in this study were entered in Microsoft excel (MS Excel) and analyzed with STATA 11 (Strata Corp, College Station, TX) to address the objectives of the study. The strategies taken to analyze the data involved descriptive statistics

including estimating totals, arithmetic means, frequencies, ranges and prevalence rates of the study variables.

Significant differences, associations and interrelationships of the variables were also assessed at a level of $p=0.05$. Where it was required, results from diagnostic tests were validated by estimating the sensitivities, specificities, positive and negative predictive values. The reliability of the methods was established by computing the kappa statistics. Specific analysis was carried out to determine:

- The prevalence of *M. ulcerans* infection among enrolled BU cases
- The performance of the various smear preparation methods which were evaluated based on the level of positivity, clarity and contrast and release of acid fast bacilli (AFB) from matrix (menstruum). These were scored and ranked based on AFB detection criteria. The expected total score for a given evaluation criteria was 12. A high score was indicative of the performance of the method applied.
- The activities of the selected chemical agents investigated were evaluated based on:
 - i. The spectrum and proportion of inhibition on clinical isolates of potential microbial skin contaminants expressed in mean counts of colonies
 - ii. The number of test samples with reduced contamination post chemical agent exposure
 - iii. The number of tests with *M. ulcerans* growth and the number of tests with contamination. The positivity rates defined by the number of tests with growth per the total number of tests were evaluated for each chemical agent.

The contamination rate defined by the number of tests with growth of microbes other than *M. ulcerans* colonies, and also with an over 50 % growth on media surface. The performance of each modified procedure was evaluated in comparison, to the conventional chemical agent used for decontamination (5% oxalic acid) as the standard. The associations between the experimental and the improved methods were determined using the chi-square test of association, and based on 95% confidence interval.

The tests were assessed using STATA statistical package version 11 software for validation (sensitivity, specificity, positive predictive and negative predictive values) and reliability (kappa estimation).

CHAPTER FOUR

RESULTS

4.1 Baseline demographic characteristics of study population

4.1.1 Baseline data on the demographic features of study population

A total of 446 swabs and 12 fine needle aspirates (FNA) were collected from various lesions of 135 clinically diagnosed Buruli ulcer patients from June 2010 to April 2012 for the laboratory confirmation of Buruli ulcer disease in Suhum-Krabo-Coaltar and Akuapem South districts of the Eastern region of Ghana. An average of 4 swabs were collected from each lesion.

Table 4.1 Age Range and Sex distribution of BU Cases (n=135)

Age range	# Males	# Females	Total #	Relative frequency (%)
0-14	20	15	35	26
15-29	11	14	25	18
30-44	11	10	21	16
45-59	13	20	33	24.5
>60	8	13	21	15.5
Totals	63	72	135	100

#=number

>=more than

n=number samples

Seventy nine patients, representing 59% of the total population enrolled in the current study were from the Paakro health Centre in the Akuapem South district, while the remaining 56 patients (41%) were from Asuboi in Suhum-Krabo-Coaltar, all in the Eastern region of Ghana. Seventy-two (53%) of the total number of patients were females, with the remaining 47% being males (Table 4.1). The age of the patients ranged from 1 to 92 years, with a mean age of 39 years. Patients within age range between 0-14 years were the highest of the recorded cases. The age range of 0-14 and 45-59 recorded higher number of males and females respectively (Table 4.1). The categories of age ranges and sex of the enrolled persons are presented in Table 4.1.

4.1.2 Clinical Epidemiology of Study Population

One hundred and twenty (91%) of the clinically diagnosed cases presented with ulcerative lesions with the remaining 12(9%) at the pre-ulcerative stages. Eighty four (62%) of the 135 had lesions on the lower limb and 40 (30%) on the upper limb (Figure 4.1b), whilst the rest were on other anatomical sites including the neck, cheek, breast and ears (Figure 4.1b). A majority of the lesions had advanced extensively, with eighty five (63%) having lesion sizes more than 15cm whilst 30 (22%) of them had their lesion size between 5 and 15cm. Twenty (15%) of the lesion had sizes less than 5cm. One hundred and thirty one (97%) of the cases were new, whilst old/recurrent cases were 4 (3%). Seventy four (55%) of the cases had applied herbal medicine to their lesions prior to reporting to the clinic.

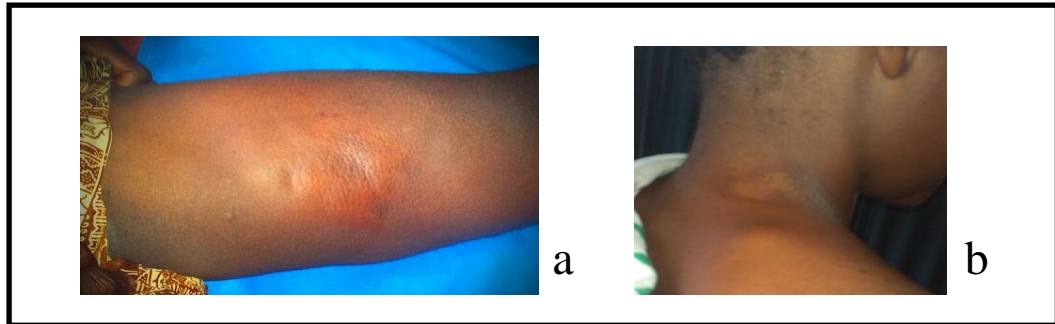


Figure 4.1 Clinical presentation of lesions on different parts of the body of patients (a) plague under the arm (b) nodules on the shoulder

4.2 BUD Case Confirmation by Conventional Laboratory Procedures

One hundred and thirty-five (135) clinically diagnosed BUD cases were analysed by three microbiological methods; PCR, microscopy and culture. PCR was included to serve as a standard for the other microbiological methods. One hundred and twenty-one (121) cases (90%) were confirmed by PCR. Sixty-eight 68 cases representing 50% were confirmed by microscopy and 57 representing 42% by culture. Five of the 12 FNA samples were culture positive, 10 were PCR positive and 6 were microscopy positive. The observed differences between the three methods was statistically significant ($p < 0.001$). A summary of the total number of positive cases detected by PCR, Microscopy and culture from swab and FNA specimens is presented in Table 4.

Table 4.2 Confirmation of Buruli ulcer by Conventional Laboratory Procedures

(N=135, swabs (n) =446 from 123 cases, FNA (n) =12 from 12 cases)

Diagnostic Procedures	Results						
	Total# Positives	% Positives	Se	Sp	PPV	NPV	p-value (fisher)
PCR	121	90					
Microscopy	68	50	55.8	0.93	0.89	0.49	<0.0001
Culture	57	42	47	0.68	0.9	0.57	<0.0001

Key

PCR=polymerase chain reaction

PPV=positive predictive value

NPV= negative predictive value

;

Se= sensitivity

Sp=specificity

#= number

;

Cases presenting with ulcerative lesions had swab specimens collected for the confirmation of Buruli ulcer disease. Table 4.3 shows the outcome of Buruli ulcer after examination of swabs. Out of 123 cases presented, PCR detected the highest with 111 cases, followed by microscopy with 62 positive cases and then culture was able to detect 52 positive cases (Table 4.3). The observed differences between the three methods was statistically significant ($p < 0.001$)

Table 4.3 *M. ulcerans* detection from Swabs by Conventional Laboratory Procedures

Diagnostic Procedures	Results						
	# MU positives	Statistics					
		% Positives	Se.	Sp.	PPV	NPV	p-value
PCR	111	90					
Microscopy	62	50	0.68	0.85	0.90	0.49	<0.0001
Culture	52	42	0.64	0.83	0.90	0.57	<0.0001

Key

PCR=polymerase chain reaction

PPV=positive predictive value

NPV=negative predictive value

M. ulcerans=*Mycobacterium ulcerans*

#=number

%=percentage

Se=sensitivity

Sp=specificity

N=number of BU cases =123

n=number of swabs =446

N=number of BU cases =123

Fine needle aspirates (FNA) was collected from cases presenting with pre-ulcerative lesions for confirmation of Buruli ulcer disease. The outcome of Buruli ulcer detection after examination of FNA is displayed in Table 4.4. Out of 12 cases, PCR detected the highest with 10 cases, followed by microscopy with 6 positive cases, while laboratory diagnosis from culture detected 5 positive cases (Table 4.4). A statistically significant difference was observed between the three methods ($p < 0.001$).

Table 4.4 *M. ulcerans* detection from FNA by Conventional Laboratory Procedures

Diagnostic Procedures	Diagnostic specimens (FNA n=12)						
	<i>Results</i>	<i>Statistics</i>					
	# <i>M.ulcerans</i> positive	% positive	Se. (%)	Sp. (%)	PPV	NPV	p-value
PCR	10	83					
Microscopy	6	50	66	77	0.83	0.5	<0.0001
Culture	5	42	62	75	0.83	0.58	<0.0001

Key

PCR=polymerase chain reaction

#=number

N=number of BU cases =12

PPV=positive predictive value

%=percentage

n=number of FNA =12

NPV=negative predictive value

Se=sensitivity

Sp=specificity

M. ulcerans=*Mycobacterium ulcerans*

4.3 Modified laboratory procedures for the confirmation of BUD

4.3.1 Modified Laboratory Procedures for the confirmation of Buruli ulcer

BU samples, processed by modifications of the conventional culture and microscopy procedures were analyzed based on appropriately indicated evaluation criteria (see appendices D.1,D.2 & D.3).

4.3.1.1 AFB Detection by Modified Smear Preparation Methods

Smear preparation protocols (one conventional and seven modified) for the detection AFB were analyzed and scored. The score was based on the following criteria: i) Number of AFBs per 100 fields (P), ii) Clarity plus contrast; indicating the extent of brightness in field (C) and iii) Release of AFBs from the sample matrix or host material (R) for easy visibility after ZN staining and examination by microscopy under oil immersion at 1000x (Table 4.5). Each smear was rated 0-4 for every criterion. The expected maximum rating per protocol per test was 12. This is as a result of scoring a maximum value of 4 each for the three aforementioned criteria. Each protocol was tested with 40 slides in duplicates, making a total number of 80 tests for a single protocol. Therefore, after adding the score of 80 tests for a protocol, the maximum score a single protocol can attain from all the three criteria was 960.

Table 4.5 Performance of smear preparation protocols based on evaluation criteria(AFB)

Methods and Evaluation Protocol	Score Per Criteria			Total Score / 960*
	Positivity / 320 #	Clarity and Contrast /320 #	Release from matrix / 320 #	
1	36	96	26	158
2 [!]	79	99	51	229
3	65	91	50	206
4	81	103	61	245
5	42	111	32	185
6	27	101	22	150
7 [#]	99	147	85	331
8	80	124	97	301

Key

* = (Positivity score + Clarity and Contrast score + Release from matrix)

n = Total number of tests for each protocol = 40 x 2 = 80 (#) = m x n

m = maximum score for a criteria = 4 ! = conventional

=best protocol

Smears prepared from BU samples processed by the protocols indicated

1(DISW) =direct from swab

5(COHOSE) = 3.5% hypochlorite & gravitational sedimentation

2(DISU) =direct from sample suspension

6(COHOCE) = 3.5% hypochlorite& centrifugation

3(COSUSE) = sample concentrated by gravitational sedimentation

7(COPHSE) =5% phenol /4% ammonium sulphate and gravitational sedimentation

4(COSUCE) = sample concentrated by centrifugation

8(COPHCE)= 5%phenol/4% ammonium sulphate and centrifugation

A summary of the total scores obtained by the 8 different protocols is presented in Table 4.5. The total scores obtained by the protocols per criteria ranged from 27 to 99 for positivity. Protocol with the highest total positivity score was smear from phenol ammonium sulphate and gravitational sedimentation, and the lowest was smear from hypochlorite and centrifugation. For clarity and contrast, the total score ranged from 91 to 147. Smear from phenol ammonium sulphate and gravitational sedimentation scored the highest for this criterion whiles smear from gravitational sedimentation scored the lowest. The total score for release from matrix criterion ranged from 22 to 97. Smear from phenol ammonium sulphate and centrifugation recorded the highest whiles smear from hypochlorite and centrifugation recorded the lowest score for this criterion. The total score for the protocols ranged from 150 to 331. Smear from phenol ammonium sulphate and gravitational sedimentation had the highest total score per protocol and proved to be the best performing (Table 4.5).

4.3.1.2 Analyses of Smear Preparation Protocols

The results were also expressed as comparable estimates between protocols including the observed geometric differences and the associated levels of significance. The comparisons were between the modified protocols and the conventional, using the conventional protocol as a standard for comparison. The validity of the various protocols as diagnostic tests were also assessed based on the computed sensitivity and specificity. The reliability/reproducibility or level of agreement of the various test protocols were also estimated as the computed kappa (κ). The results are summarized in Table 4.6.

Eight (8) different smear preparation protocols were employed in the current study (Table 4.6). Among the protocols used two (DISW & DISU) were directly applied. Other four protocols (COHOSE, COHOCE, CPHSE & CPHCE) which involved concentration techniques and chemical treatment of the samples were also used, while the remaining two protocols (COSUSE & COSUCE) involved concentrations without chemical treatment. Out of the 8 protocols, CPHSE recorded the highest percentage sensitivity (97%) and the lowest percentage specificity (40.0%). Meanwhile, DISW had the lowest sensitivity and a specificity value of 96% (Table 4.6).

Among the four (4) protocols which involved concentration with chemical treatments, two (2) recorded higher percentage sensitivity, with 96.7% and 97% for CPHCE and CPHSE respectively. The other two recorded relatively high percentage sensitivity (81% and 64.3% for COHOSE and COHOCE respectively), when compared to the percentage sensitivity of the direct protocols (Table 4.6). Generally, the protocols which involved concentration had higher percentage sensitivity (64.3-97), as compared to the direct protocols employed in the current study.

Table 4.6 Comparative Analysis of Smear Preparation Protocols (conventional & modified)

Smear preparation protocols	Total Score /protocol	Mean \pm sd (CI)*	% Se	% Sp	κ -stats	p-value
1 (DISW)	360	4.5 \pm 0.3(3.9 -5.1)	60	96	0.576	p= 0.0001
2 [!] (DISU)	472	5.9 \pm 0.3(5.4 – 6.4)	Std	Std	Std	Std
3 (COSUSE)	518	6.5 \pm 0.3(5.9 -7.0)	91	90	0.817	p=0.0700
4 (COSUCE)	550	6.9 \pm 0.3(6.3 -7.5)	96	75	0.698	p= 0.0010
5 (COHOSE)	430	5.4 \pm 0.3(4.8 – 6.0)	81	97	0.83	p= 0.1330
6 (COHOCE)	376	4.7 \pm 0.3(4.1 - 5.2)	64	97	0.60	p=0.001
7 [#] (COPHSE)	686	8.6 \pm 0.2(8.2 – 9.0)	97	40	0.250	p= 0.0000
8 (COPHCE)	662	8.3 \pm 0.2(7.9 – 8.6)	96	45	0.324	p= 0.0000

Key

Smears prepared from BU samples processed by the protocols indicated

1(DISW) =direct from swab

5(COHOSE) = 3.5% hypochlorite & gravitational sedimentation

2(DISU) =direct from sample suspension

6(COHOCE) = 3.5% hypochlorite& centrifugation

3(COSUSE) = sample concentrated by gravitational sedimentation

7(COPHSE) =5% phenol /4% ammonium sulphate and gravitational sedimentation

4(COSUCE) = sample concentrated by centrifugation

8(COPHCE)= 5%phenol/4% ammonium sulphate and centrifugation

Mean \pm sd (CI)* implied mean scores \pm standard error (confidence interval)

n =Total number of tests for each protocol

κ value=kappa inter-rater statistics

Se=sensitivity

[!] = conventional

Sp=specificity

[#] =best protocol

Micrographs of ZN stained smears from the different protocols were examined for AFB and results presented in Figure 4.1. For The first protocol, which involved smear prepared directly from swab, AFB were scantily distributed, with background line made from direct application of the swab (Figure 4.1a). The protocols which had smear made from BU specimen suspension showed homogeneity in the field with relatively more AFB in matrix material (Figure 4.1b). The third protocol which involved smear prepared from sedimented BU sample suspension displayed a clumpy distribution of AFB in the field with a lot of matrix material hampering viewing (Figure 4.1c). Smear prepared from centrifuged BU sample suspension (COSUCE) showed AFB distributed in clumps with few scattered with clearer background, a view that is not significantly different from that of Figure 4.1c (Figure 4.1d). The protocol which involved preparation of smear from hypochlorite treated BU sample concentrated by gravitational sedimentation (COHOSE) showed a dark background and fissured obscuring AFB (Figure 4.1e). Smear prepared from hypochlorite treated BU sample concentrated by centrifugation (COHOCE) also showed a fissured background but relatively brighter field (Figure 4.1f). Smear prepared from sedimented (gravitational) phenol ammonium sulphate (PhAS) treated BU sample (COPHSE) had a clear background making smear examination easier. AFB dispersedly distributed in that smear (Figure 4.1g). Finally, Figure 4.3h, which was prepared from centrifuged phenol ammonium sulphate (PhAS) treated BU sample (COPHCE) produced a slide, displaying the clearing of matrix material for the release of clumps of AFB in field. It also showed a Polka dot appearance background which is characteristic of PhAS processing. AFB has been showed with black arrow (Figure 4.1)

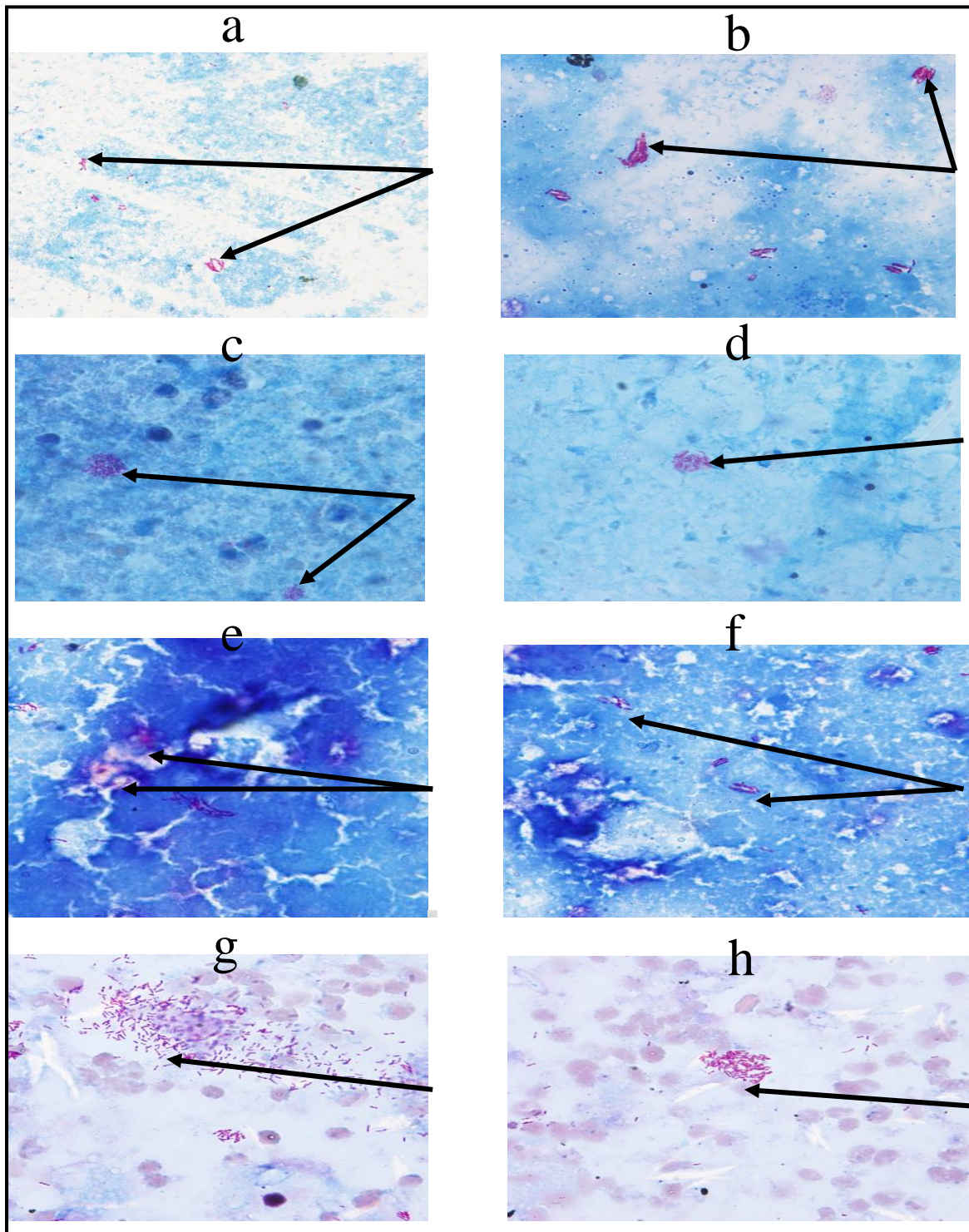


Figure 4.2 ZN stained smears of modified procedures on AFB detection (a) ZN stained smear prepared directly from swab (b) Smear from BU specimen suspension (c) Smear prepared from sedimented BU sample suspension. (d) Smear prepared from centrifuged BU sample suspension (e) Smear from Hypochlorite treated BU sample concentrated by gravitational sedimentation (f) Smear from Hypochlorite treated BU sample concentrated by centrifugation (g) Sedimented (gravitational) phenol ammonium sulphate (PhAS) treated (h) Sedimented (centrifugation) phenol ammonium sulphate (PhAS) treated.

4.3.2 *M. ulcerans* Culture by Modified Methods

4.3.2.1 Inhibition of Potential Microbial Skin Contaminants by Decontamination Protocols

The inhibitory property of five chemical agents with different concentrations having disinfection, lysis and decontamination properties was assessed on nine clinical isolates of potential skin contaminants (Table 4.7). The chemical agents were evaluated based on the level of growth inhibition of the tested microbes' *in-vitro*. All the microbes tested against 0.5% & 1% povidone iodine (PI), 10% oxalic acid and 2% CPC / 4% NaCl and 1% virkon were susceptible to the chemical agents as indicated in Table 4.7. Three of the isolates (*Pseudomonas aeruginosa*, *Bacillus cereus* and *Aspergillus niger*) showed resistance to 20% Benzalkonium chloride (Timsen). Forty percent (40%) Benzalkonium chloride (Timsen) on the other hand could not inhibit two of these isolates (*Pseudomonas aeruginosa* and *Aspergillus niger*). Five percent 5% Oxalic acid did not have inhibitory effect on *Bacillus cereus* and *Aspergillus niger* whiles 1% CPC/2% NaCl could not inhibit *Escherichia coli* and *Salmonella typhi*. Virkon (0.5%) disinfectant did not also inhibit *Aspergillus niger*.

The selected chemical agents preliminarily screened against nine clinical isolates of microbial agents showed various inhibitory effects on the tested microbes. Amongst them, 0.5% PI, 1% PI, 2% CPC / 4% NaCl, 10% Oxalic acid and 1 % Virkon disinfectant inhibited growth of all the nine microbes tested. 1% CPC/2% NaCl, 40% BC (Timsen) and 5% Oxalic acid could not inhibit the growth of two of the microbes tested. Of all the chemical agents tested 20% BC (Timsen) performed the least as it was unable to inhibit the growth of three of the nine microbes. Of the microbes tested *Bacillus cereus*, *Aspergillus niger*, *Escherichia coli*,

Salmonella typhi and *Pseudomonas aeruginosa* exhibited various degrees of resistance to some of the chemical agents (Table 4.7).

Table 4.7 Inhibitory effect of selected chemical agents on clinical isolates of Potential skin contaminants

Chemical Agents	In-vitro resistance of Microbes to chemical agents.
(I) 0.5% PI	None of the microbes tested
(II) 1% PI	None of the microbes tested
(III) 1% CPC/2% NaCl	<i>Escherichia coli</i> & <i>Salmonella typhi</i>
(IV) 2% CPC / 4% NaCl	None of the microbes tested
(V) 5% Oxalic acid	<i>Bacillus cereus</i> & <i>Aspergillus niger</i>
(VI) 10% Oxalic acid	None of the microbes tested
(VII) 0.5% Virkon disinfectant	<i>Aspergillus niger</i>
(VIII) 1 % Virkon disinfectant	None of the microbes tested
(IX) 20% BC (Timsen)	<i>Pseudomonas aeruginosa</i> , <i>Bacillus cereus</i> & <i>Aspergillus niger</i>
(X) 40% BC (Timsen)	<i>Pseudomonas aeruginosa</i> & <i>Aspergillus niger</i>

Key

BC=Benzalkonium chloride
Aspergillus niger,
Pseudomonas aeruginosa
Salmonella typhi

PI=povidone iodine
Escherichia coli
Candida albicans
Bacillus cereus

CPC=cetylpyridinium chloride
Staphylococcus epidermidis
Staphylococcus aureus
Klebsiella pneumoniae

4. 3.2.2 Inhibitory Activities of Chemical Agents on Microbial Contaminants in BU Samples

The selected chemical agents assessed against microbial contaminants in the thirty-five BU samples tested in duplicates, making a total of 70 tests showed varying levels of inhibitory activities against the contaminants. The maximum level was shown at 98% by 1% virkon and 1% Povidone iodine; whilst the minimum of 85% was by 20% 'Timsen'. Details of the results are shown in Table 4.8.

Table 4.8 Effect of chemical agents on microbial contaminants in BU samples

Total number of test plates =70		
<i>Chemical agents</i>	<i>Test plates showing no growth</i>	<i>Estimated Activity levels of Chemical agents (%)</i>
0.5% PI	68	97
1% PI	69	99
1% CPC/2% NaCl	65	92
2% CPC/4% NaCl	67	95
5% Oxalic acid	64	91
10% Oxalic acid	67	96
0.5% Virkon	67	96
1% Virkon	69	99
20% BC (Timsen)	60	86
40% BC (Timsen)	63	90

Key

BC=Benzalkonium chloride (Timsen)

PI=Povidone iodine

CA=chemical agent

CPC=cetylpyridinium chloride

NaCl=sodium chloride

4.3.2.3 Performances of selected decontamination protocols

Performances of the selected decontamination protocols for the primary isolation of *M. ulcerans* are summarized in Table 4.9.

Eight selected decontamination protocols were investigated for the primary isolation of *M. ulcerans* on Lowenstein-Jensen (L-J) media. The best performances were exhibited by 2% CPC/4% NaCl (*M. ulcerans* growth on 37 out of 70 culture tubes) and 0.5% virkon (growth on 36 of the 70 culture tubes). This performance was closely followed by 1% CPC/2% NaCl (growth on 33 out of 70 L-J tubes) and then by 5% Oxalic acid (conventional decontamination method for *M. ulcerans* culture. Povidone iodine at concentrations of 0.5% and 1% were decontamination procedures that did not exhibit *M. ulcerans* growth on L-J media.

Table 4.9 Comparative Analysis of *M. ulcerans* Recovery after Treatment with Selected Decontamination Protocols (conventional & modified)

Decontamination Protocols	# of tubes with <i>M. ulcerans</i> growth per protocol	Mean \pm sd (CI)*	% Se	% Sp	P- value
0.5% PI	0	0.07 \pm 0.03 (0.0-0.1)	Nd	Nd	p=0.000
1% PI [†]	0	0	Nd	Nd	Nd
1% CPC/ 2% NaCl	33	2.71 \pm 0.62 (1.5 -4.0)	80	86	p=0.000
2% CPC/4% NaCl	37	3.42 \pm 0.65 (2.1 -4.7)	80	90	p= 0.7608
5% Oxalic acid	31	2.60 \pm 0.53 (1.5 – 3.6)	Std	Std	p= 0.0718
10% Oxalic acid	10	1.02 \pm 0.30 (0.4 -1.6)	17	79	p= 0.0002
0.5% Virkon	36	2.94 \pm 0.2 (1.75 –4.13)	80	94	p= 0.4070
1% Virkon	6	0.58 \pm 0.18(0.22 -0.95)	01	79	p= 0.0000

Key

PI= povidone iodine

Se= sensitivity

CPC=cetyl pyridinium chloride

NaCl=sodium chloride

Sp=specificity

n= total number of tests =35 x 2 =70

nd=not determined

Std=standard

M. ulcerans=*Mycobacterium ulcerans*

4.3.2.4 Analysis of Microbial Contamination after Decontamination Protocols

The effectiveness of the decontamination protocols were also determined based on the level of growth of the fast growing contaminants after processing the samples with the selected decontamination protocols and the results are contained in 4.9.1

The conventional method (5% oxalic acid) recorded the highest number of contamination with 20 tubes at a contamination rate of 29%. This was followed closely by 1% cetylpyridinium chloride/2% sodium chloride with 15 contaminated tubes at 21%. (Table 4.9.1). There was no growth of fast growers on the two concentrations (0.5% and 1%) povidone iodine (PI) (Table 4.9.1).

Table 4.9.1 Comparative Analysis of Levels of Microbial Contamination after Treatment with Decontamination Protocols (conventional & modified).

Decontamination Protocols	Number of tubes with Microbial contamination / protocol	Microbial Contamination rate / protocol (%)	Mean \pm sd (CI)*	P – Value
1 (0.5% PI)	0	0	0	0
2 (1% PI)	0	0	0	0
3 (1% CPC/2% NaCl)	15	21	0.2 \pm 0.05(0.1-0.3)-	p> 0.001
4 (2% CPC /4% NaCl)	10	14	0.1 \pm 0.0 (0.05 -0.2)	p> 0.001
5! (5% Oxalic acid)	20	29	0.3 \pm 0.05 (0.2 – 0.4)	0
6 (10% Oxalic acid)	12	17	0.2 \pm 0.05 (0.08 -0.26)	p> 0.001
7 (0.5% Virkon)	12	17	0.2 \pm 0.05 (0.08 –0.26)	p> 0.0001
8 (1% Virkon)	7	10	0.1 \pm 0.03(0.03 -0.17)	p> 0.000

Key

PI = povidone iodine

NaCl =sodium chloride

M.ulcerans=*Mycobacterium ulcerans*

CPC=cetylpyridinium chloride

Std=standard

!=conventional method

p=probability

n= total number of test

nd=not determined

n = total number of tests = 35 x 2 =70

4.3.2.5 Performances of protocols in comparison to conventional procedure for a particular laboratory diagnostic method

The overall performances of the various methods and procedures investigated in this study were analysed in comparison with the appropriate conventional methods peculiar to a diagnostic method (Table 4.10).

Table 4.10 Performances of protocols in comparison to conventional procedure for a particular laboratory diagnostic method

Diagnostic methods	<i>Estimated valuation per protocol (%)</i>							
	1	2	3	4	5	6	7	8
Microscopy	39.1	*49.1	53.9	57.2	44.7	37.5	71.4	68.9
Culture	0.0	0.0	47.1	52.8	*44.0	14.0	51.4	8.0

*=conventional procedure for a particular laboratory diagnostic method

Protocols for microscopy

Smears prepared from BU samples processed by the protocols indicated

1(DISW) =direct from swab

5(COHOSE) = 3.5% hypochlorite & gravitational sedimentation

2(DISU) =direct from sample suspension

6(COHOCE) = 3.5% hypochlorite& centrifugation

3(COSUSE) = sample concentrated by gravitational sedimentation

7(COPHSE) =5% phenol /4% ammonium sulphate and gravitational sedimentation

4(COSUCE) = sample concentrated by centrifugation

8(COPHCE)= 5%phenol/4% ammonium sulphate and centrifugation

Decontamination Protocols for culture

1=0.5% Povidone iodine

5= 5% oxalic acid

2=1% Povidone iodine

6=10% oxalic acid

3=1% cetyl pyridinium chloride / 2% sodium chloride

7=0.5% virkon disinfectant

4=2% cetyl pyridinium chloride / 4% sodium chloride

8= 1% virkon disinfectant

Microscopic examination of smear from phenol ammonium sulphate and gravitational sedimentation recorded the highest valuation percentage of 71.4 against the conventional procedure which involved microscopic examination direct smear from sample suspension (Table 4.10). For culture, smear from centrifuged concentration sample recorded the highest valuation percentage (52.8). Meanwhile, both microscopic examination and culture of direct smear from swab recorded the lowest valuation of 39.1% and 0 % respectively (Table 4.10).

4.3.2.6 Drug susceptibility profiles of *M. ulcerans* isolates

Drug susceptibility tests of 34 isolates of *Mycobacterium ulcerans* (tests in duplicates) was determined against rifampicin (RIF) and streptomycin (STR) using the Canetti's indirect proportion method for determining DST in *Mycobacterium*. Six of the 34 isolates tested for susceptibility to rifampicin (40µg/ml) were resistant whilst only one showed resistance to 4µg/ml of Streptomycin (Table 2.11). No isolate exhibited resistance against both rifampicin and streptomycin.

Table 4.11 In-vitro Susceptibility of Clinical Isolates of 34 *M. ulcerans* to Rifampicin And Streptomycin (n=34)

Outcomes	Tests (antibiotics)	
	Rifampicin (40µg/ml)	Streptomycin(4µg/ml)
<i>Growth</i>	6	1
<i>No growth</i>	28	33
<i>Total</i>	34	34

CHAPTER FIVE

DISCUSSION

5.1 Confirmation of BUD cases

The prevalence of Buruli ulcer disease, one of many neglected tropical skin conditions remains high, despite efforts at disease control measures. The associated morbidity has long term socio-economic implications on endemic communities. These communities are characteristically rural, especially in developing low income countries including Ghana.

The study was conducted in Asuboi in Suhum-Kraboaa-Coaltar and Paakro in the Akuapem South, both districts of the Eastern Region of Ghana. These districts were selected for this study because BUD endemic communities can be located in these districts. (http://en.wikipedia.org/wiki/File:Eastern_Ghana_districtdistricts.png). Endemicity of BU disease within these communities could be attributed to the livelihood of the inhabitants and the environment. Fifty-three percent (53%) of the total number of patients enrolled were females, with the remaining 47% being males (Table 4.1). This ratio indicates that, BU case used as the sample population was almost evenly distributed in terms of gender.

In this study, PCR confirmed over 90% of clinically diagnosed Buruli ulcer cases, while conventional microscopy and culture methods confirmed 51% and 41% respectively. The high detection rate of *M. ulcerans* by PCR emphasizes its high sensitivity when compared to microscopy and culture. This is in accordance with other related studies. For instance in a study by Herbinger *et al*, the overall sensitivities for PCR was 85%, microscopy at 57% and culture at 51% (Herbinger *et al*, 2009). In another study conducted in Togo the overall

sensitivities seemed relatively lower than that reported, as PCR confirmed 54% (109/202) clinically diagnosed cases and microscopy confirmed 29.9% (43/144) cases. The investigators, Bretzel *et al* attributed the observed low detection rate, to manpower challenges amongst other factors such as low concordance rate and a high percentage of false negative results for microscopy. (Herbinger *et al*, 2009; Bretzel *et al*, 2007). All the same, WHO standards (case confirmation rate > 50%) were met in both the current study and that of Bretzel *et al*. The relatively low positivity (51% and 41%) rate observed for microscopy and culture respectively in this study as compared to Herbinger could be attributed to the quality of samples collected. Swabs and Fine needle aspirates tend to have yields relatively lower than that for tissue.

Most (26%) of the cases confirmed with the disease were individuals less than fifteen years, whilst more females were confirmed with the disease than males, validating findings from other studies where more females were reported in their studies than males (WHO, 2008; Aiga *et al*, 2004; Debacker *et al*,2003). This could be due to similar activities undertaken by both women and children such as frequenting water related environments both for household and recreational purposes. The association with water bodies regarding Buruli ulcer has been severally hypothesized, and the first isolation of the organism from the environment was also water related (Marsollier *et al*, 2003; Raghunathan *et al*,2005 ; Portaels *et al*,2008)

In this study more lesions were located on the lower limbs than on the upper limbs whilst very few were found on other anatomical sites. Similar observations were made by other studies conducted in Ghana (Hospers *et al*, 2005). This also confirms many reports that unlike MTB, *M. ulcerans* transmission necessitates skin exposure and contact (W H O, 2001; Phillips *et al*,2005).

5.2 Detection of AFBs by microscopy

This study represents the first of an in-depth evaluation of laboratory procedures aimed at improving the detection of *M. ulcerans* from BU samples for the early detection of the disease. Eight smear preparation protocols based on the conventional procedures were evaluated by comparing their performance with that of the conventionally used direct smear method. The performance criteria used were based on the criteria set for the study (appendix D.3). Performance levels were also based on total geometric scores obtained per criteria.

The differences observed between the various protocols and the conventional method were found to be statistically significant by the student's t-test statistical inferential tool. The highest score of 686 was by protocol 7, a method which involved the treatment of the samples with 5% phenol 4% ammonium sulphate solution, and concentrated by gravitational sedimentation. This is a protocol that has been investigated and found to be useful in MTB studies. In 2002, Selvakumar *et al* reported sensitivities (85%) and specificities (83%) that were higher than the direct smear method. He also found out that in MTB diagnosis it was a more user-friendly method and better accepted by laboratory technicians. Equally favorable results have been reported on the performance of phenol ammonium sulphate where it was used in conjunction with basic fuchsin and also in overnight sedimentation for the detection of AFB for MTB diagnosis (Selvakumar *et al*,2002).The current study also reported similar performance in comparison to the direct method. Smears from this protocol appeared relatively clearer with good contrast. In comparison to the conventional method (protocol 2)

the 5%/4% PhAS performed higher (rank score 686). Protocol 1, which involved the direct application of swabbed exudates from lesion on a slide, recorded the lowest rank score of 374. As far as this study is concerned, method for protocol 1 is not routinely used. It was however included in the study as a possible alternative for conventional method where samples collected would have to be processed to obtain sample suspension. It was envisaged that if outcomes from this protocol was favorable, then it could conveniently serve as a relatively rapid method with a quicker turnaround time for sample processing. This is because BU sample processing for conventional smear preparation takes time. Even though as far as this study is concerned, no such protocol has been reported, this study assessed the possibility of introducing it since the conventional method requires an elution process, which is quite time consuming in comparison to protocol 1. This poor performance of protocol 1 could be as a result of the poor release of the exudates from the tip of the swab and it would therefore be appropriate in subsequent studies to apply substantial moisture before preparing the smear. The conventional method (protocol 2) ranked fifth at a score of 472. The differences observed among all the protocols in comparison to the conventional method were all statistically significant ($p < 0.05$) except for protocols 3 (BU sample concentration by gravitational sedimentation) and 5 (BU sample concentration by gravitational sedimentation after hypochlorite treatment). This study also reported a relatively better performance between the concentration methods (protocols 3 and 4) and the conventional method (direct-Protocol 2). This is a widely observed phenomenon reported by workers in both MTB and *M. ulcerans* studies including Bruchfeld in 2000, Gebre-Selassie in 2003 for MTB and Yeboah-Manu *et al* in 2011 for *M. ulcerans*. (Bruchfeld *et al*, 2000; Gebre-Selassie *et al*, 2003; Yeboah-Manu *et al*, 2011). The present study also reported a difference between samples concentrated by either sedimentation or centrifugation. The observed differences were however not

statistically significant ($p > 0.05$). Outcomes from similar studies on MTB diagnosis however showed that concentration by centrifugation recorded the highest average number of AFBs per microscopic field. Amongst samples concentrated by gravitational sedimentation which included protocols 3, 5, and 7, the observed differences were found to be significant ($p < 0.001$). The influencing factor could be attributable to the chemical agent used in a particular protocol. Whilst PhAS treated samples performed well, HOCl did not do so- well. The differences were not only indicated by the scored ranks, but by the micrographs of the prepared smears

Micrographs of smears prepared from PhAS showed a clearly visible field, though interspersed with polka-dot elements. In a study similar to this, Selvakumar *et al* reported sputum samples treated with phenol ammonium sulphate had sensitivities similar to that of the conventional methods. The PhAS method had a relatively shorter slide reading period. This served as an added advantage. He also reported that even though they observed the washed-off effect. This did not happen in their study. Similar observations were made in this study. In this study smears made from PhAS processed samples remained intact. This was in comparison to smears from hypochlorite processed samples.

In the case of smears processed with hypochlorite, the field appeared relatively darker and visibly fissured (Van Deun *et al*, 2000; Ängeby *et al*, 2004). In this study samples treated with hypochlorite before various concentration methods did not perform as well as the other protocols. Even though others had reported on the method improvement after bleach treatment (Chew *et al*, 2011), studies working with hypochlorite and MTB had also reported

improvements. (Miörner *et al*, 1996; Selvakumar *et al* 2002 ; Frimpong *et al*, 2005). The outcomes have generally been subjective for bleach treatment particularly in MTB studies (Gebre-Selassie, 2003; Miörner *et al*, 1996; Habeenzu *et al* 1998).

The observed differences in smear reading in terms of clarity, may be due to procedural differences; whilst this study used a concentration of 3.5% hypochlorite, Frimpong and his colleagues used 1% hypochlorite whilst working on MTB (Frimpong *et al.*, 2005). In a related study, Chew *et al* showed that bleach sedimentation significantly decreased the number of acid fast bacilli visualised, compared with conventional smears. In that study although smear made from bleach sedimentation was more rapid and the inter-observer agreement was lower than the conventional method, they also observed that strong AFB positive smears were misread as negative, partly explaining the contradictory reportage of bleach treated smears. In this study, micrographs of hypochlorite treated smears appeared dark with visibly fissured background. Observed AFB seemed obscured within the smear. This may probably explain the findings by Chew *et al*, which reported that hypochlorite sedimentation seemed to have a ten-fold decrease in AFB present. (bleach sed.=185;conv=205) (Chew *et al*, 2011). Relatively better outcome with the sedimentation procedure makes it a likely method that can be conveniently used at peripheral health facilities where there may be no centrifuge. The observed effect of the hypochlorite used in this study, could be as result of characteristic properties of sodium hypochlorite. Sodium hypochlorite has bleaching properties. This makes it a useful stain remover. Additionally, it is a useful disinfectant at lower concentrations. It also has defatting and saponification properties. The observed fissured or “cracked” background could be as a result of the

cracking effect on the smear. In furtherance to this, the study by Frimpong *et al*, must have found the method effective because the concentration of hypochlorite used was 1%. This study used 3.5%. The only limitation to the use of the sedimentation method would be the delay in diagnosis due to the overnight sedimentation, but the clarity of the slides produced by this method will reduce the slide reading period and so compensate for the delays. Overall results obtained from this study have shown that BU specimens concurrently processed physically and chemically produced relatively higher AFB detection rates. Another challenge is that sodium hypochlorite treated samples appear “washed off” after staining and so care should be taken when staining. The “washed off” effect can be reduced if (i) the concentration of hypochlorite used is relatively reduced. (ii) The exposure period for the sample and the hypochlorite is also reduced. This is in accordance with other findings including a systematic review of 83 publications on the subject which concluded that, compared with direct smears, concentration of sample after chemical treatment was better (Steingart *et al*, 2006; Chew *et al*, 2011). This is also a convenient method for the presumptive diagnosis of *Mycobacterium ulcerans* infection, especially in endemic under-resourced communities where the lack of adequate infrastructure, technical and financial resources hampers the application of a more sensitive method such as PCR for routine laboratory confirmation of the disease. The findings from this study will also reduce some peculiar limitations associated with the detection of AFB, especially with swab specimens from paucibacillary lesions. These findings will clearly improve smear microscopy, the most widely used test for BUD diagnosis and the generation of false negative results will be relatively reduced. This will ultimately enhance disease control measures and public health. Increasing sensitivity of AFB detection by microscopy is, therefore, of public health priority (Farnia, 2002).

5.3 Decontamination protocols in *M. ulcerans* culture

The presence of contaminants in specimens for primary isolation of *Mycobacterium ulcerans* is a frequent occurrence and poses a challenge in *M. ulcerans* culture.

In this study, preliminary investigation of eight selected decontamination protocols on nine clinical isolates of potential skin contaminants including *Bacillus cereus*, *Candida albicans*, *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella species*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Staphylococcus epidermidis*, , *Staphylococcus aureus* was done.

Five of the ten chemical agents were able to totally inhibit growth of all the test microbes. The least effective was 20% benzalkonium chloride which was not able to inhibit growth of three out of nine of the potential contaminants. Among the potential contaminants, *Pseudomonas aeruginosa* and *Aspergillus niger* were the two most challenging to eliminate. *Pseudomonas aeruginosa* is generally an environmental organism and a very successful opportunistic pathogen. Its pathogenicity is mediated by the production of toxin A. This toxin kills host cells by inhibiting protein synthesis and the production of several proteolytic substances and haemolysins. In addition, *P. aeruginosa* can survive under harsh conditions and displays intrinsic characteristics that enable it to exhibit resistance to a wide range of antimicrobials; this facilitates the ability to survive in hospital environments, and same reason could be attributed to the resistance recorded.

In the case of *Aspergillus niger*, while a considerable amount of information is available about fungal resistance, data on the mechanisms of fungal resistance to chemical biocides is very limited. One commonly accepted theory about the mechanism of fungal resistance to

biocides involves natural (intrinsic) resistance. Generally, vegetative fungi and fungal spores are more resistant than vegetative bacteria to decontamination agents. It is often assumed that fungi are inactivated in a similar or identical fashion to vegetative bacteria. However, the different structural and chemical properties of bacteria and fungi might challenge this assumption. It is likely that the first interaction between biocide and fungus occurs at the cell surface, and then the biocide crosses the cell wall (or outer membrane) to reach its target sites within the cell. Some biocides are likely to have a predominate effect on the outer layers. However, few antimicrobial agents are intended to focus on the fungal cell wall as a major, or sole target and this could account for the challenge in eliminating *Aspergillus niger* with the chemical agents used in the current study.

In a related study by McClean *et al*, 5% oxalic acid alone and in combination with NaOH was more effective in eliminating the contaminants. They also found that the most predominant contaminant was *Pseudomonas aeruginosa* (McClean *et al*, 2011). The differences observed between this study and that of McClean could be due to the microorganisms used, for whilst this study used stored clinical isolates, the target contaminants were found in association with the sample.

The current study further investigated the inhibitory effects of the decontamination protocols on the contaminants in BU samples. The highest activity of 98% was exhibited by 1% povidone iodine and 1% virkon, highlighting their effectiveness as decontamination agents. The lowest inhibitory activity was by 20% benzalkonium chloride at 86%.

5.4 Culture of *M. ulcerans* after decontamination

Of the eight decontamination protocols investigated, the highest mean recovery of *M. ulcerans* from culture was from 2% cetyl pyridinium chloride /4% sodium chloride (53.0%) and 0.5% virkon (51.0%) treated samples and the lowest from 0.5% and 1% povidone iodine (0%). The recovery from 1% cetyl pyridinium chloride / 2% sodium chloride and 5% oxalic acid were comparable at 44% and 41 % respectively. These two decontamination protocols have been extensively used.

In 1975 Smithwick found 1% cetyl pyridinium chloride / 2% sodium chloride useful for the transport of samples from peripheral to reference laboratories. He found it as useful as the conventional N-acetyl-L-cysteine in use. In this study its activity was also found to be useful. Whilst the 1% povidone iodine protocol did not yield any *M. ulcerans* from culture it recorded no contamination. This was also observed for 1% virkon disinfectant. To the best of our knowledge, this use of povidone iodine and virkon as a decontamination agent in the primary isolation of *Mycobacterium ulcerans* represents the first attempt.

The concentration of povidone iodine used in this study appeared to have affected the viability of the *M. ulcerans*. This implies that the decontamination protocols utilising the two concentrations of povidone iodine seems to affect not only the viability of the contaminating microbes, but it also inhibits the growth of the *M. ulcerans* in culture as well.

The conventional protocol which utilizes 5% oxalic acid had an effect that was comparable to 1% cetyl pyridinium chloride / 2% sodium chloride, though the contamination rate for the 5%

oxalic acid was relatively higher than that of 1% cetyl pyridinium chloride / 2% sodium chloride.

In a study conducted by Palomino & Portaels, they observed a marked reduction in the growth rate of *M. ulcerans* in culture due to the harmful effect of oxalic acid on the culture of *M. ulcerans* from the BACTEC system (Yajko *et al*, 1993 ;Palomino and Portaels, 1998) but others continued to use it. A study conducted in Ghana using of 5% oxalic acid gave an *M. ulcerans* isolation rate of 78% from tissue (Yajko *et al.*, 1993). This study also showed that a concentration of 5% oxalic acid seemed to have a marked effect on the viability of the *M. ulcerans*, this shows that an optimal concentration will be one between 5% and 10%.

Several factors may have contributed to the observed differences among which are the types of specimens used, contaminant load and the type of lesion. A paucibacillary lesion would be a limitation for the recovery of *M. ulcerans* in culture. Another important factor is that swab specimens collected from large open ulcers are prone to contamination by fungal spores among others which can only be removed by chemical agents with antifungal properties.

Studies have shown that cetylpyridinium chloride has antifungal properties (Phillips and Kaplan, 1976 ; Pardini *et al*, 2005). It does not only serve as a good decontamination agent but can be used effectively for the storage of specimens being transported from the peripheral centres to the reference laboratories for analysis especially in under-resourced endemic areas (Smithwick *et al*, 1975).

0.5% virkon had similar results and even though cetyl pyridinium has been used in the field of Mycobacteriology, 0.5% virkon in this arena. This study reports the first effective use of 0.5% virkon, a broad spectrum disinfectant for the isolation of *M. ulcerans* from swabs. The most important challenge associated with this study was that, the investigation required more assays for the same quantity of sample suspension and this affected the yield particularly in paucibacillary samples.

The importance of effectively controlling BUD in patients emphasizes the need to have an effective, inexpensive decontaminating agent for the processing of specimens for the isolation of *M. ulcerans* from swabs. The successful generation of more isolates will provide information on clinical epidemiology of the disease and treatment outcomes. This will also help with policy formulation and efficient health delivery service especially for the BU endemic communities by the Ghana Health Service and the National Buruli Ulcer Control Programme (NBUCP). In all of the diagnostic tests the observed trends were that the best performing protocols had relatively high sensitivities, generally higher than the corresponding specificities.

5.5 Drug susceptibility profile of *M. ulcerans* isolates

Following the successful clinical trials of rifampicin and streptomycin for the management of Buruli ulcer, antibiotic treatment has gradually replaced surgery as the definitive treatment for the condition (Etuafu *et al*, 2003; WHO, 2004).

This study determined the susceptibility profiles of thirty four *M. ulcerans* isolates to rifampicin and streptomycin *in-vitro* using the Canetti agar proportion method (Canetti *et al*, 1963). Twenty eight of the *M. ulcerans* isolates were susceptible to rifampicin at a media incorporation concentrations of 40µg/ml whilst one isolate was resistant to streptomycin at 4µg/ml. None of the isolates tested showed resistance to both rifampicin and streptomycin. Several studies have shown similar trends in susceptibility patterns both *in-vitro* and *in-vivo* (Dega *et al*, 2000; Thangaranj *et al*, 2002; Espey *et al*, 2002).

One important challenge associated with this study was that, the clumpy, aggregative nature of the *M. ulcerans* colonies on the Lowenstein-Jensen media made the determination of CFU difficult. A strict adherence to Canetti's less than 1% proportion evaluation criterion was difficult to achieve. A slight modification to the criterion was made in which an observation of no growth on drug incorporated media was the criteria for susceptibility of isolate to the test drug and whilst growth on drug-incorporated medium was compared to controls set up.

In-vitro determination of the susceptibility profiles of isolates obtained from clinical samples is an essential component for effective case management and public health (Marsollier *et al*, 2003; Asiedu and Wansbrough-Jones, 2007).The present effective use of rifampicin and streptomycin with particularly large lesions (size > 15 cm) raises expectations about the continuous use of antibiotics for effective management of the disease (Thangaranj *et al*, 2000).

This study however detected isolates that were resistant to rifampicin whilst others were resistant to streptomycin *in-vitro*. Though the study found no isolates that were resistant to both antibiotics, this information is indicative of the continuous need for vigilance on the

possible emergence of resistant *M. ulcerans* strains in the endemic communities. This could be as a result of antibiotic abuse. The emergence of resistant strains will have some long-term implication for disease control, particularly where individuals with Buruli ulcer may also be harbouring MTB in these communities, considering the fact that these communities are not only endemic for Buruli ulcer, but also for tuberculosis. The usefulness of rifampicin and streptomycin for BU treatment is evidenced by reported short healing durations ranging from 2-4 weeks (Chauty *et al*, 2007,).

Study limitations and solutions

1. Intra and inter-specific validation challenges as a result of subjective evaluation of the detection of AFB by microscopy. This was solved when readings were made by principal investigator and validated by results from two other laboratory workers (kappa statistics).
2. The characteristically clumpy and aggregative nature of *M. ulcerans* affected quantum of AFB detected per smear field per evaluated procedure. Solved by keeping materials for inoculums preparation on ice to facilitate dispersion when vortexed.
3. The problem was partially solved with quality control checks with inter-observer tools like the use of Kappa statistics
4. Due to the comprehensive procedures applied, coupled with limited incubator space, the sample size for the modified methods had to be limited. This, however did not significantly affect results

5. Some slow growing *M. ulcerans* strains could have growth periods exceeding stipulated 12 week observation period
6. Solving limitations associated modified culture techniques was challenging due to limited study period.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS AND FUTURE PROJECTIONS

6.1 Conclusions

New cases of Buruli ulcer are reported each year, some with large ulcers leading to severe consequences for patients. The control of the disease must therefore be a public health priority. The challenges associated with the diagnosis are real and must be addressed. Some of the ways by which this problem can be addressed include the adoption and the improvement of much simpler laboratory diagnostic methods that can easily be applied in health facilities within endemic communities. Microscopy for the detection of AFBs represents a simple method which can be used in almost all laboratories with a microscope and trained staff.

From this study BU specimens processed with phenol ammonium sulphate with an overnight sedimentation provides slides which are clear and easy to examine. In the peripheral centres where equipments like centrifuge may not be readily available, this sedimentation procedure can be used with adequate training. This is a simple procedure which technical staff at the peripheral level can perform. This strategy has been successfully applied in the National TB control where personnel were trained to acquire skills for AFB detection by microscopy.

Findings from this study also indicated that preparations of 2% cetyl pyridinium chloride / 4% sodium chloride and 0.5% virkon were effective decontamination agents and could easily

be used in the isolation of *M. ulcerans* from swabs. These preparations produced relatively lower contamination rates in *M. ulcerans* culture than the current conventional method in use.

Furthermore, 2% cetyl pyridinium chloride / 4% sodium chloride, a known fungicide can control fungi and their spores in *M. ulcerans* culture. Studies have shown that it is also being effectively used for the storage of MTB specimens and can also be used for the storage of BU specimens during transportation of specimens from peripheral to reference centres for laboratory analysis. This study therefore recommends the use of 2% cetyl pyridinium chloride / 4% sodium chloride in routine laboratory decontamination of BU specimens, particularly swabs.

Decontamination protocol with the use of 0.5% Virkon, can also be considered for the isolation of *M. ulcerans*, after further investigations have been conducted into its usefulness in the field situation alongside the conventional protocol especially with swab samples.

Investigating the drug susceptibility profile of the isolates can give important information on evolving resistant strains. This study recommends the regular susceptibility testing of isolates and surveillance of susceptibility results (as is being done for *Mycobacterium tuberculosis*). For all isolates obtained for the necessary remedial measures to be taken pertaining to administration of antibiotics.

6.2. Recommendations

In consideration of the outcomes from this investigation, it is recommended that policy makers and implementers on the control of Buruli ulcer disease should consider these propositions .

- ❑ The Ministry of Health through the National Buruli ulcer control program (NBUCP) should support programmes aimed at early BU disease detection. This can be done by initiating a laboratory diagnostic program that incorporates the use of this modified method in tandem with the current conventional methods used. This can help authenticate its usefulness in field settings.
- ❑ That further studies be conducted with lower concentrations of povidone iodine which was found to be effective at inhibiting the growth of the contaminants in the Buruli ulcer samples.
- ❑ The Ministry and the control programme can fund the provision of items like simple light microscopes, materials and reagents in laboratories within the endemic communities. The provision of basic laboratory training for personnel can serve a useful purpose.
- ❑ In view of the late reporting attitudes of the cases, more ulcers are seen at the clinics making swabbing a convenient specimen collection method. Swab specimens from open ulcers would potentially carry more contaminants including bacterial and fungal spores which sporulate under favourable conditions. The use of 2% CPC / 4% NaCl as a decontaminant can also be used for specimen processing alongside the conventional 5% oxalic acid method.

- ❑ In furtherance to this, education on the disease should be intensified in the endemic areas because despite years of community sensitization, patients continue to report late to the clinic.
- ❑ That a surveillance team should be set up to monitor the use /misuse of streptomycin and rifampicin and its potential effect on the emerging resistant *M. ulcerans* strains. This team can start by obtaining a collection of *M. ulcerans* isolates obtained in all of the endemic communities in the country per year and determine their sensitivity to the recommended drugs. This can be done in a reference laboratory such as the Public Health Reference Laboratory of the Korle-Bu Teaching Hospital.

6.3 Future Projections

Microscopy as a diagnostic tool for the laboratory diagnosis of Buruli ulcer represents a very essential component in the health delivery system especially in developing countries including Ghana.

Though this has been widely explored in the area of MTB studies, *M. ulcerans* studies in this area remains vastly unexplored. It is projected that a lot of work will be conducted along with publications and many more technicians and students trained in this area.

In line with this, more studies will be conducted aimed at improving basic diagnostic tools especially in the area of BUD diagnosis.

REFERENCES

- Bayley A.C.** (1971). Buruli ulcer in Ghana. *Br Med J* 2, 401–402.
- Addo P., Owusu E., Adu-Addai B., Quartey M., Abbas M., Dodoo A. and Ofori-Adjei D.** (2005). Findings from a Buruli ulcer mouse model study. *Ghana Medical Journal* 39, 55-63.
- Adjei O. and Asiedu A.** (1998). Phenytoin in the treatment of Buruli ulcer. *Trans. Soc. Trop. Med. Hyg*, 92, 108-109.
- Adusumilli S., Mve-Obiang A., Sparer T., Meyers W., Hayman J. and Small P.L.** (2005). *Mycobacterium ulcerans* toxic macrolide, mycolactone modulates the host immune response and cellular location of *M. ulcerans* in vitro and in vivo. *Cell Microbiol.* 7, 1295-1304.
- Aiga H., Amano T., Cairncross S., Adomako J. and Nanas O.K.** (2004). Assessing water-related risk factors for Buruli ulcer: A case-control study in Ghana. *Am J Trop Med Hyg*, 71, 387–392.
- Allen B. W. and Mitchison D. A.** (1992). Counts of viable tubercle bacilli in sputum related and culture grading. *Med. Lab. Sci*, 49, 94-98.
- American Thoracic Society** (1981). Diagnostic standards and classification of tuberculosis and other mycobacterial diseases. *Am. Rev. Respir. Dis.* 123, 343–358.
- Amofah G., Bonsu F., Tetteh C., Okrah J. and Asamoah K.** (2002). Buruli ulcer in Ghana: Results of a national case search. *Emerg. Infect. Dis.* 8 167–170.

Amofah G.K., Sagoe-Moses C., Adjei-Acquah C. and Frimpong E.H. (1993). Epidemiology of Buruli ulcer in Amansie West district of Ghana. *Trans. R. Soc. Trop. Med. Hyg.* 87, 644-645.

Ängeby K.A., Alvarado-Galvez C., Pineda-Garcia L and Hoffner S.E. (2000). Improved sputum microscopy for a more sensitive diagnosis of pulmonary tuberculosis. *J Tuberc Lung Dis.* 4, 684-687.

Ängeby K.A., Hoffner S.E. and Diwan V.K. (2004). Should the ‘bleach microscopy method’ be recommended for improved case detection of tuberculosis? Literature review and key person analysis. *Int J Tuberc Lung Dis.* 8, 806-815.

Asiedu K. and Etuaful S. (1998). Socioeconomic implications of Buruli ulcer in Ghana: a three-year review. *Am J Trop Med Hyg.* 59, 1015–1022.

Asiedu K., Meyers W. and Agbenorku P. Clinical Features and treatment in Buruli ulcer. K. Asiedu, R. Sherpbier, AND M. Ravliogne (ED).(2000). Buruli ulcer. *Mycobacterium ulcerans* infection. *Global Buruli Ulcer Initiative, World Health Organization, Geneva, Switzerland*

Asiedu K. and Wansbrough-Jones M. (2007) *Mycobacterium ulcerans* infection (Buruli or Bairnsdale ulcer): challenges in developing management strategies. *Med J Aust;* 186:55–6.

Atlas R.M. (1995). Microorganisms of our World. *Mosby –Year Book Inc, USA.*

Aujoulat I., Zinsou J.C., Guedenon A. and Portaels F. (2003). Psychosocial aspects of health seeking behaviours of patients with Buruli ulcer in southern Benin. *Trop Med Int Health* 8, 750–759

Barker D.J. (1972). The distribution of Buruli disease in Uganda. *Trans R Soc Trop Med Hyg.* 66:867.

Barksdale L. and Kim K. S. (1977). *Mycobacterium*. *Bacteriol Rev.* 41(1): 217–372.

Baron E.J., Peterson L.R., and Finegold S.M. (1994). Bailey and Scott's Diagnostic Microbiology. *Mosby Year book Inc. Publishing, USA*, 168-183.

BCG (1969). BCG vaccination against *mycobacterium ulcerans* infection (Buruli ulcer). First results of a trial in Uganda. *Lancet*, 1, 111.

Benowitz A.B., Fidanze S., Small P.L. and Kishi Y. (2001). Stereochemistry of the core structure of the mycolactones. *J Am Chem Soc*; 123:5128.

Bretzel G., Siegmund V., Niesche J., Herbing K.H., Thompson W., Klutse E., Crofts K., Massavon W., Etuaful S., Thompson R., Asamoah-Opore K., Racz Pvloten F., Van Berberich C., Kruppa T., Ampadu E., Fleischer F. and Adjei O. (2007). A stepwise approach to the laboratory diagnosis of Buruli ulcer disease. *Trop.Med.Int.Health.* 12, 89-96.

Bruchfeld J., Aderaye G., Palme I.B., Bjorvatn B., Källenius G. and Lindquist L. (2000). Sputum concentration improves diagnosis of tuberculosis in a setting with a high prevalence of HIV. *Trans R Soc Trop Med Hyg.* 94:677-680

Cadapan L.D., Arslanian R.A., Carney J.R., Zavala S.M., Small P.L. and Licari P. (2001). Suspension cultivation of *Mycobacterium ulcerans* for the production of mycolactones. *FEMS Microbiol. Lett* 205, 385-389

Canetti G., Fox W., Khomenko A., Mahler H.T., Menon N.K., Mitchison D.A., Rist N.A. and Smelev N.A. (1963). Advances in techniques of testing mycobacterium drug sensitivity,

and the use of the sensitivity tests in tuberculosis control program. *Bulletin of the World Health Organization*, 41, 21- 43.

Cassisa V., Chauty A., Marion E., Ardant M.F., Eyangoh S., Cottin J., Aubry J., Kousseimou H., Lelievre B., Ferec S., Tekaiia F., Johnson C. and Marsollier L.(2010) Use of fine needle aspiration for diagnosis of Mycobacterium ulcerans infection. *J Clin Microbiol.* 48(6):2263-4, PMID20375229 , PMCID PMC2884471 .

Cattamanchi A., Davis J., Pai M., Huang L., Hopewell P.C. and Steingart K.R.. (2010). Does bleach-processing increase the accuracy of sputum smear microscopy for diagnosing pulmonary tuberculosis?. *J Clin Microbiol*, 48, 2433-2439.

Cattamanchi A., Dowdy D.W., Davis J.L., Worodria W., Yoo S., Joloba M., Matovu J., Hiopewell P.C. and Huang L. (2009) Sensitivity of direct versus concentrated sputum smear microscopy In HIV-infected patients suspected of having pulmonary tuberculosis. *BMC Infectious Diseases*, 9, 153.

Chauty A. (2007). Treatment of Buruli ulcer with the combination rifampicin and streptomycin in Be´nin. . *Report of the 8th Annual World Health Organization Advisory Group Meeting on Buruli ulcer. World Health Organization, Geneva, Switzerland.* <http://www.who.int/gtb-buruli/publications/index.html>, in press.

Chew R., Calderon C., Schumacher S.G., Sherman J.M., Caviedes L., Patricia F., Coronel J., Valencia T., Herrera B., Zimic M., Huaroto L., Sabogal I., Rod E.A., Gilman R.H. and Evans C.A. (2011). Evaluation of bleach-sedimentation for sterilising and concentrating Mycobacterium tuberculosis in sputum specimens. *BMC Infectious Diseases*, 11, 269.

Chukwuekezie O., Ampadu E, Sopoh G., Dossou A., Tiendrebeogo A., Sadiq ., Portaels F. and Asiedu K. (2007). Buruli ulcer, Nigeria. *Emerg. Infect.Dis.* 13, 782–783.

Clancy J., Dodge R. and Lunn H.F. (1962). Study of a Mycobacterium causing skin ulceration in Uganda. *Ann.Soc.Belg.Med.Trop*, 42, 585-590.

Clancy JK., Dodge O.G., Lunn H.F. and Odouri M.L. (1961). Mycobacterial skin ulcers in Uganda. *Lancet*, i, 951-954.

Collins C., Grange J. and Yates M.D. (1997). Tuberculosis bacteriology (organization and practice) *Reed Educational and professional Publishing Ltd UK.*, 2nd ed.

Connors D.H. and Lunn F. (1965). Mycobacterium ulcerans infection. *Int. J. Leprosy*, 33, 698.

Connor D.H. and Lunn F. (1966). Buruli ulceration: a clinicopathologic study of 38 Ugandans with *M. ulcerans* ulceration. *Arch. Pathol*, 81, 183.

Cook, A., Makerere Medical School Library. (1897). Mengo hospital notes. *Br Med .J*, 2, 378-9.

Cornet L., Richard-Kadio M., N'Guessen H.A., Yapo P., Hossoko H., Dick R. and Casanelli J.M.(1992.) Treatment of Buruli ulcers by excision-graft. *Bull. Soc. Pathol. Exot.* :85, 355-358.

Corper H.J. and Stoner R.E. (1946). Improved procedure for the diagnostic culture of mammalian tubercle bacilli. *J Lab Clin Med*, 31, 1364.

Corper H.J. and Nelsen C.R. (1949). Methods for concentrating acid fast bacilli *American Journal of Clinical Pathology*, 19, 269-273.

Daley P., Michael J.S., Kalaiselven S., Latha A., Mathai D., John K.R. and Pai M. (2009) A pilot study of short-duration sputum pre-treatment procedures for optimising smear microscopy for tuberculosis. *PLoS One*, 4:e5626.

Debacker M., Zinsou C, Aguiar J, Meyers W.M. and Portaels F. (2003). First case of *Mycobacterium ulcerans* disease (Buruli ulcer) following a human bite. *Clin.Infect.Dis*, 36, e67-e68.

Dega H., Bentoucha A, Robert J., Jarlier V. and Grosset J. (2002). Bactericidal activity of rifampin amikacin against *Mycobacterium ulcerans* in mice. *Antimicrob. Agents Chemother.*, 46, 3193–3196.

Dega H., Robert J., Bonnafous P., Jarlier V. and Grosset J. (2000). Activities of several antimicrobials against *Mycobacterium ulcerans* infection in mice. *Antimicrob.*

Agents Chemother, 44, 2367–2372.

Dodge O.G. (1964). Mycobacterial skin ulcer in Uganda: histopathological and experimental aspects. *J. Pathol. Bacteriol*, 88, 167.

Eddyani M., Debacker M., Martin A., Aguiar J., Johnson R.C., Uwizeye C., Fissette K. and Portaels F. (2008). Primary culture of *Mycobacterium ulcerans* from human tissues after storage in semi-solid Transport medium *Journal of Clinical Microbiology*, A, 4669-72.

Eddyani M., Fraga A., Schmitt F., Uwizeye C., Fissette K., Johnson R.C., Aguiar Sopoh G., Barogui Y., Meyers W.M., Pedrosa J. and Portaels F. (2009). Fine needle aspiration, an efficient sampling technique for bacteriological diagnosis of non-ulcerative Buruli ulcer. *Journal of Clinical Microbiology*, 47, 1700-1704.

Ersoz C., Polat A., Serin M.S., Soylu L. and Demircan O. (1998). Fine needle aspiration (FNA) cytology in tuberculous lymphadenitis. *Cytopathology*, 9, 201–207.

Espey D.K., Djomand G., Diomande I., Dosso M., Saki M. Z., Kanga J. M., Spiegel R. A., Marston B. J., Gorelkin L., Meyers W.M., Portaels F., Deming M.S., Horsburgh C.R. Jr. (2002). A pilot study of treatment of Buruli ulcer with rifampin and dapson. *Int. J. Infect. Dis.* 6, 60-65.

Etuaful, E.K., Quarshie E., Van Der Werf T.S., Van der Graaf W.T., King C.H. and Ashford D.A. (2003). Histopathologic features of *Mycobacterium ulcerans* infection. *Emerg. Infect. Dis*, 9, 651–656.

Etuaful, S. Carbonnelle. B., Grosset J, Lucas S., Horsfield C., Phillips, R. Evans M., Ofori Adjei D., Klustse E., Owusu-Boateng J., Amedofu G. K., Awuah P., Ampadu E., Amofah G., Asiedu K. and Wansbrough-Jones M. (2003). Bactericidal activity of rifampicin and streptomycin treatment for early human *M. ulcerans* lesions. *In: Report of the 6th WHO advisory group meeting on Buruli ulcer; Geneva, Switzerland Geneva: World Health Organization.*

Evans, M. R., Phillips R., Etuaful S. N., Amofah G., Adomaku J., Adjei O., Dennis-Antwi J., Lucas S.B. and Wansbrough-Jones M.H. (2003). An outreach education and treatment project in Ghana for the early stage of *Mycobacterium ulcerans* disease. *Trans. R. Soc. Trop. Med. Hyg.* , 97, 159-160.

Farnia P.I. (2002). Improving Sensitivity of Direct Microscopy for Detection of Acid Fast Bacilli in sputum Use Chitin in Mucus Digestion. *Journal of Clinical Microbiology*, 508-511.

Fyfe J.A.M., Caroline J.L., Paul D.R., Maria G, Aina S, Joseph A. and Timothy P.S. (2007). Development and application of two multiplex real-time PCR assays for the detection of *Mycobacterium ulcerans* in clinical and environmental samples. *Applied and Environmental Microbiology* 73, no. 15 (August): 4733-4740. doi:10.1128/AEM.02971-06.

Fletcher R.H., Fletcher S.W. and Wagner E.H. (1982). Clinical epidemiology - the essentials. *Baltimore: Williams and Wilkins*,: 41-58.

Flood P., Street A., O'Brien P. and Hayman J. (1994). Mycobacterium ulcerans infection on Phillip Island, Victoria. *Med J Aust*;, 160, 160.

Frimpong E.H., Adukpo R. and Owusu-Darko K. (2005). Evaluation of two novel Ziehl Neelsen methods for tuberculosis diagnosis. *WAJM*, 24, 316-320.

Gasparini R., Pozzi T., Magnelli R., Fatighenti D., Giotti E., Polisen G., Pratelli M., Severini R., Bonanni P. and De Feo L. (1995) Evaluation of in vitro efficacy of the disinfectant Virkon. *European Journal of Epidemiology*, 11 (2); 193-197.

Gebre-Selassie S. (2003). Evaluation of the concentration sputum smear technique for the laboratory diagnosis of pulmonary tuberculosis. *Trop Doct*, 33, 160-162.

George K.M., Chatterjee D., Gunawardana G., Welty D., Hayman J., Lee R. and Small P.L. (1999). Mycolactone: a polyketide toxin from Mycobacterium ulcerans required for virulence. *Science* 283, 854-857.

Gooding T.M., Kemp A.S., Robins-Browne R.M., Smith M. and Johnson P.D. (2003). Acquired T-Helper 1 lymphocyte anergy following infection with *mycobacterium ulcerans*.. *Clin Infect Dis*, 36.

Gooding T.M., Johnson P.D., Campbell D.E., Hayman J.A., Hartland E.L., Kemp A. S. and Robins-Browne R. M. (2001). Immune response to infection with *Mycobacterium ulcerans*. *Infect. Immun*, 69, 1704-1707.

Gooding T.M., Kemp A.S., Robins-Browne R.M., Smith M. and Johnson P.D. (2003). Acquired T-helper 1 lymphocyte anergy following infection with *Mycobacterium ulcerans*. *Clin Infect Dis* 36: 1076–1077

Grandjean L., Martin L., Gilman R., Valencia T., Herrera B., Quino W., Ramos E., Rivero M., Montoya R., Escombe A., Coleman D., Mitchison D. and Evans CA. (2008), TB & MDRTB testing by direct sputum culture in selective broth without decontamination or centrifugation. *J Clin Microbiol* 46:2339-44.

Grange J.M., (1988). *Mycobacteria* and human disease. Edward Arnold (Publishers) Pty Ltd. London. Great Britain

Guarner J., Bartlett J., Whitney E.A., Raghunathan P.L., Stienstra Y., Asamoah K., Etuaful S., Klutse E., Quarshie E., van der Werf T.S., van der Graaf W.T., King C.H. and Ashford D.A. (2003). Histopathologic features of *Mycobacterium ulcerans* infection. *Emerg. Infect. Dis* 9, 651–656.

Habeenzu C., Lubasi D. and Fleming A.F. (1998). Improved sensitivity of direct microscopy for detection of acid-fast bacilli in sputum in developing countries. *Trans R Soc Trop Med Hyg*, 92, 415-416.

Havel A. and Pattyn S.R. (1975). Activity of rifampicin on *Mycobacterium ulcerans*. *Ann. Soc. Belg. Med. Trop.* , 55:, 105-108.

Hayman J. (2003). *M. ulcerans* disease in Australia. In *Report of the 6th WHO Advisory Group Meeting on Buruli ulcer* ,10-13 March ,WHO headquarters ,Geneva ,Switzerland Geneva, World Health Organization 2003:48 (WHO/CDS/CPE/GBUI/2003.8).

Hayman J. (1993). Out of Africa: observations on the histopathology of *Mycobacterium ulcerans* infection. *J. Clin. Pathol*, 46, 5.

Hayman J. and McQueen A.(1985). The pathology of *Mycobacterium ulcerans* infection. *Pathology*,17,594-600.

Herbinger K.H., Adjei O., Awua –Boateng N.Y., Nienhuis W.A., Kunaa L., Siegmund V., Nitschke J., Thompson W., Klutse. E., Agbenorku P., Schipf A., Reu S., Racz P., Fleischer B., Beissner M., Fleischmann E., Helfrich K., Van der Werf T.S., Loscher T. and Bretzel G. (2009). Comparative study of the sensitivity of different diagnostic methods for the laboratory diagnosis of Buruli ulcer disease. *Clin. Infect. Diseases*, 48, 1055-1064.

Hernandez A., Martro E., Matas L., Martin M. and Ausina V. (2000). Assessment of in-vitro efficacy of 1% Virkon against bacteria, fungi, viruses and spores by means of AFNOR guidelines". *Journal of Hospital Infection*, 46 (3), 203–209.

Hockmeyer W.T., Krieg R.E., Reich M. and Johnson R.D. (1978). Further characterization of *Mycobacterium ulcerans* toxin. *Infect. Immun.*, 21, 124.

Horsburgh R.C. Jr. and Meyers W.M. in: Horsburgh CR, Jr, Nelson AM, EDS: - (1997). Pathology of Emerging Infections Buruli ulcer. *American Society for Microbiology*., 119, 134. .

Hospers I.C., Wiersma I., Dijkstra P.U., Stienstra Y., Etuaful S., Ampadu E.O. and Van der Graaf T.S. (2005). Distribution of Buruli ulcer lesions over body surface area in a large case series in Ghana: uncovering clues for transmission. *Trans R Soc Trop Med Hyg*, 99, 196.

Huygen K. (2003). Prospects for vaccine development against Buruli disease. *Expert. Rev. Vaccines*, 2, 561-569.

Johnson P.D.R., Stinear T., Small P.L.C., Pluschke G., Merrit R.W., Portaels F., Huygen K., Hayman J.A. and Asiedu K. (2005). Buruli ulcer (*M. ulcerans* Infection): New insights, new hope for disease control. *DOI:10.1371/journal.pmed. 0020173.PLoSMedicine*, 2, 5, e173.

Junghans T., Um Boock A., Vogel M., Schuette D., Weinlaeder H. and Pluschke G. (2009). Phase change material for thermotherapy of Buruli ulcer: a prospective observational single centre proof-of-principle trial. *PLoS Negl Trop Dis*, 3, e380.

Reimer K. and Fleischer W. A. (1997). Povidone-iodine in antiseptics — State of the art. *Dermatology*, 195(Suppl 2) 3–9

Karl-Heinz Z. (2012). Ammonium Compounds in Ullmann's Encyclopedia of Industrial Chemistry Wiley-VCH, Weinheim. doi:10.1002/14356007.a02_243.

Kaser M., Ruf M.T., Hauser J., Marsollier L. and Pluschke G. (2009). Optimized method for preparation of DNA from pathogenic and environmental mycobacteria. *Appl Environ Microbiol* 75: 414-418.

Kent P.T. and Kubica G. (1985). *Public health mycobacteriology: A guide for the level III laboratory.*, Atlanta, Centers for Disease Control.

Krieg R.E., Wolcott J.H. and Meyers W.M. (1979). Mycobacterium ulcerans infection: treatment with rifampin, hyperbaric oxygenation and heat. *Aviat. Space Environ. Med*, 50, 888-892.

Krieg R.E., Hockmeyer W.T., and Connor D.H., (1974). Toxin of *Mycobacterium ulcerans*: production and effects in guinea pig skin. *Arch. Dermatol.*, 110, 783..

Kubica G.P. (1980). Correlation of acid-fast staining methods with culture results for mycobacteria. *Bull. Int. Union Tuberc.*, 55, 117–124.

Lennette E., Ballows A., Hausler W. Jr. and Shadomy J.R.H. (1985). *Manual of Clinical Microbiology. 4th ed*, American Society for Microbiology. Washington DC. USA.

Lester J. and Layfield M.D. (2007). Fine-needle aspiration in the diagnosis of head and neck lesions: a review and discussion of problems in differential diagnosis. *Diagn. Cytopathol.* **35**:798–805.

Lumb R., Ardian M., Waramori G., Syahrial H., Tjitra E., Maguire G.P., Anstey N.M. and Kelly P.M. (.2006). An alternative method for sputum storage and transport for Mycobacterium tuberculosis drug resistance surveys. *Int. J. Tuberc. Lung Dis*, 10, 172–177.

MacCallum P., Tolhurst J.C., Buckle G. and Sissons H.A. (1948). A new mycobacterial infection in man. *J Pathol Bacteriol*, 60, 93–122.

Marsollier L., Prevot G., Honore N., Legras P., Manceau A.L., Payan C., Kouakou H. and Carbonnelle B. (2003). Susceptibility of Mycobacterium ulcerans to a combination of amikacin/rifampin. *Int. J. Antimicrob. Agents*, 22, 562–566.

Marsollier L., Honore´ N., Legras P., Manceau A.L., Kouakou H., Carbonnelle B. and Cole S.T. (2003). Isolation of three Mycobacterium ulcerans strains resistant to rifampin after experimental chemotherapy of mice. *Antimicrob. Agents Chemother.* **47**, 1228–1232.

Marsollier L., Robert R., Aubry J., Andre J.S., Kouakou H., Aubry J., Legras P., Kouakou H., Manceau A., Mahaza C. and Carbonnelle B. (2002). Aquatic insects as a vector for *Mycobacterium ulcerans*. *Appl Environ Microbiol* 68, 4623–4628.

Marsollier L., Severin T., Aubry J., Merritt R.W., Saint J. P., Andre J.S., Legras P., Manceau A.L., Chauty A., Carbonnelle B. and Cole S. T. (2004). Aquatic snails, passive hosts of *Mycobacterium ulcerans*. *Appl. Environ. Microbiol*, 70, 6296-6298.

Marston B.J., Diallo M.O., Horsburgh C.R. Jr., Diomande I., Saki M.Z., Kanga J.M., Patrice G., Lipman H.B., Ostroff S.M. and Good R.C. (1995). Emergence of Buruli ulcer disease in the Daloa region of Côte d'Ivoire. *Am. J. Trop. Med. Hyg*, 52, 219 224.

McClellan M., Stanley T., Stanley S., Maeda Y., Goldsmith C.E., Shepard R., Millar B.C., Dooley J.S. and Moore J.E. (2011). Identification and characterization of breakthrough contaminants associated with the conventional isolation of *Mycobacterium tuberculosis*. *Journal of Medical Microbiology*, 60, 1292-1298.

McOrist S., Jerret I.V., Andersen M. and Hayman J. (1985). Cutaneous and respiratory tract infection with *Mycobacterium ulcerans* in two koalas (*Phascolarctos cinereus*) *J Wildl. Dis*:21:171-3

Meyers W.M., Priuli G.B. and Portaels F. (1996). *Mycobacterium ulcerans* infection (Buruli ulcer): First reported patients in Togo. *Br J Dermatol* 134, 1116–1121.

Meyers WM., Shelley W.M. and Connor D.H. (1974). Heat treatment of *Mycobacterium ulcerans* without surgical excision. *Am J Trop Med Hyg* 23, 924-929.

Miørner H., Ganlov G., Yohannes Z. and Adane Y. (1996). Improved sensitivity of direct microscopy for acid-fast bacilli: sedimentation as an alternative to centrifugation for concentration of tubercle bacilli. *J Clin Microbiol*, 34, 3206-3207.

Molyneux D., Hotez P. and Fenwick A. (2005). "Rapid-impact interventions": how a policy of integrated control for Africa's neglected tropical diseases could benefit the poor. *PLoS Med*, 2, e336.

Monson M.H., Gibson D.W., Connor D.H., Kappes R. and Hienz H.A. (1984). *Mycobacterium ulcerans* in Liberia; a clinicopathological study of patients with buruli ulcer. *Acta Tropica*, 41, 165-72.

Muelder K. and Nourou A. (1990). Buruli ulcer in Benin. *Lancet*, 336, 1109–1111.

Mve-Obiang A., Lee R.E., Portaels F. and Small P.L. (2003). Heterogeneity of mycolactones produced by clinical isolates of *Mycobacterium ulcerans*: implications for virulence. *Infect Immun*, 71, 774.

Oluwasanmi J.O., Solankee T.F., Olurin E.O., Itayemi S.O., Alabi G.O. and Lucas A.O. (1976). *Mycobacterium ulcerans* (Buruli) skin ulceration in Nigeria. *Am.J.Trop.Med.Hyg*, 25, :122-128.

Pahlevan A.A., Wright D.J., Andrews C., George M., Small P.L. and Foxwel B.M L.(1999). The inhibitory action of *Mycobacterium ulcerans* soluble factor on monocyte/T-cell cytokine production and NF-kappa B function.*J.Immunol*, 163, 3928-3935.

Palomino J.C. and Portaels F. (1998). Effects of decontamination methods and culture conditions on viability of *Mycobacterium ulcerans* in the BACTEC system. *J. Clin. Microbiol*, 36, 402–408.

Pardini M., Varaine F., Iona E., Arzumanian E., Checchi F., Oggioni M.R., Orefici G., and Fattorini L. (2005). Cetyl-pyridinium chloride is useful for isolation of *Mycobacterium tuberculosis* from sputa subjected to long-term storage. *J. Clin. Microbiol*, 43, 442–444.

Pettit J.H., Marchette N.J. and Rees R. J. (1966). *Mycobacterium ulcerans* infection. Clinical and bacteriological study of the first cases recognized in South East Asia.*Br. J. Dermatol.*, 78, 187–197.

Phillips B.J. and Kaplan W. (1976). Effect of cetylpyridinium chloride on pathogenic fungi and *Nocardia asteroides* in sputum. *J. Clin. Microbiol.*, 3, :272–276.

Phillips R., Horsfield C., Kuijper S., Lartey A., Tetteh I., Etuaful S., Nyamekye B., Awuah P., Nyarko K.M., Osei-Sarpong F., Lucas S., Kolk A.H. and Wansbrough-Jones M. (2005). Sensitivity of PCR targeting the IS2404 insertion sequence of *Mycobacterium*

ulcerans in an assay using punch biopsy specimens for diagnosis of Buruli ulcer. *J. Clin. Microbiol.* **43**:3650–3656

Phillips R.O., Sarfo F.S., Osei-Sarpong F., Boateng A., Tetteh I., Lartey A., Adentwe E., Wansbrough-Jones M. (2009). Sensitivity of PCR targeting *Mycobacterium ulcerans* by use of fine needle aspirates for diagnosis Of buruli ulcer. Sensitivity of PCR targeting *Mycobacterium ulcerans* by use of fine needle Aspirates for diagnosis of Buruli ulcer *J. Clin. Microbiol* 47, 924-926.

Portaels F., Aguiar J. and Debacker M. (2004). *Mycobacterium bovis* BCG vaccination as prophylaxis against *Mycobacterium ulcerans* osteomyelitis in Buruli ulcer disease. *Infect Immun*, 72, 62.

Portaels F. and Hernandez P.R. (2006). The local immune response in ulcerative lesions of Buruli disease. *Clin.Exp.Immunol*, 143, 445-451.

Portaels F., Aguiar J., Debacker M., Steunou C., Zinsou C., Guedenon A. and Meyers WM. (2002). Prophylactic effect of *Mycobacterium bovis* BCG vaccination against osteomyelitis in children with *Mycobacterium ulcerans* disease (Buruli Ulcer).*Clin.Diagn.LabImmunol*, 9, 1389-1391.

Portaels F., Agular J., Fisette K., Fonteyne P.A., De Beenhouwer H., de Rijk P., Guedenon A., Lemans R., Steunou C., Zinsou C., Dumonceau J.M. and Meyers W.M.

(1997). Direct detection and identification of *Mycobacterium ulcerans* in clinical specimens by PCR and oligonucleotide-specific capture plate hybridization. *J.Clin.Microbiol.* 35:1097-1100

Portaels F., Meyers W.M., Ablordey A., Castro A.G., Chemlal K., de Rijk P., Elsen P., Fissette K., Fraga A.G., Lee R., Mahrous E., Small P.L., Stragier P., Torrado E., VanAerde A., Silva M.T. and Pedrosa J. (2008). First cultivation and characterization of *Mycobacterium ulcerans* from the environment. *PLoS Negl Trop Dis*, 2, e178.

Portaels F., Johnson P. and Meyers W.E. (2001). Buruli ulcer: diagnosis of *Mycobacterium ulcerans* disease. *World Health Organization, Geneva, Switzerland.*

Prevot G., Bourreau E., Pascalis H., Pradinaud R., Tanghe A., Huygen K. and Launois A.P. (2004). Differential production of systemic and intralesional gamma interferon and interleukin-10 in nodular and ulcerative forms of Buruli disease. *Infect.Immun.* 72, 958-965.

Pszolla N., Sarkar M.R., Strecker W., Kern P., Kinzl L., Meyers W.M. and Portaels F. (2003). Buruli ulcer: a systemic disease. *Clin.Infect.Dis*, 37, e78-e82.

Pradinaud R. (2003). Buruli ulcer situation in French Guiana. *In: Report of the 6th WHO Advisory Group Meeting on Buruli ulcer, 10-13 March, WHO headquarters, Geneva, Switzerland.*

Raghunathan P.L., Whitney E.A.S., Asamoah K., Stienstra Y., Taylor T.H. Jr., Amofah G.K., Ofori-Adjei D., Dobos K., Guarner J., Martin S., Pathak S., Klutse E., Etuaful S., van der Graaf W.T.A., van der Werf T., King C.H., Tappero J.W. and Ashford D.A. (2005) Risk factors for Buruli ulcer Disease (*Mycobacterium ulcerans* Infection): Results from a case-control study in Ghana. *Clinical infectious diseases*.40:1445-53

Read J.K., Heggie C.M., Meyers W.M. and Connor D.H. (1974). Cytotoxic activity of *Mycobacterium ulcerans*. *Infect. Immun*, 9, 1114.

Riemenschneider W. and Tanifuji M. (2002). Oxalic acid". in *Ullmann's Encyclopedia of Industrial Chemistry*, Wiley-VCH Weinheim. doi: 10.1002/14356007.a18_247.

Rondini S., Horsfield C., Mensah-Quainoo E., Junghanss T., Lucas S. and Pluschke G. (2005). Contiguous spread of *Mycobacterium ulcerans* in Buruliulcerlesionsanalysed by histopathology and real-time PCR quantification of mycobacterialDNA. *J.Pathol*

Ross B.C., Johnson P. D., Oppedisano F., Marino L., Sievers A., Stinear T.,

Hayman J. A., Veitch M. G. and Robins-Browne R. M. (1997). Detection of *Mycobacterium ulcerans* in environmental samples during an outbreak of ulcerative disease. *Appl. Environ. Microbiol*, 63, 4135-4138.

Satoskar R.L., Bhandarkar S.D. and Ainapure S.S. (2001). *Pharmacology and Pharmacotherapeutics*, Gobsons Papers Pvt. Ltd. India.

Selvakumar N., Rahman F., Gard R., Rajasekaran S., Mohan N.S., Thyagarajan K., Sundaram V., Santha T., Frieden T.R. and Narayanan P.R. (2002). Evaluation of the phenol Ammonium Sulphate sedimentation smears microscopy method for Diagnosis of Pulmonary Tuberculosis. *Journal of clinical Microbiology*, 3017-3020.

Sensi P., Margalith P. and Timbal M.T. (1959). Rifomycin, a new antibiotic—preliminary report". *Farmaco Ed Sci.*, 14, 146–147.

Siegmund V, Adjei O., Racz P, Berberich C. and Klutse E. (2005). Dry-reagent-based PCR as a novel tool for laboratory confirmation of clinically diagnosed *Mycobacterium ulcerans*-associated disease in areas in the tropics where *M. ulcerans* is endemic. *J. Clin. Microbiol*, 43, 271–276.

Singh B. and Mitchison D. (1954). Bactericidal Activity of Streptomycin and Isoniazid Against Tubercle Bacilli". *British Medical Journal*: . doi:10.1136/bmj.1.4854.130. PMC 2084433. PMID 13106497., 1 (4854), 130–132

Sizaire V., Nackers F., Comte E. and Portaels F. (2006). *Mycobacterium ulcerans* infection: control, diagnosis, and treatment. *Lancet Infect Dis*; 6:288–96.

Smith P.G., Reville W.D.I., Lukwago E. and Rykushin Y.P. (1976). The protective effect of BCG against *Mycobacterium ulcerans* disease: A controlled trial in an endemic area of Uganda. *Trans. R. Soc. Trop. Med. Hyg.*, 70, 449–457.

Smithwick R.W., Stratigos C.B. and David H. L. (1975). Use of cetylpyridinium chloride and sodium chloride for the decontamination of sputum specimens that are transported to the laboratory for the isolation of *Mycobacterium tuberculosis*. *J. Clin. Microbiol*, 1, 411–413.

Steingart K.R., Vivienne Ng., Henry M., Hopewell PC., Ramsay A., Cunningham J., Urbanczik R., Perkins MD., Mohamed A.A., Pai M. (2006). Sputum processing Methods to improve the sensitivity of smear microscopy for Tuberculosis: a systematic review., *Lancet Infect Dis*, 6, 664-674.

Stienstra Y., Van der Werf W.T., Asamoah K., Van der Werf T.S. (2002). Beliefs and attitudes toward Buruli ulcer in Ghana. *Am J Trop Med Hyg*, 67, 207–213.

Stinear T.P., Mve-Obiang A., Small P. L., Frigui W., Pryor M. J., Brosch R., Jenkin G. A., Johnson P. D., Davies J. K., Lee R. E., Adusumilli S., Garnier T., Haydock SF, Leadlay PF. And Cole ST. (2004). Giant plasmid- encoded polyketide syntheses produce the macrolide toxin of *Mycobacterium ulcerans*. *Proc. Natl. Acad. Sci. USA* 101, 1345-1349.

Thangaranj H.S., Adjei O., Allen B.W., Portaels F., Evans M.R.W., Banerjee D. K. and Wansbrough-Jones M.H. (2000). In vitro activity of ciprofloxacin, sparfloxacin, ofloxacin, amikacin and rifampicin against Ghanaian isolates of *Mycobacterium ulcerans*. *J. Antimicrob. Chemother.*, 45, 231–233.

Thangaranj H.S., Evans M.R.W. and Wansbrough-Jones M.H. (1999). *Mycobacterium ulcerans* disease; Buruli ulcer. *Trans. Royal Soc. Trop. Med. and Hyg.* 93: 337-340.

Thornton C.G., MacLellan K.M., Brink L.T. Jr., Lockwood D. E., Romagnoli M., Turner J., Merz W.G., Schwalbe R.S., Moody M., Lue Y. and Passen S. (1998) Novel Method for Processing Respiratory Specimens for Detection of Mycobacteria by Using C18 Carboxypropylbetaine: Blinded Study. *J. Clin. Microbiol.* 36 (7):1996.

Tuberculosis Division. International Union Against Tuberculosis and Lung Disease. (2005). Tuberculosis bacteriology priorities and indications in high prevalence countries: position of the technical staff of the Tuberculosis Division of the International Union Against Tuberculosis and Lung Disease. *Int.J.Tuberc. Lung Dis.*, 9, 355–361.

Van der Werf T.S., Van der Graaf W. T., Groothuis D.G. and Knell A. J. (1989). *Mycobacterium ulcerans* infection in Ashanti region, Ghana. *Trans.R.Soc.Trop.Med.Hyg.*, 83, 410-413.

Van der Werf T. S., Van der Graaf W.T., Groothuis D.G. and Knell A.J. (1999) *Mycobacterium ulcerans* infection in Ashanti Region, Ghana *Trans R Soc Trop Med Hyg*,410, 13- 21.

Van Deun A., Maug A.K., Cooreman E., Hossain M.A., Chambuganj N., Rema V., Marandi H., Kawria A. and Portaels F. (2000). Bleach-sedimentation method for

increased sensitivity Of sputum smear microscopy: does it work?. *Int J Tuberc Lung Dis*, 4, 371-376.

Vasanthakumari R. (1988). Concentrated sputum smear microscopy, a simple approach to better case detection. *Indian Journal of Tuberculosis*, 35, 80-84.

Veitch M.G., Johnson P. D., Flood P. E., Leslie D. E., Street A. C. and Hayman J. A.(1997). A large localized outbreak of Mycobacterium ulcerans infection on a temperate southern Australian island. *Epidemiol.Infect*, 119, 313-318.

Wells A.F. (1984). Structural Inorganic Chemistry. *Oxford: Clarendon Press. ISBN 0-198553706.*

Wilkinson D. and Sturm A.W. (1997). Diagnosing tuberculosis in a resource-poor setting: the value of sputum concentration. *Trans R Soc Trop Med Hyg*, 91, 420-421.

World Health Organization. (2003). Clinical features and treatment of pre-ulcerative Buruli lesions (Mycobacterium ulcerans infection). Report II of the Uganda Buruli Group. *British Medical Journal*, (1970)Geneva,:43 (WHO/CDS/CPE/GBUI/2003.8), 2:, 390–393.

World Health Organization. Portaels F, J. P., Meyers W.M, EDITORS. (2001). Buruli ulcer. Diagnosis of *Mycobacterium ulcerans* disease. A manual for health care providers.WHO/CDS/CPE/GBUI/2001.4. Geneva: The Organization.

World Health Organization. (2004). Provisional guidance on the role of specific antibiotics in the management of *Mycobacterium ulcerans* disease (Buruli ulcer). . Geneva: World Health Organization.

World Health Organization. (2001). *Buruli ulcer. Diagnosis of Mycobacterium ulcerans disease. A manual for health care providers*, WHO/CDS/CPE/GBUI/2001.4. Geneva: .

World Health Organisation. (2001). Buruli ulcer. Management of Mycobacterium ulcerans disease. . Geneva: World Health Organization,. (Accessed on February 12, 2008).Global Buruli Ulcer Initiative (GBUI).Available at: www.who.int/buruli/en

Yajko D.M., Nassos P. S., Sanders C. A., Gonzalez P. C., Reingold A. L., Horsburgh C. R. Jr, Hopewell P. C., Chin D. P. and Hadley W. K. (1993). Comparison of four decontamination methods for recovery of Mycobacterium avium complex from stools. *J. Clin. Microbiol.*, 31, 302–306.

Yeboah-Manu D., Bodmer T., Mensah-Quainoo E., Owusu S., and Ofori-Adjei D. (2004). Evaluation of decontamination methods and growth media for primary isolation of *Mycobacterium ulcerans* from surgical specimens. *J. Clin. Microbiol*, 42, 5875–5876.

Zumla A. and Grange J. (2002). Infection and disease caused by environmental mycobacteria. *Curr Opin Pulm Med*, 8(3), 166-72/gmdrt/foreword.html.Part III.

APPENDICES

APPENDIX A

A.1. Questionnaires administered to suspected Buruli ulcer cases in the Akuapem south and the Suhum- Kraboa-Coaltar district of the eastern region of Ghana

QUESTIONNAIRE FOR BU STUDY

I. General information

1. Name of treatment facility: _____
2. Name of interviewer _____
4. Name of patient: _____
5. Patient identification number (OPD and/or admission number): _____
5. Age (yrs): _____
6. Sex: M F
7. Location of patient (village or town): _____
8. District: _____

Lesions

11. Clinical form:

- I Nodule (N)
- II Plaque (Q)
- III Oedema (E)

IV Ulcer (U)

12. Size of lesion _____

13. Number of lesions _____

14. Location of lesions _____

15. Classification: New Recurrent

16 Patient on antibiotic treatment

I Yes

II No

17. Duration/period of clinical signs _____ -

18. Name of Physician requesting test _____ --

19. _____ Date _____ of _____ test
request _____ signed _____

20. Name of test requested

Reasons for requesting laboratory confirmation

I Diagnosis of a new case

II Follow up recurrent case

III Follow-up re-infection

IV Others

Specimen collection

21. Date specimen was collected _____

22. Laboratory specimen number _____

23. Specimen collection methods

- I Swabbing
- II Fine needle aspiration (FNA)
- III Punch
- IV Surgical excision

24. Specimen collected by _____

Specimen type and source

25. Type of specimens:

- I Exudates from ulcers
- II aspirates from nodules, papules, plaques or ulcers
- III Punch biopsy
- IV Surgical biopsy

26. Points within lesion where specimen was collected

Distal central proximal

27. Transport medium used for specimen

PBS Dubos broth P5 no medium

28. Date and time for which specimen was received for processing _____

29. Specimen received by _____

30. Specimen storage condition before processing

Room temperature 4° C

31. Type of microbiological examination(s)

- I ZN
- II PCR

III Culture

IV Histopathology

32. Indicate time for the commencement of specimen processing

33. Results

	Microbiological analysis		
Specimen	ZN(1)	PCR	Culture
Swabs(C)			
FNA (D)			

34. Name of laboratory scientist providing the results: _____ signature-----

35. Date: ___/___/___

36.

Comments:

37. Name of laboratory: _____

A.2. A copy of the consent form used in this study

CONSENT FORM

Principal Investigator: Enid Owusu

Address: School of Public Health, College of Health Sciences, University of Ghana

P.O.Box LG 13

Legon-Accra, Ghana

Buruli ulcer disease (BU) is a chronic debilitating skin disease caused by *Mycobacterium ulcerans*. BU presents in various forms including, nodules, papules and ulcers. In late stages ulcers may affect daily activities of the person. Spontaneous healing may occur and leaves patients disfigured or even disabled. Within West-African countries Ghana carries a large part of the disease burden. Amongst the district of Ghana, Suhum- Kraboa -Coaltar is highly affected by the disease. Unfortunately the exact way of disease transmission and disease reservoirs are not known.

Therefore we are conducting a joint study focusing on:

- Distribution of the disease within communities along the Densu river in order to contribute to the elucidation of transmission pathways and environmental reservoirs
- Identification of possible aspects influencing treatment seeking behavior
- Improvement of laboratory procedures to enable cultivation and subsequent identification of environmental reservoirs of the disease
- Improvement and assessment of BU treatment

Your involvement will require that you answer some personal questions and we would like to know more about your knowledge regarding the BUD. Your participation also includes registration of your residential coordinates. This will help us to identify possible factors that make people get the disease and also areas that are mainly affected by the disease. We hope that the results of our study will bring benefits to the community by contributing to the BU Control Program targeting communities that are severely affected. We will protect the information provided by you to the best of our ability. Your name will not appear in any report. However, all the ACBridge PhD students may sometimes look at the research records. You may leave the research at any time you wish. If you choose to take part, you can change your mind at any time and withdraw. Please tell the interviewer whenever you wish to withdraw.

If you have a problem or have other questions, please call _____ on _____ if you have questions about the research.

The above document describing the benefits, risks and procedures for the research _____ in Suhum-Krabo-Coaltar has been read and explained to me. I have been given an opportunity to have any questions about the research answered to my satisfaction. I agree to participate as a volunteer.

Date

Signature or mark of volunteer

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

I was present while the benefits, risks and procedures were read to the volunteer. All questions were answered and the volunteer has agreed to take part in the research.

Date

Signature of witness

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

Date

Signature of Person who obtained Consent

APPENDIX B:

B.1 Media, reagents and equipment used in this study

B.1.1 Preparation of agar media and broths for culture, bacteria identification: The following media, broths and standard solutions were prepared using sterile distilled water. Where appropriate the solutions were either autoclaved at 121°C for periods between 15-20 minutes at 1.12 kgcm⁻² pressure or filter sterilized with 0.22 or 0.45µm filter units (MILLEX-HA, Molsheim, France).

For agar plates, dehydrated powders were dissolved in appropriate volumes of distilled water according to the manufacturer's instructions and autoclaved. When cooled to about 45-55°C, approximately 25ml volumes were dispensed into 90cm sterile Petri dishes and allowed to set. For slopes like L-J various components were appropriately mixed and dispensed into 25 ml screw-capped test tubes and allowed to set by inspissations.

Sterility and quality of media after each preparation were controlled by respectively leaving a slope or plate on a bench overnight and also inoculating randomly selected media with positive and negative controlled organisms.

B.1.2 Preparation of Löwenstein-Jensen medium

Step I. Preparation of mineral solution			
Component	Quantity		
	1600ml	800ml	400ml
Potassium dihydrogen phosphate anhydrous (SAME AS: Monopotassium dihydrogen phosphate anhydrous) [KH ₂ PO ₄]	2.40g	1.20g	0.6g
Magnesium sulphate [MgSO ₄ . 7H ₂ O]	0.24g	0.12g	0.06g
Magnesium citrate	0.60g	0.30g	0.15g
Asparagine	3.60g	1.80g	0.9g
Glycerol [Reagent grade]	12ml	6ml	3ml
Distilled water	600ml	300ml	150ml
1. Dissolve the ingredients following the above order, in distilled water, by heating. 2. Autoclave the solution at 121°C for 30 minutes to sterilize. 3. Cool to room temperature. May be stored in suitable amounts in the refrigerator			
<i>Step ii. Preparation of malachite green solution</i>			
	Final quantity of L-J medium		
	1600ml	800ml	400ml
Malachite green dye	2g	1g	0.5g
Sterile distilled water	100ml	50ml	25ml
Using aseptic techniques, dissolve the dye in sterile distilled water by placing the solution in an incubator for 1 to 2 hours. Fresh solution to be prepared at each time			
<i>Step iii. Preparation of homogenized whole eggs</i>			

<u>Wash hands thoroughly before handling eggs</u>			
1. Scrub eggs with a hard brush in warm water with plain alkaline soap			
2. Soak scrubbed eggs in soap solution for 30 minutes			
3. Rinse eggs thoroughly with sterile water			
4. Soak rinsed eggs in 70% alcohol for 15 minutes			
5. Crack eggs with sterile knife.			
6. Whisk eggs in sterile blender			
	Final quantity of LJ medium		
	1600ml	800ml	400ml
Whisked eggs	1000ml	500ml	250ml
<i>Step iv. Preparation of the complete medium</i>			
	Final quantity of LJ medium		
	1600ml	800ml	400ml
Mineral solution	600ml	300ml	150ml
Malachite green solution	20ml	10ml	5ml
Homogenized eggs [20-25 eggs]	1000ml	500	250ml

B.1.3 Preparation of Mueller-Hinton (MH) agar (per liter)

<i>Media</i>	<i>Components</i>	<i>Procedure</i>
Mueller Hinton Agar	2.0 g beef infusion solids 17.5g Acid Hydrolysed Casein 1.5g Starch 17.0g Agar No.150-100 mg /L Calcium ions 20-35 mg/l Magnesium ion pH 7.3+ or – 0.1	Prepared according to the manufacturer's (BIOTEC) instructions. When cooled to about 55°C, approximately 25 ml volumes were dispensed into 90 cm sterile Petri dishes and allowed to set.

B.2 Chemicals & Reagents**B.2.1. Chemical agents 1: microscopy**

Chemical agents	Composition	Procedure
1. 3.5% Hypochlorite solution	3.5g hypochlorite 100ml distilled water	Mix components by shaking .*This was commercially acquired as household bleach
2. Phenol Ammonium sulphate	5g phenol crystals 4g Ammonium sulphate 95 ml sterile distilled water,	Weigh components into reagents and mix by shaking with sterile distilled water. Store at room temperature until ready to be used

B.2.2 Chemical Agents 2: Culture

Chemical agents	Composition	Preparation procedure
5% oxalic acid	5g oxalic acid 100ml distilled water	Weigh 5g of oxalic acid crystals and mix with 100ml of distilled water. Mix thoroughly by shaking. Autoclave reagent at 121°C for 15 minutes and store at 4°C until use.
10% Oxalic acid	5g oxalic acid 100ml distilled water	Weigh 10g of oxalic acid crystals and mix with 100ml of distilled water. Mix thoroughly by shaking. Autoclave reagent at 121°C for 15 minutes and store at 4°C until use.
1% CPC/2% NaCl	<ul style="list-style-type: none"> • 1g CPC • 2g NaCl • 100ml sterile distilled water. (Smithwick, Stratigos, and David 1975). 	Weigh 1g CPC and 2g of NaCl into a dark sterile reagent bottle and mix by shaking with 100ml sterile distilled water.(self sterilizing, does not require sterilization
0.5% CPC/1%NaCl	5ml 1% CPC/2% NaCl 5ml sterile distilled water	Mix components to obtain 1:2 dilution of agent
1% Povidone iodine	1g iodine crystals 100ml distilled water	Commercially acquired at 1% dilution
0.5% Povidone iodine	5ml 1% povidone iodine 5ml sterile distilled water	Thoroughly mix the two components in a sterile container
1% virkon	1g virkon powder 100ml distilled water	Weigh 1g of virkon disinfectant into a sterile container and Add 100 ml of sterile distilled water
0.5% virkon	5ml 1% virkon 5ml distilled water	Mix two components by shaking.(self sterilizing agent)

- **CPC**-Cetyl-pyridinium chloride ;**PI**- Povidone iodine

B.3. Antibiotics used in this study: Reagent grade rifampicin and streptomycin were obtained from Sigma Chemicals through the funding agency (DAAD) rifampicin, streptomycin.

PANTA (polymixin B, amphotericin B, nalidixic acid, trimethoprim, azloxycillin) media supplement.

Antibiotics	Diluents	Stock concentration	Volume	Procedure
Streptomycin	Sterile distilled water	1mg/ml	2ml	Weigh 2 mg of Streptomycin into a 15ml falcon tube and mix with 2ml of sterile distilled water
Rifampicin	Absolute methanol	10mg/ml	2ml	Weigh 20mg of rifampicin into a 15ml sterile falcon tube and mix with 2ml of absolute methanol

B.4. The incorporation of antibiotics into Lowenstein-Jensen (L-J) medium.

Antibiotic	Stock concentration	Required incorporation concentration	Volume required	Procedure
Rifampicin	10mg/ml	40ug/ml	400ml	Add 1.6 ml of stock rifampicin to 398.4ml of prepared L-J media. Mix by swirling, Distribute into sterile receptacles, solidify by inspissating <i>85°C for 55 minutes</i>
Streptomycin	1mg/ml	4ug/ml	400ml	

APPENDIX C**C.1. Equipments used in this study**

Equipment	Model	Manufacturer
Light microscope	Olympus CX 31 brightfield biological microscope	Olympus Corporation Tokyo Japan
Refrigerated centrifuge	Falcon 6/300	CAC, Lab net International, Inc.
Vortex mixer	Stuart vortex mixer-sa8	SI-Scientific Industries Inc.USA
-20° C refrigerator	NORLAKE scientific under counter freezer - 20C NSLF051WMW	Norlake Inc, Standex Food Service Group,USA
Autoclave	Market -forge STME -L- Autoclave	Market Forge, Alfa Medical, New Yorke,USA
Heating block	Stuart Block heater-SBH 200D	Stuart bench top science equipments, bibby scientific limited .UK.
Inspissator /coagulator		
Electric pipette	Soft ouch Electronic pipettes	Hamilton Company USA
Weighing balance	Mettler Toledoa1204 laboratory balance	Fisher Scientific
Bio safety cabinet	Laggard ES Biological Safety Cabinet	Nuaire Corporate

APPENDIX D: Microbiological Procedures

D.1. Ziehl-Neelsen (ZN) staining procedure for AFB (acid fast bacilli)

- Put two drops of sample suspension on a labelled slide.
- Allow to air –dry
- Fix the smear by passing the slide 3 times over a flame (do not burn)
- Flood the slide with Carbol Fuchsin stain to cover the smear.
- Apply heat beneath the slide until steam appears. (Do not boil)
- Leave it for 5 minutes applying heat intermittently
- Wash off the excess carbol fuchsin with tap water and drain off excess water
- Decolorize by covering the slide with 20% H₂SO₄
- Leave for 2-5 minutes, if traces of Carbol Fuchsin are seen decolorize again to clear and Wash with water.
- Counter-stain with 3% methylene blue stain
- Leave for 2 minutes.
- Wash with plenty of water, drain and leave to dry.
- Observe with the light microscope under oil immersion

D.2. Evaluation criteria for AFB (acid fast bacilli)

Interpretation of Microscopy Result (International Union of Against Tuberculosis and Lung Diseases .1996 Tuberculosis Guide for Low Income Countries 4th ed. International Union against Tuberculosis and Lung Diseases, Paris, France)

Observations (Readings)	Estimation (grading)
No AFBs per 100 fields	Negative
1-9 AFBs per 100 oil immersion fields	Scanty
10-99 AFBs per in 100 oil immersion fields	+1
1-10 AFBs per oil immersion field	+2
More than 10 AFBs per oil immersion field in at least 20 fields	+3

D.3 Evaluation criteria for modified protocols for the detection of acid fast bacilli (AFB) by microscopy

P (Positivity) AFBs count per 100 fields: -

P 0- Negative (no visible AFBs per 100 fields; P1= scanty P2=1+ P3=2+ and P4=3+

C (Contrast and Clarity) –

C0-dark and AFBs indistinguishable; C1=Slight illumination of slide and relatively improved contrast; C2=brighter field and AFBs distinguishable; C3=Bright field, good contrast and AFBs clearly visible; C4=Very bright clear background, AFBs' visibility enhanced.

R (Release of AFBs embedded in matrix):

R0-AFB totally embedded in matrix material; R1-AFBs partially detached from matrix material; R2-AFBs detached from matrix; R3= AFBs distinguishably detached from matrix
R4= AFBs totally detached and aids visible detection.

D.4. Decontamination protocols in culture***D.4.1 Conventional Procedure using 5% oxalic acid*****Procedure**

- i) Add 2ml of specimen suspension to 2ml of 5% oxalic acid.
- ii) Mix by vortexing
- iii) Leave standing at room temperature for 30minutes. (With intermittent mixing)
- iv) Neutralize with 40ml. of sterile PBS.
- v) Centrifuge at 3000g for 30 minutes.
- vi) Resuspend pellet in 1ml. of PBS.
- vii) Inoculate onto L-J media.

D.4.2 Modified Procedures with selected decontamination protocols

- i) Add 500ul of specimen suspension to 500ul of test agents (see agents in appendix B4).
- ii) Mix by vortexing
- iii) Leave standing at room temperature for 30mins. (with intermittent mixing)
- iv) Neutralize with 5ml of distilled water

- v) Centrifuge at 3000g for 30 minutes.
- vi) Resuspend pellet in 200ul of PBS.
- vii) Inoculate onto L-J media.

D.4.3. Evaluation criteria for *M. ulcerans* culture: Colonies appearing after 8 weeks on L-J media

Readings	Estimation (grading)
1-19 colonies	Few colonies
20 or more but fewer than 100 colonies	+1
Innumerable colonies	+2
Confluent growth	+3

APPENDIX E:

E.1 List of microbes used in this study

Clinical / Reference isolates

Klebsiella pneumonia

Staphylococcus epidermidis

Bacillus species

Staphylococcus aureus

Escherichia coli (ATCC 25922)

Klebsiella species

Escherichia coli

Salmonella typhi

Pseudomonas aeruginosa

Candida albicans

Aspergillus niger

M. ulcerans (A5)-reference

M. tuberculosis

MU 35 clinical isolates

APPENDIX F:**F.1. Microscopy for the detection of AFB**

Index Smears preparation protocols

DISW: - Direct swab smear

DISU: - Direct sample suspension smear

COSUSE: - Sample suspension concentration by sedimentation smear

COSUCE: - Sample suspension concentration by centrifugation smear

COHOSE:- Hypochlorite (HOCl) treated sample concentration by sedimentation smear

COHOCE:- Hypochlorite (HOCl) treated sample concentration by centrifugation smear;

COPHSE:- Phenol Ammonium Sulphate (PHAS) treated suspension/ sedimented;

COPHCE:- Phenol Ammonium Sulphate (PhAS) treated suspension /centrifuged

F.2. Tables and figures for results

F. 2.1 Tables

Table F1: Selection of Chemical Decontamination Agents

<i>Chemical agent</i>	<i>Contemporary uses</i>	<i>Decontaminating activity</i>
1. <i>1% povidone iodine</i>	Wound dressing, antiseptic antimicrobial	+
2. <i>1% Cetyl pyridinium chloride/ 2 % sodium chloride</i>	Decontamination, liquefaction and concentration of sputum specimens in TB Mycobacteriology, antimicrobial	(+/-)
3. <i>5% oxalic acid</i>	Decontamination agent for primary isolation of <i>Mycobacterium ulcerans</i> , antimicrobial	(+/-)
4. <i>1% virkon</i>	Disinfectant and antimicrobial	+

Legend

+ =Activity present

-=Activity absent

(+/-)= partial Activity

Table F2: Susceptibility of Potential Skin Contaminants to Selective Decontamination Chemical Agents

Microbial agents		Chemical decontaminating agents (%) vol/vol								
			<i>Povidone iodine</i>		<i>Cetyl pyridinium Chloride</i>		<i>Oxalic acid</i>		<i>Virkon disinfectant</i>	
		0	0.5%	1%	1%/2%	2%/4%	5%	10%	0.5%	1%
<i>Test microbes</i>	0	NG	NG	NG	NG	NG	NG	NG	NG	NG
	1. <i>Klebsiella pneumonia</i>	G	NG	NG	NG	NG	NG	NG	NG	NG
	2. <i>Staphylococcus epidermidis</i>	G	NG	NG	NG	NG	NG	NG	NG	NG
	3. <i>Staphylococcus aureus</i>	G	NG	NG	NG	NG	NG	NG	NG	NG
	4. <i>Escherichia coli</i>	G	NG	NG	G	NG	NG	NG	NG	NG
	5. <i>Salmonella typhi</i>	G	NG	NG	G	NG	NG	NG	NG	NG
	6. <i>Pseudomonas aeruginosa</i>	G	NG	NG	NG	NG	NG	NG	NG	NG
	7. <i>Bacillus cereus</i>	G	NG	NG	NG	NG	NG	NG	NG	NG
Fungal agents	8. <i>Candida albicans</i>	G	NG	NG	NG	NG	G	NG	NG	NG
	9. <i>Aspergillus niger</i>	G	G	NG	NG	NG	G	NG	G	NG

key-

G=indicates visible microbial growth on media plate

NG=indicates no growth on media plate

F2.2. Figures

Figure 1: Chart showing trends in the means of the smear preparation protocols

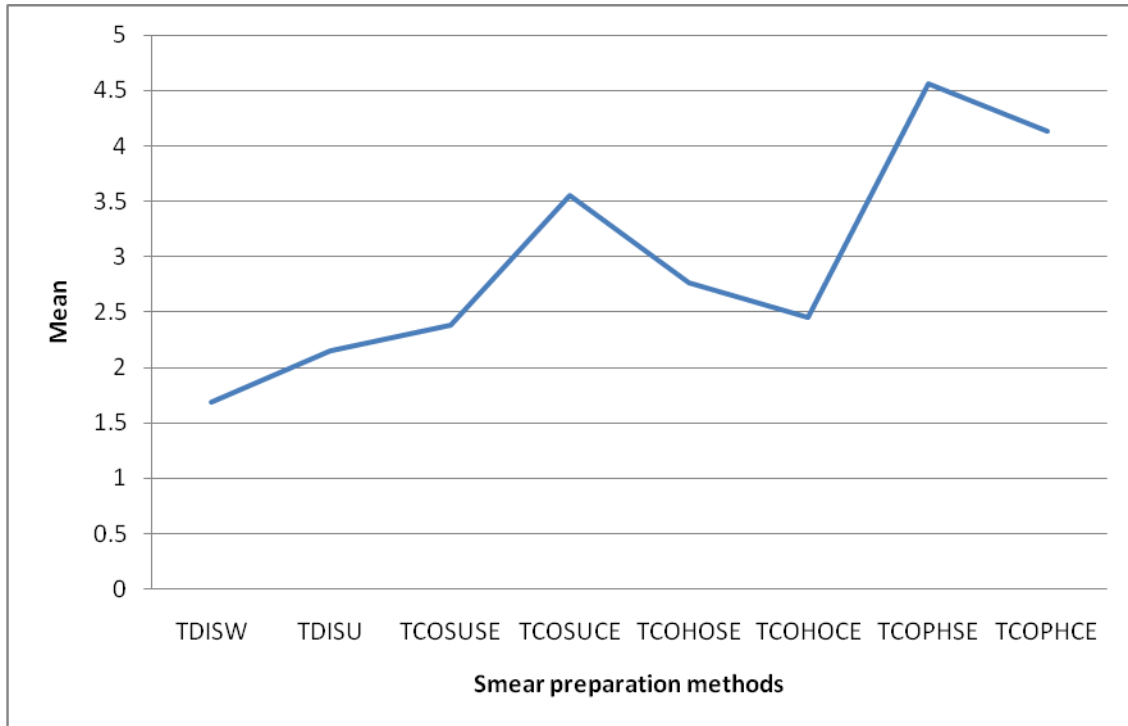
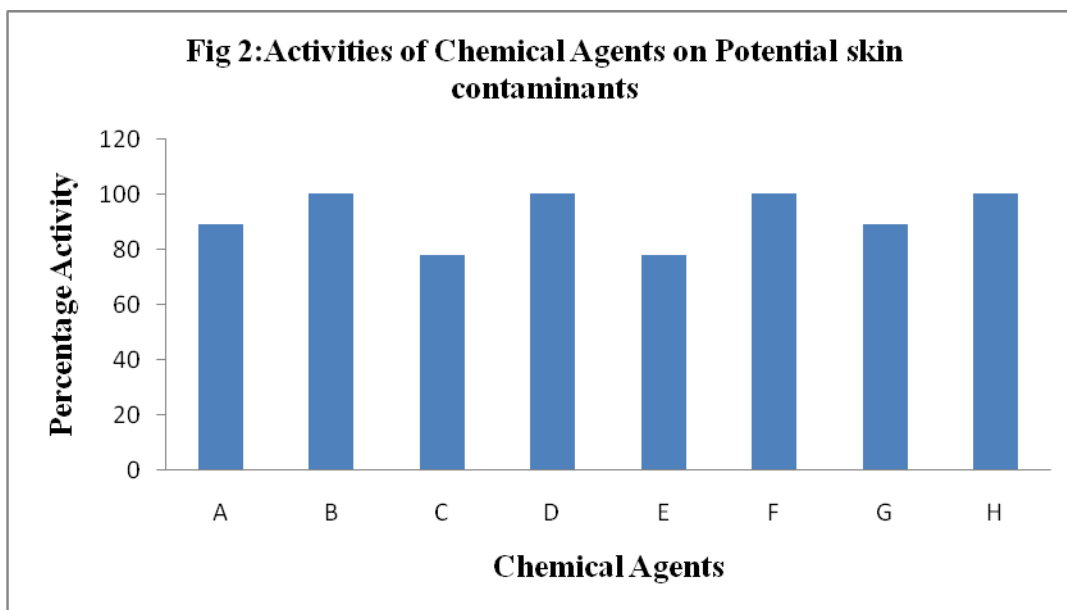


Figure 2: showing results on the inhibitory activities of chemical agents on selected microbial organisms



Key

A= 0.5% Povidone iodine

B= 1% Povidone iodine

C= 1% Cetyl pyridinium chloride/2% sodium chloride

D= 2% Cetyl pyridinium chloride /4% sodium chloride

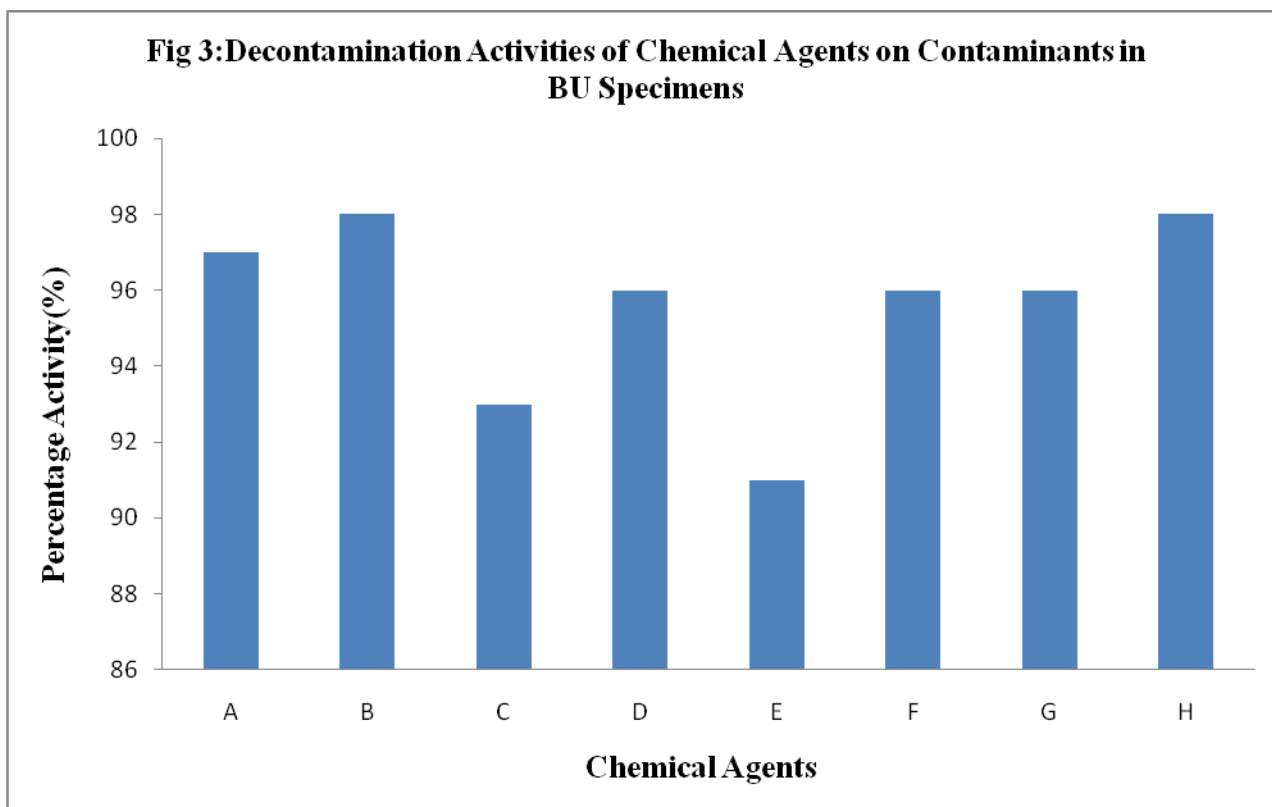
E=5% Oxalic acid

F=10% Oxalic acid;

G=0.5% Virkon disinfectant

H= 1% Virkon disinfectant

Figure 3: Results of decontamination activities of chemical agents on contaminants in BU samples



Legend A=0.5% Povidone iodine;

B=1% Povidone iodine;

C=1% Cetyl pyridinium chloride/2% sodium chloride

D=2% Cetyl pyridinium chloride /4% sodium chloride

E=5% Oxalic acid

F=0% Oxalic acid

G=0.5% Virkon disinfectant

H=1% Virkon disinfectant