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OCCURRENCE AND DISTRIBUTION OF MYCOBACTERIUM ULCERANS ALONG THE DENSU AND OFFIN RIVER BASINS OF GHANA

By

SAMUEL YAW ABOAGYE

(10289360)

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DECLARATION

I hereby declare that this thesis is a result of my own research work carried out under the supervision of Prof. Dorothy Yeboah-Manu and Dr. Dzidzo Yirenya-Tawiah and that references made to other people's work have been duly acknowledged. I further declare that this work has not been submitted in part or whole to any other institution for the purpose of acquiring a degree.

SAMUEL YAW ABOAGYE
(Candidate)

DATE……

PROFESSOR DOROTHY YEBOAH-MANU
(Supervisor)

DATE……

DOCTOR DZIDZO YIRENYA-TAWIAH
(Co-Supervisor)

DATE……

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Abstract

Background: Buruli ulcer (BU), caused by *Mycobacterium ulcerans* (MU), is the third most important mycobacterial disease after tuberculosis and leprosy. Many features of MU ecology, including distribution within the environment, reservoir hosts and niche preference are still an enigma. The exact route of its transmission is also not known, although it has been identified as an environmental pathogen. A major drawback is the difficulty to isolate viable MU from the environment due to the slow growth rate and the complex mix of fast growing bacteria and fungi that outgrow culture media. This thesis sought to detect and isolate *M. ulcerans* from the environment.

Methodology: Samples were collected from ten communities along the Offin and Densu river bodies at different seasons. Following DNA extraction, the presence of MU was determined by gene targets, IS*2404*, IS*2606* and the Ketoreductase (KR) gene. Samples were first analysed for IS*2404*, after which IS*2404* positive samples with CT ≤ 35 were multiplexed for the MU specific markers IS*2606* and KR gene. Four decontamination solutions and supplemented media were optimized after which the best were used for MU culture. The isolates were identified on the basis of specific genetic sequences, including heat shock protein (hsp) 65, IS*2404*, IS*2606*, rpoB, and the KR gene. Presumptive BU cases from active case search and passive reports were collected and analysed by IS*2404* PCR. A case control study was further conducted to assess potential environmental and behavioural risk factors of BU.

Results: One thousand six hundred environmental samples were analyzed along the Densu (434; 27%) and Offin (1166; 73%) river basins and MU DNA was detected in 139 (9%) of the combined samples. The positivity of MU DNA along the Densu river basin

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was 89/434 (20.5%) whilst that of the Offin river basin was 50/1166 (4.3%). The DNA was detected mainly in snails 5/6 (83%), moss 8/40 (20%), soil 55/586 (9%) and vegetation 55/675 (8%). The proportion of MU positive samples recorded was higher during the months with higher rainfall levels (126/1175 [11%]) than during the dry season months (13/425 [3%]). Among the methods evaluated, decontamination with 1 M NaOH followed by 5% oxalic acid gave the highest rate of recovery of mycobacteria (50.0%) and the lowest rate of contamination (15.6%). The cultivation medium that supported the highest rate of recovery of mycobacteria was polymyxin B-amphotericin B-nalidixic acidtrimethoprim-azlocillin–supplemented medium (34.4%), followed by isoniazid-supplemented medium (28.1%). Among the 139 samples cultivated in the main analysis, 58 (41.7%) yielded mycobacterial growth, 70 (50.4%) had no growth, and 11 (7.9%) had all inoculated tubes contaminated. A total of 25 different mycobacterial species were identified. Fifteen species (60%) were slowl growing (e.g., *Mycobacterium ulcerans*, *Mycobacterium avium*, and *Mycobacterium malmoense*), and 10 (40%) were rapidl growing (e.g., *Mycobacterium chelonae*, and *Mycobacterium abscessus*). The occurrence of mycobacterial species in the various environmental samples analysed was as follows: soil, 16 species (43.2%); vegetation, 14 species (38.0%); water, 3 species (8.0%); moss, 2 species (5.4%); snail, 1 species (2.7%); fungi, 1 species (2.7%). Out of 84 presumptive BU cases analyzed, 32 (38.1%) were laboratory confirmed by IS*2404* PCR; 24 (75%) were from Densu, and 8 (25%) from Offin. A total of 176 cases and 176 controls were enrolled in the case-control study. Multivariate conditional logistic regression analysis identified farming in swampy areas (OR = 4.10, 95% CI = 3.82-7.18), farming while wearing short clothing (OR = 1734.1, 95% CI = 68.1-44120.9), insect bite

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(OR = 988.3, 95% CI = 31.4-31115.6) and application of leaves on wounds (OR = 6.23, 95% CI = 4.74-18.11) as potential risk factors. Farming in long clothing (OR = 0.000, 95% CI = 0.00-0.14), washing wound with water and soap (OR = 0.37, 95% CI = 0.29-0.98) and application of adhesive bandage on wounds (OR = 0.31, 95% CI = 0.15-0.82) were found to be protective against BU infection.

Conclusion: The study confirmed the abundance of *M. ulcerans* in the environment which is influenced by seasonality. This study established an efficient and effective in-house decontamination procedure (NaOH-OA) and cultivation media that are supplemented with various antimicrobials. This study is the first to report on the *in vitro* isolation of *M. ulcerans* from environmental samples. Lastly, the study identified potential risk and protective factors for BU along the Densu river basin which can be integrated in BU control measures in Ghana.

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DEDICATION

This thesis is dedicated to all BU patients

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LIST OF ABBREVIATIONS

1. µl	Microlitre
2. mg	Milligram
3. ml	Milliliters
4. AFB	Aid-fast bacilli
5. BCG	Bacillus Calmette-Guerin
6. BLAST	Basic Local Alignment Search Tool
7. BU	Buruli Ulcer
8. BS2	Biosafety Level 2
9. CBVS	Community Based Surveillance Volunteers
10. CDS	Coding Sequences
11. CT	Critical Threshold
12. DF	Drug Free
13. DNA	Deoxyribonucleic acid
14. EB	Ethambutol
15. ER	Enoyl Reductase
16. FNA	Fine Needle Aspirate
17. GPS	Global Positioning System
18. HCl	Hydrochloric Acid
19. hsp65	Heat shock protein 65
20. HPC	1-Hexadecylpyridinium Chloride
21. IU/L	International Units/liter
22. IPC	Internal Positive Control

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23. INH	Isoniazid
24. IS	Insertion Sequence
25. IUALTD	International Union against Tuberculosis and Lung Diseases
26. KR	Ketoreductase B domain
27. LJ	Löwenstein-Jensen
28. MGIT	Mycobacteria Growth Indicator Tubes
29. MU	*Mycobacterium ulcerans*
30. NALC	N-acetyl-L-cysteine
31. NaOH	Sodium Hydroxide
32. NCBI	National Center for Biotechnology Information
33. NTM	Nontuberculous Mycobacteria
34. NBUCP	National BU Control Programme
35. OA	Oxalic Acid
36. OADC	Oleic Albumin Dextrose Catalase
37. PANTA	Polymyxin B Amphotericin B Nalidixic acid Trimethoprim Azlocillin
38. PCR	Polymerase Chain Reaction
39. PBS	Phosphate Buffered Saline
40. PM	PANTA-mycobactin J
41. SDS	Sodium Dodecyl Sulfate
42. TSB	Tryptic Soy Broth
43. TBE	Tris-Borate EDTA

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44. UV	Ultraviolet
45. WHO	World Health Organization
46. ZN	Ziehl-Neelsen

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CHAPTER ONE

Introduction

1.1 Background

Buruli ulcer (BU), caused by *Mycobacterium ulcerans*, belongs to the genus Mycobacterium. *Mycobacterium ulcerans* (MU) is a member of the group of mycobacterial species classified as nontuberculous mycobacteria (NTM) or atypical mycobacteria or mycobacteria other than tuberculosis (Cook 2010). Buruli ulcer is the third most important mycobacterial disease globally after tuberculosis and leprosy (Johnson *et al.,* 2005; van der Werf *et al.,* 2005). The disease has been reported in 33 countries globally with the highest disease burden found in West African countries along the Gulf of Guinea (Hotez *et al.,* 2007). In West-Africa, BU is the second after tuberculosis and in worst affected communities like the Amansie-West district of Ghana; BU is the leading mycobacterial disease that affects immune-competent individuals (Asiedu *et al.,* 2000).

The BU disease may manifest initially as a pre-ulcerative nodule, plaque or a rapidly progressing oedematous lesion. Over a few weeks, these lesions break down to form characteristic ulcers with undermined edges, which are associated with extensive necrosis alongside minimal inflammatory response (Portaels, 2000; Portaels *et al.,* 2008; George *et al.,* 1999). The extensive skin damage resulting from the cytopathic action of the virulence factor mycolactone could lead to loss of vital organs including the eye and amputation of limbs (WHO, 2004). The standard specific treatment currently available involves 8 weeks of combination therapy with streptomycin (15 mg/kg) and rifampicin (10 mg/kg) (WHO, 2001; Etuaful *et al.,* 2005).

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Many aspects of the disease including epidemiology, immune response and pathogen ecology as well as mode of transmission are still not fully understood. The epidemiology of BU in endemic countries is not fully known due to the focal distribution of cases where endemic and non-endemic communities are separated by few kilometres (Yeboah-Manu *et al.*, 2012). Nevertheless, epidemiological evidences have linked BU incidences to slow flowing or stagnant waters and disturbed environment (Weir, 2002). Rapid changes in landscape such as deforestation, flooding, construction of dams, mining activities, construction of artificial lakes for irrigation, and extending swamps for growing rice and fish breeding have been suggested to have a great impact on the emergence of the disease (Merritt *et al.*, 2005; Weir, 2002). The BU disease affects equally both sexes; all age groups can be affected, but currently children below age 15 years accounts for close to 50% of all reported cases (Bratschi *et al.*, 2013).

One major factor that has limited the prevention and control of BU is the lack of understanding of the ecology and mode of transmission of the causative agent. Many features of *M. ulcerans* ecology, including distribution within the environment, niche adaptation, and host range (s) are still not fully known (Doig *et al.*, 2012). Theories that have been propounded to explain the mechanism of *M. ulcerans* transmission included and 1) contamination of existing wound or site of trauma by the environment such as soil, vegetation and water among others (Portaels *et al.*, 2001; Duker *et al.*, 2006), 2) inhalation of aerosolized *M. ulcerans* from contaminated water (Hayman, 1991), and 3) acquisition of *M. ulcerans* through an insect or vector bite (Portaels *et al.*, 1999), however none of these theories have been confirmed.

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A major factor that has also limited the understanding of *M. ulcerans* ecology is the inability to isolate viable *M. ulcerans* from the environment. The isolation challenges are enormous; as the environment contains other fast growing bacteria and fungi. While other bacteria such as *Escherichia coli* grow very fast to attain macroscopic growth within 18 hours, it can take up to six months or more to isolate *M. ulcerans* from clinical samples. Thus the culture media are often times contaminated by other fast growing bacteria and fungi. The main determinant of growth rate is the number of rRNA operons; *M. ulcerans* possess one operon and other fast growing microorganisms such as *E. coli* have seven operons (Condon *et al.,* 1995; Klappenbach, *et al.,* 2000; Cook *et al.,* 2009). To address contamination challenges during culture, various chemical agents including acids and bases are being used to decontaminate both clinical and environmental samples. Additionally, antimicrobials (PANTA) and antifungals (Mycobactin J) are also added to cultivation media to further eliminate the growth of unwanted bacteria.

The completion of the *M. ulcerans* genome sequence provided specific targets for DNA-based detection methods such as the insertion sequence IS*2404*, IS*2606* and the plasmid encoding mycolactone, ketoreductase B domain (KR) (Fyfe *et al.,* 2007). Such methods have been used to study the prevalence of *M. ulcerans* in various environmental samples including soil, insects, vegetation and water. Some of such studies conducted in Australia for example have implicated the tree dwelling possums and mosquitoes as possible reservoirs (Fyfe *et al.,* 2010) and potential vectors (Lavender *et al.,* 2011) respectively in South-Eastern Australia. However until now no potential reservoirs and vectors have been identified in Africa, which harbours most of the BU disease burden.

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Understanding the mode of transmission and pathogen ecology is crucial to contribute to design of control and prevention strategies.

While the influence of seasonal variation and occurrence of some infectious diseases is well studied, effect of seasonality on BU is not known. Seasonal changes affect both pathogens and vectors in ways that modifies their basic reproductive numbers (Altizer *et al.*, 2006), which determines the rate at which they could infect a susceptible host. Seasonal changes therefore affects disease incidence of many pathogens in humans ranging from increases in cases of influenza and respiratory infections during the winter months (Moura *et al.*, 2009; Fuhrmann, 2010) to peaks in the incidence of malaria (Cairns *et al.*, 2015) and diarrhea (Rebaudet *et al.*, 2013) following seasonal rains in the tropics.

1.2 Problem Statement and Significance

Ghana is the second most endemic country after Ivory Coast (WHO, 2016). The mode of transmission of the causative agent still remains unknown, even though epidemiological evidence strongly associates the disease with wetlands, swamps, and disturbed environment. An important aspect in the control of infectious disease is identification of pathogen ecological niches, reservoirs and vectors that aid in transmission. This is essential so as to develop measures to limit host contact with the pathogen. For instance, understanding the pathogen ecology would give insight into environmental variables such as climatic conditions that might favour the thriving of the pathogen within a specific location.

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Moreover, immune response to *M. ulcerans* is also not fully known and this has ultimately affected the development of vaccines. In the absence of a clear transmission mechanism, the current control strategy is by case detection and treatment; this has reduced the number of BU cases. However, there is a greater cause for concern as similar strategy adopted for leprosy (mycobacteria disease) decades ago reduced the number of cases but recently new cases are being identified within endemic zones, an indication of the neeed for more preventive strategy. Even though the current antibiotic treatment regimen for BU has proven efficacious; the rapid development of resistance to most other antibiotics poses a major threat, as there is currently no available substitute should there be any resistance to the existing antibiotics.

Studies that have implicated insects as vectors in the transmission of *M. ulcerans* (Portaels *et al.,* 1999; Marsollier *et al.,* 2002) were based solely on detection of DNA in these vectors, however DNA does not indicate a viable bacteria, on this basis, vector studies cannot be based alone on PCR since it is likely for the DNA to be picked from the environment by these vectors. More so, the low concentration of DNA in the environment does not allow for detailed genomic analyses.

Until this thesis, only one study has reported of isolation of *M. ulcerans* from the environment (Portaels *et al.,* 2008). The isolate was obtained after 15-day incubation in BACTEC 12b broth and three successive passages in mouse footpad P1, P2 and P3 for 9 months, 6 months and 12 months, followed by culture on 37 Löwenstein-Jensen for two months (Portaels *et al.,* 2008). This method took much longer time (approximately 3

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years) and not optimum for large-scale environmental analysis. The techniques used by Portaels *et al.* require special expertise infrastructure such as animal experimentation unit and animal models for infection assays, making this approach almost impossible to be conducted in low resource countries where BU is endemic. At the same time, more isolates are needed for micro-epidemiological studies to better under the transmission of *M. ulcerans* in the environment.

1.3 Hypothesis

Mycobacterium ulcerans has a unique and characteristic thicker cell wall made up of peptidoglycan, arabinogalactan and mycolic acids surrounded by a non-covalently linked outer capsule of proteins and polysaccharides. The lipids in the cell wall form a bilayer or outer membrane that prevents the entry of many chemical agents. Thicker cell walls and unique mycolic acids contribute to the impermeability, hydrophobicity, and slow growth of *M. ulcerans*.

I hypothesize that;

1. the thicker, lipid rich cell wall nature of *M. ulcerans* will allow it to withstand harsher solutions and resist more antimycobacterials in culture media, and incorporation of antibiotics and antimycobacterials such as isoniazid in culture media will facilitate its isolation from the environment
2. climatic conditions such as rainfall will influence the occurrence of *M. ulcerans* in the environment

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1.4 Aim

The aim of this thesis was to detect and isolate *M. ulcerans* from the environment.

1.5 Specific Objectives

The specific objectives were to;

1). Detect *M. ulcerans* from the environment

2). Detect *M. ulcerans* from clinical samples

3). Optimize decontamination methods to isolate viable *M. ulcerans* from environmental samples

4). Determine the environmental and behavioral risk factors of BU

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CHAPTER TWO

Literature Review

2.1 Historical Facts and Global burden of Buruli ulcer

Sir Albert Cook, a British physician in Mengo Hospital Kampala, Uganda in 1879 was the first to describe Buruli ulcer (BU) (Lunn *et al.*, 1965). Cases of chronic skin conditions in Bairnsdale, Australia were also reported in 1935 (Alsop, 1972). In 1948, the etiologic agent of Buruli ulcer was first isolated from a farmer in Bairnsdale Australia, by MacCallum and his colleague who named the agent as *Mycobacterium ulcerans* (MacCallum and Tolhurst, 1948). Subsequently, in1960's, the Buruli County in Uganda recorded the largest BU cases which led to the designation of the name Buruli ulcer (BU) (Clancy *et al.*, 1962). By the 1980's, Buruli ulcer had emerged as a public health issue with the largest number of cases reported in West Africa, especially countries along the Gulf of Guinea (Benin, Cameroon, Côte d'Ivoire, Democratic Republic of the Congo and Ghana). The World Health Organization (WHO) launched a Global Buruli Ulcer Initiative with the aim of coordinating control and increasing research efforts in 1998. Subsequently, the World Health Assembly adopted a resolution to increase disease surveillance, control and intensified research to develop tools for diagnosis, treatment and prevention of the disease in 2004 (WHO, 1998).

Currently more than 33 countries mainly in tropical and subtropical climates including Africa, South America and Western Pacific regions have reported BU disease (Figure 2.1) (WHO, 2015). The disease is considered the third most common mycobacterial disease after tuberculosis and leprosy (Walsh *et al.*, 2010). The African sub-region

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reports 98% of cases followed by Australia (1.5%), Papua New Guinea (0.2%), Japan (0.1%) and French Guiana (0.1%). In 2014 alone, of the 2,251 new BU cases reported globally 2,151 (95.5%) were from the African sub-region (Table 2.1).

Early case reporting is undermined by the focal distribution of BU cases, and lack of accessibility to biomedical care in BU endemic villages in Africa.There is a seemingly decline in BU cases in some endemic since 2010 with the exception of few countries including Australia, Nigeria and Togo (Table 2.2). Côte d'Ivoire and Ghana for instance, reported 2,533 and 1,048 new cases respectively in 2010, by 2014 cases reported were 827 and 443 respectively (WHO, 2015). This may be a reflection of the actual annual cases after treating the backlog of cases that accumulated over the years when there was no control strategy in place. Portaels *et al.* (2009) also suggested that, some countries might have over reported cases as a result of diagnosis based on clinical judgment alone.

The BU disease affects equally both sexes and all age groups and no ethnic preference has been reported (Debacker *et al.,* 2004). The clinical and epidemiological aspects of cases vary considerably within and across different geographical settings, especially in Africa children 15 years of age or less constitute about 48% of all cases, whereas in Australia, 10% are children under 15 years and in Japan 19% are children under 15 years (WHO, 2016). Case epidemiology shows that the proportion of children below five years seems to be less among patients and sero-epidemiological studies indicate that *M. ulcerans* exposure occurs after the first five years when individuals starts moving out of their homes (Röltgen *et al.,* 2014; Amaph *et al.,* 2016).

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Among the reported cases about 80% of lesions are located on the limbs, most commonly on the lower extremities; 35% on the upper limbs, 55% on the lower limbs, and 10% on the other parts of the body (WHO, 2016). Analyses of lesion location shows that lesions in adults mostly occur on the extremities whereas in children, the trunk, head and neck are also affected (Portaels *et al.*, 2009). The BU disease is classified into categories based on the severity and the anatomical site. Among the global reported cases, the proportions of each category presented are; category I (single lesion < 5 cm) (32%), category II (single lesion measuring 5–15 cm) (35%) and category III (single lesion > 15 cm) (33%). In the African sub region, lesions reported are 31%, 35% and 33% for category I, II, and III lesions respectively whereas in Australia lesion reported are 90%, 5% and 5% for categories I, II, and III lesions respectively (WHO, 2016).

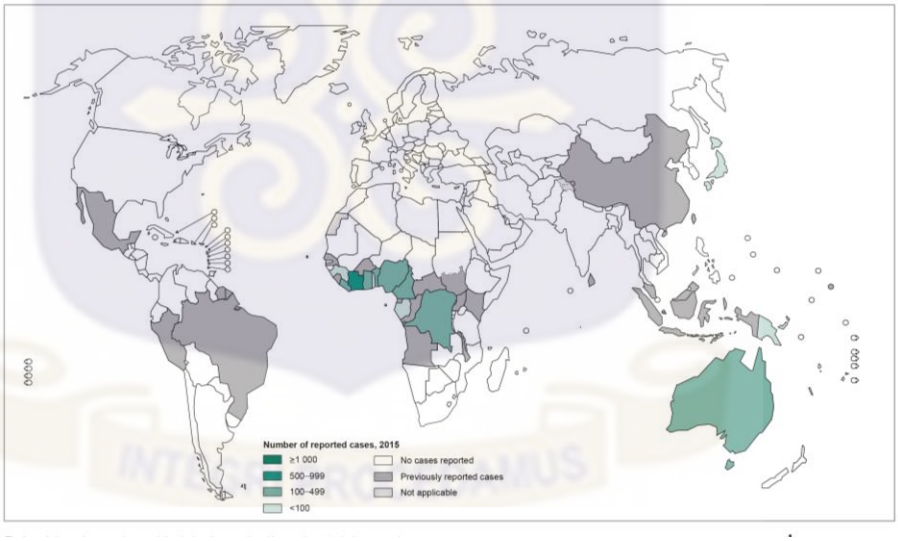

Figure 2.1 Global distribution of BU. Global map representing countries that have reported cases of BU in 2015 (WHO, 2015).

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Table 2.1 Total reported BU cases worldwide between 2004 and 2014

Countries	**2004**	**2005**	**2006**	**2007**	**2008**	**2009**	**2010**	**2011**	**2012**	**2013**	**2014**	**Total**
Ivory Coast	1153	1564	1872	2191	2242	2679	2533	1659	1386	1039	827	19145
Ghana	1157	1005	1096	668	986	853	1048	971	632	550	443	9409
Benin	925	1045	1195	1203	897	674	572	492	365	378	330	8076
Cameroon	914	265	271	230	312	323	287	256	160	133	126	3277
DRC	487	51	74	340	260	172	136	209	284	214	192	2419
Togo	800	317	40	141	95	52	67	52	51	37	67	1719
Congo	235	53	370	99	126	147	107	56	38	6	Nd	1237
Guinea	146	208	279	Nd	80	61	24	59	82	96	54	1089
Australia	34	47	72	61	40	35	42	143	105	74	89	742
Gabon	43	91	54	32	53	41	65	59	45	59	47	589
Nigeria	Nd	Nd	9	Nd	Nd	24	7	4	40	23	65	172
Uganda	7	72	5	31	24	3	Nd	Nd	Nd	Nd	Nd	142
PNG	31	Nd	Nd	26	24	8	5	8	Nd	Nd	3	105
South Sudan	4	24	38	8	3	5	4	Nd	Nd	Nd	Nd	86
Japan	1	1	1	3	2	5	9	10	4	10	7	53
French Guiana	17	2	2	2	8	2	7	3	2	3	1	49
Liberia	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	21	8	Nd	29
Sierra Leon	Nd	Nd	Nd	Nd	1	Nd	Nd	28	Nd	Nd	Nd	29
CAR	Nd	Nd	Nd	Nd	3	Nd	Nd	Nd	Nd	Nd	Nd	3
EG	Nd	3	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	3
Total	**5954**	**4748**	**5378**	**5035**	**5156**	**5084**	**4913**	**4009**	**3215**	**2630**	**2251**	**48373**

DRC= Democratic Republic of Congo, PNG= Papua New Guinea, CAR= Central African Republic EG= Equatorial Guinea, Nd= No data

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2.1.1 Buruli ulcer in Ghana

Ghana is the second most BU endemic country after Ivory Coast (Table 2.1). In Ghana, the first BU case was detected in a patient from Amasaman at the Korle-Bu Teaching Hospital in 1971 (Bayley, 1971). Subsequent to this first case detection, communities along the Densu River and its tributaries were routinely surveyed for likelihood of additional cases. Additionally, van der Werf *et al.* described 96 BU cases in the Afram valley at Agogo in the Asante Akim North district of Ashanti region (van der Werf *et al.*, 1989). Between 1993 and 1998, the first passive surveillance system reported a total of 1,200 BU cases.

The BU disease was previously linked to swampy and tropical rain forest zones, but a nationwide active case surveillance conducted in 1999 reported BU in all regions of Ghana (Amofah *et al.*, 1993). After the survey, an overall crude national prevalence rate of active lesions was estimated to be 20.7 per 100,000 (Amofah *et al.*, 1993). The Central Region reported the highest overall prevalence rate of active cases (59.2 per 100,000); followed by the Ashanti Region (30.8 per 100,000); the Northern and Upper West Regions had the lowest prevalence rates (7.4 per 100,000). Out of the 110 districts, cases were identified in 90 (81.8%) of them. Amansie West had the highest rate (prevalence 150.8 per 100,000), followed by Asante Akim North (prevalence 131.5 per 100,000) and Upper Denkyira (prevalence 114.7 per 100,000) (Amofah *et al.*, 1993).

With the advent of antibiotic therapy, laboratory confirmation of BU cases became necessary to avert unnecessary antibiotic use. However, Ghana Health Services lacked

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the capacity (infrastructure and expertise) to perform the gold standard PCR method. NMIMR responded to serve as reference laboratory for the routine laboratory confirmation of all presumptive cases. Between 2004 and 2014, Ghana reported a total of 9,409 cases of BU (Table 2.1) (Huygen *et al.*, 2009), and between 2007 to 2016, NMIMR alone analyzed by PCR 2,179 BU cases from seven regions of Ghana including Greater Accra, Eastern, Ashanti, Brong Ahafo, Central, Northern and Volta with an average annual positivity rate of 46.5%; ranging between 8.7% and 76.2%. Majority of the samples (71.4%) were received from Greater Accra region followed by Eastern region (13.3%).

2.2 Causative agent of Buruli ulcer

Mycobacterium ulcerans is the causative agent of Buruli ulcer with a necrotizing skin infection. *M. ulcerans* is shown to have evolved from *M. marinum* known to live in different aquatic and intracellular environments but causes granulomatous skin lesions in human sporadically (Tobin and Ramakrishnan, 2008). Although *M. ulcerans* shares 98.3% average genomic DNA sequence identity with *M. marinum*, within the course of evolution, *M. ulcerans* has acquired a virulence plasmid pMUM (Stinear *et al.*, 2007).

Mycobacterium ulcerans belong to the phylum, Actinobacteria, order actinomycetals, suborder corynebacteriaceae and the genus mycobacterium. *M. ulcerans* is a member of the non-tuberculous mycobacteria (NTM), also referred to as atypical mycobacteria or environmental mycobacteria (Mirsaeidi *et al.*, 2014; Cook, 2010; Dawson, 2000).

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2.2.1 Biology of *Mycobacterium ulcerans*

Mycobacterium ulcerans is a slow growing bacterium and like any other species of the genus Mycobacterium, has a high G + C DNA content (65%) and also a unique characteristic rigid and thick cell wall. These features allow the bacteria to be resistant to hydrophilic compounds, disinfectants and common antibiotics. The unique cell wall characteristics confers on it an acid fast property; the ability to resist decolourization by acids during staining procedures. Optimally, *M. ulcerans* grows between 31^{O}C to 33^{O}C, with a pH of 5.4 -7.4 (Palomino *et al.*, 1998). The bacterium has a doubling time that ranges between 24-84 hours under laboratory conditions on standard mycobacterial media (Pidot *et al.*, 2010; Mve-Obiang, *et al.*, 2003).

2.2.2. Genome of *Mycobacterium ulcerans*

Stinear *et al.* were the first to sequence and assemble the whole genome of a clinical *M. ulcerans* strain called Agy99 isolated from a Ghanaian patient in 1999 (Stinear *et al.*, 2007). The Agy99 genome is made up of two circular replicons (Fig 2.2), a chromosome of 5,631,606 bp with a 174,155 bp virulence plasmid called pMUM001. The chromosome consist of protein-coding genes (4,160), pseudogenes (771), two prophages phiMU01 (18 kb, 18CDS) and phiMU02 (24 kb, 17CDS) and many copies of insertion sequence (IS) elements, IS*2404* (209 copies) and IS*2606* (83 copies) (Stinear *et al.*, 2007). The virulence plasmid also consists of protein coding DNA sequences (81), copies of IS*2404* (4) and copies of IS*2606* (8). The G+C content of the chromosome (65%) is slightly higher than that of the plasmid which is 62.5%. IS*2404* sequences account for 6% of the genome of *M. ulcerans*. The insertion sequence (IS) elements are

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mobile genetic entities that encodes transposases which catalyze DNA copying or movement. The IS*2404* is 1,368 bp long, containing 41 bp perfect inverted repeats and producing 10 bp target-site duplications whereas IS*2606* is 1,438 bp long, with 31 bp imperfect inverted repeats and producing target site duplications of 7 bp (Stinear *et al.*, 2005).

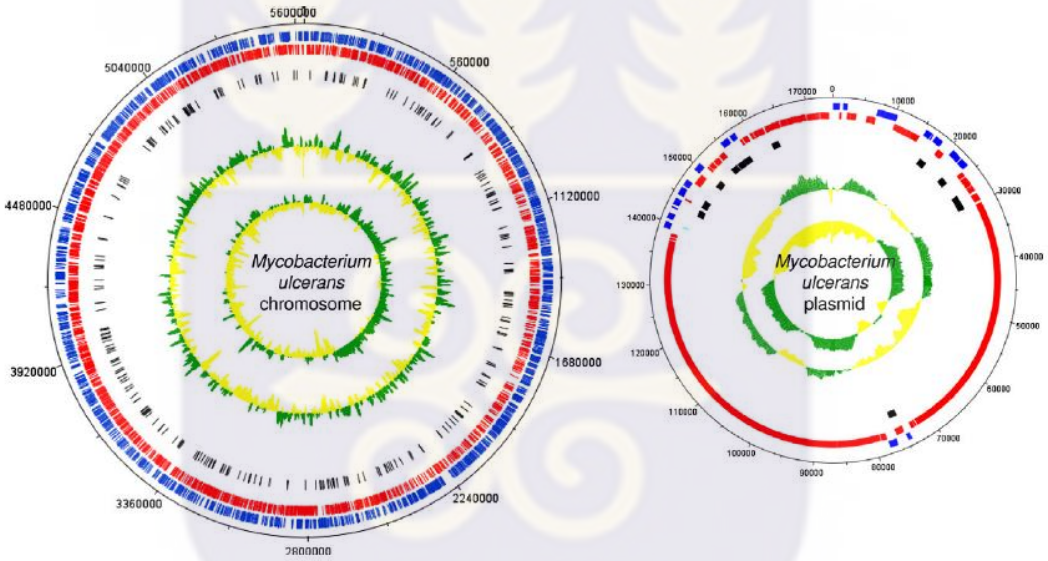

Figure 2.2 Circular representation of the *M. ulcerans* Agy99 chromosome and plasmid. The outer black circle shows the scale in bases. The next two inner circles illustrate the forward and reverse strand CDS in blue and red, respectively. IS element regions are displayed in the next inner circle in black. The innermost circles show GC plot and GC skew. The graph was designed using DNA Plotter Release 1.4 (Carver *et al.*, 2009).

2.2.3. The Mycobacteria Cell wall

Mycobacterium has a characteristic lipid rich thicker cell wall than those of many other bacteria. The mycobacterial cell wall functions as a permeability barrier for many

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molecules, including many antibiotics and detergents (Brennan and Nikaido, 1995). It also provides a physical protection that helps the bacteria survive from the harsh environment inside infected cells such as macrophages (Wang *et al.*, 2000). This atypical cell wall confers a characteristic acid-fast staining property to the bacteria; cells stained with a primary dye (basic fuschin) are resistant to acid-alcohol decolourisation (Brennan and Nikaido, 1995; Danilchanka *et al.*, 2008). The *Mycobacterium* cell has two segments upper and lower (Fig 2.3). The cell wall complex comprises peptidoglycan attached directly the membrane and linked covalently to an arabinogalactan layer and mycolic acids forming the mycolyl arabinogalactan–peptidoglycan complex. The uniqueness of mycolic acids confers on the bacterium lipid rich properties and makes it impermeable to hydrophilic antibiotics (Brennan and Nikaido, 1995). The upper segment of the cell wall is composed of free lipids interspersed in such a manner to allow for processing of lipoarabinomannan and other lipids. These components give the cell wall a hydrophobic feature which contributes substantially to the hardiness of the genus, their resistance to common disinfectants and high intrinsic resistance of mycobacteria to many drugs (Hoffmann *et al.*, 2008).

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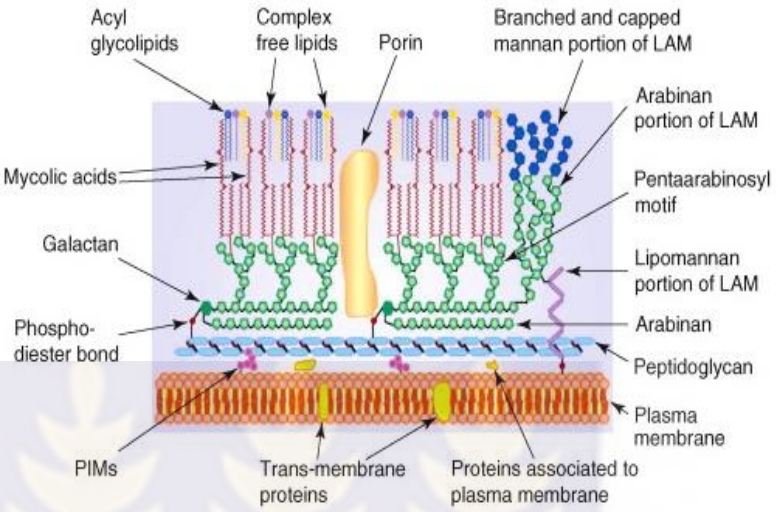

Figure 2.3. The Mycobacterium cell wall (Source: Trends in Microbiology, 2001)

2.3 Pathogenesis

The portal of entry of *M. ulcerans* into the host is not clear. Studies have suggested that *M. ulcerans* may be introduced into the host via insect bite (Portaels *et al.*, 1999; Marsollier *et al.*, 2002), puncture of the skin by contaminated environment or entry through a pre-existing wound upon trauma, or aerosol (Hayman, 1991; Ross *et al.*, 1997); however, none of these portals of entries has been confirmed.

Upon a successful entry of *M. ulcerans* into the host, it is confined under the skin (Fig 2.4). The long incubation period of *M. ulcerans* between 2-4.5 months favours proliferation within the dermis. Moreover, the temperature requirement of *M. ulcerans* also offers optimal conditions for the development of lesions in cooler tissues particularly the skin and subcutaneous tissues (Merritt *et al.*, 2010; Demangel *et al.*,

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2009). In BU infection there is minimal inflammatory response despite extensive tissue necrosis in ulcerated lesions that results from the production of mycolactone (Connor and Lunn, 1965; Torrado *et al.*, 2007). Histopathological analysis of biopsies obtained from active lesions shows *M. ulcerans* as extracellular clusters of bacilli lying within areas of coagulative necrosis (Forbes *et al.*, 1954; Torrado *et al.*, 2007).

Mycolactone, the virulence factor for *M. ulcerans*, is a polyketide composed of an invariant 12-membered lactone ring to which two polyketide-derived highly unsaturated acyl side chains are attached (Fig 2.5) (George *et al.*, 1999). The upper chain is invariant, whereas variations in the lower chain give rise to different congeners with variations in virulence (George *et al.*, 1999; Mve-Obiang *et al.*, 2003) (Fig 2.4). Pathogenic human strains from Africa, Australia and China predominantly produce mycolactone A/B, C and D, respectively (Mve-Obiang *et al.*, 2003, Walsh *et al.*, 2011).

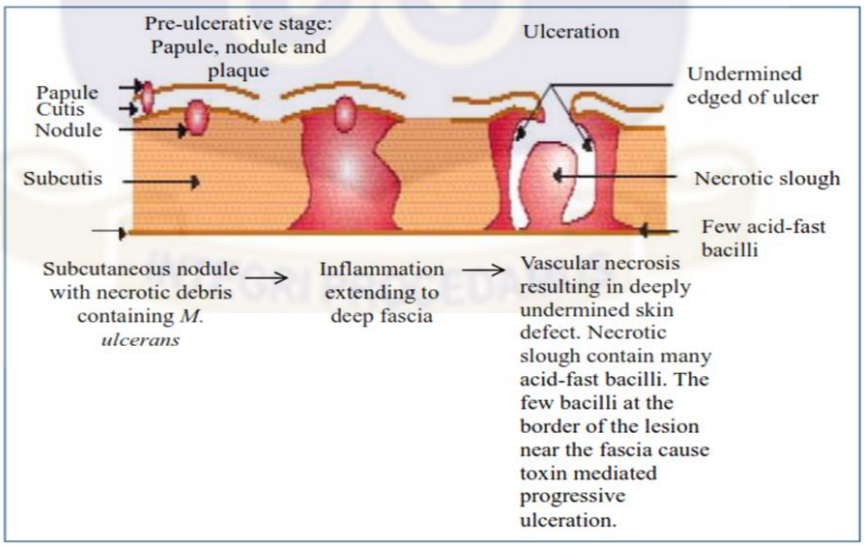

Figure 2.4 Changes in the skin caused by Buruli ulcer infection (van der Werf *et al.*, 1999).

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The most cytotoxic congener in vitro is mycolactone A/B. Strains from Asia are less virulent compared to strains from Africa and Australia (Mve-Obiang *et al.*, 2003). Also, other genetically related organisms pathogenic to fish and frogs can also produce variants of mycolactone E and F (Mve-Obiang *et al.*, 2005; Ranger *et al.*, 2006; Hong *et al.*, 2008). In *M. ulcerans* infection just as in other mycobacterial infections (e.g. *tuberculosis*), the immune system may fail to recognize common mycobacterial antigens thereby facilitating disease progression (van der Werf *et al.*, 1999). Both local and systemic immune responses through the effect of macrophages, dendritic cells, monocytes, T-cells and B-cells are suppressed (Hall *et al.*, 2014; Pahlevan *et al.*, 1999).

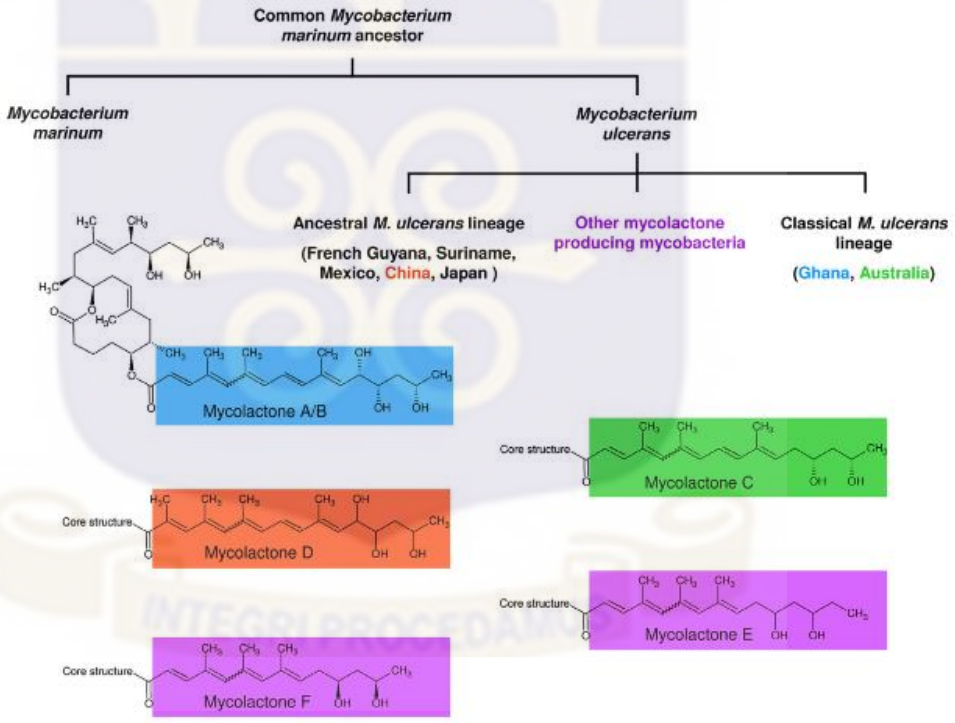

Figure 2.5 Simplified family tree of *M. marinum, M. ulcerans* and other MPM strains. Variants of mycolactone are shown with the corresponding color codes: mycolactone A/B produced by African *M. ulcerans* strains (blue); mycolactone C formed by *M. ulcerans* strains from Australia (green); mycolactone D produced by an *M. ulcerans* strain from China; mycolactone E and F formed by other MPM strains (George *et al.*, 1999).

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The state of an individual's immune system plays a role in the development of the disease (Van der Werf *et al.,* 1999). According to Gooding *et al* those who develop BU may already have (i) immune deviation resulting in down-regulation of Th1 and increased Th2 or (ii) have inherent defect in the immune system that leads to failure to develop any strong response to mycobacterial antigens (Gooding *et al*., 2002; Gooding *et al*., 2001). According to Johnson *et al*, although the mycolactone results in the development of BU, it may not be the only virulence factor in the pathogenesis of infection (Johnson *et al.*, 2005). Host susceptibility factors, which could be genetic or environmental, need to be explored in order to understand the reason for the development of BU (Stienstra *et al.*, 2002).

2.4 Diagnosis of Buruli ulcer

BU is presumably diagnosed based on clinical evidence by a health worker. However, a number of skin conditions present with symptoms similar to BU. In order to limit misdiagnosis, there is therefore the need for laboratory confirmation of clinically diagnosed BU cases. There are four different methods including, microscopic detection of acid-fast bacilli (AFB) in a stained smear, polymerase chain reaction (PCR) for the detection of *M. ulcerans* DNA, and isolation of viable bacilli by culture and histopathological analysis of biopsy specimen for laboratory confirmation (Fig 2.6). These four diagnostic methods have varied sensitivity, specificity, rapidity and cost effectiveness.

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2.4.1 Microscopy

Although direct smear examination of swab specimen from undermined edges of ulcers stained by ZN is the simplest and the easiest accessible diagnostic tool, the sensitivity is as low as 40%. Also it lacks specificity as a number of other mycobacterial species can cause skin lesions, therefore detection of AFB alone does not establish *M. ulcerans* as the cause of the infection (Yeboah-Manu *et al.*, 2011).

2.4.2 Culture

Cultivation of viable *M. ulcerans* from BU lesion is the full proof method for confirming the pathogen as causing the disease. However, the slow growth nature of the bacteria, which may take about 8 to 12 weeks or more, makes culture not suitable for diagnosis, as patients may be required to wait for longer periods before undergoing treatment. Furthermore, contaminations of cultures due to the presence of fast growing bacteria and fungi greatly affect the recovery of *M. ulcerans*. Variable *M. ulcerans* recovery rates have been reported; for tissue specimen, as high as 75% positivity has been obtained (Yeboah-Manu *et al.*, 2004), 41% for fine-needle aspirate (FNA) and 43% for swab samples (Yeboah-Manu *et al.*, 2011).

2.4.3 PCR

Polymerase chain reaction (PCR) assay targeting the insertion sequence IS*2404* is currently the gold standard for BU diagnosis in reference laboratories with specificity close to 100%. However, issues with false-positive results have recently been raised which require efficient quality control measures to ensure accurate results (Eddyani *et al.*, 2014). Although PCR technique is highly sensitive (98%), rapid and results can be obtained the same day; it is expensive and requires experienced personnel, expensive

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equipment and elaborate infrastructure (Yeboah-Manu *et al.*, 2011; de Souza *et al.*, 2012).

2.4.4 Histopathological Analyses

Histopathological features such as clumps of extracellular acid-fast bacilli surrounded by large areas of necrosis of the deep dermal and adipose tissue are used in the confirmation of BU cases, however these characteristics are not specific and may evolve with time as lesions erode from the pre-ulcerative stage to the ulcerative stages (Ruf, *et al.*, 2011). Moreover, highly experienced personnel and expensive equipment are required in the utilization of this diagnostic tool making it unsuitable as a point-of-care rapid diagnostic technique in low resource settings.

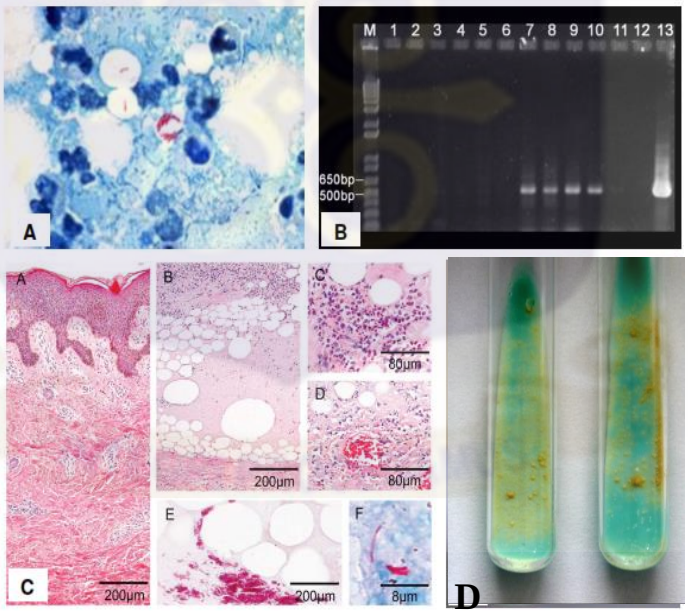

Figure 2.6 Laboratory techniques for diagnosing clinical BU cases. Direct smear examination (A) (Yeboah-Manu *et al.*, 2006); Agarose gel showing *M. ulcerans* insertion sequence *2404* PCR bands for positive samples (7-10 and 13) (B); Histological characteristics associated with untreated BU lesion (Schutte *et al.*, 2007) (C); Colonies of *M. ulcerans* on Lowenstein-Jensen medium (D).

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2.5 Treatment of Buruli ulcer

Until 2006, the main treatment of BU was surgical debridement of infected necrotic tissues including removal of portions of healthy tissues to minimize the rate of relapse and subsequent skin grafting to correct deformities that may result (Etuaful *et al.*, 2005). However, due to lack of surgical facility in most endemic areas, treatment was centralized, which required patients to be referred and treated in reference hospitals outside their domicile. The surgical cost and the prolonged hospitalization after surgery; approximately 3 months often times discouraged a number of patients to seek treatment (Asiedu and Etuaful, 1998). Since the extent of excision was the sole prerogative of the clinician, some bacilli could remain leading to relapse. Relapse after surgical treatment was as high as 30% (WHO, 2001; Kibadi *et al.*, 2009). The recurrence of cases further burdens the patients in terms of treatment cost and loss of trust in the health care system.

WHO in response to a pilot study that evaluated the efficacy of rifampicin and streptomycin on early lesions (Etuaful *et al.*, 2005; Chauty *et al.*, 2007), released a guideline in 2004 and recommended chemotherapy of oral rifampicin (10mg/kg) and intramuscular administration of streptomycin (15mg/kg) daily for 8 weeks (WHO, 2004). This was implemented in most of the affected countries by 2006 and has greatly improved healing of BU wounds and reduced relapses to less than 2% (Chauty *et al.*, 2007; Sarfo *et al.*, 2010). Despite the efficacy of the antibiotic therapy killing the infecting pathogen, debridement and skin grafting is often required, due to late reporting and patients presenting with large wounds that could not heal on its own. Physiotherapy is also provided at BU centers to reduce the risk of permanent sequelae from joint

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contracture and poor scaring (Walsh *et al.*, 2010; Walsh *et al.*, 2011) as well as education to improve early case reporting.

While rifampicin and streptomycin regimen have proved successful, the daily injection is quite invasive and uncomfortable especially considering that most of the affected are children. Thus an all-oral regimen is currently on clinical trial. An observational study in Ghana showed no difference in outcome when rifampicin and streptomycin were given for only 2 weeks followed by rifampicin and clarithromycin for 6 weeks (Philips *et al.*, 2014). The findings indicate that rifampin and clarithromycin can replace rifampin and streptomycin for the continuation phase after rifampin and streptomycin administration for 2 weeks without any apparent loss of efficacy (Philips *et al.*, 2014). Furthermore, observational studies conducted in both Benin and Australia indicate that oral chemotherapy using rifampicin plus clarithromycin during an 8-week period are clinically and microbiologically effective (Chauty *et al.*, 2011; Gordon *et al.*, 2010). Junghanss *et al.* have taken advantage of the temperature sensitivity of *M. ulcerans* into consideration and applied heat in the form of phase change material as a treatment option (Junghanss *et al.*, 2009). In their proof of principle study, this method was highly effective, however limited number of BU patients were used (Junghanss *et al.*, 2009).

2.6 Ecology and Transmission of *Mycobacterium ulcerans*

The emergence of BU has been strongly linked to aquatic ecosystems such as stagnant waters or river basins and changes in the environment (Fig 2.7). Disturbances in the environment resulting from changes in landscape through man-made and climatic events such as deforestation, flooding, construction of dams, mining activities, construction of

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artificial lakes for irrigation, and extending swamps for growing rice and fish breeding have been reported to impact greatly on the emergence of the BU disease (Weir, 2002; Merritt *et al.*, 2005). The evidence for the presence of *M. ulcerans* in the environment has been provided solely by the detection of *M. ulcerans*-specific DNA sequences such as IS*2404*, IS*2606* and ketoreductase B domain (KR) (Fyfe *et al.*, 2007) in many biotic components of aquatic ecosystems, such as plants, snails, fish, insects, water, fish, aquatic insects, detritus, leeches, crustaceans, and mosquitoes (Eddyani *et al.*, 2004; Johnson *et al.*, 2007; Kotlowski *et al.*, 2004; Marsollier *et al.*, 2002; Williamson *et al.*, 2008). However, the presence of *M. ulcerans* DNA in the environment is not necessarily indicative of a potential reservoir; but may rather confirm the ubiquitous presence of *M. ulcerans* in these environments.

Figure 2.7 Buruli ulcer endemic riverine sites along the Densu river basin (A and B) Mining site along the Offin river basin (C) Deforested palm plantation for pond construction along the Densu river basin (D).

Establishing the ecology of *M. ulcerans* has been a major challenge due to the extremely slow growth rate; cultivation from potential environmental sources are frequently

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contaminated with faster growing bacteria and fungi. Until this thesis, only one study has reported on the isolation of an *M. ulcerans* strain from an environmental source, an aquatic insect collected in Benin, after several passages in mouse footpads (Portaels *et al.*, 2008).

In South-Eastern Australia, testing of environmental samples using DNA based methods from BU endemic areas have been able to detect high concentrations of *M. ulcerans* DNA in the faeces of possums, small tree-dwelling arboreal marsupials (Fyfe *et al.,* 2010). Subsequent analyses of these animals showed that a large proportion of them had developed *M. ulcerans* skin lesions, indicating their potential as infectious reservoir host (Fyfe *et al.,* 2010; Carson *et al.,* 2014). In the BU endemic regions of Africa where there are no possums; analyses of faecal material in small animals did not lead to the identification of *M. ulcerans* DNA (Durnez *et al.,* 2010). In a more recent study, Tobias et al. (2016) made an attempt to identify source reservoirs of *M. ulcerans* in faecal matter of domestic animals in BU endemic villages of Ghana. All the test samples were negative for the *M. ulcerans* targets IS*2404* and KR. Their findings suggest that domestic animals living in and around humans do not shed *M. ulcerans* in their faeces, an indication that unlike Australian possums, domestic animals in BU endemic villages of Ghana are unlikely to be a major reservoir of *M. ulcerans*.

A recent longitudinal study conducted in Mapé basin of Cameroon suggests an aquatic niche environment as *M. ulcerans* DNA was persistently detected in a village water site (Bratschi *et al.,* 2014). Over more than two years period, underwater detritus at a localized position of the water hole was repeatedly tested positive for *M. ulcerans*-

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specific DNA sequences after all local BU cases had been treated, an indication that *M. ulcerans* has adapted to survive in a restricted niche environment (Bratschi *et al.*, 2014). Multiple studies have also reported that *M. ulcerans* is unlikely to be free-living and may persist in the environment as a commensal, associated with another protective organism such as amoeboid protozoa or inhabitants of biofilms in stagnant water bodies (Eddyani *et al.*, 2008; Gryseels *et al.*, 2012; Amissah *et al.*, 2014). Genome analyses have also shown that there is deletion or inactivation of genes required for pigment biosynthesis (light inducible carotenoids) and anaerobiosis (Stinear *et al.*, 2007). These evidences indicate that *M. ulcerans* is adapting to a new, probably relatively protected and stable ecological niche (Doig *et al.*, 2012; Stinear *et al.*, 2007; Qi *et al.*, 2009).

The mode of transmission of *M. ulcerans* still remains unknown even though routes of transmission including insect bite, contamination by the environment through puncture and aerosol have been proposed. Epidemiological reports from Australia describing the presence of *M. ulcerans* specific DNA in adult mosquitoes have led to the hypothesis that mosquitoes may play an important role in the transmission of *M. ulcerans* (Johnson *et al.*, 2007; Lavender *et al.*, 2011). In Point Lonsdale, examination of possums between 2008 and 2009 show that 38 % of ringtail possums and 24 % of brushtail possums had laboratory confirmed BU lesions and their faecal matter were PCR-positive for *M. ulcerans* (Fyfe *et al.*, 2010). It is believed that possums may take up *M. ulcerans* from environmental sources or may be infected by an insect vector and pass the bacteria back into the environment (Fyfe *et al.*, 2010). However, it is not clear how *M. ulcerans* may be transmitted from possums to humans.

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Sero-epidemiological studies conducted both in Ghana and Cameroon that analyzed antibody titers against the 18-kDa-small heat shock protein of *M. ulcerans* in healthy persons living in BU endemic areas found that children below the ages of 5 years are less exposed to *M. ulcerans* than older children (Yeboah-Manu *et al.*, 2012, Röltgen *et al.*, 2014, Ampah *et al.*, 2016). This indicates that exposure to *M. ulcerans* might occur outside of the relatively small movement range of very young children and increases in older children with more direct environmental contacts further away from their homes (Röltgen *et al.*, 2014). Other recent studies have also hypothesized that humans with active BU lesions may play a role in the spread of *M. ulcerans* into the environment (Bratschi *et al.*, 2014). This was noted after a decline in BU incidence in zones with improved BU surveillance and early treatment such as Australia, where patients are treated at early stages of the disease.

2.7 Plausible Risk Factors for the Buruli Ulcer disease

Over the years, studies have suggested different vectors, environmental and animal reservoirs to be involved in the transmission of *M. ulcerans*. It is also likely that different transmission pathway other than the already proposed theories may be involved. Studies conducted on the plausible risk factors have looked at the demographic, socio-economic, environment, host behaviours as well as host genetics (Table 2.3) to contribute to better understanding of the transmission of *M. ulcerans.*

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Children below 15 years are known to be the most high risk group. Debacker *et al.* found a higher rate of BU among children aged 5–14 years in Benin (Debacker *et al.*, 2006). Bratschi *et al.* in a survey in Cameroon found the highest risk in children between the ages of 4 and 14 years and as well as among 50 years and above (Bratschi *et al.*, 2013). In a study from Togo and others from Australia, the risks were reported to be higher among adults than in children (Johnsonet *et al.*, 2007; Quek *et al.*, 2007; James *et al.*, 2003; Veitch *et al.*, 1997). It appears that between the ages of 4 and 15 years, children are prone to intense environmental contacts as they move away from their homes (Röltgen *et al.*, 2014). A nearly equal sex distribution has been reported among cases in studies from both African and Australia (Amofah *et al.*, 2002; Marston *et al.*, 1995; Debacker *et al.*, 2004; O'Brien *et al.*, 2014).

Assessments of socioeconomic factors for BU have been reported with varying results. While one study found increased risk among individuals from households with low ownership rates (Nackers *et al.*, 2007); in another study no such association was found (Pouillot *et al.*, 2007). Participation in agricultural activities such as farming was reported to increase the risk of BU in a study conducted in Benin (Debacker *et al.*, 2006), however no such association was found in other studies (Aiga *et al.*, 2004; Pouillot *et al.*, 2007; Nackers *et al.*, 2007). On education, adults with only primary education or no education were found to be at a higher risk of BU (Pouillot *et al.*, 2007), however Stienstra *et al.* observed no such association (Stienstra *et al.*, 2002). Wetlands and swamps have also been linked to the incidence of BU (Raghunathan *et al.*, 2005; Debacker *et al.*, 2006; Merritt *et al.*, 2005). A recent study reported on the persistence *of M. ulcerans* specific

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DNA sequences over two years period at a water contact location in Cameroon (Bratschi *et al.,* 2014). Furthermore, BU incidence in Australia was attributed to dammed water for irrigating golf course; no further cases were reported after the irrigation ceased (Veitch *et al*., 1997).

Host behavioral factors have been found to play a major role in the acquisition of BU. Studies have reported that wound care and hygiene practices (Kenu *et al.,* 2014; Quek *et al.,* 2007; Nackers *et al.,* 2007) as well as wearing protective clothing (Kenu *et al.,* 2014; Raghunathan *et al.,* 2005; . Pouillot *et al.,* 2007) have a reduced risk for BU. Quek *et al.* found exposure to mosquitoes as a risk factor for BU; this lead to the assumption that *M. ulcerans* may be transmitted by mosquitoes (Quek *et al.,* 2007). Landier *et al.* reported that the use of bed net use may significantly decrease the risk of BU (Landier *et al.,* 2011). Furthermore, host genetic factors could predispose some individuals to the BU disease. The only reported human host genetic study showed that the susceptibility to BU may be associated with a polymorphism in a NRAMP gene (Stienstra *et al.,* 2006). Similar association to this gene has been reported in leprosy and tuberculosis.

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Table 2.2 Risk factors for Buruli ulcer identified from comparative case-control studies

Country (Study period)	**No of cases**	**No of controls (background)**	**Assessed risk factors**	**Identified risk factors**	**Protective factors identified**	**Reference**
Australia (1998-2005)	49	609 (community)	Lifestyle and insect exposure	Mosquito bites on lower legs and lower arms	Use of insect repellent, wearing protective clothing, washing of wounds	Quek *et al.*, 2007
Ghana (1999)	51	51 (community, matched by age, sex and BCG	Water related activities	Swimming in rivers on a habitual basis	None	Aiga *et al.*, 2004
Ghana (2000)	116	116 (community, matched by age and village)	Environment and behaviour	Wadding in a river	Wearing a shirt while farming, bathing with toilet soap, sharing indoor living space with livestock	Raghunathan *et al.*, 2005
Ghana (2010-2011)	113	113 (community, matched by age, sex and village)	Demography, socio-economy, health and hygiene, environment	Presence of wetland, insect bites in water, washing in the river, wading in the river, use of adhesive when injured	Use of alcohol for injuries, covering limbs during farming	Kenu *et al.*, 2014

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Benin (2002-2003)	324	1,173 (neighborhood, matched by age and sex)	Environmental and health related behaviour	Frequent contact with stagnant water	Regular use of soap for washing, treating injuries with soap, antibiotic powder	Nackers *et al.*, 2007
Benin (2002-2003)	279	988 (neighborhood, matched by age and sex)	BCG vaccination	None	None	Nackers *et al.*, 2006
Benin (1997-2003)	2399	1444 (hospital unmatched)	Age, sex, place of residence, BCG vaccination, water type for domestic usage, occupation	Age (children below 15 years and adults over 49 years), use of water from swamps,	None	Debacker *et al.*, 2006
Cameroon (2007-2009)	77	153 (community, matched by age, sex and village)	Activities and habits	Bathing in the river	Systematic use of bed net, cleansing wound with soap	Landier *et al.*, 2011
Cameroon (2006)	163	163 (community, matched by age and village) 118 (family)	Demography, environment and behaviour	Low level of education, swamp wading, wearing shorts, lower body clothing while farming, leaving near cocoa plantation or woods	Washing clothes, using bed nets, treating wounds with alcohol or leaves	Pouillot *et al.*, 2007
Côte d'Ivoire	116	116	116 (community, matched by age, sex	Socio demographic characteristics, BCG	No history of	Ahoua *et al.*,

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(2001)			and village)	vaccine status, water related activities, environment	BCG Having river, lake or dam near housing, place for fishing	2001
Côte d'Ivoire (2012)	51	102		Regular contact with unprotected surface water, absence of protective equipment during agricultural activities	Good knowledge about the risks of BU, perception about the causes of BU disease	N'krumah *et al.*, 2016

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2.8 Detection of *Mycobacterium ulcerans* from the environment

The currently available detection methods are aimed at detection of the mycobacteria DNA from environmental samples.

2. 8.1 PCR Based Targets for Detection of *M. ulcerans* from the environment

Several PCR methods for detection of *M. ulcerans* have been reported (Mve-Obianget *et al.*, 2005; Phillips *et al.*, 2005; Ablordey *et al.*, 2005; Stragier *et al.*, 2005). The most common, IS*2404* PCR, however is not specific in its application making it unsuitable for detection of *M. ulcerans* from environmental samples since other closely related *Mycobacterium* strains including *M. liflandii, and M. pseudoshottsii*, (Stinear *et al.*, 1999; Stragier *et al.*, 2007) have been found to harbor the IS*2404* sequence. Other molecular targets found on the *M. ulcerans* chromosome (IS*2606*) and plasmid (Ketoreductase (KR), enoyl reductase (ER)) have also been targeted for detection (Fyfe et al., 2007; Williamson *et al.*, 2012). The development of TaqMan assay (Fyfe *et al.*, 2007) which targets IS*2404* multiplexed with an internal positive control to monitor inhibition, and multiplexing of IS*2606* and KR have enhanced the specificity of the IS*2404* PCR for the analysis of a variety of environmental samples. Assaying for these three targets has resulted in the detection of *M. ulcerans* DNA in a large number of environmental samples including soil, sediment, and mosquito (Fyfe *et al.*, 2007).

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2.8.2 Culture

Cultivation of mycobacteria is the "gold standard" for detection of mycobacteria in a sample. Culture of mycobacteria is done on either solid or liquid media or both; however, cultivation on solid allow for the observation of colony morphology. Other members of NTM have been readily isolated from various environmental sources including drinking water distribution systems (Thomson *et al.,* 2013), hot tubs (Marras *et al.*, 2005), potting soils (De Groote *et al.,* 2006), showerheads (Falkinham *et al.,* 2008), feeds, animal feaces, raw milk, powdered infant milk and retail cheese (Ayele *et al.,* 2005), however, *M. ulcerans* has only once been isolated from the environment; after several passages through mouse footpad (Portaels *et al.,* 2008), a process that took almost three years. Furthermore, the complex mixture of other fast-growing bacteria and fungi in environmental samples, which contaminate the culture medium coupled with the slow growth rate of *M. ulcerans* possess a challenge to its isolation in pure culture. The *M. ulcerans* thicker cell walls nature and the unique mycolic acids contribute to its impermeability to certain chemical agents and antimycobacterials. Exploiting this feature of the cell wall by subjecting it to various chemical agents prior to culture is essential to limit the growth of unwanted microorganisms while maximizing the recovery of *M. ulcerans* in pure culture.

2.8.2.1 Decontaminating Agents

To eliminate unwanted microorganisms in primary cultivation; chemical agents (acids, bases, and detergents) have been utilized in decontaminations steps since the thicker cell wall and mycolic acids contents make *M. ulcerans* resistant these chemicals (Burdz *et al.*,

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2003). Some of these agents destroy both target organisms along with the contaminants, while others are too weak to destroy them (Steingart *et al.,* 2006; Burdz *et al*., 2003; de Kantor and Laszlo, 1998). Therefore a balance between the two is required to enhance isolation of target mycobacteria. Furthermore, introduction of antimicrobial agents such as malachite green and cycloheximide during the decontamination step to suppress contaminants also enhances recovery rate (Falkinham III, 1996; Gumber and Whittington, 2007; Whittington *et al.,* 1999). The timing allowed for samples to decontaminate also contributes to the killing action on both contaminants and target mycobacteria (Falkinham III, 1996; Pfyffer *et al.,* 2003).

A number of chemical agents that have been used in various decontamination steps include oxalic acid (OA) for clinical BU samples (Yeboah-Manu *et al.,* 2004; Yeboah-Manu *et al.,* 2011), N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) for sputum specimen (Kubica *et al,* 1963; Buijtels and Petit, 2005; Heifets and Good, 1994), sodium dodecyl (lauryl) sulfate–NaOH (SDS-NaOH) for sputum (Pfyffer *et al.,* 1994), NaOH-Malachite green-Cycloheximide for soil samples (Portaels *et al.,* 1988), and 1-Hexadecylpyridinium Chloride (HPC) for sputum (Holanda *et al,* 2002; Smithwick *et al.,* 1975).

The acids and bases such as OA and NaOH are lethal to contaminating microorganisms by disrupting their intracellular pH; however, mycobacteria are able to resist maintaining their internal pH significantly better than non-mycobacterial organisms (Rao *et al.,* 2001). The sodium dodecyl (lauryl) sulfate (SDS) on the other hand act as an

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anionic surfactant that aids in lysing cells and for denaturing proteins, (Chung *et al.*, 2013). N-acetyl-L-cysteine (NALC) is a mucolytic agent that has the ability to reduce disulphide linkages between proteins (Kubica *et al.*, 1964). Also HPC is quaternary ammonia compounds that disrupt the lipid bilayer membranes of microorganisms leading to generalized and progressive leakage of cytoplasmic materials (Gilbert and Moore, 2005).

Few studies have compared different decontamination steps in the recovery of mycobacteria from environmental samples (Engbaek *et al.*, 1967; Falkinham *et al.*, 1980; Kamala *et al.*, 1994). Engbaek *et al.* evaluated five different decontamination steps and found sodium lauryl sulfate method as the most suitable for mycobacteria recovery (Engbaek *et al.*, 1967). Kamala *et al.* also evaluated six decontamination steps each for isolation of mycobacteria from soil and water samples taken in Madras in southern India (Kamala *et al.*, 1994). They found 1% NaOH and 3% sodium lauryl sulphate to be effective in isolating mycobacteria than higher concentrations of NaOH and other decontamination procedures (Kamala *et al.*, 1994). When this decontamination procedure was employed by Parashar *et al.* contamination could not be removed from any of specimens. This could be due to the differences between the ranges of contaminants present in the samples collected. However, when the decontamination step was further modified by using different concentrations of reagents from 1 to 4% NaOH and 1 to 2% cetrimide, contamination with other organisms was completely eliminated leading to mycobacteria recovery (Parashar *et al.*, 2009).

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Portaels *et al*. also compared different decontamination steps and culture media for the isolation of mycobacteria from soil specimens collected in Louisiana. The best results was obtained from pre-incubation of the soil samples in tryptic soy broth (TSB), followed by decontamination with malachite green, cycloheximide and NaOH and by inoculation of the samples onto Ogawa medium containing cycloheximide (Portaels *et al*., 1988). These varied findings clearly indicate that there is no standard decontamination procedure for recovery of mycobacteria from the environment, however, factors such as sample location and type must be considered to achieve optimal recovery.

2.8.2.2 Growth Media for *Mycobacterium ulcerans*

Over the years, culture media have been formulated in the form of egg-based, agar-based, or liquid media. Both liquid and solid media can be made selective for mycobacteria via addition of antibiotics (Parish and Stoker, 1998) to eliminate growth by contaminants. The recommended solid media include either egg based media such as Löwenstein-Jensen medium (LJ) and agar-based media such as Middlebrook 7H10 and 7H11 agar which contains malachite green and are enriched with Oleic Albumin Dextrose Catalase (OADC) to enhance mycobacteria recovery.

The first egg-based medium was developed by Dorset in 1903, since then egg yolk has been used to enrich media to recover mycobacteria (Twort and Ingram., 1912). Other media formulations such as LJ, American Trudeau Society, Ogawa, and Petragnani have been produced. Löwenstein-Jensen medium was originally formulated by Lowenstein; it contained congo red and malachite green dyes (Lowenstein, 1931). Jensen later modified

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Lowenstein's medium by altering the citrate and phosphate contents, eliminating the congo red dye and by increasing the malachite green concentration to inhibit growth of contaminating organisms (Jensen, 1932). The main sources of nitrogen and vitamin in LJ medium are L-Asparagine and potato flour. The mono-potassium phosphate and magnesium sulfate component of the medium enhance organism growth and act as buffers. Glycerol and the egg suspension provide fatty acids and protein that are required for the metabolism of mycobacteria. The coagulation of the egg albumin during sterilization provides a solid medium for inoculation purposes. Löwenstein-Jensen medium is now the recommended medium for culture of mycobacteria (WHO, 1998). Löwenstein-Jensen medium requires incubation between 2-8 weeks and even more than 6 months for the isolation of slow growing *M. ulcerans* (Hosek *et al.,* 2006; Sharp *et al.,* 2000).

Current liquid cultivation of mycobacteria requires the use of BACTEC MGIT (Mycobacteria Growth Indicator Tubes) which is composed of 7 ml of modified Middlebrook 7H9 Broth base enriched with OADC and PANTA antibiotic mixture.

This literature review highlights a number of missing gaps including the ecological niche (s) and adaptation preference, lack of direct isolation methods of *M. ulcerans* from the environment and drivers (environmental and behavioural) that are likely to predispose individuals to BU infection that limit efforts towards understanding to the ecology of *M. ulcerans* of which this thesis sought to out to address.

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CHAPTER THREE

Materials and Methods

3.1 Ethics Statement

This study was part of the Stop Buruli (BU) project at the Noguchi Memorial Institute for Medical Research (NMIMR). The scientific merit of the study and ethical clearance was respectively obtained from the Scientific and Technical Committee and the Institutional Review Board (Federal-wide Assurance number FWA00001824, NMIMR-RIB CPN 099/11-12) of the Noguchi Memorial Institute for Medical Research (NMIMR). Most of the analysis done within this study involved environmental samples. Within the case epidemiology analysis, clinical samples were obtained from presumptive BU cases for laboratory confirmation. Sampling and case management followed the national guidelines.

Written informed consent was sought from all study participants. For participants that were minors, informed consent was sought through their parents or guardians and all those who consented for participation either signed or thumb printed an informed consent form. Participants were assured of confidentiality for their involvement in the study. The objectives and methodology were fully explained to participants involved in the behavioural study. Study participants were made aware that their involvement was totally voluntary and they reserve the right to withdraw at any time. Participants enjoyed no direct benefits but the study finding would aid in identification of potential risk factors for BU within their communities.

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3.2 Study design and Site

The study was an environmental epidemiological study and consisted of two major sections involving different experimental designs. 1) The first part was a cross-sectioinal environmental assessement that sought to detect *M. ulcerans* by both molecular and conventional methods, assessment of seasonal influence on the occurrence of *M. ulcerans* DNA in the environment and detection of human BU cases from the selected communities and 2) The second part was a case-control study which was conducted only in the communities along Densu river basin which sought to assess possible environmental and behavioral risk factors for BU. In this section both cases and their controls were interviewed using structured questionnaires.

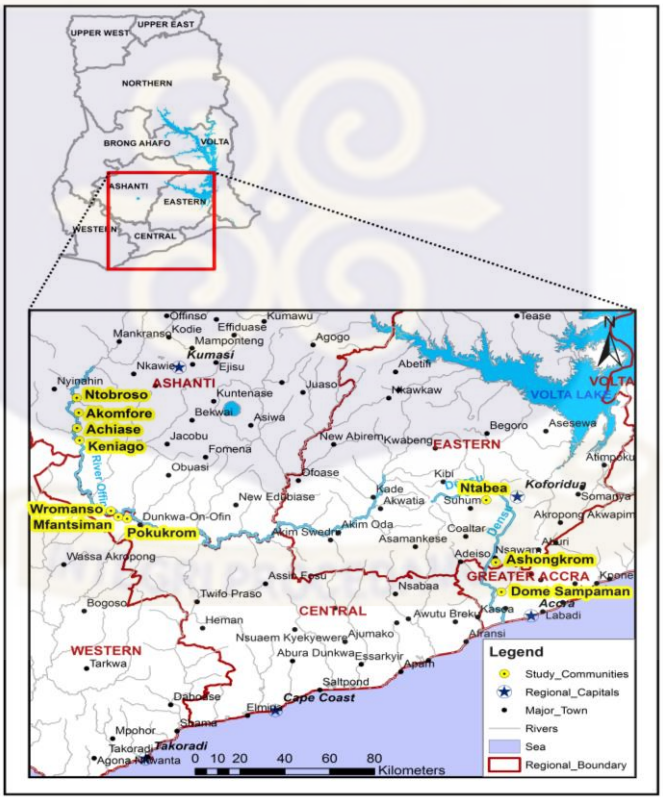

Figure 3.1 Map of the Ghana showing studied communities along both Densu and Offin river basins. Map was purposely generated for this study using Arc Map (Environmental Systems Resource Institute ver 10.2).

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The study was conducted in ten selected communities along two major river bodies (Densu and Offin) of Ghana (Fig 3.1). The Densu River is 116 km long and is the main source of drinking water supply for the inhabitants. Agriculture is the predominant occupation of the people in the communities absorbing about 70% of the total population within the districts. The Offin River is 130 km long and drains through two regions, the Ashanti and Central. Inhabitants of the communities upstream of the river are mainly engaged in agricultural activities while inhabitants at both mid and downstream are generally engaged in agricultural and mining activities. These river bodies were selected for the study because previous sero-epidemiological studies have shown high exposure of community member to *M. ulcerans*' 18Kda heat shock protein 65 (Yeboah-Manu *et al.*, 2012; Roltgen *et al.*, 2014; Ampah *et al.*, 2016a). Moreover most of the communities within the study sites continually report BU cases to the National BU Control Programme (NBUCP) (Amofah *et al.*, 2002).

The procedure that was used for site selection was as done by Ampah *et al.*, (2016). The communities were selected from a total of 199 communities using a randomization tool embedded in the ArcGIS 10.2 mapping software. Along the Densu river basin, the following communities were included; Ntabea in the East-Akim district, upstream of the river; Ashongkrom in the Akwapim South district and midstream of the river; and Domesampaman in the Ga-West Municipality of the Greater Accra region, downstream of the river.

Along the Offin river basin, seven communities were randomly selected. These included, Ntobroso in the Atwima district which lies upstream of the river (Ashanti region),

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Akomfore in the Amansie West district (Ashanti region) and Achiase, Wromanso and Keniago also in the Atwima district but located midstream of the river (Ashanti region) and finally Mfantsiman and Pokukrom both in the Upper Denkyira district and located downstream of the river (Central Region).

3.3 Environmental sample collection

Prior to environmental sample collection, all the selected communities were mapped using the GPS co-ordinates from NBUCP and with the ArcGIS 10.2 mapping software. Sites of frequent human activities such as water bodies which are regularly utilized for domestic purposes such as washing and cooking, communal bathing areas utilized by household members, school compounds particularly playgrounds, agricultural farms, market grounds, community centres, sources of drinking water such as boreholes, streams, rivers and water from storage tanks in homes were used as reference points (Fig 3.2). Each community was divided into grids and sampling points were randomly selected using a randomization tool embedded within the ArcGIS 10.2.

Convenience sampling was used to collect environmental samples from three communities (Ntabea, Ashongkrom, and Domesampaman) along the Densu river basin in 2011, 2013 and 2014. This was purposely done to capture specific zones within the environment where there is likelihood of frequent human interactions considering the community sizes. All the communities were mapped and divided into grids and 239 sampling points were randomly selected using a randomization tool embedded within the

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ArcGIS 10.0. During the sampling, myself together with two research assistants walked through the communities and collected environmental samples from specific reference points generated where there seem to be frequent human interactions.

At each sampling location, a distance of at least 10 m was allowed between sampling points and GPS coordinates were taken at each collection point. Solid samples such as animal faeces (sheep, lizard and chicken), terrestrial insects (using insect net) and soil (approximately 5g) and fungi found growing in the soil and on dead and decaying log were collected separately into sterile labelled 50 ml falcon tubes (BD Biosciences) and sealed tightly to avoid cross-contamination of samples. Snail samples were kept in labelled sealable plastic bags. Moss and vegetation parts were collected and pressed separately into labelled 50 ml falcon tubes (BD Biosciences) and later released into sealable plastic bags from which biofilm suspensions were prepared. Water samples (45 ml) were collected into sterile labelled 50 ml falcon tubes (BD Biosciences) and sealed tightly. All samples were clearly labelled immediately, kept in a cool pack at $4^{O}C$ after collection and transported to the laboratory within 6 hours from the time of collection. Samples were processed on the same day of collection and analyzed by Zeihl-Neelson staining, real time PCR and culture within 48 hours of collection.

Along the Offin river basin, sampling was conducted in 2013 and 2014 from seven communities (Achiase, Akomfore, Keniago, Mfantsiman, Ntobroso, Pokukrom and Wromanso), as conducted along the Densu river basin. All the communities were mapped and divided into grids and 487 sampling points were randomly selected using a randomization tool embedded within the ArcGIS 10.2. Samples were collected from each of the 487 points generated and treated in the same way as those from the Densu river

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basin and transported to the laboratory. Rainfall levels data were collected from the Ghana Meteorological Agency, Accra after monthly rainfall levels data from the meteorological sub-stations within the study sites have been reported.

Figure 3.2 Sites of sample collection and sample preparation in the laboratory (A) Community river for domestic use (B) Stream used for washing (C) A stream in BU endemic village along the Densu (D) Environmental samples being processed in the laboratory.

3.4 Environmental Sample Processing

All samples were processed on a clean bench at a biosafety level 2 (BS2) laboratory. Each of snail, feacal and fungi samples was diced separately with sterile disposable surgical blade (Swann-Morton) and homogenized using sterile porcelain and pestle (Cole-Parmer). The contents were suspended in 10 ml of phosphate buffered saline (PBS) (Sigma-Aldrich).

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Soil samples were prepared using a modified version of Parashar *et al.* (2009) method. Approximately 5 g of soil samples were suspended in 30 ml of PBS in sterile 50 ml falcon tubes (BD Biosciences). Samples were shaken vigorously for 1 minute and centrifuged at 600 x g for 5 min at 4°C to pellet the soil particles. The turbid supernatant (15 ml) was transferred into new sterile 50 ml falcon tubes (BD Biosciences) and centrifuged at 7, 000 × *g* for 10 min at 4°C and the pellets suspended in 10 ml PBS (Sigma-Aldrich). Biofilms were washed from moss and vegetation parts using modified version of Gryseels *et al.* (2012) method. Samples were emptied into sterile plastic re-sealable bags and 50 ml of PBS (Sigma-Aldrich) was added to each. The content of the bag was vigorously agitated to dislodge the biofilms into solution. The suspension was poured into sterile 50 ml falcon tubes (BD Biosciences) and centrifuged at 1700 x g for 30 minutes to sediment all suspended bacteria. The supernatant was decanted and the resulting pellet was suspended in 10 ml PBS (Sigma-Aldrich). Water samples were vortexed to mix homogenously and centrifuged at 1700 x g for 30 minutes to sediment all suspended bacteria (Parashar *et al.,* 2009). The supernatant was decanted and the resulting pellet was suspended in 10 ml PBS (Sigma-Aldrich). Prepared sample suspensions were kept at 4^{O}C until ready for analysis.

3.5 Molecular detection of *Mycobacterium ulcerans*

3.5.1 Genomic DNA Extraction

Genomic DNA was extracted directly from 1,600 environmental samples using the FastDNA SPIN kit for soil with the FastPrep-24^{TM} instrument (MP Biomedicals) according to the manufacturer's instructions. Processed samples (400 ul) were each added

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into lysing matrix E tubes provided in the kit. Lysis buffer (800 µl) and 200 µl of protein precipitation solution (PPS) were added to the sample and vortexed at full speed for two minutes. The suspension was further homogenized to break the bacterial cell wall using the fast prep machine for 40 seconds at a speed of 6 m/s. The mixture was centrifuged at 14000 0 g for 10 minutes to sediment the soil, cell wall and other debris. Supernatant containing (200 ul) of DNA was transferred into 2ml microcentrifuge tube and 500 µl of binding matrix solution was added and briefly vortexed to bind to the released DNA. The mixture was then transferred into a spin column provided in the kit using transfer pipette and centrifuged at 14000 g for 1 minute to wash away excess diluent. The buffer SEWS-M (500 ul) (containing ethanol) was added gently to the bound DNA in the column for further DNA purification and also centrifuged at 1400 x g for 1 minute. The bound DNA in the column was centrifuged at 1400 x g for 2 minutes again without any solvent to dry the matrix after which it was air-dried for 5 minutes. Bound DNA was gently re-suspended in 100 µl of Dnase/Pyrogen-free water (DES) and incubated for 5 minutes at room temperature. DNA was eluted from the column by centrifugation at 14000 g for 1 minute into a sterile new catch tube. The eluted DNA was stored at -20 °C until ready for PCR. Negative controls were included at each point of DNA extraction.

3.5.2 Screening of samples by Real Time PCR (RT-PCR)

Three independent gene targets; IS*2404* (present in over 205 copies in the *M. ulcerans* genome), IS*2606* (present in over 90 copies in the *M. ulcerans* genome) and ketoreductase-B domain (KR) (Fyfe *et al.*, 2007; Stinear *et al.*, 1999) were screened. Primers and TaqMan MGB probes from Applied Biosystems that were selected from

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regions of the sequences for IS*2404*, IS*2606*, and KR used as shown (Table 3.1) (Fyfe *et al.,* 2007). Probes IS*2404*TP and KRTP were labelled with the fluorescent dye 6-carboxyfluorescein (FAM) at the 5′ end and a nonfluorescent quencher at the 3′ end. Probe IS*2606*TP was labelled with the fluorescent dye VIC at the 5′ end and a nonfluorescent quencher at the 3′ end (Fyfe *et al.,* 2007).

The extracted DNA was first screened for IS*2404* by RT-PCR using Rotor Gene Q (Qiagen). The IS*2404* RT-PCR mixtures contained 1 µl of template DNA, 0.9-µM concentrations of each primer, a 0.25 µM concentration of the probe, SensiFast (500 nM) mix (Bioline), and TaqMan exogenous internal positive control (IPC) reagents (Applied Biosystems) in a total volume of 20 µl. Amplification and detection were performed using the Gene Q sequence detection system (Qiagen) according to the following program: 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 15 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. DNA extracts were tested in at least duplicate, and negative controls were included in each assay. All samples positive for IS*2404* target with critical threshold (C_T) value 35 and below were multiplexed (without IPC) for IS*2606* and KR assays. Amplification and detection were performed using the Gene Q sequence detection system (Qiagen) according to the following program: 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 15 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. DNA extracts were tested in at least duplicate, and at each PCR run, two each of negative and positive controls were added. All samples that were positive for both IS*2606* and KR after the multiplex assay were classified as *M. ulcerans* confirmed.

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Table 3.1 Primers and probes for real-time PCR assays targeting IS*2404*, IS*2606*, and

Primers and Probes	**Sequence 5'-3'**	**Reference**
IS*2404* TF	AAAGCACCACGCAGCATCT	Fyfe *et al.*, 2007
IS*2404* TR	AGCGACCCCAGTGGATTG	
IS*2404* TP	6 FAM-CGTCCAACGCGATC-MGBNFQ	
IS*2606* TF	CCGTCACAGACCAGGAAGAAG	Fyfe *et al.*, 2007
IS*2606* TR	TGCTGACGGAGTTGAAAAACC	
IS*2606* TP	VIC-TGTCGGCCACGCCG-MGBNFQ	
KRTF	TCACGGCCTGCGATATCA	Fyfe *et al.*, 2007
KRTR	TTGTGTGGGCACTGAATTGAC	
KRTP	6 FAM-ACCCCGAAGCACTG-MGBNFQ	

3.6 Direct Smear Microscopy

Direct smear examination was conducted to directly detect acid fast bacilli (AFB) in the processed samples. Frosted glass slides (Sigma-Aldrich) were labeled with sample identification number and date. Smear was prepared by transferring 100 µl each of processed samples onto respective labeled slides. The smear was allowed to air-dry in a level 2 biosafety cabinet to prevent risk of infection. The air-dried slides were heat fixed by passing the slide over flame 3 times. Heat fixed slides were arranged on a staining rack, leaving enough space between each slide to prevent cross contamination during the staining process. Slides were stained by Ziehl-Neelsen procedure.

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Briefly, each slide was flooded with filtered carbol fuchsin which contains phenol and heated underside to steam but not to boil. The purpose was to break open the cell wall for the stain to penetrate the lipid rich thick cell wall of mycobacteria. The slides were left at room temperature for 5 minutes. Slides were rinsed off the excess carbol fuchsin stain with tap water; the smears were decolorized by flooding with 20% H_2SO_4 solution to remove the carbol fuchsin from the non-acid-fast bacilli cell wall for 5 minutes and then gently rinsed in tap water. The slides were counter stained with 0.1% methylene blue solution for 1 minute and air dried before observing under the microscope. Mycobacteria appeared bright reddish-pink in colour while other cells stained blue. The slides were observed using a light microscope under oil immersion and were graded using the International Union against Tuberculosis and Lung Diseases (IUALTD) grading scale. A smear was declared negative only after reading at least 100 microscopic visual fields and confirmed by a second reader.

3.7 Cultivation of *Mycobacterium ulcerans*

3.7.1 Preparation of Selective Growth media for *M. ulcerans* culture

Lowenstein-Jensen (LJ) medium was prepared by weighing the mineral salts (Table 3.2) and added to 100 ml of distilled water in flat-bottomed flask. The medium base was sterilized in an autoclave at 121 ^{0} C for 15 minutes and allowed to cool to 46 ^{O}C.

Fresh eggs were broken, homogenized in a sterile beaker and the homogenate was filtered through sterile gauze. One litre of the homogenate was aseptically and slowly added to the LJ base to reduce creation of bubbles inside a class 2 microbiological safety cabinet. Filtered malachite green solution was added to the media to prevent the growth of fast

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growing bacteria. The LJ medium mixture was supplemented with various antibiotics such as PANTA (Polymixin B (2000,000 IUL^{-1}), Amphotericin (10 mgL^{-1}, Nalidixic acid (10 mgL^{-1}), Trimethoprim (10 mgL^{-1}/l), Azlocillin (10 mgL^{-1}) (BD) (P); Isoniazid (0.02 mgl^{-1}) (INH) (Sigma-Aldrich) and Ethambutol (0.2 mgl^{-1}) (EB) (Sigma-Aldrich) and without drug supplement (drug free/DF) respectively and antifungal (mycobactin J) as indicated (Table 3.3). The medium was thoroughly mixed, allowed to settle to reduce bubbles and distributed into sterile 30-ml universal bottles in 5 ml aliquots. The tubes were placed in a slanted position and inspissated at 80 ^{0}C for 50 minutes to coagulate the medium. The prepared media were allowed to cool to room temperature and stored at 4 ^{0}C until ready to be used.

Table 3.2 Components of the Lowenstein-Jensen media

Components	**Lowenstein-Jensen media**
Monopotassium dihydrophosphate (KH_2PO_4), anhydrous	2.4 g
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.24 g
Magnesium citrate	0.6 g
L-Asparagine	3.6 g
Distilled water	600 ml
Glycerol (ml)	12 ml
Egg homogenate	1000 ml
Malachite green (2%)	20 ml
pH (about)	7

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Table 3.3 Composition of antibiotics supplementing Lowenstein-Jensen media

Antibiotics	Composition
PANTA	For 1000 ml of LJ prepared medium, 3ml of sterile distilled water was added aseptically to each PANTA bottle and mix thoroughly after which 20 ml of PANTA was added to medium mixture
Isoniazid	For 1000 ml of LJ prepared medium, 10 ml of 0.02mg/ml of isoniazid was added
Ethambutol	For 1000 ml of LJ prepared medium, 10 ml of 0.2mg/ml of isoniazid was added
Mycobactin J	For 1000 ml of LJ prepared medium, 2 mgL^{-1} mycobactin J was dissolved by adding 0.5 ml of absolute ethanol and thoroughly mixed and the mixture was added to the medium

3.7.2 Optimization of Decontamination Procedures for Mycobacterial Culture

3.7.2.1 Preparation of Spiked Environmental Samples

A suspension of in-house reference clinical *M. ulcerans* isolate NM209 was subcultured and harvested mid-log phase and put into suspension with PBS at a concentration of 10^8 ml^{-1} and was mixed with 1ml of 10^7 ml^{-1} each of *E. coli* suspension obtained from stool sample, *Pseudomonas aeurginosa* from wound cultures, representing gram negative bacteria and *Bacillus sp* (gram positive rods) obtained from an open plate culture. Two millilitre aliquots of the pooled suspension were then prepared and used for isolation of *M. ulcerans*. Eight environmental samples (4 water and 4 wet soil samples) were spiked with 10^8 ml^{-1} of *M. ulcerans* pool suspension and decontaminated.

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3.7.2.2 Evaluation of the Decontamination Step

To optimize the decontamination procedure for isolation of *M. ulcerans* from environmental samples, an in-house decontamination method, sodium hydroxide/ Oxalic Acid, (NaOH-OA) was evaluated, together with three other standard decontamination procedures that are commonly used in TB laboratories namely; sodium dodecyl sulphate /Sodium hydroxide (SDS/NaOH) (Parashar *et al*., 2009), sodium hydroxide / Malachite green-Cycloheximide (NaOH/Mal) (Wollinsky and Rynearson, 1968) and N-Acetyl-L-Cysteine-Sodium Hydroxide (NaLC-NaOH) (Selkon *et al.,* 1966) using the spiked samples following the steps outlined in the flow chart (Fig 3.3).

3.7.2.2.1 NaOH-Oxalic acid

Two volumes of 1M NaOH was added to spiked sample suspension and allowed to incubate at room temperature with intermittent vortexing for 20 mins. The reaction was neutralized by addition of sterile distilled water to the 45 ml mark of the 50 ml falcon tube. The sample was centrifuged (Eppendorf 5810 R) at 3800 rpm for 30 minutes and the supernatant carefully decanted after which 2 ml of 5% oxalic was added to the pellet and allowed to incubate at room temperature for another 30 minutes at 4°C with intermittent vortexing. Decontamination reaction was finally terminated by the neutralization of sterile distilled water. The suspension was centrifuged at 3800 rpm for another 30 minutes at 4°C and the supernatant carefully decanted leaving the precipitate.

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3.7.2.2.2 SDS-NaOH

Two millilitres of 3% SDS and 4% NaOH was added to 2 ml of spiked sample suspension and allowed to incubate at room temperature with intermittent vortexing for 30 minutes. The decontamination reaction was stopped by the neutralization of the mixture with sterilized distilled water to the 45 ml mark of the 50 ml falcon tube. The suspension was then centrifuged (Eppendorf 5810 R) at 3800 rpm for 30 minutes at 4°C after which the supernatant was decanted.

3.7.2.2.3 NaOH- Malachite green-Cycloheximide

For each of 2% NaOH and 0.3% malachite green decontaminant, 2.5 ml was added to 2ml spiked sample suspension and allowed to incubate at room temperature with intermittent vortexing for 30 mins. The decontamination reaction was stopped by the neutralization of the mixture with 1N HCl. The suspension was centrifuged at 3800 rpm for 30 minutes at 4°C and the supernatant carefully decanted leaving the precipitate after which 2.5 ml 0.15% cycloheximide was added to the pellet and allowed to incubate at room temperature for another 30 minutes at 4°C with intermittent vortexing. Decontamination reaction was finally terminated by the neutralization of sterile distilled water. The suspension was centrifuged at 3800 rpm for another 30 minutes at 4°C and the supernatant carefully decanted leaving the precipitate.

3.7.2.2.4 N-Acetyl-L-Cysteine-Sodium Hydroxide (NALC-NaOH)

An equal volume of 3 ml 2% Nalc-NaOH solution prepared was added to spiked sample suspension and allowed to incubate at room temperature for 20 minutes with intermittent vortexing. Decontamination reaction was terminated by neutralizing mixture with sterile

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distilled water to the 45 ml mark of the 50 ml falcon tube. The suspension was centrifuged at 3800 rpm for 30 minutes at 4°C and the supernatant carefully decanted leaving the precipitate.

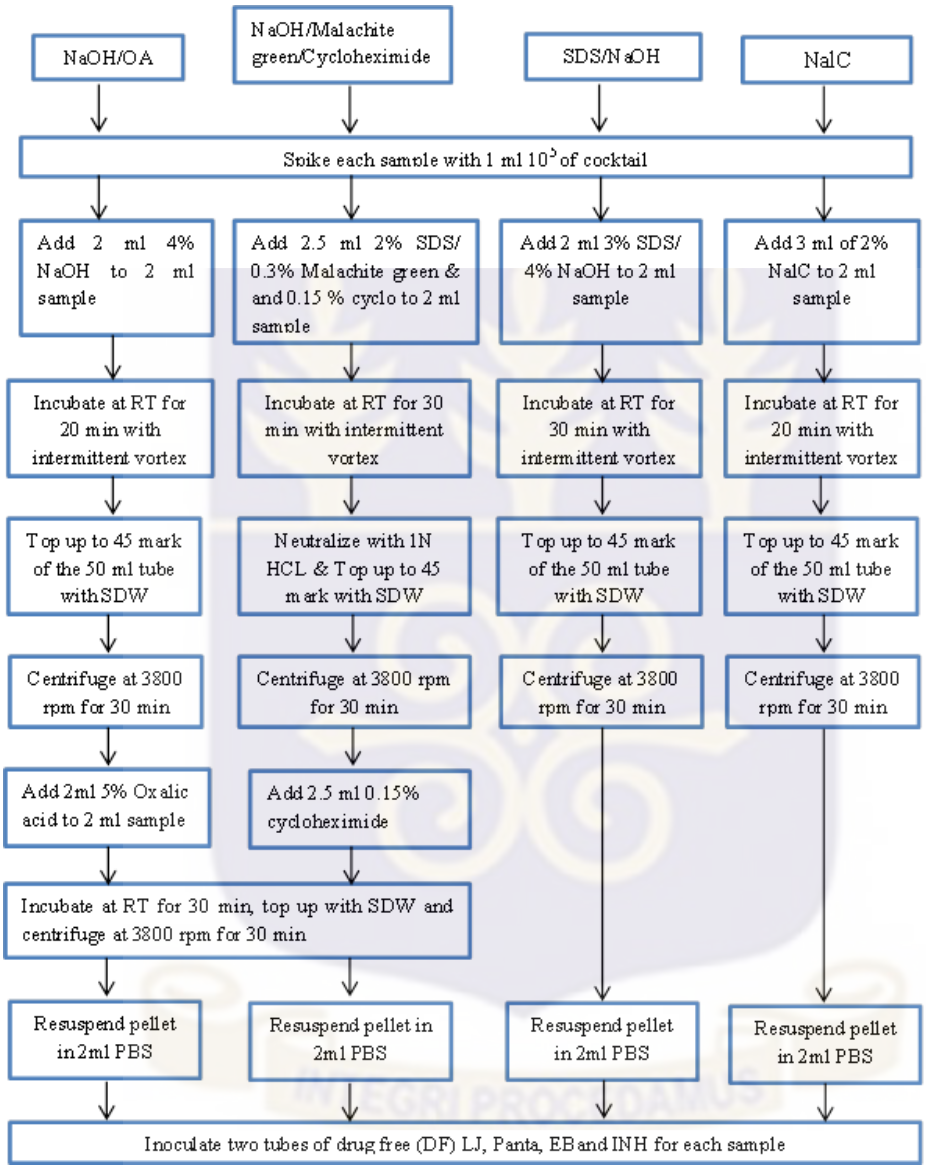

Figure 3.3 Flow chat detailing step by step decontamination process (SDW; sterilized distilled water, RT; room temperature).

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3.7.2.3 Primary Isolation of Mycobacterial species from spiked environmental samples

In order to determine the most effective cultivation medium for the recovery of mycobacteria species, 1 ml of sterile PBS was added to the precipitate of individual decontaminated samples. The content was mixed uniformly and 0.1 ml of the pellet-PBS suspension was then inoculated in duplicates on LJ prepared media supplemented with various antibiotics as previously and LJ medium without drug supplement. The inoculated culture tubes were then incubated at 32°C and observed for macroscopic growth until 6 months after which they were discarded.

3.7.2.4 Purification and Amplification of Mycobacterial Colonies by Sub-Culture

A loopful of mycobacterial growth was taken from distinct colony on a culture tube with an inoculating loop into a sterile culture tube containing six of three millimetre (3mm) glass beads and 2 drops of PBS. The content was vortexed for about two minutes until the colonies are completely emulsified. The tube was left for 5 minutes to settle all created aerosol and 500 µl PBS added. A transfer pipette was used to transfer 100 µl of the inoculum onto 2 LJ slants, incubated at 32^0C and observed weekly until confluent growth was achieved. A drop of the prepared suspension was also used to prepare a smear for ZN staining to confirm the acid-fastness of the microbial growth as previously.

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3.7.2.5 Identification of AFB Positive Isolates by GenoType Mycobacterium CM/AS

Genomic DNA from heat killed AFB isolates was extracted as previously. The GenoType Mycobacterium CM/AS assay (Hain Lifesciences) was performed following manufacturers protocol (Gitti *et al.*, 2006). The reaction mixture consisted of 2 µl of $MgCl_2$, 5 µl of 10X buffer, 35 µl of biotinylated primer- nucleotide mix (PNM), 0.2 µl HotStar Taq (Qiagen, Hilden Germany), 2.8 µl of nuclease free water and 5 µl of DNA in a total volume of 50 µl. The PCR was performed at 95^{O}C for 15 mins, 10 cycles of 95^{O}C for 30 secondss, 58^{O}C for 2 mins, 20 cycles of 95^{O}C for 25 seconds, 53^{O}C for 40 seconds, 70^{O}C for 8 mins. Hybridization and detection was done using Twincubator (Hain Lifescience GmbH) following manufacturers protocol (Gitti *et al.*, 2006).

3.7.3 Isolation of *M. ulcerans* from Environmental Samples

Based on the findings from the spiked decontamination evaluation, a total of 139 *M. ulcerans* confirmed samples by RT-PCR were cultivated using the most effective decontamination method; 1M NaOH and 5% oxalic acid as previously. During the mock experimentation, fungal growth on cultures inoculated on PANTA was observed. To overcome this fungal contamination, mycobactin J (2 mgL^{-1}) (IDVet), an antifungal was added to the LJ PANTA supplemented medium (PM). Suspensions were then inoculated in duplicates on the best in-house LJ prepared media supplemented media with INH, PM and without supplement DF respectively. The inoculated culture tubes were then incubated at 32°C and observed for macroscopic growth until 6 months after which they were discarded. Culture tubes were read daily during the first week for the isolation of rapid growers, and there after weekly. The cultures that showed growth, the week of

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growth was recorded and the growth quantified. Tubes that showed positive growth were purified by sub-culturing individual single colonies onto drug free media for species identification. AFB was detected by preparing smears of bacteria colony and stained for AFB as shown previously.

3.7.3.1 Species Identification of Mycobacteria Isolates

The obtained isolates after confirming as AFB positive were harvested, heat-killed by heating at 95 °C for 30 mins and used for genomic DNA extraction (Käser *et al.*, 2009). Cell wall lysis was achieved first by the addition of 300 µl of lysis buffer to bacteria pellets followed by the addition of 50 µl of lysozyme (10 mg ml^{-1}) (Fluka), 100µl of SDS (0.04 mg ml^{-1}) and 40 µl of Proteinase K (0.2 mg ml^{-1}) (Roche). The mixture was incubated at 37°C for one hour after which 200 µl of zirconia beads (Sigma-Aldrich) were added and homogenised using FastPrep-24™ (MP Biomedicals) at 4700 *g* for 4 minutes. The mix was spun at 5000 x g for 5 mins and supernatant containing released DNA was transferred to new eppendorf tubes. Protein and other cell debris were removed by two steps of phenol and alcohol extraction using 500 µl of Phenol: Chloroform: Isoamyl alcohol (Sigma) to the mix and centrifuged at 5000 *g for* 10 min. DNA precipitation was achieved by addition of 20 µl of 3M sodium acetate (Sigma) to the mix followed by addition of 500 µl of absolute ethanol (Sigma-Aldrich). The mix was frozen at -20°C overnight, and spun at 5000 *g* for 30 minutes after which the pellets were dried at 50°C. The genomic DNA was suspended in 100 µl of nuclease free water. The concentrations of the DNA were checked using Nano Drop 2000C (Thermo Scientific).

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A 441-bp portion of the mycobacterial heat shock protein 65 (*hsp*65) was amplified using the mycobacteria-genus-specific primers' TB-11 and TB-12 forward and reverse primers as shown in Table 3.4 (Telenti *et al.,* 1993) as previously described (Shinnick, 1987). The PCR mixture contained 5 µl of a 1/100 dilution of template DNA, 0.25 µM concentrations of each primer, 6 µl of Q-solution, 3 µl of 10X buffer, 200 µM (each) dATP, dCTP, dGTP, and dTTP (Pharmacia Biotech), 1.5 mM $MgCl_2$, and 0.5 U of Fire *Taq* polymerase in a total volume of 100 µl. Amplification was performed using the following program: for 32 cycles of 5 min at 94°C, 30 sec at 94°C, 30 sec at 60°C, 1 min at 72°C and 10 min at 72°C in a 2720 thermal cycler (Applied Biosystems). Amplified product (10 µl) was confirmed by gel electrophoresis. The amplified PCR product (40 µl) was sequenced by outsourcing. The generated sequences were edited using codon code aligner (6.0.2) software to remove vector sequences and species identified by NCBI Microbial Nucleotide BLAST using default settings (Benson, 2015).

3.7.3.2 Confirmation of Isolated *Mycobacterium ulcerans* by IS*2404*, IS2606, KR and rpoB

Isolate suspected as *M. ulcerans* was confirmed in addition to the hsp65 sequence using gel PCR detecting the following gene targets, IS*2404* (Bratschi *et al.,* 2014), IS*2606* (Käser *et al.,* 2009), KR and rpoB. The primers for KR and rpoB were designed using the Primer3 software (Bratschi *et al.,* 2014). The primer sets used for the amplification are shown (Table 3.4). The reaction mixture for all the targets IS*2404*, IS*2606*, KR and rpoB contained 10 µM concentration of each primer, a 200-µM concentration of each (each) dATP, dCTP, dGTP, and dTTP, 19 µl of nuclease free water, 25 mM $MgCl_2$, 3 µl of 10X

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buffer, 1 U Fire PolTaq (Solis BIODYNE) and 1 µl of template DNA (5 ng) in a total volume of 30 µl. Amplification was performed using the following program: for 32 cycles of 5 min at 94°C, 30 sec at 94°C, 30 sec at 60°C, 1 min at 72°C and 10 min at 72°C in a 2720 thermal cycler (Applied Biosystems). Amplified DNA (10 µl) were subjected to electrophoresis in a 2% agarose gel prepared with Tris-Borate EDTA (TBE) buffer detected by ethidium bromide staining and ultraviolet (UV) transillumination as previously.

Table 3.4 Primers used for confirmation of mycobacterial DNA

No.	Primer	Sequence (5'-3')	Reference
1	TB-11F	ACCAACGATGGTGTGTCCAT	Telenti *et al.,* 1993
2	TB-12R	CTTGTCGAACCGCATACCCT	
3	IS2404-F	GGCAGTTACTTCACTGCACA	Käser *et al.,* 2009
4	IS2404-R	CGGTGATCAAGCGTTCACGA	
5	IS2606-F	GGTGCGGTTCCATTGAGA	Bratschi *et al.,* 2014
6	IS2606-R	GTCGTAGATGTGGGCGAAAT	
7	KR-F	TTCTGTTCCCGCAGTTCTTT	From this work
8	KR-R	CTAGCTCGGAAGTGGTCAGC	
9	rpoB-F	GTGGGTCGGTACAAGGTCAA	From this work
10	rpoB-R	GCGGTCAGGTAGTGGATCTC	

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3.8 Detection of Buruli Ulcer Cases

Presumptive BU cases were detected through both active case search and passive reporting to health facilities. Active case search to detect BU cases was conducted using community outreach program and monthly household visits by community-based surveillance volunteers (CBSV) after consultations with community leaders, management of the health facilities and the NBUCP. Community outreach education was conducted once every three months in all the selected communities based on the presumed incubation time for *M. ulcerans* (Trubiano *et al.*, 2013). During the outreach program, community members were educated on the transmission, early case detection and treatment of BU by showing videos on BU documentaries and interaction with community members through questions and answers.

Presumptive cases that were detected at the community during the active case search and those that passively report at the health facilities were then sampled for laboratory confirmation. Two swab specimens were collected from the undermined edges of ulcerative lesions and one fine needle aspirate (FNA) was collected into 500 µl phosphate buffered saline (PBS) as previously described (Eddyani *et al.*, 2009) from pre ulcerative lesions and samples were sent to the NMIMR for laboratory confirmation.

Monthly household-visit based surveillance was introduced as an additional tool to rapidly detect cases. Within each community, one CBSV was trained and equipped with android phone (HTC wildfire S) pre-loaded with a BU surveillance questionnaire. The questionnaire used for the study was designed as previously described (Ampah *et al.*, 2016). Starting from August 2013 to December 2014, the CBSVs were mandated to visit

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all households monthly and record any presumptive case using the mobile application. Any presumptive case detected during the monthly surveillance was referred to the nearest health facility for sample to be taken and sent through a courier services for confirmation at NMIMR.

3.8.1 Laboratory Confirmation of BU Cases

All samples received from presumptive cases from both active case search and passive reporting were laboratory confirmed by IS*2404* PCR after DNA extraction using Qiagen miniprep kit (Lavender and Fyfe, 2013). Briefly, swab samples from the same lesions were pooled together into glass tubes with beads containing 2 ml of PBS. The content was vortex for 5 minutes until all the trapped specimen have been released into solution. The suspension was put into 2ml tubes and centrifuged at 3,000 x g for 10 minutes to concentrate the cells. The supernatant was then decanted and the sediment mixed with PBS. Final volumes of 400 µl suspension from both swab and FNA were used for the extraction of *M. ulcerans* DNA (Lavender and Fyfe, 2013).

Amplification of a 515-bp product was performed using the primers MU1-new (5′-GAT CAA GCG TTC ACG AGT GA-3′) and MU2 (5′-GGC AGT TAC TTC ACT GCA CA-3′). The 50-µl PCR contained 5 µl 10× PCR buffer with 15 mM $MgCl_2$ (QIAGEN), primers MU1 and MU2 at a final concentration of 1 µM, deoxynucleoside triphosphates (200 µM each) and 2.5 U *Taq* polymerase (QIAGEN), and 1 ng of *M. ulcerans* genomic DNA. Amplification was performed in an Eppendorf gradient thermocycler using the following cycling conditions: 1 cycle of 94°C for 4 min, 35 cycles of 94°C for 40

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seconds, 60°C for 40 seconds, and 72°C for 40 seconds, and a final extension of 72°C for 5 minutes. Amplified DNA (10 µl) were subjected to electrophoresis in a 2% agarose gel prepared with Tris-Borate EDTA (TBE) buffer detected by ethidium bromide staining and ultraviolet (UV) transillumination.

3.9 Case-Control Study

3.9.1 Definitions

A BU Case was defined as any person living within the selected communities along the Densu river basin, presenting with active or inactive BU and have been diagnosed for Buruli ulcer according to the WHO clinical definition, thus confirmed by IS*2404* polymerase chain reaction (PCR).

A Control was defined as an individual who resided in the community/household where the case come from but has never been diagnosed of Buruli ulcer. Controls for children and adult case patients were randomly selected within the communities and matched to cases to the nearest age (± 5 years), sex, and residence.

3.9.2 Selection Procedure for the Case-Control Study

Buruli ulcer cases were recruited retrospectively from NMIMR BU laboratory confirmed database. Cases that were laboratory confirmed between January, 2012 and December, 2015 were selected following the selection procedure by Ampah *et al.* (2016). Together with two research assistants and two community volunteers, homes of all the selected cases were visited to seek consent for recruitment into the study. Having explained the purpose of the study to each case and consent was given, a matching control was

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randomly selected from within the case household or the nearest house the case has interaction with and consent was again sought same way as done for the case. Both the case and control were made to sign or thumbprint the written consent form for participation in the study. Prior to questionnaire administration, cases were asked to indicate the locations within their environment they are in frequent contact. The findings were incorporated in the questionnaire design for the study. Standardized study questionnaires were administered to eligible participants for demographic information (age, sex, education, and marital status), environmental (agricultural, fishing, and mining activities) and other behavioural practices (clothing worn, precautions taken against insect bites, the form of treatments given to traumas, regular contact with fauna within households, occupational activities, and contact with water bodies). Cases were asked to restrict their responses to the year before the onset of the disease or during the past year for control participants. Questionnaires were interpreted into languages participants could easily understand.

3.10 Data Analysis

For the human BU cases, environmental sampling and the case-control study, data collected were entered into a Microsoft Excel 2010 spread sheet and analyzed using R statistical software (R Development Core Team, 2008). The proportion of *M. ulcerans* positive at 95% confidence interval for different categories of the samples was estimated. Logistic regression was used to account for the cluster-sampling by including random effects for location and community. From the analyses stratified by site or adjusting for calendar month, the numbers were too small to allow the random effects model to

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converge and so adjusting for clustering was not feasible. The sensitivity of the four decontamination methods and cultivation media were evaluated. The colonial morphology and length of time before visible colonies appeared were recorded during culture reading. A culture was said to be positive if at least one tube from the same treatment had confirmed AFB growth. Culture was said to be negative if none of the culture tubes has microbial growth after 6 months and culture classified as contaminated when all tubes had more than half of the tube with other bacterial growth or liquefied. The performance of decontamination methods and cultivation media were calculated using the *t*-test. For the environmental and behavioural assessment, the demography of case and control participants was compared by using 2-sample t-test. Variables that attained significance at a P value < 0.05 were retained for multivariate analyses using multiple conditional logistic regressions. A step down backward elimination process was used to identify factors that are significantly associated with BU and also to control possible confounding factors in the model. P values ≤ 0.05 was considered statistically significant.

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CHAPTER FOUR

Results

4.1 Occurrence of *Mycobacterium ulcerans* in Environmental samples

4.1.1Enviornmental Positivity of *Mycobacterium ulcerans* by Real Time PCR

One thousand six hundred environmental samples were collected from 726 locations; 239 from three communities along the Densu and 487 from seven communities along the Offin river basins. At least two composite environmental samples were collected from each of the locations sampled. The samples were screened for three independent molecular markers; IS*2404*, IS*2606* and KR within the *M. ulcerans* genome. A sample was classified as *M. ulcerans* confirmed if it tested positive for all the three targets. Overall, 139 (9%) samples were positive for *M. ulcerans* DNA (Table 4.1).

Mycobacterium ulcerans DNA was found to be broadly distributed in majority of the sample categories from all the communities along both river basins (Table 4.2). Along the Densu river basin, sample positivity for *M. ulcerans* was higher at Ntabea (21/37 [57%]), followed by Ashongkrom (61/293 [22%]) and the least was at Domesampaman (7/104 [7%]) (Table 4.2). Among the seven communities along the Offin river basin, Wromanso (16/142 [11%]) had the highest *M. ulcerans* positivity and the least was recorded for Akomfore (2/139 [1%]) and Keniago (2/177 [1%]) (Table 4.2).

Mycobacterium ulcerans DNA was also found among all the nine sample types analysed along the Densu river basin with positivity ranging from 83% among snails to 9% in faecal samples as indicated in Table 4.1. However, in the Offin river basin, *M. ulcerans* detections were observed among only moss (25%), soil (5.2%), vegetation biofilm (3.9%) and water (2.6) as indicated in Table 4.1. *Mycobacterium ulcerans* was detected in at least one sample each of vegetation and soil at every sampling period in both river basins.

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Table 4.1 *M. ulcerans* DNA positivity among samples analyzed from communities along Densu and Offin river basins

	Densu		Offin		Total MU Confirmed	
Samples	**No. of Samples**	**MU Confirmed (%)**	**No. of Samples**	**MU Confirmed (%)**	**No. of Samples**	**MU Confirmed (%)**
Detritus	2	1 (50.0)	21	0 (0.0)	23	1 (4.3)
Faeces	44	4 (9.1)	36	0 (0.0)	80	4 (5.0)
Fungi	8	1 (12.5)	3	0 (0.0)	11	1 (9.1)
Insect	8	1 (12.5)	28	0 (0.0)	36	1 (2.7)
Moss	35	7 (19.4)	4	1 (25.0)	40	8 (20.0)
Water	67	7 (10.4)	76	2 (2.6)	143	9 (6.2)
Soil	143	30 (20.9)	443	24 (5.2)	586	54 (9.0)
Vegetation biofilm	121	33 (27.5)	555	23 (3.9)	675	56 (8.1)
Snail	6	5 (83.3)	-	-	6	5 (83.3)
TOTAL	**434**	**89 (20.5)**	**1166**	**50 (4.2)**	**1600**	**139 (8.7)**

Real time PCR targeting three insertion sequences was used to screen environmental samples from communities along the two river basins. The overall numbers and percentages of samples confirmed for *M. ulcerans* DNA are shown under the total section.

The occurrence of *M. ulcerans* DNA was significantly higher along the Densu (89: 21%) than at Offin (50: 4%) river basin ($p < 0.001$) (Table 4.1). Among the samples confirmed to contain *M. ulcerans* DNA, the highest proportion was found in soil and vegetation from agricultural farms (45%), followed by water sources within the communities (36%) and the least found soil near households (19%).

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The IS*2404*: IS*2606* copy number ratios (2.37) differentiate *M. ulcerans* from the other mycolactone producing mycobacterium and of the 139 samples confirmed by RT-PCR to contain *M. ulcerans* DNA, about 80% were found to be below 2.37, the expected ratio for *M. ulcerans*. Among the *M. ulcerans* confirmed samples from Densu (89), 73 (88%) met the expected IS*2404*: IS*2606* copy number ratio representing soil (29%), vegetation biofilm (27%), moss (28.5%) and water (3.5%) whereas at Offin, thirty-seven (74%) out of the fifty *M. ulcerans* confirmed samples met the IS*2404*: IS*2606* copy number ratio representing soil (36%) and vegetation (38%).

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Table 4.2 Occurrence and Prevalence of *M. ulcerans* DNA among study communities

Site	No. of Samples	MU Positive	MU Prevalence rate (%)
‡Ntabea	37	21	57
‡Ashongkrom	293	61	22
‡Domesampaman	104	7	7
*Achiase	184	11	6
*Akomfore	139	2	1
*Keniago	177	2	1
*Mfantsiman	198	13	2
*Ntobroso	238	4	2
*Pokukrom	88	2	2
*Wromanso	142	16	11
Total	**1600**	**139**	**8.7**

Distribution of *M. ulcerans* DNA among environmental samples from the ten communities along the Densu and Offin river basins. Communities along the ‡Densu and those along *Offin river basin respectively

4.1.2 Acid Fast Bacilli Positivity by Microscopy

Direct smear analysis of the one-hundred and thirty-nine RT-PCR positive samples that were cultivated identified only 12 (8.6%) to contain AFB. The acid-fast bacilli were detected mainly among snail 1/5 (20.0%) moss 1/8 (12.5%), soil 6/54 (11.1%) and vegetation biofilm 4/56 (7.1%) as indicated in Table 4.3. Also shown are slides of some the AFBs that were directly detected in soil, moss, snail and vegetation biofilm samples (Fig 4.2).

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Table 4.3 Rates of positivity for acid-fast bacilli by direct smear analysis and isolation of mycobacterial species

Sample Type	No. of Samples	Acid-fast bacilli Positivity n (%)	No. of Samples yielding mycobacteria isolate
Fungi	4	0/4 (0%)	1/4
Snail	5	1/5 (20%)	1/5
Moss	8	1/8 (12.5%)	3/8
Soil	54	6/54 (11.1%)	30/54
Vegetation biofilm	56	4/56 (7.1%)	21/56
Water	12	0/12 (0%)	2/13
Total	**139**	**12/139 (8.6%)**	**58**

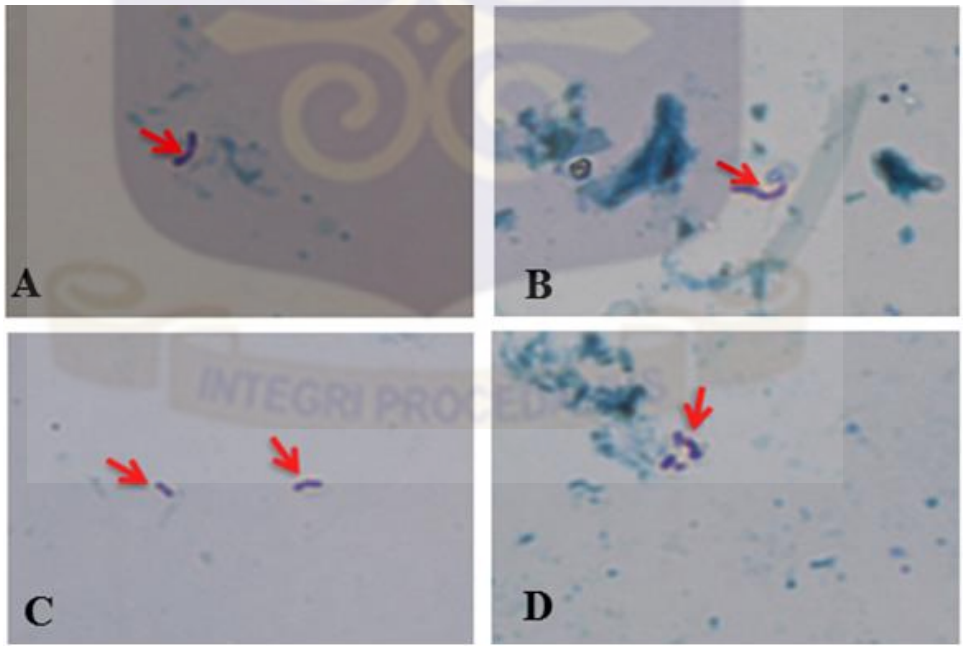

Figure 4.1 Direct smear analyses, using the Ziehl-Neelsen procedure, of environmental samples from soil (A), moss (B), vegetation biofilm (C), and snail (D). Red arrows, acid-fast bacilli. Magnification, X 1,000.

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4.1.3 Isolation of *Mycobacterium ulcerans* by Culture

4.1.3.1 Performance of Decontamination Methods and Growth Media Using Spiked Samples

Using the four decontamination procedures and four different culture media, a total of 128 cultures were carried out for the 8 samples (4 water and 4 soils) analyzed. As indicated in Table 4.4, the decontamination procedure that resulted in the highest recovery of *mycobacteria* was NaOH-OA 16 (50.0%) followed by NaLC 9 (28.1%) and NaOH-Mal-C 7 (21.8%). Among the samples decontaminated with SDS-NaOH method, no mycobacteria were recovered. Likewise, the least number of contaminated tubes was from NaOH-OA 5 (15.6%); followed by NaOH-Mal-C 11 (34.4%). The SDS-NaOH 28 (87.5%) recorded the highest contaminated tubes as indicated in Table 4.3. Among the four decontamination procedures evaluated, NaOH-Mal-C 14 (43.7%) gave the highest no-growth and SDS-NaOH 4 (12.5%) gave the least no-growth as shown in Table 4.4.

The cultivating medium that supported the highest number of mycobacterial growth was PANTA supplemented medium 11 (34.4%), followed by INH 9 (28.1%), drug free 7 (21.8%) and the least was EB 5 (15.6%). The supplementation of cultivation media with various antimicrobials was to enhance the elimination of unwanted microorganisms, however the drug free medium 10 (31.3%) without antimicrobial supplement performed better than cultivation medium supplemented with EB 18 (56.3%) in elimination of unwanted microorganism as indicated in Table 4.4. Acid-fast bacilli isolates from two out of the 8 samples were confirmed as *M. ulcerans* and in both cases the isolation medium was PANTA supplemented medium.

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Table 4.4 Decontamination Methods and Cultivation Media for the Recovery of *Mycobacterium ulcerans*

Decontamination methods [a]	No. of Cultures [b]														
	Positive					Contaminated					No mycobacterial growth				
	DF	INH	EB	PANTA	Total (*n=32*)	DF	INH	EB	PANTA	Total (*n=32*)	DF	INH	EB	PANTA	Total (*n=32*)
SDS-NaOH	0	0	0	0	0	6	8	8	6	28	2	0	0	2	4
NaOH-Mal-C	2	0	0	5	7	4	1	5	1	11	2	2	3	7	14
NaOH-OA	3	5	3	5	16	1	1	3	0	5	4	2	2	3	11
NALC	2	4	2	1	9	3	4	3	2	12	3	3	3	2	11

[a] SDS-NaOH, sodium dodecyl sulfate-sodium hydroxide; NaOH-Mal-C, NaOH-malachite green-cycloheximide; NaOH-OA, NaOH-oxalic acid; NALC-NaOH, N-acetyl-L-cysteine–sodium hydroxide. The NaOH-oxalic acid method was the most effective among the evaluated procedures. [b] DF, drug-free; INH, isoniazid; EB, ethambutol. The cultivation medium with the highest mycobacterial growth rate was PANTA-supplemented medium, followed by INH supplemented medium, and finally DF medium. EB-supplemented medium had the lowest number of positive cultures and the highest number of contaminated cultures.

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4.1.3.2 Performance of Optimized Procedures

Based on the performance of the evaluated decontamination methods and cultivation media, the best decontamination method (NaOH-OA) and the best cultivation media (P, INH and drug free [DF]) that enhanced mycobacteria recovery were used for the actual decontamination of samples. Of the 139 RT-PCR positive environmental samples that were cultivated, 58 (41.7%) yielded mycobacterial growth, 70 (50.4%) had no bacterial growth and 11 (7.9%) had all the inoculated tubes contaminated.

The performance of the different growth media as indicated in Table 4.5 showed that the best cultivating medium supporting the highest recovery of mycobacterial growth was LJ medium supplemented with PANTA and mycobactin-J, PM 75 (26.9%), followed by INH supplemented medium 63 (22.7%), and finally drug free, DF 24 (8.6%). The least number of contaminated tubes was recorded by the mycobactin-J and PANTA supplement, 111 (39.9%), followed by INH, 132 (47.5%) drug free medium, DF, 224 (80.6%) giving the highest contaminated tubes ($p < 0.001$) (Table 4.5). Among the three cultivation media, PM 92 (33.1%) gave the highest no-growth, followed by INH 83 (29.8%); the drug free 30 (10.8%) gave the least no-growth as shown in Table 4.5. Shown below are primary culture tubes with mycobacterial colonies from decontaminated environmental samples in culture (Fig 4.1).

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Table 4.5 Performance of different growth media in isolating *Mycobacterium spp*. from the environment

Medium	Number of culture tubes			
	Positive	**Contamination**	**No Growth**	**Total Tubes**
DF	24	224	30	**278**
INH	63	132	83	**278**
PM	75	111	92	**278**
Total	**162**	**467**	**205**	**834**

DF, drug-free; INH, isoniazid; PM, PANTA-mycobactin J. The cultivation medium with the highest mycobacterial growth rate was PM medium, followed by INH supplemented medium and finally DF medium.

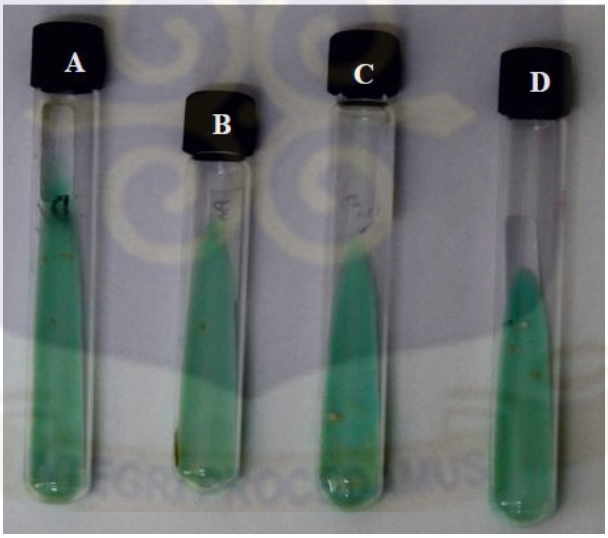

Figure 4.2 Primary Culture tubes showing distinct mycobacterial colonies on LJ. Tube (A), 25 PM NaOH-OA, (B), 50 PM NaOH-OA, (C), 50 INH NaOH-OA and (D), 50 DF SDS-NaOH. NaOH-OA, NaOH-Oxalic Acid; PM, PANTA-Mycobactin J; INH, Isoniazid and SDS-NaOH, sodium dodecyl sulfate-sodium hydroxide

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4.1.3.2.1 Mycobacterial Species Isolated

Based on colonial morphology, growth rate characteristics and growth medium, a total of 162 AFB positive isolates were collated for identification. Analysis of hsp65 sequences identified twenty-five different mycobacteria species as shown in Fig 4.3. The mycobacteria species isolated were from soil 16/30, vegetation 14/21, water 2/2, moss 2/3, snail 1/1 and fungi 1/1. Classification of the isolated mycobacterial species based on growth rate and sequenced analyses identified 15 (60%) to be slow growing mycobacteria and 10 (40%) to be rapidly growing mycobacteria. Among the cultivated samples, soil (17) gave the highest mycobacteria recovery followed by vegetation biofilm (14) and the least reported for fungi (1) and snail (1) as shown in Fig 4.3.

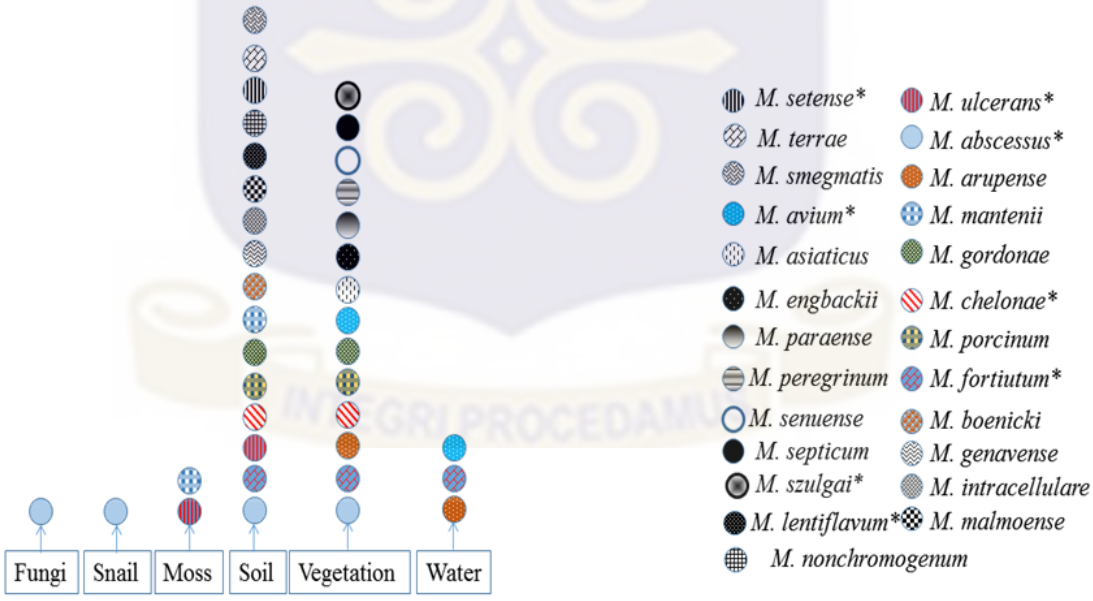

Figure 4.3 Heat shock protein 65 analyses reveals twenty-five different mycobacteria species isolated from environmental sample from BU endemic communities. *Causes skin and soft tissues infections

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Among the identified mycobacteria species were two *M. ulcerans* isolated each from moss and soil samples from Ashongkrom, a BU endemic community along the Densu river basin. Other slow growing mycobacteria including *M. avium, M. mantenii, M. senuense, M. lentiflavum and M. malmoense* and rapidly growing mycobacteria such as *M. chelonae, M. abscessus, M. septicum, M. arupense and M. fortuitum* were among the many mycobacterial species isolated as represented in Table 4.6.

Performance of cultivation media in the recovery of mycobacterial species was evaluated as shown in Table 4.6 Among the media tested, LJ supplemented with both PANTA and mycobactin-J (PM) gave the highest recovery of mycobacteria species 16 (42.1%) followed by INH supplemented medium 13 (34.2%) and the least was from drug free LJ medium (DF) 12 (28.9%). Among the 15 slow growing mycobacterial species identified, 10 (*M. ulcerans, M. mantenii, M. szulgai, M. senuense, M. tarrae M. nonchromogenicum, M. malmoense M. intracellulare, M. paraense* and *M. genavense*) were isolated from single medium and 5 (*M. avium, M. lentiflavum, M. engbaekii, M. gordonae* and *M. asiaticus*) were from different cultivation medium as shown in Table 4.6. Of the 10 rapidly growing mycobacteria identified, only 3 (*M. peregrinum, M. arupense and M. porcinum)* were isolated from a single medium and the remaining 7 (*Chelonae, M. abscessus, M. gordonae* M. *boenickei*a, *M. setense, M. smegmatis* and *M. septicum*) were isolated from different medium (Table 4.6).

Eight (32%) of the mycobacterial species were from PM only, 3 (12%) from INH only and 2 (8%) from DF only as indicated in Table 4.7. Whilst 4 (16%) mycobacteria species were isolated from all the three cultivation media (PM/INH/DF), 2 (8%) were isolated

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from PM/DF and PM/INH respectively and 4 (16%) from isolated from DF/INH (Table 4.7).

Table 4.6 *Mycobacterium species*, Growth rate characteristics and isolation media

Growth feature	***Mycobacterium sp.***	**Isolation medium(a)[a]**
Slowly growing	*M. ulcerans*[b]	PM
	M. szulgai[b]	PM
	M. mantenii	PM
	M. senuense	PM
	M. lentiflavum[b]	PM, DF
	M. avium[b]	PM, DF, INH
	M. engbaekii	PM, DF
	M. gordonae	PM, INH, DF
	M. paraense	PM
	M. terrae[b]	DF
	M. asiaticus	DF, INH
	M. nonchromogenicum[b]	DF
	M. intracellulare	INH
	M. genavense	INH
	M. malmoense	PM
Rapidly growing	*M. peregrinum*	PM
	M. chelonae[b]	PM, DF, INH
	M. abscessus[b]	PM, DF, INH
	M. fortuitum[b]	DF, INH
	M. smegmatis	DF, INH
	M. septicum	PM, INH
	M. porcinum	INH
	M. setense[b]	DF, INH
	M. boenickei[b]	PM, INH
	M. arupense	PM

[a] PM, PANTA-mycobactin J; DF, drug-free; INH, isoniazid. [b] A mycobacterial species that causes skin and soft tissue infections.

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Table 4.7 Cultivation media and isolated mycobacteria species

Cultivation Medium (a)[a]	No. (%) of mycobacteria species isolated
PM	8 (32%)
INH	3 (12%)
DF	2 (8%)
PM, DF	2 (8%)
PM, INH	2 (8%)
DF, INH	4 (16%)
PM, INH, DF	4 (16%)

[a] PM, PANTA-mycobactin J; DF, drug-free; INH, isoniazid.

4.1.3.2.2 The *M. ulcerans* Isolates

The *M. ulcerans* that were isolated from both moss and soil samples were further analysed using different genetic markers by gel based PCR analyses. As shown below in Fig 4.4 is the gel picture indicating the presence of IS*2404*, IS*2606* sequences with specific markers for MU as well as MU specific rpoB gene sequence. For the IS*2404* and IS*2606* gel electrophoresis, product sizes of 492 bp and 332 bp respectively were detected; ketoreductase product size of 615 bp and rpoB product size of 606 bp were also detected. More importantly these isolates were submitted for whole genome sequcing and have been both confirmed as *M. ulcerans* (Prof. Gerd Pluschke, Swiss TPH)

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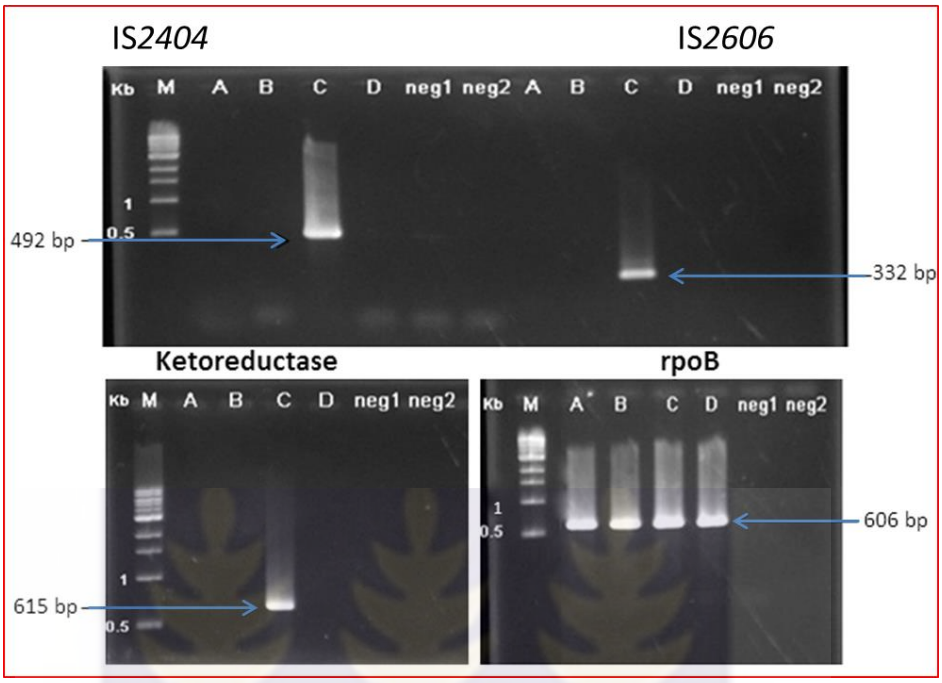

Figure 4.4 Confirmation of *M. ulcerans* by PCR detection and amplification of IS*2404*, IS*2606*, ketoreductase B domain, and rpoB. Lane M, size ladder (Gibco); lane A, *M. avium* isolated from a cocoa pod; lane B, *M. gordonae* isolated from soil; lane C, *M. ulcerans* isolated from moss; lane D, *M. chelonae* isolated from soil.

4.1.4 Sensitivities of Microbiological Detection Methods Employed

Out of the 1,600 environmental samples analyzed by RT-PCR, the gold standard for clinical BU diagnosis, 139 (9%) were confirmed as *M. ulcerans*. Direct smear detection of AFB by ZN method from the 139 PCR positive samples showed rather low positivity (8.6%). Mycobacterial culture which is a full proof of viable mycobacterial identified two *M. ulcerans* isolates from moss and soil samples out of the 25 mycobacterial species identified in the study. Although varied sensitivities and specificities have been observed, the combination of all these methods is essential for the recovery of viable *M. ulcerans* from varied environmental samples.

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4.2 Seasonal Pattern and *M. ulcerans* Occurrence

The monthly *M. ulcerans* positivity by RT-PCR was compared with monthly rainfall levels using data obtained from the meteorological sub-stations within the study communities after approval from the Ghana Meteorological Agency, Accra to gain better understanding of the seasonal drivers for *M. ulcerans* distribution in the environment. Throughout the sampling period, *M. ulcerans* DNA was detected in at least one sample type. Out of the total 1,600 environmental samples analyzed, the proportions of samples that were positive for *M. ulcerans* was higher during the months with higher rainfall levels (above 100 mm) (126/1175 [10.7%]) than during the dry season months (13/425 [3.1%]), ($p < 0.001$).

Along the Densu river basin in 2011, high *M. ulcerans* positivity was recorded for the minor rainy seasons, September 23/34 (67%) and October (13/17, 76%), however, rainfall level at 130.1mm seemed to favour *M. ulcerans* positivity (76%) compared to 268.1 mm with positivity *M. ulcerans* of 67%. During the dry season, December (2/15; 13%), there was a decline in *M. ulcerans* positivity with a corresponding decrease in rainfall levels 22.2 mm as indicated ($R = 0.94$) (Table 4.8 and Fig 4.5). In both 2013 and 2014, a similar pattern was also observed. A drastic decline in *M. ulcerans* positivity was observed during both the minor and rainy seasons and even the dry season in both 2013 and 2014 when compared to the positivity recorded in 2011 ($p = 0.002$; 95%, CI = 1,7-6.4) (Table 4.8). At rainfall level 176.6 mm, corresponding *M. ulcerans* positivity of 54% was observed whereas rainfall level 20.6 mm lead to positivity of 11% in 2013. Similarly, rainfall levels at 103.3 mm corresponded to *M. ulcerans* positivity of 21% whereas 45 mm lead to positivity of 3% in 2014 as shown in Table 4.8.

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Table 4.8 Rainfall levels and Monthly *M. ulcerans* DNA positivity along the Densu river basin

Period of Sampling	Rainfall levels (mm)	No. of Samples	*M. ulcerans* Positive	*M. ulcerans* Positivity Rate (%)
Sep 2011	268.1	34	22	67
Oct 2011	130.1	17	13	76
Dec 2011	22.2	15	2	13
Jul 2013	176.6	24	13	54
Aug 2013	20.6	18	2	11
Oct 2013	146.2	20	6	30
Dec 2013	26.7	22	2	9
Jul 2014	103.3	28	6	21
Aug 2014	108.9	36	8	22
Sep 2014	102.4	42	6	14
Oct 2014	45.3	61	2	3
Nov 2014	95.5	41	5	12
Dec 2014	26.2	76	2	3
TOTAL	-	**434**	**89**	**20.5**

Monthly positivity rate of *M. ulcerans* DNA detected from environmental samples along the Densu river basins. Gradual decline in *M. ulcerans* DNA positivity observed over the course of the study period

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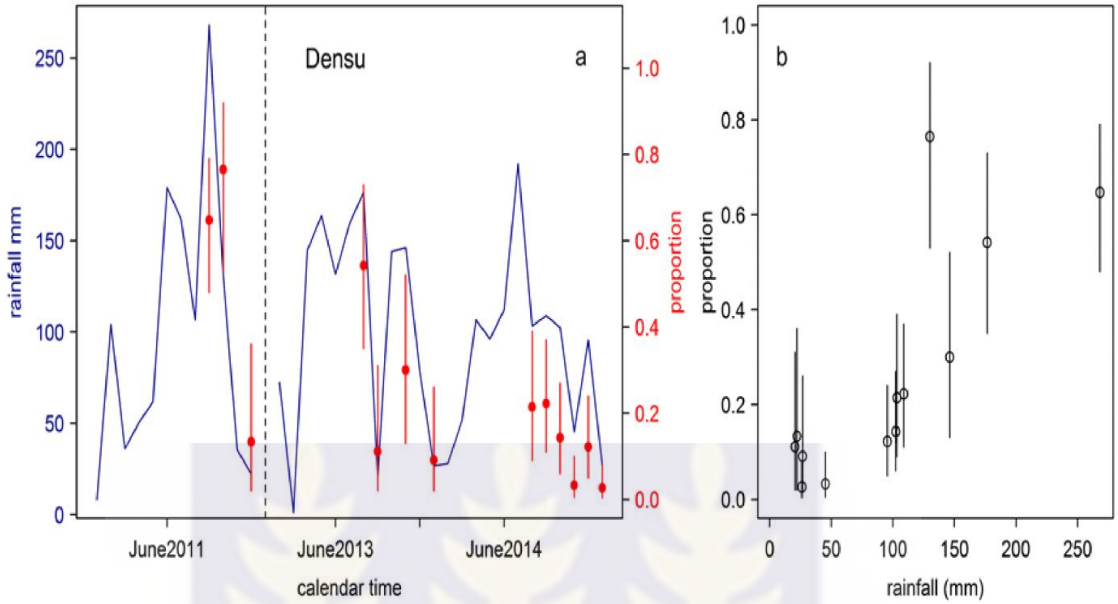

Figure 4.5 Rainfall levels and *M. ulcerans* distribution along the Densu river basin. *M. ulcerans* positivity was compared with recorded monthly rainfall levels within the study period. High proportions of *M. ulcerans* positivity seem to be associated with increased rainfall levels of rainfall.

Along the Offin river basin, the study finding also suggests that rainfall over a low threshold is associated with the increased *M. ulcerans* positivity (Table 4.9 and Fig 4.6), but the linear trend observed in the Densu was not apparent in this situation. For instance in both 2013 and 2014, *M. ulcerans* positivity for both the minor and major rainy seasons was low as observed along the Densu river basin. Rainfall levels at 115.4 mm and 149.4 mm for August and October lead to an observed *M. ulcerans* positivity of 13% and 7% respectively. A more drastic decline in rainfall levels 24.1 mm in December resulted in *M. ulcerans* positivity of 0.5%.

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Table 4.9 Rainfall levels and Monthly *M. ulcerans* DNA positivity along Offin river basin

Period of Sampling	Rainfall levels (mm)	No. of Samples	*M. ulcerans* Positive	*M. ulcerans* Positivity Rate (%)
Aug 2013	114.5	64	8	13
Oct 2013	149.4	292	21	7
Dec 2013	24.1	208	1	0.5
Jul 2014	270.9	292	10	2
Sep 2014	176.4	78	8	10
Oct 2014	39.5	98	1	1
Dec 2014	38.3	63	1	2
TOTAL	-	**1166**	**50**	**4.1**

Monthly positivity rate of *M. ulcerans* DNA detected from environmental samples along the Offin river basin. Drastic decline in *M. ulcerans* DNA positivity also observed over the course of the study period.

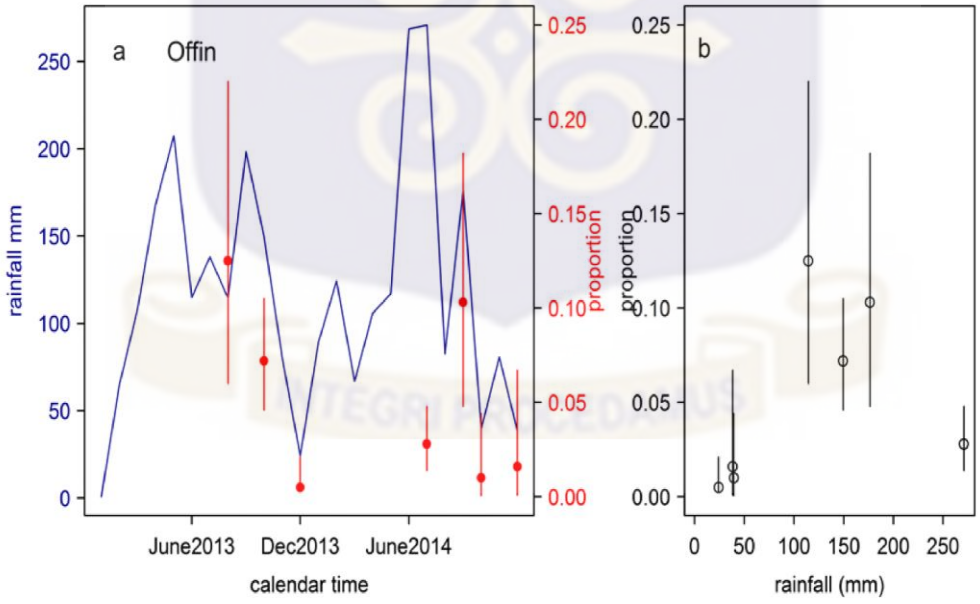

Figure 4.6 Rainfall levels and *M. ulcerans* distribution in along the Offin river basin. *M. ulcerans* DNA positivity was compared with recorded monthly rainfall levels within the study period. The proportions of *M. ulcerans* positivity are associated with increased rainfall levels at a certain threshold

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4.3 Findings from Epidemiological studies

4.3.1 Prevalence of BU in the Studied Communities

An overall population of 10,851 from ten communities; three along the Densu 1,217 (11.2%) and seven along the Offin 9,634 (88.8%) were assessed. Along the Densu were 45.6% females and 54.4% males. At Offin 49.6% were females and 50.4% were males were studied as shown Table 4.10.

Eighty-four presumptive BU cases were detected in all communities inlcuded; 56 (66.7%) from Densu and 28 (33.3%) from Offin. Thirty-two (38.1%) were laboratory confirmed by IS*2404* PCR, of which 24 (75%) were from Densu, and 8 (25%) from the Offin river basin. At Densu, 15 (62.5%) of the confirmed cases were from passive reports. The confirmed cases were detected in Ashongkrom 16 (66.7%) and Domesampaman 8 (33.3%) with a prevalence of 14.3% and 3.6% respectively whilst no case was detected in Ntabea. At Offin, 7 (87.5%) of the confirmed cases were reported through an active case search. The eight cases were detected in three communities; Achiase 4(62.5%), Ntobroso 3 (37.5%) and Akomfore 1 (12.5%). The recorded prevalence was 3.8% for Achiase (3.8%), Ntobroso (1.6%) and Akomfore (3.0%) as shown Table 4.10.

Of the laboratory confirmed cases, 19 (59.4%) were males and 13 (40.6%) were females, aged between 3 and 70 years, with a mean age of 26 years. Lesions presented by cases were in the early stages; 25 (78.2%) were detected with pre-ulcerative lesion and 7 (21.9%) presented with category II lesions.

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The monthly recorded BU incidence for Ashongkrom and Achiase along the Densu and Offin river basin respectively is shown in Fig 4.7. Of the 16 reported cases in Ashongkrom, 3 (18.8%) were detected in 2012; November (2) and December (1), and 13 (81.2%) detected in in 2014; September (2), October (3), November (4) and December (4). At Achiase, of the 4 BU cases, 1 was detected in November 2013 and the remaining cases were detected 2014 in the months January (1), April (1) and December (1).

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Table 4.10 Demographic characteristics and Identified BU Cases in studied Communities

Variables	Community									
	Densu				Offin					
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**
Population (n)	378	512	327	1,900	1,016	3,350	303	1,949	900	216
No. of households	52	64	48	295	157	413	85	251	121	41
Sex: Females, n (%)	184	228	143	939	499	1,719	141	972	409	106
	(48.6)	(44.5)	(43.7)	(49.4)	(49.1)	(51.3)	(46.5)	(49.9)	(45.4)	(49.1)
Presumptive BU cases	29	27	0	6	3	3	3	7	4	2
Lab confirmed	16	8	0	4	1	0	0	3	0	0
BU prevalence (%)	14.3	3.6	0	3.1	3	1.2	8.9	1.6	1.3	2.3

Community: Ashongkrom **(A),** Domesampaman **(B),** Ntabea **(C),** Achiase **(D),** Akomfore **(E),** Keniago **(F),** Mfantsiman **(G),** Ntobroso **(H),** Pokukrom **(I),** Wromanso **(J).** [+]Prevalence rate is given as the total prevalence comprising both active and healed lesions.

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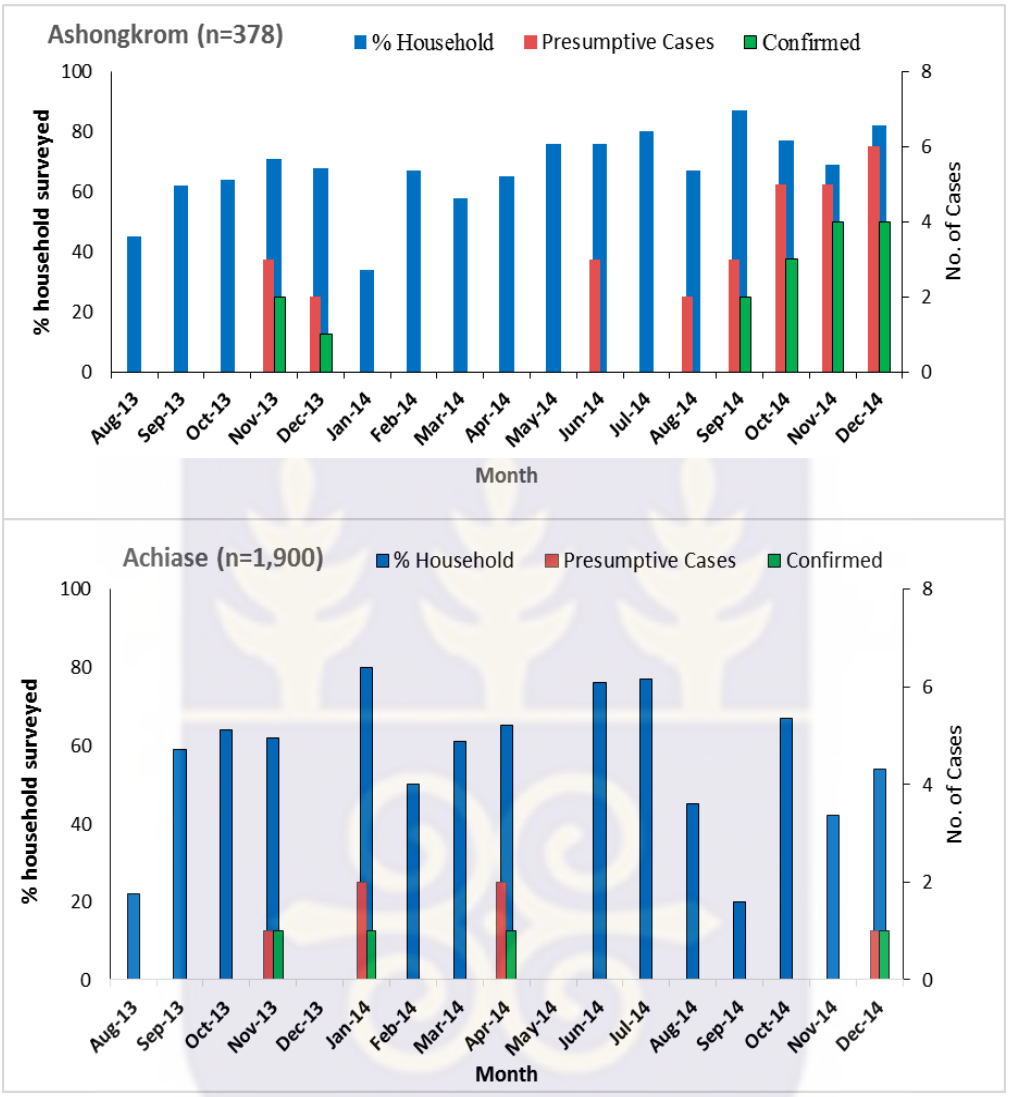

Figure 4.7 Monthly active surveillance of BU for Ashongkrom and Achiase

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4.3.2 Risk Factors Assessment

4.3.2.1 Demographic Characteristics of Participants

Overall, equal numbers of cases (176) and controls (176) were matched by age, village and neighborhood. The demographic characteristics of both cases and controls as well as clinical characteristics of cases are shown respectively in Table 4.11 and Table 4.12. Among the 176 case participants, 86 (49%) were males and 90 (51%) were males. The age of the case participants were in the range (9-51 years), median (28 years) and mean (27 years). The case participants aged 15 and below were 38 (21.6%) and above 15 years were 138 (78.4%) with majority of cases (68; [38%]) in the age 25-35 years. Among the 176 control participants, the age of the controls ranged between 8 and 54 years with a median age of 28 years. Out of the 176, 86 (47%) were males and 90 (51%) were females with a mean age of 28 years (Table 4.11).

All the cases that participated in the study had been confirmed for BU by PCR following the World Health Organisation guidelines and were recruited from laboratory confirmed BU database at NMIMR. More than half of the cases 115 (65%) presented an ulcerative BU lesion. BU lesions frequently occurred on the lower limbs 77 (44%) followed by the upper limbs (38%). Lesions that occurred on the abdomen were less 8 (4%) compared to the head and trunk 24 (14%). The lesion size of the case patients were categorized as: 27 (15%) category I, 55 (31%) category II plus 95 (53%) category III (Table 4.12).

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Table 4.11 Demographic characteristics of cases and controls

Characteristics	**No. of Cases n (%)**	**No. of Controls n (%)**
	176 (100)	**176 (100)**
Sex		
Male	86 (49)	86 (49)
Female	90 (51)	90 (51)
Age in years (median, range)	**28 (9-51)**	**28 (8-54)**
≤15	38 (22)	34 (19)
[16-24]	33 (19)	34 (19)
[25-35]	68 (38)	77 (44)
≥36	37 (21)	31 (18)
Marital status		
Single	99 (56)	100 (57)
Married	76 (43)	74 (42)
Divorced	1 (0.5)	2 (1)

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Table 4.12 Clinical characteristics of BU cases

Characteristics	No. of Cases n (%) 176 (100)
Lesion type	
Ulcerative	115 (65)
Non-ulcerative	61 (35)
Location of lesion	
Lower limbs	77 (44)
Upper limbs	67 (38)
Head and Trunk	24 (14)
Abdomen	8 (4)
Category of lesion	
Category I (<5 Cm)	27 (15)
Category II (5–15 Cm)	55 (31)
Category III (> 15 Cm)	94 (53)

4.3.3 Factors Associated with the Risk of Contracting BU by Univaraite Analysis

4.3.3.1 Socio-economic factors associated with contracting BU

Socioeconomic status was assessed by both the participants' educational level and occupation type; and these variables were found to be lower in the cases than controls. There was a significant association between educational level and risk of BU (OR = 2.67, 95% CI = 1.46-5.03). Among the study population, individuals with tertiary level of education (higher education) were protected from developing BU as compared to those without education. Occupation type was also found to influence the chances of

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contracting BU. While agricultural farmers were at risk for contracting BU (OR = 2.9, 95% CI = 1.37-3.53), civil servants were protected from BU (OR = 0.24, 95% CI = 0.09-0.56) (Table 4.13).

4.3.3.2 Environmental and Behavioural factors for contracting BU

Cases reported staying in mud walls, having agricultural plantation, having bush and woods in their immediate environment more than the controls. The study found staying in mud walls and mud floors as well as closeness of households to river bodies was negatively associated with BU. Moreover, there was no evidence for association between risk of BU and the presence of agricultural farms, bush, swamps and woods in the immediate environment. Also, sources of drinking, bathing and cooking water were similar for both cases and controls and no evidence was observed for an association between the risk of BU and the water sources.

Washing of clothes around river bodies (OR = 2.12, 95% CI = 1.12-4.11) and passing through the rivers to individual destinations (OR = 2.16, 95% CI = 1.29-3.64) were reported more frequently in cases than the controls and these activities were positively associated with BU (Table 4.13).

Farming or mining activities in swamps (OR = 3.30, 95% CI = 2.08-5.29) was significantly associated with BU however, farming around water bodies showed no such association. While wearing of short upper body clothing (OR = 39.43, 95% CI = 19.91-83.58) and short dress (OR = 14.1, 95% CI = 0.08-25.5) during farming or mining activities were found to be positively associated with BU, the wearing of long upper body cloth (OR = 0.31, 95% CI = 0.14-0.63) and trousers (OR = 0.28, 95% CI = 0.17-0.45)

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during such activities were found to be protective for BU (Table 4.12). Wrapping wounds with leaves were reported more frequently in case patient than controls and it positively associated with BU (OR = 3.91, 95% CI = 2.32-6.74). Washing of wounds with water and soap (OR = 0.52, 95% CI = 0.31-0.85) and applying adhesive bandage (OR = 0.42, 95% CI = 0.26-0.66) on wounds were found to be protective for BU.

4.3.3.3 Insect bites

The risk of contracting BU was significantly associated with insect bites (OR = 227.58, 95% CI = 58.02–1999.04) and use of insect net at night appeared to be protective for BU (OR = 0.25 95% CI = 0.14-0.42) (Table 4.13).

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Table 4.13 Univariate analyses of selected variables for Buruli ulcer diseases in selected communities **along the** Densu river basin; community matched case control study.

Parameters	No. of Cases (N, %)	No. of Controls (N, %)	Univariate OR (95% CI)	p-value
Socio-economic status				
Primary	90 (51)	86 (49)	1.09 (0.71-1.70)	0.7492
Secondary	34 (19)	48 (27)	073 (0.43-1.25)	0.2525
Tertiary	7 (4)	25 (14)	0.25 (0.09-0.62)	<0.0014
No education	45 (26)	20 (11)	2.67 (1.46-5.03)	<0.0009
Occupation:				
Artisan	3 (2)	8 (5)	1.09	0.7492
Student	44 (25)	54 (31)	0.75	0.2845
Farmer	77 (44)	46 (26)	2.19 (1.37-3.53)	<0.001
Trader	14 (8)	18 (10)	0.76	0.5787
Fisherman/woman	5 (3)	3 (2)	1.68	0.7234
Miner	25 (14)	18 (10)	1.45	0.3288
Civil servant	8 (5)	29 (16)	0.24 (0.09-0.56)	<0.001
Household Environment				
Mud wall: yes/no	77 (44)	68 (39)		0.448
Mud floor: yes/no	38 (22)	39 (22)		0.699
Within < 1 km versus > 1 km to river basin	46 (26)	42 (24)		0.924
Agricultural plantation in the immediate environment: yes/no	66 (38)	65 (37)		0.912
Bush in the immediate environment: yes/no	158 (90)	152 (86)		0.324
Swamp in the immediate environment: yes/no	88 (50)	95 (54)		0.595
Woods in the immediate environment: yes/no	79 (45)	69 (39)		0.280
Source of drinking water				
Borehole	140 (80)	133 (76)		0.369
River/stream	14 (8)	10 (6)		0.526
Sachet	21 (11)	32 (18)		0.097

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Hand dug well	1 (0.6)	1 (0.6)		1.000
Source of water for bathing				
Borehole	154 (88)	154 (88)		0.893
River/stream	19 (10)	20 (11)		1.000
Hand dug well	3 (2)	2 (1)		1.000
Source of water for cooking				
Borehole	153 (87)	154 (88)		0.986
River/stream	21 (12)	20 (11)		1.000
Hand dug well	2 (1)	2 (1)		1.000
Domestic Activities				
Washing clothes around site: yes/no	36 (20)	19 (10)	2.12 (1.12-4.11)	0.013
Passing through river to destination: yes/no	60 (34)	34 (19)	2.16 (1.29-3.64)	0.004
Agricultural\Mining activities				
Farming/mining in swamps: yes/no	126 (71)	76 (43)	3.30 (2.08-5.29)	<0.001
Farming/ mining around river/Stream: yes/no	46 (26)	35 (20)		0.164
Wearing long upper body cloth to farm/mine: yes/no	13 (7)	36 (20)	0.31 (0.14-0.63)	<0.001
Wearing short upper body cloth to farm/mine: yes/no	163 (93)	42 (24)	39.43 (19.91-83.58)	<0.001
Wearing trouser to farm/mine: yes/no	80 (45)	132 (75)	0.28 (0.17-0.45)	<0.001
Wearing short dress to farm/mine: yes/no	80 (45)	150 (85)	14.1 (0.08-25.5)	<0.001
Insect bite				
Receive insect bites in river/home: yes/no	174 (99)	48 (27)	227.58 (58.02-1999.04)	<0.001
Use of mosquito net: yes/no	28 (16)	132 (75)	0.25 (0.14-0.42)	<0.001
Use of mosquito coil: yes/no	82 (46)	94 (53)		0.286
Wound management				
Washing wounds with water/soap regularly: yes/no	37 (21)	60 (34)	0.52 (0.31-0.85)	0.012
Robbing wounds with alcohol regularly: yes/no	23 (13)	38 (22)	0.55 (0.29-0.99)	0.067
Wrapping wounds with leaves frequently: yes/no	75 (43)	28 (16)	3.91 (2.32-6.74)	<0.001
Applying adhesive bandage on wounds always: yes/no	56 (32)	93 (53)	0.42 (0.26-0.66)	<0.001

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4.3.4 Confirmed Factors Associated with Risk of Contracting BU by Multivaraite Analysis

The multivariate analyses (conditional logistic regressions) found none of the socio-economic factors as a potential risk for contracting BU. However, some environmental and behavioral factors were retained. Farming activities in swamps (OR = 4.10, 95% CI = 3.82-7.18), wearing of short upper body clothes (OR = 1734.1, 95% CI = 68.1- 44120.9), wearing of short lower body clothes (OR = 14.4, 95% CI = 1.25-165.7), insect bite (OR = 988.3, 95% CI = 31.4-31115.6) and application of leaves on wounds (OR = 6.23, 95% CI = 4.74-18.11) were found to be risk factors for contracting BU. Moreover, farming in long upper body cloth (OR = 0.000, 95% CI = 0.00-0.14), farming in trousers (OR = 0.58, 95% CI = 0.21-0.97), washing wounds with water and soap (OR = 0.37, 95% CI = 0.29-0.98), and application of adhesive bandage on wounds (OR = 0.31, 95% CI = 0.15-0.82) were found to be protective against BU disease (Table 4.14).

Table 4.14 Multivariate analyses of selected variables for Buruli ulcer disease in selected communities along the Densu river basin; Community-matched case-control study

Parameters	Multivariate OR (95% CI)	p- value
Farming in swamps	4.10 (3.82-7.18)	0.001
Farming in long upper body cloth	0.000 (0.00-0.14)	0.032
Farming in short upper body cloth	1734.1 (68.1-44120.9)	<0.001
Farming in trousers	0.001 (0.000-0.14)	<0.001
Farming in short lower body cloth	14.4 (1.25-165.7)	<0.000
Insect bites in rivers or home	988.3 (31.4-31115.6)	0.001
Washing wounds with water and soap	0.37 (0.29-0.98)	0.001
Wrapping wounds with leaves	6.23 (4.74-18.11)	<0.001
Applying adhesive bandage on wounds	0.31 (0.15-0.82)	0.036

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CHAPTER FIVE

Discussion and Conclusions

5.1 Discussion

5.1.1 General Introduction

Current control of Buruli ulcer is limited by lack of understanding of the mode of transmission and the ecology of the causative pathogen. This thesis therefore, sought to understand the ecology of *Mycobacterium ulcerans* to facilitate strategies for control and prevention of BU in Ghana. The study analysed environmental samples as well as human BU samples, optimized decontamination procedures for isolation of *M. ulcerans* from environmental samples and also assessed risk factors for contracting BU.

5.1.2 Detection of *Mycobacterium ulcerans* in the Environment

Mycobacterium ulcerans in the environment was detected by three methods: RT-PCR, microscopy and culture. Using three independent molecular markers, IS*2404*, IS*2606* and KR for the RT-PCR, only 139/1600 (9%) of the samples analyzed were confirmed to contain *M. ulcerans* with varying low cycle threshold (C_T) levels in the ranges of (IS*2404*, 24.47-37.22), (IS*2606*, 26.43-41.39) and (KR, 25.91-39.18). Furthermore, direct microscopic analysis of the 139 samples identified only 12 (8.6%) to contain acid-fast bacilli (AFB), though all were confirmed by RT-PCR analysis to contain mycobacteria. The low sensitivity of Ziehl-Neelsen microscopy for detection of AFB is well documented, thus samples are only positive when they contain at least 10^4 CFU ml^{-1} of AFB. These findings probably suggest that, while *M. ulcerans* may be abundant in the environment, the bacterial concentrations are quite low. This finding may not be unique to this study, as several studies conducted in other African countries in which BU is endemic detected low C_T of *M. ulcerans*, the

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causative agent of Buruli ulcer in the environment (Vandelannoote *et al.*, 2010). In contrast, studies from Australia reported high C_T values for *M. ulcerans* in the environment (Johnson *et al.*, 2007). Exposure studies have shown quite a lot of exposed individuals do not developed active disease (Yeboah-Manu *et al.*, 2012; Röltgen *et al.*, 2014). This could be exposure to low MU concentration which may be below the infectious dose needed for establishing an active disease.

Until this study, direct isolation of *M. ulcerans* from the environment has not been possible. The challenge has been mainly due to the presence of other faster growing microorganisms that attain macroscopic growth within 18 to 48 hours, thereby contaminating cultures, albeit the mycobacteria species of interest (*M. ulcerans*) grows very slowly, requiring in some instance more than 6 months to achieve macroscopic growth. Decontamination using 1M NaOH followed by a simplified oxalic acid method (Yeboah-Manu *et al.*, 2004), led to the isolation of *M. ulcerans* from both spiked and un-spiked samples. One major important factor that is considered in the isolation of mycobacteria from the environment is the decontamination technique employed. Members of the genus mycobacterium have a thick cell wall rich in lipids that allow them to be impermeable to many acids, bases, and detergents (Jernigan and Farr, 2000); this has made decontamination with these agents a common practice in primary cultivation of mycobacteria to reduce contamination (Parashar *et al.*, 2009; Chatterjee *et al.*, 2013). Distinct agents have variable effect; while some decontaminating agents due to their harshness destroy a substantial number of mycobacterial species of interest along with the contaminants, others are too weak to eliminate them. A good decontamination agent is one, which

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effectively removes unwanted microorganisms and maximizes recovery of wanted mycobacteria.

Decontaminating 139 PCR positive samples with 1M NaOH followed by 5% oxalic acid yielded 58 (41.7%) mycobacteria positives and 70 (50.4%) no growths with only 11 (7.9%) samples getting contaminated. Although, sodium hydroxide- oxalic acid (NaOH-OA) procedure was the best among the four different decontamination procedures evaluated (Table 4.3), the proportion of no-growths indicates that is also very harsh. The harsh decontamination method probably might have also killed the *M. ulcerans*; and this could account for the low recovery rate. There is therefore the need for future work aimed to improve the decontamination step to enhance recovery. Furthermore, the introduction of the antifungal (mycobactin-J) and the antibiotic PANTA to the cultivating medium was effective in limiting contamination by fungi and other fast growing bacteria compared to drug free medium as shown in Table 4.4. Therefore, the use of these isolation methods is recommended for the recovery of *M. ulcerans* from environmental samples. The isolation from the environment will make it possible for further molecular epidemiological and animal model studies that will give insight into understanding the pathogenesis of different variants of *M. ulcerans* from the environment.

5.1.3 Distribution of *Mycobacterium ulcerans* in the environment

Mycobacterium ulcerans has been described as an environmental pathogen, which means the environmental presence outnumbers that found in humans (Stinear *et al.*, 2007); this was confirmed in this study as *M. ulcerans* DNA was found in various environmental samples analysed from all including non-endemic communities studied along both river basins (Table 4.1 and Table 4.10). In addition, the IS*2404*: IS*2606*

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copy number ratio, which differentiates *M. ulcerans* from the other mycolactone producing mycobacterium, was found in most of the positive samples to be less than 2.37. According to Fyfe *et al.* (2007), the average ΔC_T (IS*2606*–IS*2404*) for *M. ulcerans* is 2.37, while for the other mycolactone producing mycobacteria it is 7.60. Moreover, the environmental abundance of *M. ulcerans* outweighed the observed disease occurrence as found in this study. The broad distribution of *M. ulcerans* in the environmental setting, found in both BU endemic and non-endemic communities also clearly contrasts the focal distribution of human BU cases. For example, Ntabea, a community lying along the upper part of the Densu River has no record of BU (from both passive surveillance and active case detection data) but showed high environmental presence *M. ulcerans* DNA. This probably could be due to the presence of less pathogenic variants of *M. ulcerans* in the environment. A similar scenario has been observed in some strains that cause tuberculosis in Asia. An animal model study that assessed the virulence of modern versus ancient Beijing strains with similar genotypic and epidemiological features showed that the modern strains were highly pathogenic whilst the ancient sublineage displayed low virulence (Ribeiro *et al.*, 2014). This indicates that genetic changes within the modern sublineage favor selection of highly virulent bacteria. The pathogen variables that will be interesting to explore will be the genomic difference that could lead to differences in virulence between isolates obtained from this site. Furthermore, behaviour of inhabitants within the non-endemic communities may contribute to their protection from BU diseases as various studies found the use of protective clothing during farming activities to limit exposure to *M. ulcerans* (Aboagye *et al.*, 2017; Kenu *et al.*, 2014). I therefore recommend a follow-up longitudinal observation study to monitor behavioural activities of community members to better understand *M. ulcerans* transmission.

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Another major factor that could contribute to this lack of BU incidence is the host genetic factors of inhabitants; that could play a significant role by making them resistant to *M. ulcerans* disease. For instance, a study by our group on susceptibility to *M. ulcerans* infection showed two single nucleotide polymorphisms (SNPs), interferon gamma (*IFNG*) and inducible nitric oxide synthase (*iNOS*); key drivers in the host defense against BU infection. The *IFNG* activation increases the capacity of macrophages to kill intracellular mycobacteria by enhancing antimicrobial effector pathways, including *iNOS*, phagosomal maturation and autophagy, all of which play critical roles in the clearance of *M. ulcerans* (Bibert, *et al.* manuscript submitted for publication). Different sero-epidemiological studies conducted by our group in BU endemic and non-endemic regions both in Ghana and Cameroon indicated that like other mycobacterial diseases not all individuals exposed to the pathogen leads to overt disease using *M. ulcerans* specific 18kda heat shock protein, and that only a small percentage of the infected leads to BU disease (Yeboah-Manu *et al.*, 2012; Röltgen *et al.*, 2014). The first *in vitro* isolation of *M. ulcerans* from the environment has recently been published (Aboagye *et al.*, 2016) and this will pave the way for further environmental studies to enable molecular epidemiological studies using both environment and clinical isolates to better understand *M. ulcerans* transmission dynamics.

5.1.4 Seasonal influence on the presence of *Mycobacterium ulcerans* and occurrence of BU disease

A number of infectious diseases including vector, air and waterborne diseases are seasonal (Altizer *et al.*, 2006; Moura *et al.*, 2009; Luis *et al.*, 2010). At present there are no clear indications of association between BU and seasonality due to the lack of

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clear understanding of incubation period and presumed long incubation period of the disease, which is estimated between 2 to 4.5 months (Trubiano *et al.*, 2013). The findings from this study on the other hand indicated for the first time that there is a seasonal pattern for the presence of *M. ulcerans* DNA in the environment; the presence of *M. ulcerans* in the environment, which was detected using RT-PCR, was found to be higher in raining season compared to the dry season. The data from this PhD also suggest that rainfall over a certain threshold (between 100-200 mm) was associated with *M. ulcerans* positivity. This may mean that water in the ground is important probably for suspension but not so much water to wash away the bacteria. In addition, more human BU cases were detected during the monthly active case surveillance conducted in Ashongkrom after the rainy months (Fig 4.7). Similarly, in Cameroun and Papua New Guinea high rates of BU was observed during the dry season (Radford, 1974; Ravisse, 1977; Landier *et al.*, 2014), while in both Ghana and Côte d'Ivoire, the peak incidence of the BU disease has also been reported to be at the end of the rainy season (Amofah *et al.*, 1993; Marston *et al.*, 1995). I therefore propose that *M. ulcerans* cells that might have been dormant or buried in the environment particularly in the soil are exposed during the rainfall season, which increases the exposure rates. The length of the two raining seasons (April-June and September-mid November), thus supports a minimum incubation time of between 2 to 4.5 months (Radford, 1974; Ravisse, 1977; Amofah *et al.*, 1993; Trubiano *et al.*, 2013).

5.1.5 Environmental and behavioural risk factors for BU

Like many variables of BU disease, the risk factors for the disease are still enigma, which limits design of preventive measure for disease control. Therefore assessment

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of potential environmental and behavioural risk as well as protective factors for BU disease is essential not only for disease prevention but also towards understanding the transmission mechanism. Both univariate and conditional logistic regression analyses showed that farming in swampy areas, wearing of short clothing, insect bite and the application of leaves on woundsare major risk factor for BU.

Agricultural activities such as farming particularly in swampy areas put cases in constant contact with moist/ watery soil. Tian *et al* recently have shown that *M. ulcerans* strains can survive in soil for up to four months suggesting that BU could be acquired through contact of open wounds with watery soil as a transient source of infection (Tian *et al.,* 2016). Moreover, from this thesis, *M. ulcerans* was isolated from a soil sample collected from a BU endemic community along the Densu river basin of Ghana (Aboagye *et al.,* 2016). Bratschi *et al* also indicated that *M. ulcerans* may persist for many months in under water decaying organic matter (Bratschi *et al.,* 2014). These results give strong indication that farming and conducting other activities, including playing that brings one into contact with swampy areas is a risk factor for BU.

At the same time wearing of short clothing (upper and lower body) during farming activities also poses a major risk for contracting BU. The hot weather condition that is frequently experienced in most African countries encourages the population to wear less protective clothing. This behaviour results in long periods of exposure of most parts of the limbs, which is likely to facilitate infection of these body parts. Different studies showed that almost all BU lesions are located on the limbs (Amofah *et al.,* 2002; Sopoh *et al.,* 2010; Hospers *et al.,* 2005; Adu *et al.,* 2011). A study that analysed distribution of lesion location in 88 confirmed BU cases from Cameroon reported 55.7% on lower limbs and 30.7% on upper limbs (Bratschi *et al.,* 2013). A

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related study that analyzed 1,020 confirmed BU cases also reported that 57% and 38% lesion were located on lower limbs upper limbs respectively (Prof. Dorothy Yeboah-Manu, unpublished data). These observations suggest that infection by *M. ulcerans* occurs from direct contact of an exposed skin with a contaminated source.

In addition, biting insects are potential risk factor for BU infection. Studies conducted in both Ghana and Benin detected *M. ulcerans* DNA in water bugs belonging to the families Naucoridae and Belostomatidae (Portaels *et al.,* 1999). In South-Eastern Australia, Quek *et al* in a case-control study reported that more cases recalled that they were bitten by mosquitoes on the lower extremities implicating mosquitoes in the transmission of *M. ulcerans* (Quek *et al.,* 2007). Johnson et al. also screened mosquitoes in a small town during BU outbreak in South-Eastern Australia and confirmed the presence of *M. ulcerans* in a subset of pools by detection of three PCR targets (IS*2404*, IS*2606* and Ketoreductase (KR)) (Johnson *et al.,* 2007). However, no study in Ghana has detected *M. ulcerans* DNA in mosquitoes.

Lastly, direct application of leaves on open wounds is a potential risk factor for contracting BU. More recently, Aboagye *et al.* (2016) detected *M. ulcerans* DNA from vegetation biofilm from BU endemic community along the Densu river basin of Ghana. Stinear *et al* identified aquatic plants as a possible reservoir of this pathogen (Stinear *et al.,* 2000). These aquatic plants, such as algae for instance are able to secrete many organic compounds, such as amino acids and polysaccharides, which are in turn used by bacteria as substrates for growth (Jensen, 1991; Maurin *et al.,* 1997; Moore, 1964; Poulet and Martin-Jezequel, 1983). Direct application of leaves and preparations thereof may contain *M. ulcerans* as well as other microorganisms such as *Staphylococcus aureus* that also could lead to secondary infections; exacerbating the condition of the lesions as observed in BU wounds (Yeboah-Manu *et al*., 2013).

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Notwithstanding, wearing of long clothing (upper and lower body) during farming activities, washing of wounds with water and soap and application of adhesive bandages were found to offer some protection against BU (Raghunathan *et al.*, 2005; Nackers *et al.*, 2007). These protective mechanisms could be due to the washing off/ limiting access of the contaminating pathogens with water and soap off the surface of the skin whilst the long clothing and bandages serve to cover open wounds and exposed surfaces thereby limiting entry of pathogens. One can therefore speculate that productive infection requires long duration of contact with an exposed skin.

One practice that could potentially contribute to the control of BU in endemic areas is the observance of personal hygiene. Washing with soap and clean water forms a major component of the water, sanitation and hygiene (WASH) programme (UNICEF, 2015). This program is integrated into the school systems in rural derived communities; a partnership between the Ghana Health Service, Ghana Education service and the UNICEF towards meeting the MDG goal 7C (UNICEF, 2015). The goal is to half the proportion of the population without sustainable access to safe drinking water and basic sanitation. Provision of boreholes and other sanitary facilities have been made in most of the deprived rural communities of Ghana. Moreover, some non-governmental organizations (NGO) have been involved in the provision of sanitation supplies to selected rural deprived schools. The adoption of this strategy towards eradication of guinea worm in the three regions in Northern Ghana led to successful project implementations which lead to the declaration of these regions as guinea worm free (Lyons, 1972; Henderson *et al.*, 1988; Biswas *et al.*, 2013). In Cameroon for instance, BU control is integrated into that of leprosy, yaws and leishmaniasis programme. It is therefore feasible for similar integration to be achieved between the BU control program in Ghana and the WASH concept, an

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approach that can maximize the limited resources allocated for the control of this disease.

5.1.6 Ecology and Transmission of *Mycobacterium ulcerans*

While *M. ulcerans* DNA have been detected in different environmental samples, the exact ecological niche(s) still remains enigmatic. Three major theories have been propounded to explain the mechanism of *M. ulcerans* transmission (Duker *et al.*, 2006). The first hypothesis is that *M. ulcerans* is transmitted by contamination of existing wounds or sites of trauma by environment, such as soil, and vegetation (Portaels *et al.*, 2001). In support of this theory, Stinear *et al* used magnetic bead sequence capture-PCR to detect *M. ulcerans* DNA in water and plant materials from two BU endemic foci. Whiles both samples were positive, nearby sites and other unrelated sites were negative (Stinear *et al.*, 2000). Similarly, this thesis detected *M. ulcerans* DNA by RT-PCR mainly in moss, soil and vegetation biofilm and further isolated *M. ulcerans in vitro* from moss and soil samples from BU endemic area. What this means is that direct contact with environmental sources that have been contaminated by *M. ulcerans* is important for potentiating acquisition of the pathogen in humans which may subsequently lead to infection. Furthermore, cases control study conducted as part of this thesis identified farming in short clothing and swampy areas as major risks for developing BU infection. Other studies have shown that *M. ulcerans* are able to survive in soil for up to four months (Tian *et al.*, 2016), an indication that infection could only be achieved through contact with an exposed skin surface. Although contamination of existing wound or trauma to the skin seems more plausible for *M. ulcerans* acquisition, it has been suggested that BU infection through passive inoculation into an existing abrasion appears a less likely route of entry

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(Williamson *et al.*, 2013). Nevertheless, there are evidences that show that stem or leaves of plants with barbs, spines and bristles, throbs, sharp and serrated edges can cause mechanical injury to the skin (Wee and Gopalakrishnakone, 1990). Puncture of the skin by these contaminated environments could lead to deep lacerations where *M. ulcerans* could be deposited and contained within the subcutaneous tissues leading to infection. The second hypothesis states that acquisition of *M. ulcerans* is by inhalation or ingestion of aerosolized *M. ulcerans* from contaminated water (Hayman, 1991). Supporting this theory, Veitch *et al* reported on an outbreak of BU in an area near a golf course irrigation system in Australia. Limitation of the irrigation activity led to dramatic reduction in the number of new cases in the area (Veitch *et al*., 1997). The third hypothesis states that *M. ulcerans* may be acquired through an insect or animal bite (Portaels *et al.*, 1999). This theory is supported by a number of studies; by PCR, *M. ulcerans* has been detected in biting aquatic bugs (Naucoridae) (Marsollier *et al.*, 2002), mosquitoes from Australia **(**Johnson *et al.*, 2007) and the salivary glands of carnivorous insects, *Naucoris cimicoides*, **(**Marsollier *et al.*, 2005; Mosi *et al.*, 2008**).** Studies conducted in South-Eastern Australia implicated mosquitoes as vectors in the transmission of *M. ulcerans* (Johnson *et al.*, 2007; Lavender *et al.*, 2011), however findings from sero-epidemiological studies that was carried out by our group in Ghana and Cameroon doubted mosquitoes as possible vectors in *M. ulcerans* transmission (Ampah *et al.*, 2016; Röltgen *et al.*, 2014). The first serological responses to *Plasmodium falciparum* emerged around the age of one (all children below 4 years had antibodies against *Plasmodium falciparum* apical membrane antigen-1 in their sera), whereas sero-conversion for *M. ulcerans* occurred after age four (Ampah *et al.*, 2016; Röltgen *et al.*, 2014); suggesting that exposure occurs at an age which overlaps with children moving further away from their homes and having more intense

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environmental contact (Ampah *et al.,* 2016; Röltgen *et al.,* 2014). This may include schooling, expansion of their social networks (making and visiting new friends) and carrying out of chores, such as the collection of water and fire wood, which eventually increases the likelihood of them coming into contact with environment contaminated with *M. ulcerans* at the periphery of the community.

In addition, BU outbreaks in Australia led to the detection of *M. ulcerans* DNA in possums with BU-like lesions. This led to a concept that possums may act as probable reservoirs in the transmission of *M. ulcerans* (Fyfe *et al.,* 2010). However, finding similar mammalian reservoirs in African BU endemic zones have been unsuccessful up till now. Studies that involved trapping of 348 small mammals in six villages in Benin found no *M. ulcerans* positive DNA in pooled samples of the captured animal body parts or faeces (Durnez *et al.,* 2010). Although mycobacterial infections were detected in some of the 700 rodents captured in BU endemic and non-endemic regions of Uganda in 1972, none of these infections were caused by *M. ulcerans* (OIE, 2013). Also in Ghana, feacal surveys of 180 domestic animals (avian and ovine) across BU endemic and non-endemic villages yielded no *M. ulcerans* positive DNA (Tobias *et al.,* 2016).

Stinear *et al* have postulated that *M. ulcerans* may be preferentially adapting to specific ecological niches such as plant biofilms due to its inability to produce light inducible carotenoids that serve as a shield against incident sunlight (Stinear *et al.,* 2007). This thesis asserts that *M. ulcerans* has a broad ecological niche that exceeds the area of human BU presence. The positivity rates found in the environment (nearly 9 % overall) are consistent with previous studies (Garchitorena *et al.,* 2014; Marion *et*

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al., 2010; Williamson *et al.,* 2008). The high proportion of *M. ulcerans* that were detected in soil and vegetation biofilm particularly is not surprising as the hydrophobic properties of non-tuberculous mycobacteria including *M. ulcerans* confer on them the ability to attach to soil surfaces to better adapt in the environment (Tortoli, 2003). Studies by Marsollier *et al* reported that aquatic snails transiently harbour *M. ulcerans* without offering favourable conditions for its growth and survival (Marsollier *et al.,* 2004). However, the edible land African giant snail (*Achatina fulica*) from which *M. ulcerans* DNA was detected, can harbour metabolically active bacterial communities in its gut (Pawar *et al.,* 2012) during feeding on plants and soil (Rauth and Barker, 2002; Chevalier *et al.,* 2003). This further suggests that it may be possible for viable *M. ulcerans* to be released from the gut of *Achatina fulica* into the soil and vegetation surfaces during feeding which could lead to contamination of open wounds and cuts; further buttressing the first theory of *M. ulcerans* acquisition form the environment.

Critically thinking through the theories propounded to explain the transmission mechanisms of *M. ulcerans* infection; the Australians believe possums are possible reservoirs that aid in the transmission. However, no such animal reservoir has been detected in Ghana despite various studies conducted so far. Furthermore, in Australia, mosquitoes have also been implicated as possible vectors; but I dispute this claim because all diseases that are caused by mosquitoes are by haematogenous spread. And if BU infections were to be caused by mosquitoes then one would expect that the occurrence of lesions would not be restricted to only specific body parts. Also considering the location of lesions on affected individuals particularly on the limbs, one can deduce that *M. ulcerans* acquisition has probably got to do with direct

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contact. Moreover, the hot weather conditions which necessitate wearing of less protective clothing thereby exposing the skin favours contact with environmental sources contaminated with the pathogen.

This study hypothesized that *M. ulcerans* cells that are dormant or buried in the soil are exposed to the surfaces during the rainfall season due to the environmental disturbances such as erosion that occurs during the rainfall (Fig 5.1). These cells may probably collect in stagnant pools and swamps over long period of time. Moreover in BU endemic communities, agricultural activities which are mostly carried around swampy zones enhance increased environmental contact with *M. ulcerans* infested waters. The hot climatic conditions influence farmers in particular to wear less protective clothing, which may expose their body parts particularly the limbs to *M. ulcerans* that may be present in the environment. Considering the extremely long incubation period of *M. ulcerans*; susceptible host then may develop the BU disease over time (Fig 5.1).

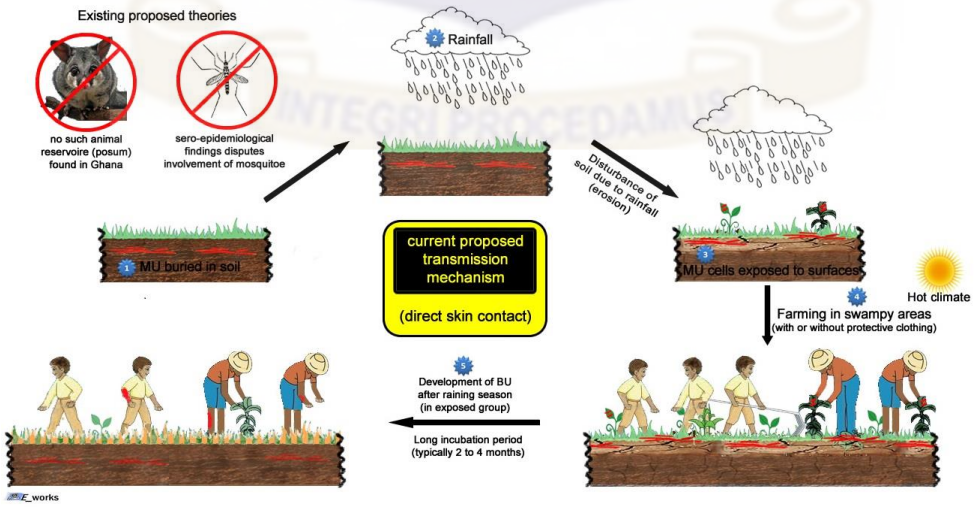

Figure 5.1 *M. ulcerans* Transmission Mechanism

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5.2 Limitations

This thesis although paramount to understanding the ecology and transmission of *M. ulcerans*, was not without limitation;

1. *In vitro* isolation of *M. ulcerans*, which is the mainstay method for indicating the presence of viable pathogen, was attained in only two out of the over hundred environmental samples that *M. ulcerans* DNA was detected using RT-PCR. This low number did not allow me to conduct further molecular epidemiological study to look for association between genotypes of environmental and clinical isolates.
2. The environmental sampling was conducted during only certain months within the study period. To better understand the seasonal patterns of *M. ulcerans*, further studies looking at longitudinal environmental sampling in BU endemic sites for spaning over 48 months is paramount.

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5.3 Conclusions

This study has provided important clues which add to existing knowledge on unraveling the ecology and the mode of transmission of *M. ulcerans* in Ghana and beyond. The most important findings and scientific achievements from this thesis were;

1. The study confirmed the abundance of *M. ulcerans* in the environment even though at lower concentrations and that its presence is influenced by seasonality
2. This study established an efficient and effective in-house decontamination procedure (NaOH-OA) and cultivation media that are supplemented with various antimicrobials which aided in the recovery of *M. ulcerans* from the environment.
3. The first ever direct *in vitro* isolation of *M. ulcerans* from environmental samples collected from BU endemic community was achieved.
4. The study identified potential risk and protective factors for BU along the Densu river basin. Farming in short clothes (upper and lower body) in swampy areas, wrapping of wounds with leaves was found as major risk of contracting the BU whereas wearing of long clothes (upper and lower body) during farming, washing of wounds with water and soap and application of adhesive bandages to wounds were protective for BU.

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5.4 Recommendations

Based on the analyses done and the obtained results from this PhD, the following are recommended for public health considerations and for future work;

1. There is the need for education and sensitization by the NBUCP, NGO's, School Health Education Program (SHEP) and Metropolitan, Municipal and District Assemblies (MMDAs) to inhabitants in BU burdened areas to take precautions such as using protective clothing (long sleeves, trousers, wellington boots etc.) during agricultural activities and rainy seasons to limit contact with *M. ulcerans* in the environment. Extensive washing of hands and bathing immediately after engaging in farming activities is paramount in removing pathogens that might have come in contact with the skin surfaces.
2. Adoption of the strategies used in WASH concept in the eradication of guinea worm in northern Ghana by the local government and the health systems to complement the existing BU control strategies to ensure rapid elimination of BU in Ghana.
3. Further studies should look at improvimng on the established in-house decontamination (NaOH-OA) procedure that led to the recovery of *M. ulcerans* from the environment. Also a lot more work needs to be done to collect more environmental samples to increase the number of isolates from the environment. Even though there were more PCR positives samples, only a few were isolated *in vitro* and together with the microscopy results suggest that the level of *M. ulcerans* in the environment is low which requires less stringent decontamination methods. Improvement on the decontamination procedure is essential so as to accrue more isolates to conduct more meaningful molecular epidemiological studies.

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4. Future works should also explore on the isolated *M. ulcerans* isolated from the environment in an exposure studies using animal models to understand the pathogenesis of the strains.

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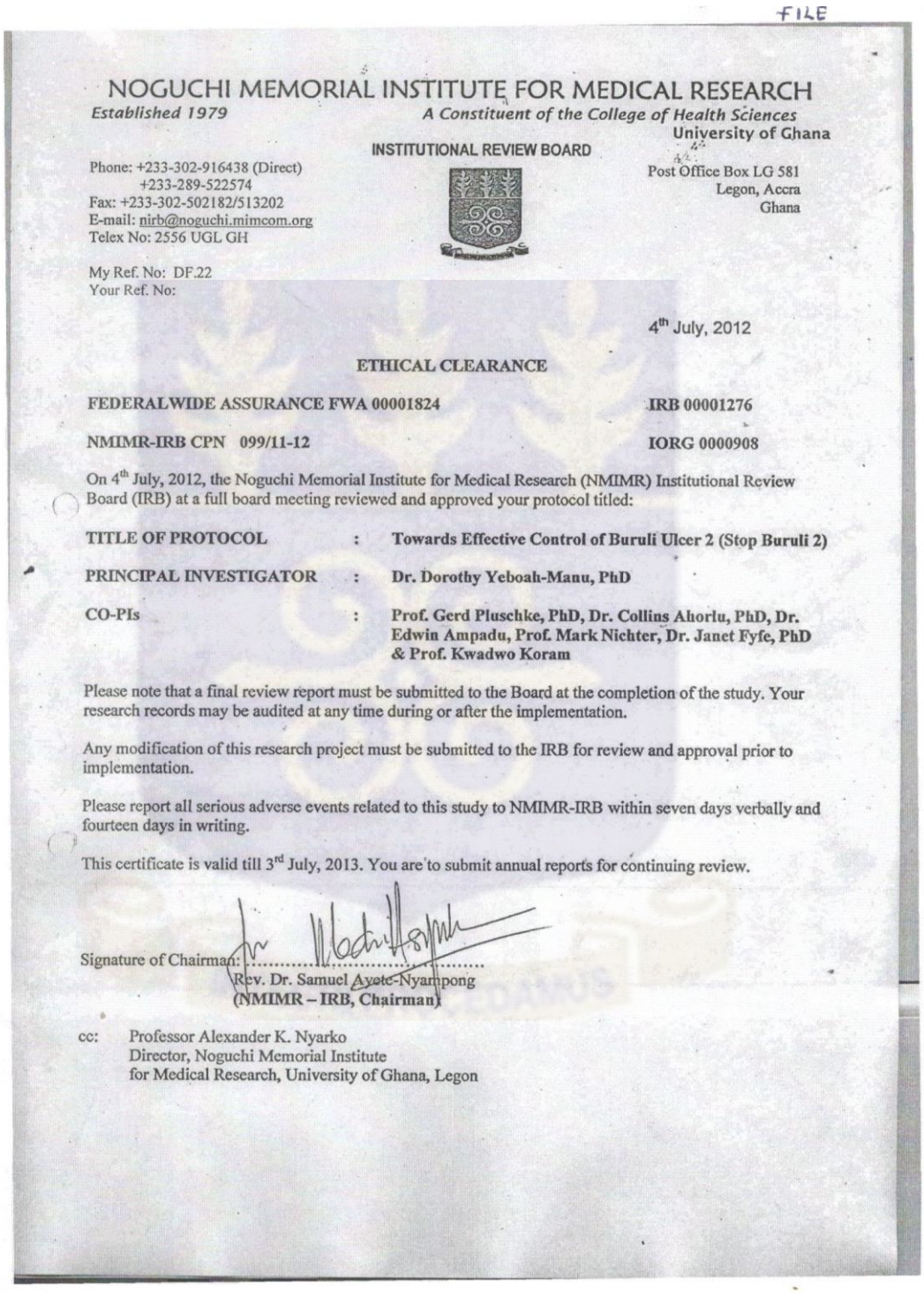
APPENDICES

Appendix A

Ethical Clearance



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Fax: +233-302-502182/513202
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Telex No: 2556 UGL GH

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Legon, Accra
Ghana

My Ref. No: DF.22
Your Ref. No:

4th July, 2012

ETHICAL CLEARANCE

FEDERALWIDE ASSURANCE FWA 00001824 **IRB 00001276**

NMIMR-IRB CPN 099/11-12 **IORG 0000908**

On 4th July, 2012, the Noguchi Memorial Institute for Medical Research (NMIMR) Institutional Review Board (IRB) at a full board meeting reviewed and approved your protocol titled:

TITLE OF PROTOCOL : **Towards Effective Control of Buruli Ulcer 2 (Stop Buruli 2)**

PRINCIPAL INVESTIGATOR : **Dr. Dorothy Yeboah-Manu, PhD**

CO-PIs : **Prof. Gerd Pluschke, PhD, Dr. Collins Ahorlu, PhD, Dr. Edwin Ampadu, Prof. Mark Nichter, Dr. Janet Fyfe, PhD & Prof. Kwadwo Koram**

Please note that a final review report must be submitted to the Board at the completion of the study. Your research records may be audited at any time during or after the implementation.

Any modification of this research project must be submitted to the IRB for review and approval prior to implementation.

Please report all serious adverse events related to this study to NMIMR-IRB within seven days verbally and fourteen days in writing.

This certificate is valid till 3rd July, 2013. You are to submit annual reports for continuing review.

Signature of Chairman: .....
Rev. Dr. Samuel Ayete-Nyampong
(NMIMR – IRB, Chairman)

cc: Professor Alexander K. Nyarko
Director, Noguchi Memorial Institute
for Medical Research, University of Ghana, Legon

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Appendix B

RT-PCR *CT*-values of MU Confirmed Samples

No.	Site	River Basin	Sample ID	Real-time PCR *CT* values			*CT* IS*2606*-IS*2404*
				IS*2404*	IS*2606*	KR	
1	Achiase	Offin	ENVSABU003B	31.55	37.28	35.73	5.73
2	Achiase	Offin	ENVSABU006A	32.74	34.67	35.37	1.93
3	Achiase	Offin	ENVSABU010B	31.73	33.56	32.86	1.83
4	Achiase	Offin	ENVSABU021A	31.13	32.37	32.05	1.24
5	Achiase	Offin	ENVSABU029B	30.56	31.33	30.87	0.77
6	Achiase	Offin	ENVSABU032A	34.46	36.22	35.07	1.76
7	Achiase	Offin	ENVSABU035C	34.39	40.44	38.34	6.05
8	Akomfore	Offin	ÊNVSAF001A	34.65	35.67	36.34	1.02
9	Keniago	Offin	ENVSKG006B	32.82	33.52	33.7	0.7
10	Mfantsiman	Offin	ENVSMF039B	29.69	31.56	31.78	1.87
11	Pokukrom	Offin	ENVSPK029A	25.84	26.43	25.91	0.59
12	Wromanso	Offin	ENVSWM010A	32.21	33.41	33.30	1.2
13	Wromanso	Offin	ENVSWM011A	31.39	34.49	33.58	3.1
14	Wromanso	Offin	ENVSWM015A	31.24	32.14	32.13	0.9
15	Wromanso	Offin	ENVSWM015B	31.52	39.92	36.70	8.4
16	Wromanso	Offin	ENVSWM016B	32.41	38.55	35.48	6.14
17	Wromanso	Offin	ENVSWM022B	31.50	33.19	32.90	1.69
18	Wromanso	Offin	ENVSWMIB	30.08	37.01	36.35	5.51
19	Wromanso	Offin	ENVSWMVIIIA	33.05	34.49	32.33	1.44
20	Ntobroso	Offin	ENVSNBU001A	34.42	35.67	37.32	1.25
21	Ntobroso	Offin	ENVSNBUS 089a	32.65	33.56	35.12	0.91
22	Ntobroso	Offin	ENVSNBUS	34.76	34.83	36.45	0.07

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			132B				
23	Ntobroso	Offin	ENVSNBUS 077a	34.29	35.41	36.34	1.12
24	Keniago	Offin	ENVSKGS073C	34.89	41.39	35.67	6.5
25	Mfantsiman	Offin	ENVSMFS 102E	34.63	35.85	35.13	1.22
26	Mfantsiman	Offin	ENVSMFS121A	34.23	36.62	34.9	2.39
27	Mfantsiman	Offin	ENVSMFS098B	34.28	36.53	35.96	2.25
28	Mfantsiman	Offin	ENVSMFS098F	34.04	35.52	34.45	1.48
29	Mfantsiman	Offin	ENVSMFS036A	34.67	36.68	35.56	2.01
30	Mfantsiman	Offin	ENVSMFS057B	33.61	40.42	39.18	6.81
31	Mfantsiman	Offin	ENVSMFS088B	32.73	35.04	33.34	2.31
32	Mfantsiman	Offin	ENVSMFS088C	34.07	35.95	34.79	1.88
33	Mfantsiman	Offin	ENVSMFS088D	34.63	36.61	37.18	1.98
34	Mfantsiman	Offin	ENVSMFS113G	34.07	38.23	34.84	4.16
35	Mfantsiman	Offin	ENVSMFS160A	33.27	34.99	32.78	1.72
36	Mfantsiman	Offin	ENVSMFS092A	34.71	36.35	34.3	1.64
37	Pokukrom	Offin	ENVSPKS046D	34.67	36.23	37.34	1.56
38	Wromanso	Offin	ENVSWMS008I	33.28	37.53	36.78	4.25
39	Wromanso	Offin	ENVSWMS018B	34.26	36.14	35.56	1.88
40	Wromanso	Offin	ENVSWMS021E	33.56	35.15	36.78	1.59
41	Wromanso	Offin	ENVSWMS078H	34.19	36.37	35.74	2.18
42	Wromanso	Offin	ENVSWMS067D	34.22	35.14	34.35	0.92
43	Wromanso	Offin	ENVSWMS027B	34.21	36.17	37.12	1.96
44	Wromanso	Offin	ENVSWMS140B	34.21	35.16	34.68	0.95
45	Wromanso	Offin	ENVSWMS160B	32.15	33.15	35.46	1
46	Achiase	Offin	ENVSABUS090I	34.76	39.86	38.57	5.1
47	Achiase	Offin	ENVSABUS073E	34.71	35.25	34.31	0.54
48	Achiase	Offin	ENVSABUS127D	34.49	35.76	35.13	2.27

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49	Achiase	Offin	ENVSABUS160A	34.33	35.89	35.45	1.56
50	Akomfore	Offin	ENVSAFS081B	32.72	37.35	36.45	4.63
51	Ntabea	Densu	NT4	27.78	28.63	28.30	0.85
52	Ntabea	Densu	NT6	32.52	33.27	33.39	0.75
53	Ntabea	Densu	NT18	28.54	30.14	29.16	1.6
54	Ntabea	Densu	NT11	32.93	33.96	34.34	1.03
55	Ntabea	Densu	NT20	27.70	28.76	28.27	1.06
56	Ntabea	Densu	NT15	37.22	28.20	27.41	0.98
57	Ntabea	Densu	NT17	30.17	31.88	31.13	1.71
58	Ntabea	Densu	NT3	32.33	33.45	32.38	1.12
59	Ntabea	Densu	NT14	30.41	36.21	36.46	5.8
60	Ntabea	Densu	NT1	34.64	35.23	35.24	0.59
61	Ntabea	Densu	NT27	30.17	31.11	30.45	0.94
62	Ntabea	Densu	NT28	28.50	29.86	29.29	1.36
63	Ntabea	Densu	NT32	31.13	32.87	31.55	1.74
64	Ntabea	Densu	NT34	25.47	26.51	26.24	1.04
65	Ntabea	Densu	NT23	30.17	31.10	30.78	0.93
66	Ntabea	Densu	NT24	27.50	39.96	28.28	12.46
67	Ntabea	Densu	NT25	29.48	30.16	30.01	0.68
68	Ntabea	Densu	NT29	34.02	39.14	37.26	5.12
69	Ntabea	Densu	NT33	30.64	34.77	32.89	4.13
70	Ntabea	Densu	NT22	33.62	34.12	33.98	0.5
71	Ntabea	Densu	NT31	32.85	34.12	33.56	1.27
72	Ashongkrom	Densu	ASH53	31.26	32.28	31.75	1.02
73	Ashongkrom	Densu	ASH57	28.49	29.83	29.58	1.34
74	Ashongkrom	Densu	ASH64	25.49	26.50	26.14	1.01
75	Ashongkrom	Densu	ASH39	26.64	28.42	28.29	1.78

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76	Ashongkrom	Densu	ASH40	30.60	31.85	31.57	1.25
77	Ashongkrom	Densu	ASH48	27.65	28.73	28.34	1.08
78	Ashongkrom	Densu	ASH42	29.48	31.25	31.27	1.77
79	Ashongkrom	Densu	ASH52	30.18	31.22	30.89	1.04
80	Ashongkrom	Densu	ASH55	34.99	36.54	35.57	1.55
81	Ashongkrom	Densu	ASH58	31.09	32.34	31.71	1.25
82	Ashongkrom	Densu	ASH46	30.10	32.33	31.54	2.23
83	Ashongkrom	Densu	ASH51	32.81	34.23	35.16	1.42
84	Ashongkrom	Densu	ASH56	33.26	34.94	34.21	1.68
85	Ashongkrom	Densu	ASH49	30.94	32.72	32.07	1.78
86	Ashongkrom	Densu	ASH50	31.45	32.84	32.42	1.39
87	Ashongkrom	Densu	ASH54	33.65	35.14	34.96	1.49
88	Ashongkrom	Densu	CC2	29.92	32.16	32.11	2.24
89	Ashongkrom	Densu	T1PL	30.33	32.44	32.17	2.11
90	Ashongkrom	Densu	CC7	28.74	30.57	30.18	1.83
91	Ashongkrom	Densu	T5 S	31.88	33.79	33.07	1.91
92	Ashongkrom	Densu	T5 PF	28.59	30.35	30.11	1.76
93	Ashongkrom	Densu	T5 F	29.86	31.25	31.13	1.39
94	Ashongkrom	Densu	T5 PL	30.50	32.42	32.10	1.92
95	Ashongkrom	Densu	CCSWM	28.30	29.81	29.68	1.51
96	Ashongkrom	Densu	HHL	31.48	33.56	33.15	2.08
97	Ashongkrom	Densu	FP5	30.08	31.75	31.21	1.67
98	Ashongkrom	Densu	PS2	29.32	33.21	33.10	3.89
99	Ashongkrom	Densu	STW7	31.62	37.92	36.92	6.8
100	Ashongkrom	Densu	STW7 STW	32.28	34.12	33.42	1.84
101	Ashongkrom	Densu	CC9	34.19	35.92	34.82	1.73
102	Ashongkrom	Densu	SWS 3	31.81	36.02	32.87	4.21

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103	Ashongkrom	Densu	SWS R	32.52	36.55	34.07	4.03
104	Ashongkrom	Densu	PPT3PL	32.63	34.15	33.20	1.52
105	Ashongkrom	Densu	PPT2PL	32.51	34.17	33.75	1.66
106	Ashongkrom	Densu	FP4S	32.51	37.66	35.87	5.15
107	Ashongkrom	Densu	PPT4PL	32.31	34.43	34.02	2.12
108	Ashongkrom	Densu	FP3	32.43	36.79	35.87	4.36
109	Ashongkrom	Densu	AS094F	30.99	39.37	37.42	8.38
110	Ashongkrom	Densu	AS098B	34.12	35.65	35.20	1.53
111	Ashongkrom	Densu	AS075C	34.53	36.40	35.66	1.87
112	Ashongkrom	Densu	AS079B	34.20	39.13	37.54	4.93
113	Ashongkrom	Densu	AS059B	34.81	36.43	35.20	1.62
114	Ashongkrom	Densu	AS057B	33.79	34.48	34.32	0.69
115	Ashongkrom	Densu	AS056A	34.05	35.83	35.92	1.78
116	Ashongkrom	Densu	AS056A1	34.06	35.83	35.12	1.79
117	Ashongkrom	Densu	AS055A	33.18	35.28	34.25	2.1
118	Ashongkrom	Densu	AS036B	34.13	35.70	35.20	1.57
119	Domesampaman	Densu	DS003A	33.64	39.64	34.26	6
120	Domesampaman	Densu	DS003B	32.72	34.67	35.21	1.95
121	Domesampaman	Densu	DS004A	32.02	33.61	35.26	1.59
122	Domesampaman	Densu	DS004B2	33.13	39.10	36.42	5.97
123	Domesampaman	Densu	DS008C	33.18	34.45	33.63	1.27
124	Domesampaman	Densu	DS009B	34.12	38.44	36.34	4.32
125	Domesampaman	Densu	DS020A	33.93	34.56	36.34	0.63
126	Ashongkrom	Densu	AS 120 B1	34.09	34.26	34.18	0.17
127	Ashongkrom	Densu	AS 121A	33.38	34.78	34.16	1.4
128	Ashongkrom	Densu	AS 121A2	34.15	35.08	34.16	0.93
129	Ashongkrom	Densu	AS 122A	34.42	36.45	3616	2.03

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130	Ashongkrom	Densu	AS 122B	33.6	34.18	34.72	1.2
131	Ashongkrom	Densu	AS 123A1	34.18	35.12	34.13	0.94
132	Ashongkrom	Densu	AS 123A2	33.16	34.98	34.71	1.82
133	Ashongkrom	Densu	AS 126A	32.64	34.52	33.92	1.88
134	Ashongkrom	Densu	AS 127A	32.18	36.12	34.18	3.94
135	Ashongkrom	Densu	AS 128A	34.76	35.18	34.64	0.58
136	Ashongkrom	Densu	AS 128B1	34.12	35.08	34.94	0.96
137	Ashongkrom	Densu	AS 133B2	34.28	36.18	34.4	1.9
138	Ashongkrom	Densu	AS 138B3	32.18	34.15	33.63	1.97
139	Ashongkrom	Densu	AS 138B4	34.66	35.41	34.98	0.75

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Appendix C

Environmental and Behavioural Assessment
Name of Community: ……

ID: ……

Section 1: Demographic information

1a. Age:

Gender: ☐ Male ☐ Female

1b. Marital status

☐ Single ☐ Married ☐ Divorced ☐ Widowed

1c Have you ever been confirmed for the BU disease?

☐ Yes ☐ No

Section 2: Education

2a. What is your level of education?

☐ Primary ☐ Secondary ☐ Tertiary ☐ No education

Section 3: Occupation

3a. What is your main occupation? (Please tick)
☐ Student ☐ Farmer ☐ Trader ☐ Fisherman/woman ☐ Miner
☐ Teacher ☐ Artisan ☐ Health worker other, specify
…….

3b. Beside your occupation, do you have any other minor occupations?
☐ Yes ☐ No

3c. What is the mode of transportation to your work place?
☐ Private transport ☐ Commercial transport ☐ Walk on foot

3d. How many hours do you spend at your job daily?
☐ Less than 1 hr ☐ 1-6 hrs ☐ 6-12 hrs ☐ More than 12 hrs

3e. Do you have commitment in other village (s) such that you reside there as well?
☐ Yes ☐ No

3f. If yes how long?
☐ Less than a week ☐ A week to month ☐ More than a month

Section 4: Household Environment

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4a. Is your house walled with mud?
☐ Yes ☐ No

4b. Is the house floored with mud?
☐ Yes ☐ No

4c. How close is your house to a river body?
☐ Less than 1 km ☐ Between 1km to 5km ☐ Over 5km

4d. Do you have agricultural plantation in the immediate neighbourhood?
☐ Yes ☐ No

4e. If yes specify the type of crop in the plantation ……..

4f. Do you have bush in the immediate neighbourhood?
☐ Yes ☐ No

4g. Do you have swamp in the immediate neighbourhood?
☐ Yes ☐ No

4h. Do you have woods in the immediate neighbourhood?
☐ Yes ☐ No

Section 5: Water use and contact

5a. What is your primary source of drinking water?
☐ Borehole ☐ River or stream ☐ Sachet ☐ Pond ☐ Hand dug well

5b. Where do you obtain water for bathing?
☐ Borehole ☐ River or stream ☐ Sachet ☐ Pond ☐ Hand dug well

5c. Where do you obtain water for cooking?
☐ Borehole ☐ River or stream ☐ Sachet ☐ Pond ☐ Hand dug well

5d. Do you wash clothes at the river site?
☐ Yes ☐ No

5e. Do you pass through any river or stream to any destinations within your community?
☐ Yes ☐ No

5f. If yes how often?
☐ Daily ☐ Once a week ☐ More than once a week

Section 6: Agricultural/ Mining activities

6a. Do you pass through swamp in the pursuit of your work?
☐ Yes ☐ No

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6b. Do you pass through river/stream in the pursuit of your work?

☐ Yes ☐ No

6c. Do you wear long upper body clothing/shirt?

☐ Yes ☐ No

6d. Do you wear short upper body clothing/shirt?

☐ Yes ☐ No

6e. Do you wear long trousers/dress?

☐ Yes ☐ No

6f. Do you wear short trousers/dress?

☐ Yes ☐ No

Section 7: Insect bite behaviour

7a. Do you receive insect bites in water/mud/farm?

☐ Yes ☐ No

7b. Do you use mosquito treated nets?

☐ Yes ☐ No

7c. Do you use mosquito coil?

☐ Yes ☐ No

Section 8: Treatment of wound when hurt

8a. Do you wash wounds with water and soap?

☐ Yes ☐ No

8b. Do you rub wound with alcohol?

☐ Yes ☐ No

8c. Do you cover wounds with leaves?

☐ Yes ☐ No

8d. Do you apply adhesive bandage on your wound?

☐ Yes ☐ No

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Appendix D

Multivariate Logistic Regression

Notes

Case Processing Summary

Unweighted Cases[a]		N	Percent
Selected Cases	Included in Analysis	352	100.0
	Missing Cases	0	.0
	Total	352	100.0
Unselected Cases		0	.0
Total		352	100.0

a. If weight is in effect, see classification table for the total number of cases.

Block 0: Beginning Block

Classification Table[a,b]

			Predicted		
			BU INCIDENCE		
	Observed		Yes	No	Percentage Correct
Step 0	BU INCIDENCE	Yes	0	176	.0
		No	0	176	100.0
	Overall Percentage				50.0

a. Constant is included in the model.

b. The cut value is .500

Variables in the Equation

		B	S.E.	Wald	df	Sig.	Exp(B)
Step 0	Constant	.000	.107	.000	1	1.000	1.000

Block 1: Method = Enter

Omnibus Tests of Model Coefficients

		Chi-square	Df	Sig.
Step 1	Step	442.071	16	.000
	Block	442.071	16	.000
	Model	442.071	16	.000

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Model Summary

Step	-2 Log likelihood	Cox & Snell R Square	Nagelkerke R Square
1	45.904[a]	.715	.954

a. Estimation terminated at iteration number 10 because parameter estimates changed by less than .001.

Classification Table[a]

			Predicted		
			BU INCIDENCE		
	Observed		Yes	No	Percentage Correct
Step 1	BU INCIDENCE	Yes	171	5	97.2
		No	2	174	98.9
	Overall Percentage				98.0

a. The cut value is .500

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Variables in the Equation

		B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for EXP(B)	
								Lower	Upper
Step 1[a]	EDUCATION			1.505	3	.681			
	EDUCATION(1)	.605	1.370	.195	1	.659	1.831	.125	26.857
	EDUCATION(2)	-.063	1.526	.002	1	.967	.939	.047	18.707
	EDUCATION(3)	-2.303	2.708	.723	1	.395	.100	.000	20.200
	OCCUPATION			1.608	6	.952			
	OCCUPATION(1)	-.149	1.768	.007	1	.933	.862	.027	27.557
	OCCUPATION(2)	.481	1.700	.080	1	.777	1.618	.058	45.348
	OCCUPATION(3)	1.753	1.949	.809	1	.368	5.773	.127	263.391
	OCCUPATION(4)	-1.413	14.146	.010	1	.920	.243	.000	267526209743.531
	OCCUPATION(5)	.683	2.956	.053	1	.817	1.981	.006	650.347
	OCCUPATION(6)	1.543	2.143	.518	1	.472	4.677	.070	311.646
	WASHCLOTHESATRIVERSITE	.965	1.260	.587	1	.444	2.625	.222	31.021
	FARMINGINSWAMP	4.245	1.432	3.768	1	.001	4.10	3.82	7.18
	WEARLONGUPPERBODYCLOTHES	2.667	1.247	4.574	1	.032	14.390	1.249	165.728
	WEARSHORTUPPERBODYCLOTH	7.458	1.651	20.400	1	.000	1734.125	68.158	44120.924
	WEARLONGTROUSERS	-7.552	1.686	20.060	1	.000	.001	.000	.014
	WEARSHORTTROUSERS	-7.860	1.849	18.081	1	.000	.000	.000	.014
	RECEIVEINSECTBITES	6.896	1.760	15.353	1	.000	988.311	31.391	31115.643
	WASHWOUNDS WATERSOA	5.873	1.632	14.236	1	.001	.3701	.0291	0.9818
	WRAPWOUNDWITLEAVES	4.743	1.324	16.235	1	.000	6.23	4.741	18.110
	APPLYBANDAGEONWOUND	7.678	1.735	20.458	1	.036	.31	.15	.821
	USEMOSQUITOENETS	-.345	1.226	.079	1	.779	.70	.064	7.837
	Constant	-4.448	4.998	.792	1	.374	.012		

a. Variable(s) entered on step 1: EDUCATION, OCCUPATION, WASHCLOTHESATRIVERSITE, WEARLONGUPPERBODYCLOTHES, WEARSHORTUPPERBODYCLOTH, WEARLONGTROUSERS, WEARSHORTTROUSERS, RECEIVEINSECTBITES, USEMOSQUITOENETS.

INTEGRI PROCEDAMUS

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Appendix E

Published Works

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ENVIRONMENTAL MICROBIOLOGY

Seasonal Pattern of Mycobacterium ulcerans, the Causative Agent of Buruli Ulcer, in the Environment in Ghana

Samuel Yaw Aboagye [1,2] & Kobina Assan Ampah [1,3,4] & Amanda Ross [3,4] & Prince Asare [1] & Isaac Darko Otchere [1] & Janet Fyfe [5] & Dorothy Yeboah-Manu [1]

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Abstract This study aimed to contribute to the understanding of Mycobacterium ulcerans (MU) ecology by analysing both clinical and environmental samples collected from ten communities along two major river basins (Offin and Densu) associated with Buruli ulcer (BU) at different seasons. We collected clinical samples from presumptive BU cases and environmental samples from ten communities. Following DNA extraction, clinical samples were confirmed by IS2404 PCR and environmental samples were confirmed by targeting MU-specific genes, IS2404, IS2606 and the ketoreductase (KR) using real-time PCR. Environmental samples were first analysed for IS2404; after which, IS2404-positive samples were multiplexed for the IS2606 and KR gene. Our findings indicate an overall decline in BU incidence along both river basins, although incidence at Densu outweighs that of Offin. Overall, 1600 environmental samples were screened along Densu (434, 27 %) and Offin (1166, 73 %) and MU was detected in 139 (9 %) of the combined samples. The positivity

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✉ Dorothy Yeboah-Manu
DYeboah-Manu@noguchi.ug.edu.gh

[1] Bacteriology Department, Noguchi Memorial Institute for Medical Research, University of Ghana, P.O. Box LG 581, Legon, Accra, Ghana

[2] Institute of Environmental and Sanitation Studies, University of Ghana, Accra, Ghana

[3] Swiss Tropical and Public Health Institute, Basel, Switzerland

[4] University of Basel, Basel, Switzerland

[5] Victorian Infectious Diseases Reference Laboratory, Melbourne, VIC, Australia

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of MU along the Densu River basin was 89/434 (20.5 %), whilst that of the Offin River basin was 50/1166 (4.3 %). The DNA was detected mainly in snails (5/6, 83 %), moss (8/40, 20 %), soil (55/586, 9 %) and vegetation (55/675, 8 %). The proportion of MU positive samples recorded was higher during the months with higher rainfall levels (126/1175, 11 %) than during the dry season months (13/425, 3 %). This study indicates for the first time that there is a seasonal pattern in the presence of MU in the environment, which may be related to recent rainfall or water in the soil.

Keywords Mycobacterium ulcerans · Buruli ulcer · Ghana

Background

Buruli ulcer (BU), caused by Mycobacterium ulcerans (MU), is the third most important mycobacterial disease of public health importance globally after tuberculosis and leprosy [1]. The disease has been reported in 32 countries worldwide mostly in the tropical regions with the greatest burden experienced in West African countries along the Gulf of Guinea [2]. Buruli ulcer, which affects the skin and its underlying soft tissues, begins usually as a painless papule or nodule under the skin at the site of trauma, but in some individuals, more severe diffuse forms occur such as a plaque and/or oedema. Failure to treat these early forms results in gradual erosion of the skin leaving a well-demarcated ulcer with wide undermined edges resulting from the cytopathic action of the plasmid-encoded macrolide toxin, mycolactone [3–5].

The epidemiology of BU in endemic countries is not fully understood. It has a focal distribution of cases where endemic and non-endemic communities are separated by few kilometres [6]. Nevertheless, various studies have linked high BU incidence to slow-flowing or stagnant waters and

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disturbed environment [7, 8]. Rapid changes in landscape [9] such as deforestation, flooding, construction of dams and artificial lakes for irrigation, mining activities and extending swamps for growing rice and fish breeding have been associated with the emergence of the disease in some communities [8, 10–13].

One of the factors limiting the prevention and control of BU is the lack of understanding of the ecology and mode of transmission of the causative agent. Many features of MU ecology, including distribution within the environment, niche adaptation and host range(s), are still not fully known [9, 14–16]. Theories that have been proposed to explain the mechanism of MU transmission include (1) inhalation of aerosolized MU from contaminated water [5], (2) acquisition of MU through an insect or vector bite [5] and (3) contamination of an existing wound or site of trauma by the environment such as soil, vegetation and water among others [17–20]; however, none of these theories have been confirmed.

A major factor that has limited the understanding of MU ecology is the inability to culture viable organisms from the environment [10, 20, 21]. However, the completion of the MU genome sequence provided specific targets for DNA-based detection methods, such as the insertion sequence IS2404, IS2606 and the plasmid encoding mycolactone, ketoreductase-B domain (KR) [22]. Such methods have been used to elucidate tree-dwelling possums and mosquitoes as possible reservoirs [23] and potential vectors [24], respectively, in South-Eastern Australia. In a previous study, certain water bugs were cited as possible vectors in hosting MU in its salivary glands [25]; however, until now, no potential reservoirs have been identified in Africa, which harbours most of BU disease burden.

Seasonal changes are cyclic and represent a major source of external variation influencing human and other natural systems [26–30] and affect diseases such as malaria [31] and diarrhoea [32]. It is not clearly known whether the incidence of BU is seasonal. Understanding the local seasonal drivers of MU in the environment which could influence incidence of BU disease may be of importance in improving the control strategies in Ghana. In this study, we surveyed the presence of MU in different environmental sources using DNA-based assays and looked at seasonality and also possible risk factors within the environment and retrospectively characterized the occurrence of BU cases for each community based on active surveillance data.

Methods

Study Site

The study was conducted in ten communities associated with BU along two major river basins (Densu and Offin) of Ghana (Fig. 1). These river bodies were selected for the study because extensive disease and sero-epidemiological studies have shown high exposure of community member to the M. ulcerans 18 KDa heat shock protein 65 [6, 33, 34], and unlike other communities in Ghana which depend on passive case report, these sites are active in reporting BU cases to the national BU control programme (NBUCP) [35].

Following the Ampah et al. [36] method, ten communities were selected by simple randomization from a total of 199 using a randomization tool embedded within the ArcGIS 10.0 software. Along the Densu River basin, the three randomly selected communities were Ntabea in the Akim East which lies upstream of the river, Ashongkrom in the Akwapim South at midstream both in the Eastern region and Domesampaman in the Ga-West Municipality of the Greater Accra region. In Offin, the seven randomly selected communities for the study were Ntobroso in the Atwima district which lies upstream of the river, Akomfore in the Amansie West district and Achiase, Wromanso and Keniago all in the Atwima district which lie midstream of the river and downstream of the river are Mfantsiman and Pokukrom in the Upper Denkyira district.

Sampling was conducted within the two major climatic seasons in Ghana, the rainy and dry season. The months of May to July are classified as the major rainy season and August to October as the minor rainy season with the remaining months constituting the dry season. The communities along the Densu River basin were predominantly hamlets with smaller coverage area, whilst those at the Offin River basin were large communities with extensive coverage area.

Buruli Ulcer Active Case Search

We conducted active case search to monitor the emergence of BU cases using community outreach programme and monthly household visits by community volunteers. Community outreach education was conducted once every 3 months in all the selected communities. During the outreach programme, we educated community members on the transmission, early case detection and treatment of BU by showing BU documentaries and interacted with community members through questions and answers. The following morning, the inhabitants were screened and those with clinically suspected BU lesions were sampled for laboratory confirmation. We also employed a monthly household visit-based surveillance as an additional tool to the surveillance by the community outreach programme. We trained and equipped one community-based surveillance volunteer (CBSV) from each of the communities with android phones (HTC wildfire S) pre-loaded with a BU surveillance questionnaire. The questionnaire used for the study was designed as previously described [36]. Starting from August 2013 to December 2014, we mandated the CBSVs to visit all households monthly and record any presumptive case using the mobile application and a notebook.

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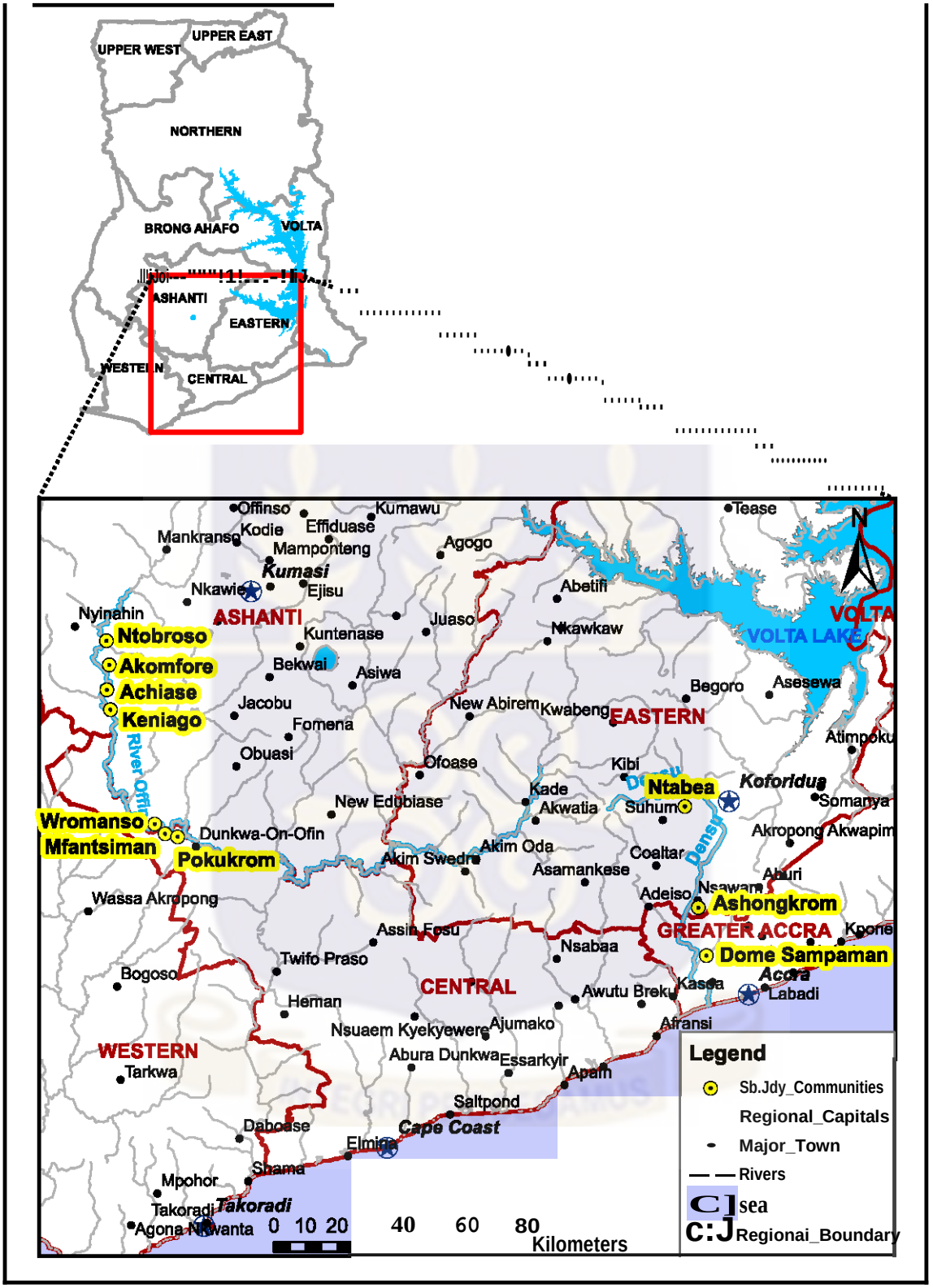

Fig. 1 Map of Ghana showing study communities along1he Densu and Offin River basins

Presumptive cases were then sampled by a local health staff, and the samples were sent to the Noguchi Memorial Institute for Medical Research (NMlMR) for laboratory confmnation.

We estimated the prevalence of BU within each of the communities using passive data obtained from the local health facilities and data from the national active case surveillance.

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Environmental Sample Collection

Two different sampling methods (convenient and random) were employed along the two river basins. Convenience sampling was conducted to collect environmental samples from the three communities (Ntabea, Ashongkrom and Domesampaman) along the Densu River basin in 2011, 2013 and 2014. We purposely used convenient sampling technique to capture specific zones within the environment where there is likelihood of human interactions. During the convenient sampling, we walked through the communities and collected environmental samples from sites of frequent human activities such as water sources including hand dug wells, ponds, streams and boreholes which are regularly utilized, communal bathing areas, school compounds, agricultural farms, market grounds and community centres. Samples were collected from 239 different locations, and at each sample location, we collected soil sample and any other sample within 1 m reach. Any other samples that were more than 1 m away from the location point were excluded from the collection. At each sampling location, a distance of about 10 m was allowed between sampling points or about 5 m where space was limited and GPS coordinates were taken at each collection point. Solid samples such as animal faeces (sheep, lizard and chicken), terrestrial insects (using insect net), snails, soil and water were collected aseptically into 50-ml Falcon tubes and vegetation parts were collected and pressed into 50-ml Falcon tubes and then released into sealable plastic bags from which biofilms were prepared. All samples were clearly labelled immediately, kept in a cool pack at 4 °C after collection and transported to the laboratory and kept frozen until analysis.

Along the Offin River basin, sampling was conducted in 2013 and 2014. Random sampling from a grid of locations was used for the collection of environmental samples from seven communities (Achiase, Akomfore, Keniago, Mfantsiman, Ntobroso Pokukrom and Wromanso). All the communities were mapped and divided into grids, and 487 sampling points were randomly selected using a randomization tool embedded within the ArcGIS 10.0. Samples were collected from each of the 487 points generated and treated in the same way as those from the Densu River basin and transported to the laboratory. We collected rainfall data from the Ghana Meteorological Agency, Accra after monthly rainfall level data from the meteorological substations within the study sites have been reported.

Sample Processing

Snail and faecal samples were diced with sterile disposable surgical blades and homogenized using sterile porcelain and pestle and suspended in phosphate-buffered saline (PBS). Soil samples were shaken vigorously in sterile distilled water and centrifuged at 600 rpm for 5 min to sediment soil particles. Biofilms were prepared from vegetation parts using a modified version of the method described by Gryseels et al. [37]. Samples were emptied into sterile plastic resealable bags, and 50 ml of PBS was added to each bag. The contents of the bags were vigorously agitated to dislodge the biofilms into solution. The suspensions were poured into sterile 50-ml Falcon tubes and centrifuged at 4000 rpm for 30 min, to sediment all suspended bacteria. The supernatant was decanted and the resulting pellet was suspended in 10 ml of PBS for the analysis. Water samples were vortexed to mix homogeneously and centrifuged at 4000 rpm for 30 min to sediment all suspended bacteria. The supernatant was decanted and the resulting pellet was suspended in PBS.

Screening of Samples by Real-Time PCR

Genomic DNA was extracted directly from 1600 environmental samples using the FastDNA SPIN kit for soil with the FastPrep-24™ instrument (MP Biomedicals) according to the manufacturer's instructions. Negative controls were included at each point of DNA extraction. Detection of MU DNA from the environment has been based solely on IS2404 PCR due to the large copy numbers present in the MU genome [38]. However, there are other organisms that also harbour this IS2404 sequence making it non-specific to MU [39]. In this study, three independent gene targets, IS2404, IS2606 and KR, within the MU genome were screened. The extracted DNA was first screened for the insertion sequence IS2404 by real-time PCR using Rotor Gene Q (Qiagen). Primers and TaqMan MGB probes from Applied Biosystems that were selected from regions of the sequences for IS2404, IS2606 and KR present on the plasmid pMUM001 were used [22]. Probes IS2404TP and KRTP were labelled with the fluorescent dye 6-carboxyfluorescein (FAM) at the 5′ end and a nonfluorescent quencher at the 3′ end. Probe IS2606TP was labelled with the fluorescent dye VIC at the 5′ end and a nonfluorescent quencher at the 3′ end [22]. The IS2404 real-time PCR mixtures contained 1 µl of template DNA, 0.9 µM concentrations of each primer, a 0.25 µM concentration of the probe, SensiFast (500 nM) mix (Bioline) and TaqMan exogenous internal positive control (IPC) reagents (Applied Biosystems) in a total volume of 20 µl. IS2606 and KR assays were performed as a multiplex assay (without IPC) for all IS2404-positive DNA with CT value below 35. At each PCR run, two each of negative and positive controls were added. Amplification and detection were performed using the Gene Q sequence detection system (Qiagen) according to the following programme: 1 cycle of 50 °C for 2 min, 1 cycle of 95 °C for 15 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. DNA extracts were tested in at least duplicate, and negative controls were included in each assay. All DNA samples that were positive for IS2404, IS2606 and KR were classified as MU confirmed. We also determined the

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IS2404/IS2606 copy number ratio which differentiates M. ulcerans from the other mycolactone=producing mycobacterium following Fyfe et al. [22].

Laboratory Confirmation of BU Cases

We confirmed presumptive BU lesions by collecting two swab specimens from the undermined edges of ulcerative lesions and one fine needle aspirate (FNA) collected into 500 µl PBS as previously described [40] for pre-ulcerative lesions. Samples were transported to NMIMR at 4 °C and confirmed by a positive IS2404 PCR laboratory test as previously described [41].

Statistical Analysis

The data collected were entered into a Microsoft Excel 2010 spreadsheet and analysed using R statistical software [42]. We used logistic regression and accounted for the cluster sampling by including random effects for location and community. We estimated the proportion positive with 95 % confidence intervals, overall and for different categories of the explanatory variables. In analyses stratified by site or adjusting for calendar month, the numbers were too small to allow the random effects model to converge and so we did not adjust for clustering.

Results

Demographic Characteristics and Identified BU Cases

An overall population of 10,851 inhabitants from communities along Densu (1217, 11.2 %) comprising 45.6 % (n = 555) females and 54.4 % (n = 662) males and Offin (9634, 88.8 %) comprising 49.6 % (n = 4785) females and 50.4 % (n = 4849) males were studied.

Within the study communities, we detected 84 presumptive BU cases; 56 (66.7 %) from Densu and 28 (33.3 %) from Offin River basins, respectively. Thirty-two (38.1 %) were laboratory confirmed by IS2404 PCR, of which 24 (75 %) were from Densu and 8 (25 %) from the Offin River basin. At Densu, 15 (62.5 %) of the confirmed cases were from passive reports. The confirmed cases were detected in Ashongkrom (16, 66.7 %) and Domesampaman (8, 33.3 %) with a prevalence of 14.3 and 3.6 %, respectively, whilst no case was detected in Ntabea. At Offin, seven (87.5 %) of the confirmed cases were actively reported. The eight cases were detected in three communities: Achiase (4, 62.5 %), Ntobroso (3, 37.5 %) and Akomfore (1, 12.5 %). The recorded prevalence was 3.8 % for Achiase, 1.6 % for Ntobroso and 3.0 % for Akomfore (Table 1).

Of the laboratory confirmed cases, 19 (59.4 %) were males and 13 (40.6 %) were females, aged between 3 and 70 years, with a mean age of 26. Lesions presented by cases were in the early stages; 25 (78.2 %) were detected with pre-ulcerative lesion and 7 (21.9 %) presented with category II lesions.

Detection and Identification of M. ulcerans by Real-Time PCR

A total of 1600 environmental samples from ten communities associated with Buruli ulcer along both the Densu (434, 27 %) and the Offin (1166, 73 %) river basins were collected, categorized and screened for MU DNA using three independent molecular markers. We sampled from 239 locations in three communities along the Densu and 487 locations in seven communities in the Offin River basins. The median number of samples per location was 1 for the communities along the Densu River basin and 2 for those along Offin River basin. Overall, 139 (9 %) samples were positive for MU DNA (Table 2).

We found MU DNA to be broadly distributed in all the communities along both river basins.

Two different sampling techniques were used in the study for both the Densu and Offin River basin. M. ulcerans positivity was significantly higher with the convenience sampling method conducted along the Densu River basin (89, 21 %) than the random sampling method at Offin (50, 4 %) river body ($p < 0.001$) (Table 2).

Along the Densu River basin, sample positivity for M. ulcerans DNA was high at Ntabea (21/37, 57 %), followed by Ashongkrom (61/293, 22 %) and the least at Domesampaman (7/104, 7 %) as shown in S1. Among the seven communities along the Offin River basin, Wromanso (16/142, 11 %) had the highest MU positivity and the least MU positivity was recorded for Akomfore (2/139, 1 %), Keniago (2/177, 1 %) and Pokukrom (2/88, 2 %), respectively (S1).

From samples collected along the Densu River basin, we found MU DNA positives among all nine sample types sampled with a positivity ranging from 83 % among snails to 9 % in animal faecal samples (Table 2). However, in the Offin River basin, detections were observed among only moss, soil, vegetation and water (Table 2). We detected MU in at least one sample each of vegetation and soil at every sampling period in both river basins. Among the samples confirmed to contain MU DNA, we found the highest proportion from agricultural farms (45 %), followed by water sources within the communities (36 %) and the least near household (19 %).

Of the 139 samples confirmed to contain MU DNA, the IS2404/IS2606 copy number ratio, which differentiates M. ulcerans from the other mycolactone producing mycobacterium, was found in most of the samples to be around 2.4, the expected ratio for MU (S2). This IS2404/IS2606 copy number

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Table 1 Demographic characteristics and identified BU cases in studied communities

Variables	Community									
	Densu			Offin						
	A	B	C	D	E	F	G	H	I	J
Population (n)	378	512	327	1900	1016	3350	303	1949	900	216
No. of households	52	64	48	295	157	413	85	251	121	41
Sex: females, n (%)	184 (48.6)	228 (44.5)	143 (43.7)	939 (49.4)	499 (49.1)	1719 (51.3)	141 (46.5)	972 (49.9)	409 (45.4)	106 (49.1)
Presumptive BU cases	29	27	0	6	3	3	3	7	4	2
Lab confirmed	16	8	0	4	1	0	0	3	0	0
BU prevalence (%)	14.3	3.6	0	3.1	3	1.2	8.9	1.6	1.3	2.3

Prevalence rate is given as the total prevalence comprising both active and healed lesions

A Ashongkrom, B Domesampaman, C Ntabea, D Achiase, E Akomfore, F Keniago, G Mfanstiman, H Ntobroso, I Pokukrom, J Wromanso

ratios were found in 73/89 (88 %) and 37/50 (74 %) positive samples along the Densu and Offin River basins, respectively.

Rainfall Pattern and MU Positivity

To better understand the seasonal drivers for the distribution of MU in the environment, we compared monthly MU positivity with monthly rainfall levels using data obtained from the meteorological substations within the study communities after approval from the Ghana Meteorological Agency, Accra.

We detected MU DNA in at least one sample in all the sampling periods throughout the study. The proportion of MU positive samples recorded was higher during the months with higher rainfall levels (126/1175, 11 %) than during the dry season months (13/425, 3 %; $p < 0.001$).

Along the Densu River basin in 2011, we recorded high MU positivity for the minor and major rainy seasons, September 23/34 (65 %) and October (13/17, 76 %), respectively, while MU positivity declined during the dry season, December (2/15, 13 %), as indicated (Table 3 and Fig. 2) ($R = 0.94$). In both 2013 and 2014, we observed a drastic decline in MU positivity in both the major and minor rainy seasons as well as the dry season (Table 3) ($p = 0.0002$, 95 % CI = 1.7–6.4).

Along the Offin River basin, our findings suggested that rainfall over a low threshold was associated with increased MU positivity (Table 4 and Fig. 3), but the linear trend observed in the Densu was not apparent. In both 2013 and 2014,

Table 2 M. ulcerans DNA positivity among samples analysed from communities along Densu and Offin River basins

Samples	Densu		Offin		Total MU confirmed	
	No. of samples	MU confirmed (%)	No. of samples	MU confirmed (%)	No. of samples	MU confirmed (%)
Detritus	2	1 (50.0)	21	0 (0.0)	23	1 (4.3)
Faeces	44	4 (9.1)	36	0 (0.0)	80	4 (5.0)
Fungi	8	1 (12.5)	3	0 (0.0)	11	1 (9.1)
Insect	8	1 (12.5)	28	0 (0.0)	36	1 (2.7)
Moss	35	7 (19.4)	4	1 (25.0)	40	8 (20.0)
Water	67	7 (10.4)	76	2 (2.6)	143	9 (6.2)
Soil	143	30 (20.9)	443	24 (5.2)	586	54 (9.0)
Vegetation biofilm	121	33 (27.5)	555	23 (3.9)	675	56 (8.1)
Snails	6	5 (83.3)	–	–	6	5 (83.3)
Total	434	89 (20.5)	1166	50 (4.2)	1600	139 (8.7)

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Table 3 Rainfall levels and monthly MU DNA positivity along the Densu River basin

Period of sampling	Rainfall levels (mm)	Densu		
		No. of samples	MU positive	MU positivity rate (%)
September 2011	268.1	34	22	65
October 2011	130.1	17	13	77
December 2011	22.2	15	2	13
July 2013	176.6	24	13	54
August 2013	20.6	18	2	11
October 2013	146.2	20	6	30
December 2013	26.7	22	2	9
July 2014	103.3	28	6	21
August 2014	108.9	36	8	22
September 2014	102.4	42	6	14
October 2014	45.3	61	2	3
November 2014	95.5	41	5	12
December 2014	26.2	76	2	3
Total		434	89	20.5

MU positivity for the minor and major rainy seasons was also low as observed along the Densu River basin.

Discussion

In this study, we looked for (1) the presence of MU DNA in the environment to contribute to understanding the pathogen ecology, (2) the associations between MU in the environment and other variables including BU endemicity and rainfall patterns and (3) the occurrence of BU cases within some selected communities. We found for the first time that there is a seasonal pattern in the presence of MU DNA in the environment, possibly related to rainfall and also more human BU cases are most likely to be detected after the raining seasons.

A number of infectious diseases including vector, air and waterborne diseases are seasonal [26–29]. At present, there are no clear indications of association between BU and seasonality due to the long incubation period of the disease which is estimated between 2 and 4 months [43]. Nevertheless, few reports from some endemic countries indicate that there may be differences in occurrence of BU between wet and dry seasons in the tropics. In Cameroun and Papua New Guinea, high rates of BU occur during the dry season [44–46], while in both Ghana and Côte d'Ivoire, the peak incidence of the BU disease has also been reported to be at the end of the rainy season [47, 48]. Our finding therefore agrees with these reports as we detected more human BU cases during the monthly active case search surveillance conducted in Ashongkrom which peaked after the rainy months (Fig. 4). We are of the view that MU cells that might have been dormant/or buried in the

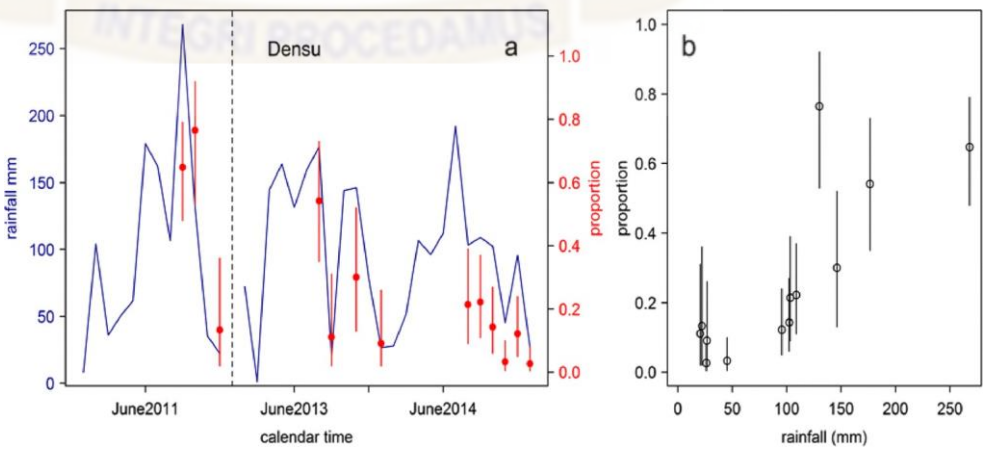

Fig. 2 a, b Rainfall levels and M. ulcerans distribution along the Densu River basin

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Table 4 Rainfall levels and monthly MU DNA positivity along Offin River basin

Period of sampling	Rainfall levels (mm)	Offin		
		No. of samples	MU positive	MU positivity rate (%)
August 2013	114.5	64	8	13
October 2013	149.4	292	21	7
December 2013	24.1	208	1	0.5
July 2014	270.9	292	10	2
September 2014	176.4	78	8	10
October 2014	39.5	98	1	1
December 2014	38.3	63	1	2
Total		1166	50	4.1

environment particularly in the soil are exposed during the rainfall season due to the erosion and other environmental disturbances that occur during the rainfalls. The exposure rates that occur during these months probably account for the high numbers of BU cases at the end of the rainy season (in the BU treatment facility at the Amasaman Hospital, Accra, Ghana) or during the dry months due to increased agricultural activities, and this also supports the suggested incubation time [43–45, 47]. This same mechanism may account for the observation of high MU positivity rate from soil and vegetation biofilm samples that were detected at each sampling period (S2). Even though rainfall seems to influence the distribution of MU in the environment, our findings suggest that rainfall within a certain threshold was associated with MU positivity. This may mean that water in the ground is important probably for suspension but not so much water to wash away the bacteria.

M. ulcerans has been described as an environmental pathogen; this was confirmed in this study by finding MU DNA in all the communities along both river basins. The copy number ratios for the insertion sequences, IS2404/IS2606, for most of the positive samples were about 2.4. According to Fyfe et al., the average ΔC_T (IS2606–IS2404) for M. ulcerans is 2.37; while for the other mycolactone-producing mycobacteria, it is 7.60 [22]. We found 79 % of the analysed samples in the expected range for M. ulcerans, an indication that M. ulcerans was being detected (S2). However, the environmental abundance of MU seems to outweigh the observed disease occurrence. This could be due to the presence of non-human pathogenic strains of MU from the environment probably resulting from mixed populations of bacteria each carrying PCR targets but not necessarily MU itself. The broad distribution of MU in the environmental setting also clearly contrasts the focal distribution of human BU cases. Ntabea for instance has reported no BU case even though community members are exposed to the 18-kDa shsp-specific antibodies of MU [6]. The monthly active case surveillance confirmed this as we detected no human BU case during the study period; however, environmental MU presence was high. This may

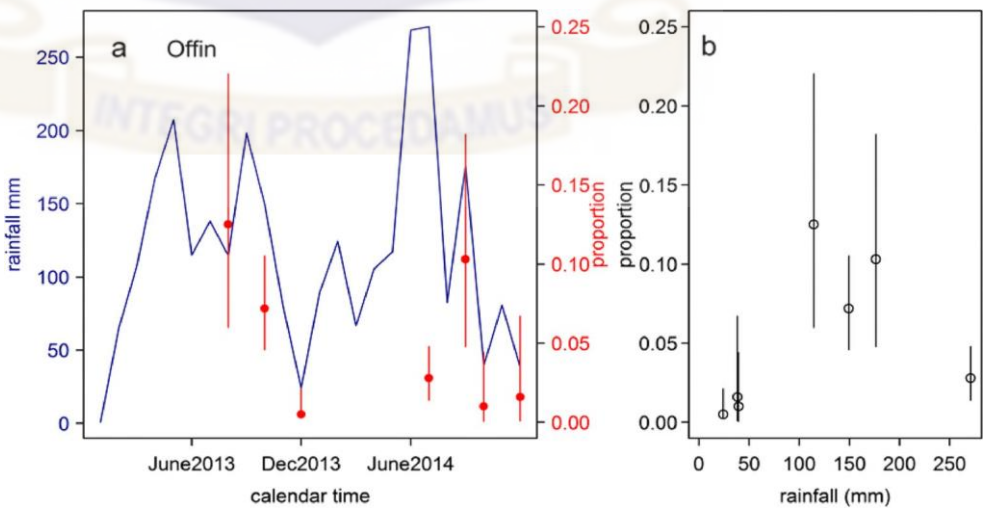

Fig. 3 a, b Rainfall levels and M. ulcerans distribution in along the Offin River basin

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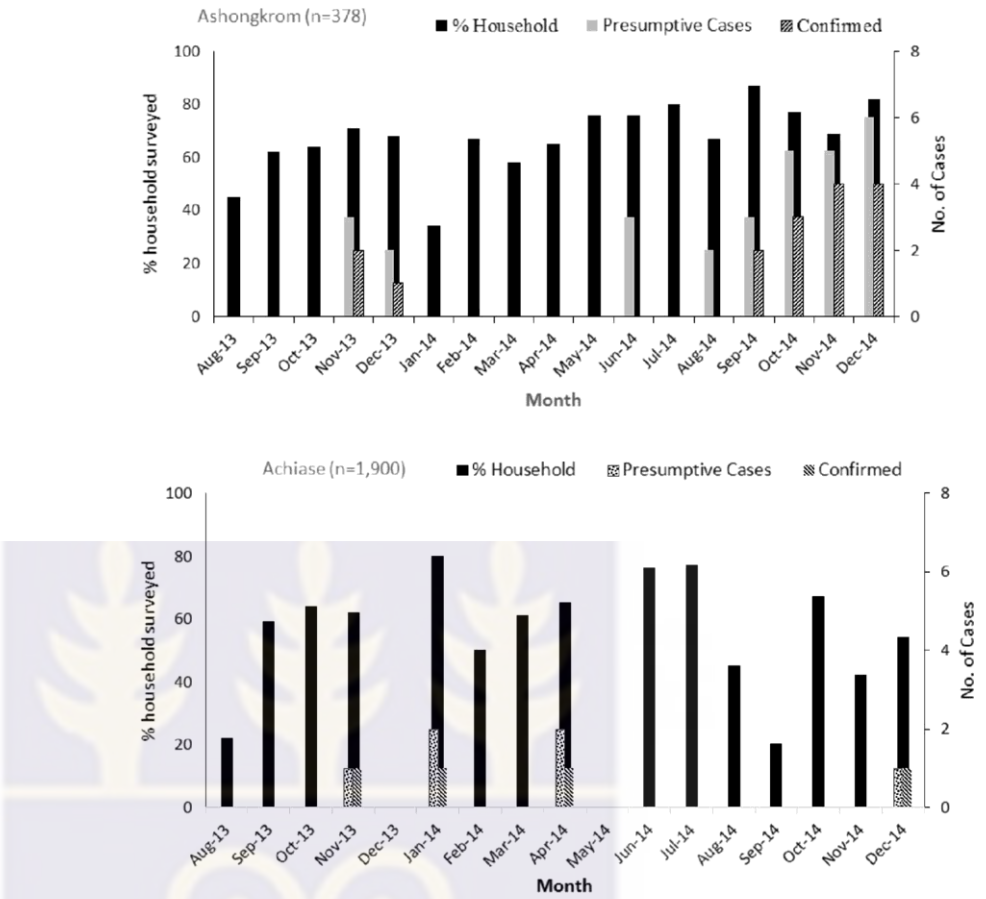

Fig. 4 Monthly BU case surveillance for Ashongkrom and Achiase

imply that community member has high inborn or genetic protection that has a reduced attack rate with BU. Furthermore, our finding suggests other pathogens and host variables may be important for the occurrence of the disease. Moreover, various studies by our group have shown that not all exposed individuals develop overt disease [6, 49]. The pathogen variables that will be interesting to explore will be the genomic difference that could lead to differences in virulence between isolates obtained from the study sites. Currently, methods for the in vitro isolation of MU from the environment have recently been published [50]. This will pave the way for comparative studies between environment and clinical samples.

Studies have postulated that MU may be preferentially adapting to specific ecological niches such as plant biofilms due to its inability to produce light inducible carotenoids that serve as a shield against incident sunlight [49]. In our study, we found high proportions of MU present in snail, moss, vegetation and soil along the Densu River basin whilst MU was more restricted to vegetation and soil at the Offin River basin sites. Aquatic snails have been reported to transiently harbour MU without offering favourable conditions for its growth and survival [51]. In contrast, the edible land African giant snail (Achatina fulica) from which we detected MU DNA can harbour metabolically active bacterial communities in its gut [52] during feeding on plants and soil [53, 54]. Considering the high MU positivity rate among the snail samples, a larger collection of snails from both river basins to further explore the presence of MU in the environment will be essential. Our finding therefore is consistent with earlier studies by Stinear et al. [49].

This study focused on the presence of MU in the environment rather than whether it was the source of human infection. However, case–control studies conducted in BU-burdened communities have identified wearing short and lower-body clothing while farming [55] as risk factors for BU and covering limbs during farming [56] as protective for BU. The proportions of vegetation biofilm and soil samples (S3) with confirmed MU DNA from this study may be relevant particularly for agricultural farmers in the tropics who often engage in activities without protective clothing due to high temperatures.

Also in most BU-burdened communities, children may swim in stagnant waters that collect during rainfall that might contain MU which may expose susceptible hosts to MU. The findings from our study confirm the presence of MU in the environment as the sero-epidemiological studies conducted along the river basins indicated that sera of individuals above age 5 contained significant amounts of 18 kDa shsp-specific

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antibodies of MU. This indicates exposure to MU from the environment as children below 5 years showed no immune response [6, 33, 34].

This study is limited by the use of two different sampling techniques for the two study sites which may have influenced the high positivity observed along the Densu site. However, we observed a similarity in higher positivity in both vegetation and soil samples irrespective of sampling technique, which again underpins the MU adaptation preferences for these particular sample types. The study is limited also by sampling during only certain months of the year. It would be desirable for sampling to cover a whole 12 months.

In conclusion, the study provides information on the presence of MU in the environment and for the first time indicates the influence of rainfall on its presence in the environment. In addition, we found clinical BU cases peaking after the raining seasons. We therefore recommend that further work on specific environmental sources may lead to human infection and potential protective measures are needed.

BU Buruli ulcer, MU Mycobacterium ulcerans, PCR polymerase chain reaction, IPC internal positive control, PBS phosphate-buffered saline, NBUCP National BU Control Programme, CBSV community-based surveillance volunteer

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Authors' Contributions SYA, DYM, JF, AR, PA, IDO, and KAA analyzed the data. DYM, SYA, JF and KAA conceived and designed the experiment. SYA, JF, and PA performed the experiments. SYA, DYM, JF, AR, PA, IDO and KAA analyzed the data. SYA, DYM, JF, and AR wrote the manuscript.

Compliance with Ethical Standards

Ethics Statement Ethical clearance for the study was obtained from the institutional review board of the Noguchi Memorial Institute for Medical Research (NMIMR) (Federal-wide Assurance number FWA00001824). Written informed consent was obtained from all individuals that participated in the study. Parents or guardians provided written consent on behalf of all participants below 18 years of age.

Consent for Publication The manuscript does not contain any individual person's data in any form. The image contained in this manuscript was generated by the authors.

Availability of Data and Material The dataset analysed during this study are included in this manuscript as supplementary information files except one dataset which will be made available from the corresponding author on request.

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Competing Interests The authors declare that they have no competing interests.

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Environmental and Behavioral Drivers of Buruli Ulcer Disease in Selected Communities along the Densu River Basin of Ghana: A Case-Control Study

Samuel Yaw Aboagye,[1,2] Prince Asare,[1] Isaac Darko Otchere,[1] Eric Koka,[1] George Ekow Mensah,[1] Dzidzo Yirenya-Tawiah,[2] and Dorothy Yeboah-Manu[1]*

[1]Noguchi Memorial Institute for Medical Research, University of Ghana, Accra, Ghana; [2]Institute of Environmental and Sanitation Studies, University of Ghana, Accra, Ghana

Abstract. The exact route of transmission of Mycobacterium ulcerans (MU) (causative agent of Buruli ulcer [BU]), risk factors, and reservoir hosts are not clearly known, although it has been identified as an environmental pathogen. This study assessed potential environmental and behavioral risk factors that influence BU infections. We conducted a case-control study where cases were matched by their demographic characteristics and place of residence. A structured questionnaire was administered to solicit information on the environmental and behavioral factors of participants that may expose them to infection. A total of 176 cases and 176 controls were enrolled into the study. Multivariate conditional logistic regression analysis identified farming in swampy areas (odds ratio [OR] = 4.10, 95% confidence interval [CI] = 3.82–7.18), farming while wearing short clothing (OR = 1,734.1, 95% CI = 68.1–44,120.9), insect bite (OR = 988.3, 95% CI = 31.4–31,115.6), and application of leaves on wounds (OR = 6.23, 95% CI = 4.74–18.11) as potential risk factors. Farming in long clothing (OR = 0.000, 95% CI = 0.00–0.14), washing wound with water and soap (OR = 0.37, 95% CI = 0.29–0.98), and application of adhesive bandage on wounds (OR = 0.31, 95% CI = 0.15–0.82) were found to be protective against BU infection. In the absence of the exact MU transmission mechanisms, education of public in BU-endemic zones on the use of protective clothing during farming activities to limit exposure of the skin and proper wound care management would be essential in the fight against BU.

INTRODUCTION

Buruli ulcer (BU) is a debilitating and necrotizing disease of the skin and soft tissues caused by Mycobacterium ulcerans (MU).[1–3] It is the third most important mycobacterial disease globally after tuberculosis and leprosy.[4] Globally, the disease has been reported in 33 countries, but the greatest disease burden is found mostly in the tropical regions of west and central Africa.[5,6] In west Africa, BU is second after tuberculosis and is the leading mycobacterial disease that affects immunocompetent individuals in highly endemic communities of this region.[1] The BU disease affects all age groups and sex, but about 50% of cases are found predominantly in children < 15 years of age.[7–9]

The first BU case was reported in Ghana in 1971 from a patient in Amasaman, a community along the Densu river basin.[10] Since then, communities along the river and its tributaries have been extensively surveyed for likelihood of other unidentified BU cases. In other parts of Ghana, such as the Afram valley at Agogo in the Asante Akim North District[11] and the Amansie West District in Ashanti region,[12] both along the Offin river basin, a number of BU cases were also reported. The BU disease which was previously perceived to persist only in swampy and tropical rain forest zones in Ghana, however, were found to be in all the 10 administrative regions of Ghana with an overall prevalence of 20.7 per 100,000 of the population,[13] after a national survey was conducted in 1999.

Though the epidemiology of BU is not fully understood,[9] studies conducted so far in most endemic regions have linked the occurrence of BU to disturbed environment[8,14,15] due to human activities including construction of dams, min-

* Address correspondence to Dorothy Yeboah-Manu, Bacteriology Department, Noguchi Memorial Institute for Medical Research, University of Ghana, P.O. Box LG 581, Legon, Accra, Ghana. E-mail: dyeboah-manu@noguchi.ug.edu.gh

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ing activities, construction of artificial lakes, and extending swamps for growing rice and fish breeding.[8,14–16]

Since the transmission of BU has remained elusive in regions burdened with BU, identifying potential behavioral and environmental risk factors may contribute to the reduction in the number of BU cases by putting in place required preventive measures. The potential risk factors and protective factors for BU seem to be geographically specific.[17–23] In Cote d'Ivoire for instance, irrigated farming and proximity to remnant rainforest patches have also been associated with higher risk of BU.[24] In Daloa region of Cote d'Ivoire, Marston and others found participation in farming activities near river bodies as a risk factor for BU infection.[25] Pouillot and others also identified swamp wading, wearing short, lower-body clothing while farming, living near a cocoa plantation or woods as potential risk factors for BU in Benin.[21] However, in southeastern Australia, frequent use of insect repellent, wearing of long trousers outdoors, and immediate washing of wounds were protective against BU.[26] Based on findings from various studies, BU risk factors tend to exhibit regional variations and this could probably be due to differences in geography, environment, and host behavior.

Water, sanitation, and hygiene (WASH) practices form a crucial component in the prevention and control of neglected tropical diseases (NTDs).[27] The link between some NTDs, including dracunculiasis, trachoma, soil-transmitted helminths, cysticercosis, and schistosomiasis, and WASH interventions has been demonstrated.[28] However, information on BU and WASH is limited even though BU incidence has been linked to aquatic systems.[14–16] Johnson and others in a recent study evaluated the level of WASH and other associated factors in a BU-endemic district in Benin[29] and found very low WASH indicators, unimproved water sources for domestic use, and poor sanitation.

In Ghana, studies conducted so far on hygiene practices and other potential risk factors for BU are very limited with variable outcomes.[18,30] Raghunathan and others found

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wading in river as a risk and bathing with clean water and soap to be protective for BU.[18] In addition, Kenu and others found the presence of wetland, insect bites in river sites, use of adhesive when injured, and washing in the river as risk factors for BU, and covering of limbs during farming as well as use of alcohol after insect bites as protective factors against BU.[22] The few case-control studies that have explored BU risk factors were limited by use of only clinical criteria without laboratory confirmation of cases. At the same time, false diagnosis based on clinical criteria alone is known. This study therefore sought to assess the potential environment and behavioral risk factors through a case-control study in selected communities along the Densu river basin of Ghana using only laboratory-confirmed cases.

MATERIALS AND METHODS

Ethics statement. Ethical clearance for the study was obtained from the institutional review board of the Noguchi Memorial Institute for Medical Research (NMIMR) (Federal-wide Assurance number FWA00001824). Written informed consent was obtained from all individuals that participated in the study. Parents or guardians provided written consent on behalf of all child participants (below 18 years of age).

Study area. The study was conducted in three BU-endemic districts, which are East Akim, Akwapim South and Ga West municipality. A common feature of the selected districts is that the Densu river, which runs through all three districts (Figure 1). The Densu river is 116 km long and is the main source of drinking water supply for the inhabitants. The studied communities are Ntabea in the East Akim District, upstream of the river; Ashongkrom in the Akwapim South District, midstream of the river; and Domesampaman in the Ga West municipality of the Greater Accra Region, downstream of the river. Agriculture is the predominant occupation of the people in the three districts absorbing about 70% of the total population within the districts. These river bodies were selected for the study because extensive disease and seroepidemiological studies have shown high exposure of community members to the MU 18-Kda heat-shock protein 65,[31–33] and unlike other communities in Ghana which depend on passive case report, these sites are active in reporting BU cases to the national BU control program.[13] Cases and controls were selected from the same communities.

Case and control definition. Case. A BU case is defined as any patient presenting with active or inactive BU, diagnosed according to the World Health Organization (WHO) clinical definition,[31] and confirmed by IS2404 polymerase chain reaction (PCR) analysis at NMIMR.

Control. A control is defined as an individual who has had no sign or symptoms of active or inactive BU. Controls for children and adult case patients were randomly selected within the communities and matched to cases to the nearest age (±5 years), sex, and residency. Enrollment in the study was voluntary.

Data collection. Cases for the study were recruited retrospectively from Noguchi Memorial Institute for Medical Research BU case database. Cases confirmed for BU from January 2013–December 2015 were randomly selected for the study using randomization software (ArcGIS version 10, Esri [Environmental Systems Research Institute], Redlands, CA). Trained resource persons visited homes of the selected cases to seek consent for study participation. Having explained the purpose of the study to the subject and after obtaining consent, a matched control was randomly selected from either the same household as the case subject or neighborhood. After obtaining informed written consent from both cases and controls, we administered standardized questionnaires to participants concerning demography (age, sex, education, and marital status), and environmental (agricultural, fishing, and mining activities) and behavioral practices. We also investigated outdoor behavior and habits including clothing worn, precautions taken against insect bites, how skin traumas were treated, natural fauna with regular contact within households, occupational activities, and activities associated with water bodies. Cases were asked to restrict their responses to the year before the onset of the BU disease or during the past year for control participants. Resource persons fluent in the native language of participants administered the questionnaires with assistance of community health volunteers. All study questionnaires were filled in at the time of the survey and double entries were made during data entry for quality.

Data analyses. The responses from the questionnaires were entered in Microsoft Excel 2010 spreadsheet and analyzed using R statistical software (R Development Core Team, 2012, Vienna, Austria). The demography of case and control participants was compared by using two-sample t test. Variables that attained significance at a P value < 0.05 were retained for multivariate analyses using multiple conditional logistic regressions. We used a step down backward elimination process to identify the factors that are significantly associated with BU and also to control possible confounding factors in the model. P values ≤ 0.05 were considered statistically significant.

RESULTS

Characteristics of participants. Overall, equal numbers of cases (176) and controls (176) were matched by age, village, and neighborhood. The demographic characteristics of all BU cases and controls are presented in Table 1. Among the 176 case participants, 86 (49%) were males and 90 (51%) were males. The age of the case participants was in the range 9–51 years, median 28 years, and mean 27 years. The case participants ≤ 15 years of age were 38 (21.6%) and > 15 years were 138 (78.4%) with majority of cases (68; [38%]) belonging to the 25–35 age group. Among the 176 control participants, the age of the controls ranged between 8 and 54 years with a median age of 28 years. Of the 176, 86 (47%) were males and 90 (51%) were females with a mean age of 28 years.

All the cases that participated in the study had been confirmed for BU by PCR following the WHO guidelines and more than half of the cases (115; 65%) presented an ulcerative BU lesion. BU lesions frequently occurred on the lower limbs (77; 44%) followed by the upper limbs (38%). Lesions that occurred on the abdomen were less (8; 4%) compared with the head and trunk (24; 14%). The lesion size presented by case patients were 27 (15%) category I, 55 (31%) category II, and 94 (53%) category III (Table 2).

Univariate analysis of factors associated with the risk of contracting BU. Socioeconomic factors associated with

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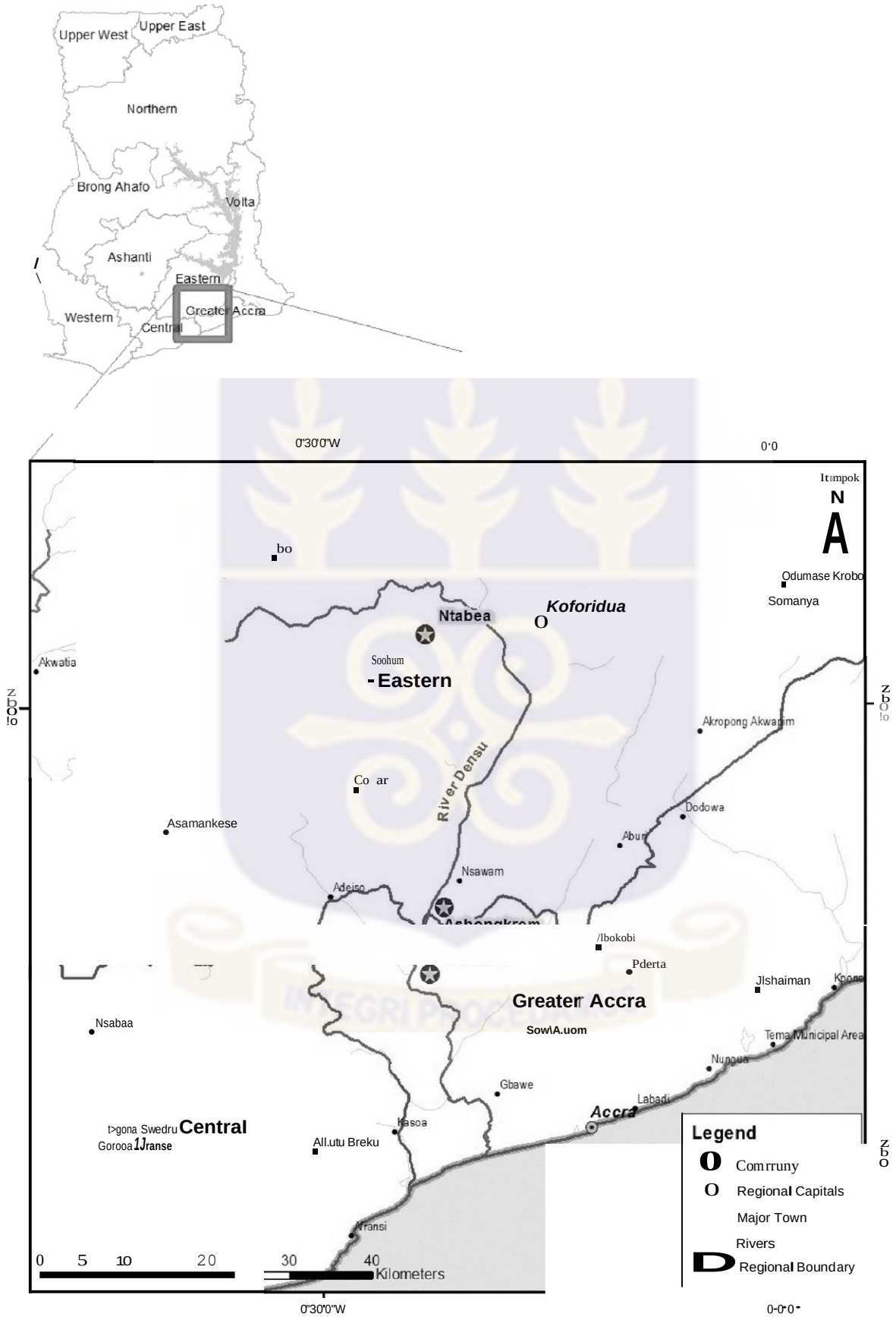

FrGURE 1. Map of Ghana, showing the Densu nver basn and selected communrties. Map created wrth ArcGIS 10.0 using GPS coordinates from the Natrona! Buruli Ulcer Control Program. This figure appears in color at www.ajtmh.org.

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TABLE 1
Demographic characteristics of cases and controls

Characteristics	No. of cases n (%) 176 (100)	No. of controls n (%) 176 (100)
Sex		
Male	86 (49)	86 (49)
Female	90 (51)	90 (51)
Age in years (median, range)	28 (9–51)	28 (8–54)
≤ 15	38 (22)	34 (19)
16–24	33 (19)	34 (19)
25–35	68 (38)	77 (44)
≥36	37 (21)	31 (18)
Marital status		
Single	99 (56)	100 (57)
Married	76 (43)	74 (42)
Divorced	1 (0.5)	2 (1)

contracting BU. Socioeconomic status was assessed by both educational level and occupation type, and these variables were found to be lower in cases than controls. There was a significant association between educational level and risk of BU (odds ratio [OR] = 2.67, 95% confidence interval [CI] = 1.46–5.03). Among the study population, individuals with tertiary level of education (higher education) were protected from developing BU as compared with those without education. Occupation type was also found to influence the chances of contracting BU. Although agricultural farmers were at risk for contracting BU (OR = 2.9, 95% CI = 1.37–3.53), civil servants were protected from BU (OR = 0.24, 95% CI = 0.09–0.56) (Table 3).

Environmental and behavioral factors for contracting BU. Cases reported staying in mud walls, having agricultural plantation, and having bushes and woods in their immediate environment more than the controls. The study found that staying in mud walls and mud floors as well as closeness of households to river bodies were negatively associated with BU. Moreover, there was no evidence for association between risk of BU and the presence of agricultural farms, bushes, swamps, and woods in the immediate environment. Also, sources of drinking, bathing, and cooking water were similar for both cases and controls and no evidence was observed for an association between the risk of BU and the water sources.

Washing of clothes around river bodies (OR = 2.12, 95% CI = 1.12–4.11) and passing through the rivers to individual destinations (OR = 2.16, 95% CI = 1.29–3.64) were reported more frequently in cases than the controls and these activities were positively associated with BU (Table 3).

Farming or mining activities in swamps (OR = 3.30, 95% CI = 2.08–5.29) was significantly associated with BU; however, farming around water bodies showed no such association. Although wearing of short upper body clothing (OR = 39.43, 95% CI = 19.91–83.58) and short dress (OR = 14.1, 95% CI = 0.08–25.5) during farming or mining activities were found to be positively associated with BU, the wearing of long upper body cloth (OR = 0.31, 95% CI = 0.14–0.63), and trousers (OR = 0.28, 95% CI = 0.17–0.45) during such activities were found to be protective for BU (Table 3).

Wrapping wounds with leaves were reported more frequently in case patients than controls and it was positively associated with BU (OR = 3.91, 95% CI = 2.32–6.74). Washing of wounds with water and soap (OR = 0.52, 95% CI = 0.31–0.85) and applying adhesive bandage (OR = 0.42, 95% CI = 0.26–0.66) on wounds were found to be protective for BU.

Insect bites. The risk of contracting BU was significantly associated with insect bites (OR = 227.58, 95% CI = 58.02–1,999.04) and use of insect net at night appeared to be protective for BU (OR = 0.25 95% CI = 0.14–0.42) (Table 3).

Multivariate analysis of factors associated with the risk of contracting BU. The multivariate analyses (conditional logistic regressions) found none of the socioeconomic factors as a potential risk for contracting BU. However, some environmental and behavioral factors were retained. Farming activities in swamps (OR = 4.10, 95% CI = 3.82–7.18), wearing of short upper body clothes (OR = 1,734.1, 95% CI = 68.1–44,120.9), wearing of short lower body clothes (OR = 14.4, 95% CI = 1.25–165.7), insect bite (OR = 988.3, 95% CI = 31.4–31,115.6), and application of leaves on wounds (OR = 6.23, 95% CI = 4.74–18.11) were found to be risk factors for contracting BU. Moreover, farming in long upper body cloth (OR = 0.000, 95% CI = 0.00–0.14), farming in trousers (OR = 0.58, 95% CI = 0.21–0.97), washing wounds with water and soap (OR = 0.37, 95% CI = 0.29–0.98), and application of adhesive bandage on wounds (OR = 0.31, 95% CI = 0.15–0.82) were found to be protective against BU disease (Table 4).

TABLE 2
Clinical characteristics of BU cases

Characteristics	No. of cases n (%) 176 (100)
Lesion type	
Ulcerative	115 (65)
Nonulcerative	61 (35)
Location of lesion	
Lower limbs	77 (44)
Upper limbs	67 (38)
Head and trunk	24 (14)
Abdomen	8 (4)
Category of lesion	
Category I (< 5 cm)	27 (15)
Category II (5–15 cm)	55 (31)
Category III (> 15 cm)	94 (53)

DISCUSSION

The exact route of MU transmission is unclear, although it is commonly presumed that infection takes place through physical contact with environmental reservoirs. This study aimed to identify potential environmental and behavioral risk factors for BU disease in some selected BU-endemic communities along the Densu river basin. We found that 1) farming in swampy areas, farming in short upper and lower body clothes, and wrapping of wounds with leaves increased the risk of contracting the BU disease and 2) farming in long upper body clothes, wearing trousers, washing wounds with water and soap, and application of adhesive bandages to wounds decreased the risk of contracting the BU disease.

The major findings from our study are in agreement with several other epidemiological studies that have been conducted in BU-burdened regions to identify risk factors associated with BU disease.[20,21,23,26,34–36] Subsistence

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TABLE 3

Univariate analysis of selected variables for Buruli ulcer disease in communities along the Densu river basin, Community-matched case-control study

Parameters	No. of cases (N, %)	No. of controls (N, %)	Univariate OR (95% CI)	P value
Socioeconomic status				
Primary	90 (51)	86 (49)	1.09 (0.71–1.70)	0.7492
Secondary	34 (19)	48 (27)	073 (0.43–1.25)	0.2525
Tertiary	7 (4)	25 (14)	0.25 (0.09–0.62)	< 0.0014
No education	45 (26)	20 (11)	2.67 (1.46–5.03)	< 0.0009
Occupation				
Artisan	3 (2)	8 (5)	1.09	0.7492
Student	44 (25)	54 (31)	0.75	0.2845
Farmer	77 (44)	46 (26)	2.19 (1.37–3.53)	< 0.001
Trader	14 (8)	18 (10)	0.76	0.5787
Fisherman/woman	5 (3)	3 (2)	1.68	0.7234
Miner	25 (14)	18 (10)	1.45	0.3288
Civil servant	8 (5)	29 (16)	0.24 (0.09–0.56)	< 0.001
Household environment				
Mud wall: yes/no	77 (44)	68 (39)		0.448
Mud floor: yes/no	38 (22)	39 (22)		0.699
Within < 1 km vs. > 1 km to river basin	46 (26)	42 (24)		0.924
Agricultural plantation in the immediate environment: yes/no	66 (38)	65 (37)		0.912
Bush in the immediate environment: yes/no	158 (90)	152 (86)		0.324
Swamp in the immediate environment: yes/no	88 (50)	95 (54)		0.595
Woods in the immediate environment: yes/no	79 (45)	69 (39)		0.280
Source of drinking water				
Borehole	140 (80)	133 (76)		0.369
River/stream	14 (8)	10 (6)		0.526
Sachet	21 (11)	32 (18)		0.097
Hand dug well	1 (0.6)	1 (0.6)		1.000
Source of water for bathing				
Borehole	154 (88)	154 (88)		0.893
River/stream	19 (10)	20 (11)		1.000
Hand dug well	3 (2)	2 (1)		1.000
Source of water for cooking				
Borehole	153 (87)	154 (88)		0.986
River/stream	21 (12)	20 (11)		1.000
Hand dug well	2 (1)	2 (1)		1.000
Domestic activities				
Washing clothes around site: yes/no	36 (20)	19 (10)	2.12 (1.12–4.11)	0.013
Passing through river to destination: yes/no	60 (34)	34 (19)	2.16 (1.29–3.64)	0.004
Agricultural\mining activities				
Farming/mining in swamps: yes/no	126 (71)	76 (43)	3.30 (2.08–5.29)	< 0.001
Farming/ mining around river/stream: yes/no	46 (26)	35 (20)		0.164
Wearing long upper body cloth to farm/mine: yes/no	13 (7)	36 (20)	0.31 (0.14–0.63)	< 0.001
Wearing short upper body cloth to farm/mine: yes/no	163 (93)	42 (24)	39.43 (19.91–83.58)	< 0.001
Wearing trouser to farm/mine: yes/no	80 (45)	132 (75)	0.28 (0.17–0.45)	< 0.001
Wearing short dress to farm/mine: yes/no	80 (45)	150 (85)	14.1 (0.08–25.5)	< 0.001
Insect bite				
Receive insect bites in river/home: yes/no	174 (99)	48 (27)	227.58 (58.02–1,999.04)	< 0.001
Use of mosquito net: yes/no	28 (16)	132 (75)	0.25 (0.14–0.42)	< 0.001
Use of mosquito coil: yes/no	82 (46)	94 (53)		0.286
Wound management				
Washing wounds with water/soap: yes/no	37 (21)	60 (34)	0.52 (0.31–0.85)	0.012
Robbing wounds with alcohol: yes/no	23 (13)	38 (22)	0.55 (0.29–0.99)	0.067
Wrapping wounds with leaves: yes/no	75 (43)	28 (16)	3.91 (2.32–6.74)	< 0.001
Applying adhesive bandage on wounds: yes/no	56 (32)	93 (53)	0.42 (0.26–0.66)	< 0.001

agriculture (44%) is the major socioeconomic activity of the BU cases in the study areas. Moreover, most cases reported farming more frequently in swampy areas than controls. Our findings using both univariate and conditional logistic regression analyses show that farming in swampy areas is a major risk factor for BU. Agricultural activities, such as farming particularly in swampy areas, puts cases in constant contact with moist/watery soil. Tian and others recently showed that MU strains can survive in soil for 4 months suggesting that BU could be acquired through contact of open wounds with watery soil as a transient source of infection.[37] Moreover, Aboagye and others recently isolated MU from soil in a BU-endemic community along the Densu river basin of Ghana.[38] Bratschi and others also indicated that MU may persist for many months in decaying organic matter under water.[39] These findings clearly confirm that farming in swampy areas is a potential risk factor for BU. Furthermore, we found wearing of long clothing (upper and lower body) during farming activities to be protective against BU, whereas wearing of short clothing (upper and lower body) was a risk factor for contracting BU. This finding is consistent with many other studies conducted in both Ghana and Cote d'Ivoire.[18,22,25] The hot weather conditions that are frequently experienced in most African countries encourage farmers to wear less protective clothes during farming to work efficiently. This behavior

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TABLE 4

Multivariate analysis of selected variables for Buruli ulcer disease in communities along the Densu river basin, Community-matched case-control study

Parameters	Multivariate OR (95% CI)	P value
Farming in swamps	4.10 (3.82–7.18)	0.001
Farming in long upper body cloth	0.000 (0.00–0.14)	0.032
Farming in short upper body cloth	1,734.1 (68.1–44,120.9)	< 0.001
Farming in trousers	0.001 (0.000–0.14)	< 0.001
Farming in short lower body cloth	14.4 (1.25–165.7)	< 0.000
Insect bites in rivers or home	988.3 (31.4–31,115.6)	0.001
Washing wounds with water and soap	0.37 (0.29–0.98)	0.001
Wrapping wounds with leaves	6.23 (4.74–18.11)	< 0.001
Applying adhesive bandage on wounds	0.31 (0.15–0.82)	0.036

results in long periods of skin exposure which is likely to facilitate infection, which is evident by different studies that majority of BU lesions are located on lower limbs than on abdomen.[13,35,39–41] In addition, exposed BU lesions could also serve as a source of infection as has been hypothesized that humans with large active ulcerative BU lesions could shed off the bacteria into the environment in the course of their daily activities.[39]

Likewise, we also found washing of wounds with water and soap and application of adhesive bandages to offer some protection against BU, which is consistent with findings by previous studies.[18,23] These protective mechanisms could be due to the washing off of the contaminating pathogens with water and soap of the surface of the skin, whereas the bandages serve as cover for the open wounds thereby limiting entry of pathogens.

One of the proposed theories is that MU may be transmitted through an insect bite.[42] Studies conducted in both Ghana and Benin by Portaels and others detected MU DNA in water bugs belonging to the families Naucoridae and Belostomatidae.[42] Moreover, Marsollier and others isolated MU from wild aquatic insects collected from a zone in the Daloa region of Ivory Coast and also showed that MU may be transmitted to laboratory mice by the bite of aquatic bugs (Naucoridae) that are infected with this organism.[43] In southeastern Australia, Quek and others, in a case-control study, reported that more cases than control individuals recalled that they were bitten by mosquitoes on the lower extremities.[26] The authors provided evidence that implicated mosquitoes in the transmission of MU in southeastern Australia.[26] Johnson and others also screened mosquitoes in a small town during a BU outbreak in southeastern Australia and confirmed the presence of MU in a subset of pools by detection of three PCR targets (IS2404, IS2606, and Ketoreductase)[44] In the present study, we found insect bite to be a potential risk for BU as confirmed by both the univariate and conditional logistic regression analyses which supports the proposed MU transmission hypothesis, although no study has confirmed PCR targets from mosquitoes in Ghana as has been shown in southeastern Australia.

In contrast to findings by Pouillot and others,[21] we found the application of leaves on open wounds to be a potential risk factor for contracting BU. Stinear and others identified aquatic plants as a possible reservoir of this pathogen.[45] More recently, Aboagye and others also detected MU DNA from vegetation biofilm from BU-endemic community along the Densu river basin of Ghana.[38] Aquatic plants, such as algae for instance, are able to secrete many organic compounds, such as amino acids and polysaccharides, which are in turn used by bacteria as substrates for growth.[46–49] Genotype analysis by Marsollier and others showed that plant-associated MU had the same profile as isolates recovered in the same region from aquatic insects and clinical specimens, an observation that seems to implicate aquatic plants as possible reservoir of MU.[50] Direct application of herbal preparations containing MU to wounds could then be a source of infection, increasing the risk of suffering from BU.

The preventive behaviors outlined in this study, particularly washing with soap and clean water are already a major component of the WASH program.[51] This has been integrated into the school systems in most deprived rural communities of Ghana through a partnership between Ghana Health Service, Ghana Education service, and UNICEF toward meeting the millennium development goals, goal 7C.[51] The partnership has made it possible for provision of boreholes and other sanitary facilities to improve hygiene conditions within the schools and the communities. Moreover, some nongovernmental organizations also provide sanitation supplies to a number of selected rural deprived schools in an effort to enhance hygiene practices. The introduction of the WASH concept facilitated in the eradication of guinea worm in northern Ghana as well as other endemic areas of Africa.[52–54] It is therefore feasible for this approach to be used toward the fight against BU disease in endemic areas. Furthermore, even though Ghana sits on the equator with almost year-round warm weather conditions, it is not far-fetched to recommend the wearing of long clothing to prevent direct contact of possible contaminated surfaces.

As part of our extension services aimed at increasing access to health care for BU patients and support for the health-care system (clinics) in the management of BU, we developed a number of interventions including community outreach to enhance early case detection and provision of transportation for BU patients,[55] which helped to improve BU management in the Obom treatment center. However, since these projects are temporal, we strongly recommend local governments and the health systems to take up these intervention measures and make provision for boreholes in rural communities that lack clean and safe water. We are of the view that sustenance of these interventions would contribute immensely to the fight against the BU disease.

This study, like other case-control studies is not exempted from limitations. Cases most often live with the BU disease for long periods before seeking medical assistance; therefore, there is the potential for recall bias that may

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influence case responses thereby impacting negatively on the study.

In conclusion, our study identified potential risk factors for BU along the Densu river basin. We found that farming in short clothes (upper and lower body) in swampy areas and wrapping of wounds with leaves were major risk factors of contracting the BU, whereas wearing of long clothes (upper and lower body) during farming, washing of wounds with water and soap, and application of adhesive bandages to wounds protective for BU. In the absence of the exact MU transmission mechanisms, education of public on the use of protective clothing during farming activities and proper wound care management are essential for the fight against BU.

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Authors' addresses: Samuel Yaw Aboagye, Prince Asare, Isaac Darko Otchere, Eric Koka, George Ekow Mensah, and Dorothy Yeboah-Manu, Department of Bacteriology, Noguchi Memorial Institute for Medical Research, University of Ghana, Accra, Ghana, E-mails: saboagye@noguchi.ug.edu.gh, pasare@noguchi.ug.edu.gh, idotchere@gmail.com, ekoka@noguchi.ug.edu.gh, gemensah@noguchi.ug.edu.gh, and dyeboah-manu@noguchi.ug.edu.gh. Dzidzo Yirenya-Tawiah, Institute of Environment and Sanitation Studies, University of Ghana, Accra, Ghana, E-mail: dzidzoy@staff.ug.edu.gh.

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Isolation of Nontuberculous Mycobacteria from the Environment of Ghanian Communities Where Buruli Ulcer Is Endemic

Samuel Yaw Aboagye,[a,c] Emelia Danso,[a] Kobina Assan Ampah,[a,b] Zuliehatu Nakobu,[a] Prince Asare,[a] Isaac Darko Otchere,[a] Katharina Röltgen,[b] Dzidzo Yirenya-Tawiah,[c] Dorothy Yeboah-Manu[a]

Noguchi Memorial Institute for Medical Research, University of Ghana, Accra, Ghana[a]; Swiss Tropical and Public Health Institute, Basel, Switzerland[b]; Institute of Environmental and Sanitation Studies, University of Ghana, Accra, Ghana[c]

ABSTRACT

This study aimed to isolate nontuberculous mycobacterial species from environmental samples obtained from some selected communities in Ghana. To optimize decontamination, spiked environmental samples were used to evaluate four decontamination solutions and supplemented media, after which the best decontamination solution and media were used for the actual analysis. The isolates obtained were identified on the basis of specific genetic sequences, including heat shock protein 65, IS*2404*, IS*2606*, *rpoB*, and the ketoreductase gene, as needed. Among the methods evaluated, decontamination with 1 M NaOH followed by 5% oxalic acid gave the highest rate of recovery of mycobacteria (50.0%) and the lowest rate of contamination (15.6%). The cultivation medium that supported the highest rate of recovery of mycobacteria was polymyxin B-amphotericin B-nalidixic acid-trimethoprim-azlocillin–supplemented medium (34.4%), followed by isoniazid-supplemented medium (28.1%). Among the 139 samples cultivated in the main analysis, 58 (41.7%) yielded mycobacterial growth, 70 (50.4%) had no growth, and 11 (7.9%) had all inoculated tubes contaminated. A total of 25 different mycobacterial species were identified. Fifteen species (60%) were slowly growing (e.g., *Mycobacterium ulcerans*, *Mycobacterium avium*, *Mycobacterium mantenii*, and *Mycobacterium malmoense*), and 10 (40%) were rapidly growing (e.g., *Mycobacterium chelonae*, *Mycobacterium fortuitum*, and *Mycobacterium abscessus*). The occurrence of mycobacterial species in the various environmental samples analyzed was as follows: soil, 16 species (43.2%); vegetation, 14 species (38.0%); water, 3 species (8.0%); moss, 2 species (5.4%); snail, 1 species (2.7%); fungi, 1 species (2.7%). This study is the first to report on the isolation of *M. ulcerans* and other medically relevant nontuberculous mycobacteria from different environmental sources in Ghana.

IMPORTANCE

Diseases caused by mycobacterial species other than those that cause tuberculosis and leprosy are increasing. Control is difficult because the current understanding of how the organisms are spread and where they live in the environment is limited, although this information is needed to design preventive measures. Growing these organisms from the environment is also difficult, because the culture medium becomes overgrown with other bacteria that also live in the environment, such as in soil and water. We aimed to improve the methods for growing these organisms from environmental sources, such as soil and water samples, for better understanding of important mycobacterial ecology.

Nontuberculous mycobacteria (NTM), also known as atypical mycobacteria or mycobacteria other than tuberculosis (TB), belong to the same genus as the pathogens that cause TB and leprosy (1). NTM are ubiquitous in the environment (2), constitute the majority of species (169 species) in the genus *Mycobacterium* (3), and are largely considered nonpathogenic. They are assuming public health importance, however, as they cause opportunistic infections of all body parts in both humans and animals (1, 4). The abilities of NTM to cause disease vary among species (5, 6). While some species have not been shown to cause disease in humans (*Mycobacterium flavescens* and *Mycobacterium moriokaense*), some produce disease occasionally and others almost always cause disease (*Mycobacterium avium* and *Mycobacterium kansasii*) (7). The reasons for these variations in pathogenicity are not known.

Although NTM can cause several types of infections in immunocompetent individuals, the emergence of the human AIDS pandemic has made NTM more relevant; *M. avium* complex was identified as a major cause of opportunistic infections in patients infected with human immunodeficiency virus (HIV) (8). Identified risk factors associated with NTM include but are not limited

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to (i) reduced immunocompetence as a result of HIV, cancer, chemotherapy, or immunosuppression linked to transplantation; (ii) alcoholism; (iii) preexisting lung diseases, including prior tuberculosis and pneumoconiosis; and (iv) smoking (9, 10). Some infections caused by NTM in immunocompetent individuals in recent times include cervical lymphadenitis and pulmonary infections caused by *M. kansasii* and *Mycobacterium intracellulare* (11–13), skin infections caused by *Mycobacterium marinum*, *Mycobacterium ulcerans*, and *Mycobacterium haemophilum* (14–16), and

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Address correspondence to Dorothy Yeboah-Manu, dyeboah-manu@noguchi.ug.edu.gh.
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nosocomial infections caused by *Mycobacterium chelonae* and *Mycobacterium fortuitum* (17).

Infection with NTM has also been shown to affect the efficacy of the *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) vaccine (9). The BCG vaccine, which is to protect against tuberculosis (TB), offers different levels of protection among adult human populations (18–21). The variations have been partly attributed to increased exposure to NTM species in some geographical regions (20, 21), reducing protection against pulmonary TB in adults (22). The mechanisms for the decreased BCG efficacy are still not fully understood; however, it has been suggested that exposure to NTM results in a broad immune response that is rapidly recruited after BCG vaccination, thereby controlling the multiplication of the vaccine strain, which is needed for the production of *Mycobacterium tuberculosis*-specific protective responses (20, 23).

The public health importance of NTM calls for an effective strategy for the prevention and control of NTM infections, which is currently lacking (24). This lack is related to a lack of understanding regarding the mode(s) of transmission. Treatment of NTM infections is complicated by the nonspecificity of the drug regimen (25); moreover, NTM are naturally resistant to some of the available antimycobacterials, and no vaccine is currently available for the prevention of NTM infections (26). Therefore, there is a need for a better understanding of the ecology of NTM, to enhance knowledge regarding disease epidemiology and to guide the design of control and prevention strategies. Different modes of transmission, including ingestion, aspiration, and inoculation of the pathogen from the environment, have been hypothesized for diseases caused by NTM, but none has been proven to date (7). Unlike TB, diseases caused by NTM are rarely, if ever, transmitted from person to person (8).

Isolation and characterization of NTM are essential to improve the understanding of pathogen ecology and transmission. Isolation of NTM from the environment is a major challenge because of the complex mixture of other fast-growing bacteria and fungi in environmental samples, which contaminate the culture medium during isolation (27). Thus, removal of the mixed microorganisms in a decontamination step is crucial for the isolation of NTM. Moreover, attributing an infection to NTM in clinical samples is not straightforward, as NTM from the laboratory or sample collection environment can contaminate the culture. Thicker cell walls and unique mycolic acids (13) contribute to the impermeability, hydrophobicity, and slow growth of both slowly and rapidly growing mycobacteria (28). These characteristic features preferentially enhance NTM attachment to surfaces (29) and resistance to disinfectants, antibiotics, acids, and bases (30), which can be used selectively in the decontamination step.

A major NTM species of public health significance in Ghana and West Africa is *M. ulcerans*, the causative agent of Buruli ulcer (BU). Like other NTM species, its control is hampered in part because of a lack of understanding of the pathogen ecology and mode of transmission. Isolation of the pathogen from different environmental samples from West Africa has not been successful. To the best of our knowledge, only one study has been reported, in which *M. ulcerans* was successfully isolated from an aquatic insect (31). Therefore, this study was conducted in communities in which BU is endemic, increasing our chances of isolating *M. ulcerans* from the environment, to isolate and to profile NTM from the environment in Ghana for the first time.

MATERIALS AND METHODS

Ethics statement. Ethical clearance for the study was obtained from the institutional review board of the Noguchi Memorial Institute for Medical Research (federal assurance no. FWA00001824).

Study sites. This study was cross-sectional and was conducted between September 2012 and November 2013, in six communities along two major river basins (Densu and Offin) in Ghana (Fig. 1). The procedure that was used for the site selection was detailed previously by Ampah et al. (32). Community designation as endemic or nonendemic was based on the passive case report data of the National Buruli Ulcer Control Programme (NBUCP) and findings from our active case search activities (32, 33). The following communities along the Densu River basin were included: Ntabea (nonendemic) in the East Akim district, upstream of the river; Ashongkrom (endemic) in the Akwapim South district, midstream of the river; and Domesampaman (endemic) in the Ga-West Municipality of the Greater Accra Region, downstream of the river. All of the studied communities along the Offin River basin were designated endemic; the communities included Ntobroso and Achiase in the Atwima district, upstream and midstream of the river, respectively, and Mfantsiman in the Upper Denkyira district, downstream of the river. Extensive seroepidemiological studies (34–36) conducted in these communities indicated high levels of serological evidence of exposure of community members to *M. ulcerans* 18-kDa small heat shock protein (hsp) (33), irrespective of the disease burden.

Sample collection. Prior to sample collection, all of the selected communities were mapped using the Global Positioning System (GPS) coordinates from the NBUCP and ArcGIS 10.0 mapping software. Sites of frequent human activities, such as bodies of water utilized regularly for domestic purposes such as washing and cooking, communal bathing areas utilized by household members, school compounds (particularly playgrounds), agricultural farms, market grounds, community centers, and sources of drinking water, such as boreholes and water from storage tanks in homes, were used as reference points. Each community was divided into grids, and sampling points were randomly selected, using a randomization tool within ArcGIS 10.0, for environmental sample collection.

Environmental samples were collected by using a convenience approach. We walked through the communities and traced the GPS coordinates from the designated reference points, using the generated grid points for environmental sample collection. At each sample location, we collected a soil sample and any other sample within a 1-m reach. Samples that were more than 1 m away from the location point were excluded from the collection. The collection of samples was carried out for 3 hours each sampling time, starting at 8:00 a.m. and ending at 11:00 a.m.

Samples such as soil (approximately 5 g), water (45 ml), and fungi found growing in the soil and on dead and decaying logs were collected separately into sterile, labeled, 50-ml Falcon tubes (BD Biosciences), which were sealed tightly to avoid cross-contamination of samples. Snail samples were kept in labeled sealable plastic bags. Moss and vegetation parts were collected and pressed separately into labeled 50-ml Falcon tubes (BD Biosciences). We collected snails because aquatic snails have been reported to harbor *M. ulcerans* transiently (37); therefore, we sought to determine whether edible terrestrial snails also could harbor *M. ulcerans*. All samples were clearly labeled immediately, were kept in a cool pack at 4°C after collection, and were transported to the laboratory within 6 h after collection. Samples were processed on the day of collection and were analyzed by Ziehl-Neelsen staining and culture within 48 h after collection.

Sample processing. Snail and fungus samples were diced separately with sterile disposable surgical blades (Swann-Morton) and were homogenized using a sterile porcelain mortar and pestle (Cole-Parmer). The contents were then suspended in 10 ml of phosphate-buffered saline (PBS) (Sigma-Aldrich). Fecal samples were processed like snail and fungus samples. Soil samples were prepared using a modified version of the method described by Parashar et al. (38). Approximately 5-g soil samples were suspended in 30 ml of PBS in sterile 50-ml Falcon tubes (BD Biosci-

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FIG 1 Map of Ghana, showing the Densu and Offin River basins and selected communities. Map created with ArcGIS 10.0 using GPS coordinates from the National Buruli Ulcer Control Programme (NBUCP).

ences). Samples were shaken vigorously for 1 min and then centrifuged at 600 × *g* for 5 min at 4°C, to pellet the soil particles. The turbid supernatants (15 ml) were transferred into new sterile 50-ml Falcon tubes (BD Biosciences) and centrifuged at 7,000 × *g* for 10 min at 4°C, and the pellets were suspended in 10 ml of PBS (Sigma-Aldrich). Biofilms were prepared from moss and vegetation parts using a modified version of the method described by Gryseels et al. (39). Samples were emptied into sterile plastic resealable bags, and 50 ml of PBS (Sigma-Aldrich) was added to each bag.

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The contents of the bags were vigorously agitated to dislodge the biofilms into solution. The suspensions were poured into sterile 50-ml Falcon tubes (BD Biosciences) and centrifuged at 1,700 × *g* for 30 min, to sediment all suspended bacteria. The supernatant was decanted and the resulting pellet was suspended in 10 ml of PBS (Sigma-Aldrich). Water samples were vortex-mixed to homogeneity and centrifuged at 1,700 × *g* for 30 min, to sediment all suspended bacteria (38). The supernatant was decanted and the resulting pellet was suspended in 10 ml of PBS (Sigma-Aldrich). Prepared sample suspensions were kept at 4°C.

Preparation of spiked environmental samples. A suspension of in-house reference *Mycobacterium ulcerans* isolate NM209 was subcultured and harvested in mid-log phase, and a suspension in PBS at a concentration of 10^8 CFU ml^{-1} was mixed with 1 ml of 10^7 CFU ml^{-1} each of an *Escherichia coli* suspension obtained from a stool sample, *Pseudomonas aeruginosa* obtained from a wound culture (representing Gram-negative bacteria), and Gram-positive rods (*Bacillus* sp.) obtained from an open plate culture. Two-milliliter aliquots of the pooled suspension were then prepared and used for isolation of *M. ulcerans*. Eight environmental samples (4 water and 4 wet soil samples) were spiked with 10^8 CFU ml^{-1} of the *M. ulcerans* pool suspension and decontaminated.

Optimization of decontamination step. To optimize the decontamination procedure for the isolation of mycobacteria from environmental samples, we evaluated an in-house decontamination method using NaOH-oxalic acid (40), together with three other standard decontamination protocols, i.e., SDS-NaOH (38), NaOH-malachite green-cycloheximide (41), and *N*-acetyl-L-cysteine–NaOH (42), using the spiked samples.

NaOH-oxalic acid method. Two volumes of 1 M NaOH were added to the spiked sample suspension, and the mixture was incubated at room temperature for 20 min, with intermittent vortex-mixing. The reaction mixture was then neutralized by the addition of sterile distilled water to the 45-ml mark of the 50-ml Falcon tube. The sample was centrifuged (Eppendorf 5810 R) at 5,000 × *g* for 30 min and the supernatant was carefully decanted, after which 2 ml of 5% oxalic was added to the pellet and the mixture was incubated at room temperature for 30 min, with intermittent vortex-mixing. The decontamination reaction was finally terminated by neutralization with sterile distilled water.

SDS-NaOH method. Two milliliters of 3% SDS-4% NaOH was added to 2 ml of the spiked sample suspension, and the mixture was incubated at room temperature for 30 min, with intermittent vortex-mixing. The decontamination reaction was stopped by neutralization of the mixture with sterilized distilled water to the 45-ml mark of the 50-ml Falcon tube. The suspension was then centrifuged (Eppendorf 5810 R) at 5,000 × *g* for 30 min at 4°C, after which it was decanted.

NaOH-malachite green-cycloheximide method. For decontamination with 2% NaOH-0.3% malachite green-0.15% cycloheximide, 2.5 ml was added to 2.0 ml of the spiked sample suspension, and the mixture was incubated at room temperature for 30 min, with intermittent vortex-mixing. The decontamination reaction was stopped by neutralization of the mixture with 1 N HCl.

***N*-Acetyl-L-cysteine–NaOH method.** A volume of 3 ml of *N*-acetyl-L-cysteine–NaOH solution was added to the spiked sample suspension, and the mixture was incubated at room temperature for 20 min, with intermittent vortex-mixing. The decontamination reaction was terminated by neutralization of the mixture with sterile distilled water to the 45-ml mark of the 50-ml Falcon tube.

Determination of best culture medium for primary isolation. In order to determine the most effective cultivation medium for the recovery of mycobacterial species, individual decontaminated samples were centrifuged at 5,000 × *g* for 30 min. The supernatant was decanted, 1 ml of sterile PBS was added, and 0.1 ml of the pellet-PBS suspension was then inoculated, in duplicate, in prepared Lowenstein-Jensen (LJ) medium (43) supplemented with polymyxin B (2,000,000 IU $liter^{-1}$)-amphotericin B (10 mg $liter^{-1}$)-nalidixic acid (10 mg $liter^{-1}$)-trimethoprim (10 mg $liter^{-1}$)-azlocillin (10 mg $liter^{-1}$) (PANTA) (BD), isoniazid (INH) (0.2 mg $liter^{-1}$) (Sigma-Aldrich), or ethambutol (EMB) (2 μg $liter^{-1}$) (Sigma-Aldrich) or not supplemented (i.e., drug-free [DF] medium). The inoculated culture tubes were incubated at 37°C and observed for macroscopic growth for 6 months, at which time they were discarded. Acid-fast bacilli (AFB) were detected by preparing smears of bacterial colonies on frosted slides (Sigma-Aldrich), which were allowed to dry and were fixed with heat. The smears were then stained using the Ziehl-Neelsen procedure (44) and graded with the International Union against Tuberculosis and Lung Diseases grading system. A slide was graded as negative only after at least 100 fields were read under oil immersion without the detection of AFB and the results were confirmed by a second reader.

Identification of AFB-positive isolates by GenoType Mycobacterium CM/AS assay. Genomic DNA from heat-killed AFB isolates was extracted. The GenoType Mycobacterium CM/AS assay (Hain Lifesciences) was performed following the manufacturer's protocol (45). The reaction mixture consisted of 2 μl of $MgCl_2$, 5 μl of 10× buffer, 35 μl of biotinylated primer-nucleotide mixture, 0.2 μl of HotStar *Taq* (Qiagen, Hilden, Germany), 2.8 μl of nuclease-free water, and 5 μl of DNA, in a total volume of 50 μl. PCR was performed as follows: 95°C for 15 min, 10 cycles of 95°C for 30 s and 58°C for 2 min, 20 cycles of 95°C for 25 s and 53°C for 40 s, and 20 cycles of 70°C for 8 min. Hybridization and detection were performed in a Twincubator (Hain Lifescience GmbH), following the manufacturer's protocol (45).

Isolation of NTM from environmental samples. Based on findings from the spiked decontamination evaluation, samples were cultivated using the most effective decontamination method, i.e., the in-house 1 M NaOH-5% oxalic acid method, as previously. During the mock experimentation, we observed fungal growth on cultures inoculated on PANTA-supplemented medium. Therefore, we added the antifungal mycobactin J (2 mg $liter^{-1}$; IDVet) to the PANTA-supplemented LJ medium (yielding PANTA-mycobactin J [PM] medium). Suspensions were then inoculated in duplicate in prepared LJ medium supplemented with INH, PM medium, or DF medium. The inoculated culture tubes were incubated at 32°C and observed for macroscopic growth for 6 months, at which time they were discarded. The choice of 32°C was to enable isolation of *M. ulcerans*, which is an important NTM in Ghana. In addition, 2 drops of each sample suspension were put on frosted slides (Sigma-Aldrich), allowed to dry, fixed with heat, and stained using the Ziehl-Neelsen procedure. Culture tubes were read daily during the first week, for the isolation of rapid growers, and thereafter were read weekly. For the cultures that showed growth, the week of growth was recorded and the growth was quantified. Tubes that showed positive growth were purified by subculture of individual colonies on drug-free medium for species identification. AFB were detected by preparing smears of bacterial colonies and staining them for AFB.

Identification of mycobacterial isolates. The isolates confirmed as AFB positive were harvested, killed by heating at 95°C for 30 min, and used for genomic DNA extraction (46). Cell wall lysis was achieved first by the addition of 300 μl of lysis buffer to bacterial pellets, followed by the addition of 50 μl of lysozyme (10 mg ml^{-1}; Fluka), 100 μl of SDS (0.04 mg ml^{-1}), and 40 μl of proteinase K (0.2 mg ml^{-1}; Roche). The mixture was incubated at 37°C for 1 h, after which 200 μl of zirconia beads (Sigma-Aldrich) was added and the mixture was homogenized at 4,700 × *g* for 4 min, using a FastPrep-24 homogenizer (MP Biomedicals). The mixture was centrifuged at 5,000 × *g* for 5 min, and the supernatant, containing released DNA, was transferred to a new Eppendorf tube. Proteins and other cellular debris were removed by two steps of phenol-alcohol extraction, with the addition of 500 μl of phenol-chloroform-isoamyl alcohol (Sigma) to the mixture and centrifugation at 5,000 × *g* for 10 min. DNA precipitation was achieved with the addition of 20 μl of 3 M sodium acetate (Sigma) to the mixture, followed by the addition of 500 μl of absolute ethanol (Sigma-Aldrich). The mixture was frozen at −20°C overnight and centrifuged at 5,000 × *g* for 30 min, after which the pellets were dried at 50°C. The genomic DNA was suspended in 100 μl of nuclease-free water. The concentrations of the DNA were checked using a NanoDrop 2000C spectrophotometer (Thermo Scientific).

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TABLE 1 Primers used for NTM detection and identification

Primer no.	Primer name	Sequence (5= to 3=)	Reference
1	TB-11F	ACCAACGATGGTGTGTCCAT	46
2	TB-12R	CTTGTCGAACCGCATACCCT	
3	IS2404-F	GGCAGTTACTTCACTGCACA	49
4	IS2404-R	CGGTGATCAAGCGTTCACGA	
5	IS2606-F	GGTGCGGTTCCATTGAGA	46
6	IS2606-R	GTCGTAGATGTGGGCGAAAT	
7	KR-F	TTCTGTTCCCGCAGTTCTTT	This work
8	KR-R	CTAGCTCGGAAGTGGTCAGC	
9	rpoB-F	GTGGGTCGGTACAAGGTCAA	This work
10	rpoB-R	GCGGTCAGGTAGTGGATCTC	

A 441-bp portion of mycobacterial heat shock protein 65 (hsp65) was amplified using the *Mycobacterium* genus-specific TB-11 and TB-12 forward and reverse primers (Table 1) (47), as described previously (48). The PCR mixture contained 5 μl of a 1:100 dilution of template DNA, 0.25 μM concentrations of each primer, 6 μl of Q-solution, 3 μl of 10X buffer, 200 μM (each) dATP, dCTP, dGTP, and dTTP (Pharmacia Biotech), 1.5 mM $MgCl_2$, and 0.5 U of Fire *Taq* polymerase, in a total volume of 100 μl. Amplification was performed using 32 cycles of 5 min at 94°C, 30 s at 94°C, 30 s at 60°C, 1 min at 72°C, and 10 min at 72°C, in an Applied Biosystems 2720 thermal cycler. Amplified product (10 μl) was confirmed by gel electrophoresis. The amplified PCR product (40 μl) was sequenced by outsourcing. The generated sequences were edited using CodonCode Aligner 6.0.2 software to remove vector sequences, and species were identified by using NCBI Microbial Nucleotide BLAST, using default settings (49).

Confirmation of *M. ulcerans* with IS*2404*, IS*2606*, ketoreductase B domain, and *rpoB*. The *M. ulcerans* isolate obtained was confirmed using PCR detecting the following gene targets: IS*2404* (50), IS*2606* (47), ketoreductase (KR) B domain, and *rpoB*. The primers for KR and *rpoB* were designed using Primer3 software (50). The primer sets used for the amplification are shown in Table 1. The reaction mixtures for all of the targets (IS*2404*, IS*2606*, KR, and *rpoB*) contained 10 μM concentrations of each primer, 200 μM (each) dATP, dCTP, dGTP, and dTTP, 19 μl of nuclease-free water, 25 mM $MgCl_2$, 3 μl of 10X buffer, 1 U Fire PolTaq (Solis BioDyne), and 1 μl of template DNA (5 ng), in a total volume of 30 μl. Amplification was performed in an Applied Biosystems 2720 thermal cycler, as follows: 32 cycles of 5 min at 94°C, 30 s at 94°C, 30 s at 60°C, 1 min at 72°C, and 10 min at 72°C. Amplified DNA (10 μl) was subjected to electrophoresis in a 2% agarose gel prepared with Tris-borate-EDTA (TBE) buffer and was detected by ethidium bromide staining and UV transillumination.

Data analysis. The sensitivities of the four decontamination methods and cultivation media were evaluated. The colony morphology and the length of time before visible colonies appeared were recorded during culture readings. Cultures were said to be positive if at least one tube from the same treatment confirmed AFB growth, cultures were said to be negative if none of the culture tubes had microbial growth after 6 months, and cultures were classified as contaminated when all of the tubes had more than one-half of the tube with other bacterial growth or liquefied contents. The performance of decontamination methods and cultivation media was assessed using the *t* test.

RESULTS

Performance of decontamination methods and growth media tested with spiked samples. Using the four decontamination procedures and four different culture media, a total of 128 cultures were performed for the 8 samples analyzed. As indicated in Table 2, the decontamination procedure that resulted in the smallest number of contaminated tubes was the NaOH-oxalic acid method (5 cultures [15.6%]); the method that gave the highest no-growth rate was the malachite green-cycloheximide method (11 cultures [34.4%]), and the SDS-NaOH method gave the largest number of contaminated tubes (28 cultures [87.5%]). The cultivation medium that supported the highest rate of mycobacterial growth was PANTA-supplemented medium, followed by INH-supplemented medium, drug-free medium, and EMB-supplemented medium. AFB isolates from 2 of the 8 samples were confirmed as *M. ulcerans*; in both cases, the medium was PANTA-supplemented medium.

Direct smear microscopy. Direct smear analysis of the 139 samples that were cultivated identified only 12 (8.6%) as containing AFB. Acid-fast bacilli were detected mainly among moss, snail, soil, and vegetation samples, as indicated in Table 3. Slides of some samples in which AFB were detected are shown in Fig. 2.

Isolation of *Mycobacterium* spp. from environmental samples. Of the 139 environmental samples cultivated, 58 (41.7%) yielded mycobacterial growth, 70 (50.4%) had no bacterial growth, and 11 (7.9%) had all of the inoculated tubes contaminated. The performance of the different growth media, as indicated in Table 4, showed that the cultivation medium supporting the greatest recovery of mycobacterial growth was LJ medium supplemented with PANTA and mycobactin J (i.e., PM medium) (75 samples [26.9%]), followed by INH-supplemented medium (63 samples [22.7%]) and finally DF medium (25 samples [8.9%]). The smallest number of contaminated tubes was recorded with the mycobactin J and PANTA supplement (111 tubes [39.9%]), followed by INH-supplemented medium (132 tubes [47.5%]) and DF medium, giving the greatest number of contaminated tubes (224 tubes [80.6%]) ($P < 0.001$).

TABLE 2 Decontamination methods and cultivation media for recovery of *Mycobacterium ulcerans*

Decontamination method[a]	No. of cultures[b]														
	Positive					Contaminated					No mycobacterial growth				
	DF	INH	EMB	PANTA	Total ($n = 32$)	DF	INH	EMB	PANTA	Total ($n = 32$)	DF	INH	EMB	PANTA	Total ($n = 32$)
SDS-NaOH	0	0	0	0	0	6	8	8	6	28	2	0	0	2	4
NaOH-Mal-C	2	0	0	5	7	4	1	5	1	11	2	2	3	7	14
NaOH-OA	3	5	3	5	16	1	1	3	0	5	4	2	2	3	11
NALC-NaOH	2	4	2	1	9	3	4	3	2	12	3	3	3	2	11

[a] SDS-NaOH, sodium dodecyl sulfate-sodium hydroxide; NaOH-Mal-C, NaOH-malachite green-cycloheximide; NaOH-OA, NaOH-oxalic acid; NALC-NaOH, *N*-acetyl-L-cysteine–sodium hydroxide. The NaOH-oxalic acid method was the most effective among the evaluated procedures.

[b] DF, drug-free; INH, isoniazid; EMB, ethambutol. The cultivation medium with the highest mycobacterial growth rate was PANTA-supplemented medium, followed by INH-supplemented medium, and finally DF medium. EMB-supplemented medium had the lowest number of positive cultures and the highest number of contaminated cultures.

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TABLE 3 Rates of positivity for acid-fast bacilli by direct smear analysis and isolation of mycobacterial species

Sample type	Total no. of samples	No. (%) of samples positive for acid-fast bacilli/total no.	No. of samples yielding mycobacterial isolate/total no.
Fungi	4	0/4 (0)	1/4
Snails	5	1/5 (20)	1/5
Moss	8	1/8 (12.5)	3/8
Soil	54	6/54 (11.1)	30/54
Vegetation	56	4/56 (7.1)	21/56
Water	12	0/12 (0)	2/13
Total	139	12/139 (8.6)	58

TABLE 4 Performance of different growth media in isolating *Mycobacterium* spp. from the environment

Medium[a]	No. of culture tubes			
	Positive	Contaminated	No growth	Total
DF	24	224	30	278
INH	63	132	83	278
PM	75	111	92	278
Total	162	467	205	834

[a] DF, drug-free; INH, isoniazid; PM, PANTA-mycobactin J. The cultivation medium with the highest mycobacterial growth rate was PM medium, followed by INH-supplemented medium and finally DF medium.

Isolated mycobacterial species. Based on colonial morphology, growth rate characteristics, and growth medium findings, a total of 162 AFB-positive isolates (obtained from 58 samples) were collected for identification. Analysis of hsp65 sequences identified 25 different mycobacterial species, as shown in Fig. 3. The mycobacterial species isolated were from soil (16/30 isolates), vegetation (14/21 isolates), water (3/2 isolates), moss (2/3 isolates), molluscs (1/1 isolate), and fungi (1/1 isolate). The isolates consisted of slowly growing mycobacteria (15 isolates [60%]) and rapidly growing mycobacteria (10 isolates [40%]), with the greatest recovery being mycobacteria found among soil and vegetation samples, as shown Fig. 3. Two *M. ulcerans* isolates were isolated from moss and soil samples from communities in which BU is endemic. Other slowly growing mycobacteria, including *M. avium*, *Mycobacterium mantenii*, and *Mycobacterium malmoense*, and rapidly growing mycobacteria such as *M. chelonae*, *Mycobacterium abscessus*, and *M. fortuitum* were among the many species isolated (Table 5).

Among the media tested, LJ medium supplemented with both PANTA and mycobactin J (PM medium) gave the highest rates of recovery of mycobacterial species (16 samples [42.1%]), followed by INH-supplemented medium (13 samples [34.2%]) and drug-free LJ medium, with the lowest rate (11 samples [28.9%]). While some mycobacterial species (*M. ulcerans*, *M. mantenii*, *Mycobacterium szulgai*, *Mycobacterium senuense*, *Mycobacterium terrae*, and *Mycobacterium genavense*) were isolated from a single medium, other species (*M. chelonae*, *M. abscessus*, *M. fortuitum*, *M. avium*, *Mycobacterium gordonae*, and *Mycobacterium septicum*) were isolated from the different cultivation medium, as shown in Table 5. Eight (32%) of the species were isolated from PM medium only, 3 (12%) from INH-supplemented medium only, and 2 (8%) from DF medium only, as indicated in Table 6. While 4 mycobacterial species (16%) were isolated from all three cultivation media (PM medium, INH-supplemented medium, and DF medium), 2 (8%) were isolated from PM medium and DF medium or PM medium and INH-supplemented medium and 4 (16%) were isolated from DF medium and INH-supplemented medium (Table 6).

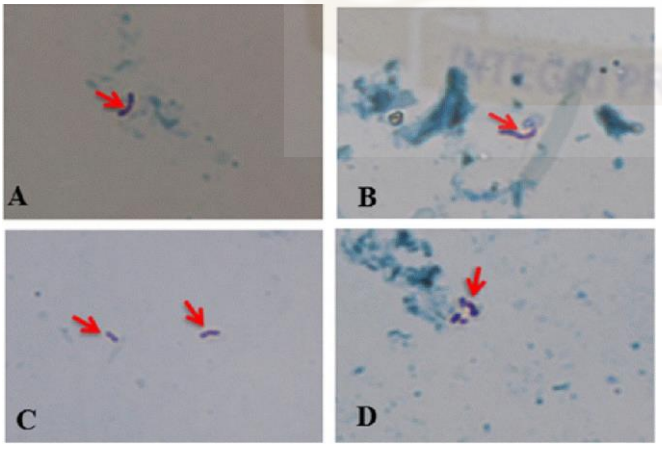

FIG 2 Direct smear analyses, using the Ziehl-Neelsen procedure, of environmental samples from water (A), moss (B), vegetation (C), and snail (D). Red arrows, acid-fast bacilli. Magnification, ×1,000.

Confirmation of *M. ulcerans*. Gel-based PCR analyses of the *M. ulcerans* isolates from moss sample using primers for the targets IS*2404*, IS*2606*, KR, and *rpoB*, which are found in all mycobacteria, showed products with their respective band sizes on electrophoresis. Product sizes of 492 bp, 332 bp, 615 bp, and 606 bp were detected for IS*2404*, IS*2606*, KR, and *rpoB*, respectively, as shown in Fig. 4.

DISCUSSION

The objective of the study was to optimize the procedure for isolation of NTM from the environment by evaluating different decontamination solutions and antibiotic-supplemented media. A secondary objective was to use the best of the evaluated methods to isolate and to profile NTM in environmental samples from communities in Ghana in which BU is endemic. The main achievement of this work was the isolation of different NTM species, including *M. ulcerans*, from various environmental sources in Ghana for the first time.

Buruli ulcer infections caused by *M. ulcerans* have afflicted many individuals, particularly in countries of West Africa, including Ghana, in which BU is endemic (15). Over the years, a number of studies aiming to elucidate the ecology and mode(s) of transmission of *M. ulcerans* have proved futile. Theories that have been propounded to explain the mechanism of *M. ulcerans* transmission include (i) inhalation or ingestion of aerosolized *M. ulcerans* from contaminated water (51), (ii) acquisition of *M. ulcerans* through an insect or animal bite (52), and (iii) contamination of existing wounds or sites of trauma by environmental factors, such as soil, vegetation, and water (53); such theories have not been proved experimentally. One challenge has been the presence of fast-growing microorganisms that attain macroscopic growth within 18 to 48 h, thereby contaminating cultures, inasmuch as the mycobacterial species of interest (*M. ulcerans*) grows very

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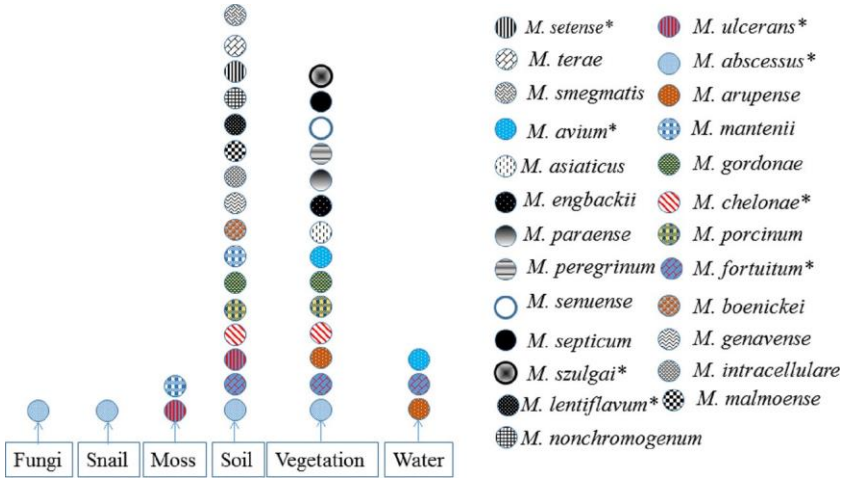

FIG 3 Heat shock protein 65 analysis, revealing 25 different mycobacterial species isolated from environmental samples from communities in which BU is endemic. *, causes skin and soft tissue infections.

slowly, requiring more than 6 months to achieve macroscopic growth in some instances. Our isolation of *M. ulcerans* from moss and soil samples from an area in which BU is endemic supports the concept that *M. ulcerans* transmission could possibly be through contamination of an open cut or wound by environmental factors, especially among children playing on the ground and agricultural farmers who are in constant contact with such factors. This achievement is a major milestone for further studies to increase the number of isolates, which will allow molecular epidemiological studies aimed toward an understanding of transmission dynamics.

Decontamination using 1 M NaOH followed by a simplified oxalic acid (40) method allowed us to isolate *M. ulcerans* from both spiked and nonspiked samples. One important factor that is considered in the isolation of mycobacteria from the environment is the decontamination technique employed. Members of the genus *Mycobacterium* have thick cell walls that are rich in lipids, which allows them to be impermeable to many acids, bases, and detergents (14); this has made decontamination with these agents a common practice in primary cultivation of mycobacteria, to reduce contamination (38, 54). As a result, samples have been decontaminated by different procedures and have been evaluated in different laboratories for decades, with different results (38, 54, 55). Various decontaminating agents, due to their harshness, destroy a substantial number of mycobacteria along with the contaminants, while others are too weak to eliminate contaminants. A good decontamination agent is one that effectively removes unwanted microorganisms and maximizes the recovery of wanted mycobacteria. Decontamination of 139 PCR-positive samples with 1 M NaOH followed by 5% oxalic acid yielded 58 samples (41.7%) that were positive for mycobacteria and 70 (50.4%) with no growth, with only 11 samples (7.9%) being contaminated. Although this procedure was the best among the four different de-

TABLE 5 Growth characteristics of and isolation media for _ycobacterium spp.

Mycobacterium sp.	Isolation medium(a)[a]
Slowly growing	
M. ulcerans[b]	PM
M. szulgai[b]	PM
M. mantenii	PM
M. senuense	PM
M. lentiflavum[b]	PM, DF
M. avium[b]	PM, DF, INH
M. engbaekii	PM, DF
M. gordonae	PM, INH, DF
M. paraense	PM
M. terrae[b]	DF
M. asiaticus	DF, INH
M. nonchromogenicum[b]	DF
M. intracellulare	INH
M. genavense	INH
M. malmoense	PM
Rapidly growing	
M. peregrinum	PM
M. chelonae[b]	PM, DF, INH
M. abscessus[b]	PM, DF, INH
M. fortuitum[b]	DF, INH
M. smegmatis	DF, INH
M. septicum	PM, INH
M. porcinum	INH
M. setense[b]	DF, INH
M. boenickei[b]	PM, INH
M. arupense	PM

[a] PM, PANTA-mycobactin J; DF, drug-free; INH, isoniazid.
[b] A mycobacterial species that causes skin and soft tissue infections.

TABLE 6 Cultivation media and isolated mycobacterial species

Cultivation medium(a)[a]	No. (%) of mycobacterial species isolated
PM	8 (32)
INH	3 (12)
DF	2 (8)
PM, DF	2 (8)
PM, INH	2 (8)
DF, INH	4 (16)
PM, INH, DF	4 (16)

[a] PM, PANTA-mycobactin J; DF, drug-free; INH, isoniazid.

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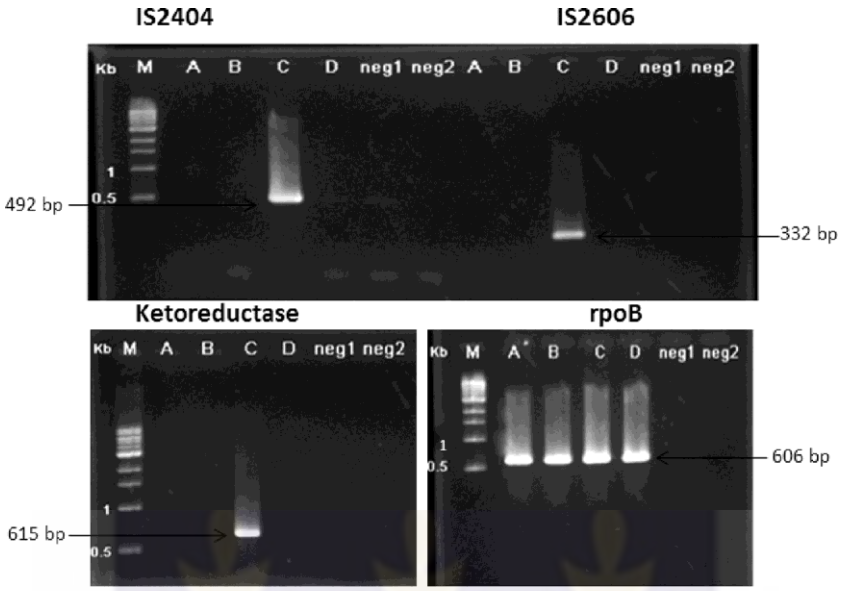

FIG 4 Confirmation of *M. ulcerans* by PCR detection and amplification of IS*2404*, IS*2606*, ketoreductase B domain, and *rpoB*. Lane M, size ladder (Gibco); lane A, *M. avium* isolated from a cocoa pod; lane B, *M. gordonae* isolated from soil; lane C, *M. ulcerans* isolated from moss; lane D, *M. chelonae* isolated from soil.

contamination procedures evaluated (Table 2), the proportion of samples with no growth indicates that it is also very harsh. In a study by Portaels et al., the use of the NaOH decontamination method and the addition of mycobactin to the culture medium did not improve the recovery of mycobacteria from soil samples (55). In our study, however, the addition of the antifungal mycobactin J and the antibiotic PANTA to the cultivation medium was effective in limiting contamination by fungi and fast-growing bacteria, compared to drug-free medium, as shown in Table 4. Isoniazid (INH) was incorporated into the selective medium because some studies have shown that some NTM (for example, *M. ulcerans*) are resistant to this antibiotic (55, 56) and it could inhibiting nontarget microorganisms; this was found to be true in our study, as INH-supplemented medium performed better than DF medium. Therefore, we recommend the use of our in-house decontamination procedure and LJ medium supplemented with PANTA and mycobactin J to enhance the recovery of NTM, particularly *M. ulcerans*, from environmental samples.

Species identification of the acid-fast isolates revealed that at least one NTM species was isolated in each type of environmental sample. This finding further implicates the environment as the natural reservoir of NTM, as has been reported from other studies (57, 58). We isolated the greatest number of mycobacterial species from soil (16 species [43.2%]), as indicated in Fig. 3. This is not surprising, as the hydrophobic nature of NTM preferentially confers on them the ability to attach to soil surfaces in order to thrive in the environment (3). Also, the high rate of NTM recovery from soil could be partly due to the abundance of organic matter in the soil, which serves as a nutrient source for the mycobacteria in the environment, since most NTM are known to be saprophytic in nature (38, 59). The high rates of the presence of NTM, especially potentially pathogenic species, in the soil are of public health concern. Agricultural farmers, miners in the pursuit of their work, and children, particularly during playtime, are constantly exposed to soil and vegetation in the environment. Therefore, it is important for persons with frequent body contact with such environmental sources to wear protective clothing, which has been shown to offer some form of protection against Buruli ulcer disease (60). In addition to *M. ulcerans*, we isolated a number of other NTM species that are known to cause disease in humans, including *M. fortuitum*, *M. chelonae*, *M. abscessus*, *M. malmoense*, and *M. avium*. A study by Kankya et al. reported on the isolation of similar NTM species of public health significance in Uganda, although *M. ulcerans* was not isolated in that study (61).

Direct microscopic analysis of the 139 samples identified only 12 (8.6%) as containing acid-fast bacilli (AFB), although all were confirmed by PCR analysis to contain mycobacteria. The low sensitivity of Ziehl-Neelsen microscopy for detection of AFB is well documented, i.e., samples are positive only when they contain at least 10^4 CFU ml^{-1} AFB. This finding suggests that, while NTM species may be abundant in the environment, the bacterial concentrations are quite low. This finding may not be unique to the isolated NTM, as several studies conducted in Ghana and other African countries in which BU is endemic detected low cycle threshold (C_T) values for *M. ulcerans*, the causative agent of Buruli ulcer, in the environment (62). In contrast, however, studies from Australia reported high C_T values for *M. ulcerans* in the environment (63).

In conclusion, we isolated *M. ulcerans* and other slow-growing and fast-growing medically relevant NTM from BU-burdened communities in Ghana. The in-house decontamination procedure (1 M NaOH followed by 5% oxalic acid) and cultivation media supplemented with various antimicrobial and antifungal agents were effective for the recovery of NTM. The isolation of at least one NTM species from each pool of environmental samples cultivated confirms the abundant distribution of NTM in the environment, but their concentrations in the environment may be low, as shown by direct smear analysis.

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