THE ROLE OF FREE-LIVING AMOEBAE AS RESERVOIR IN THE TRANSMISSION OF *MYCOBACTERIUM ULCERANS*

BY

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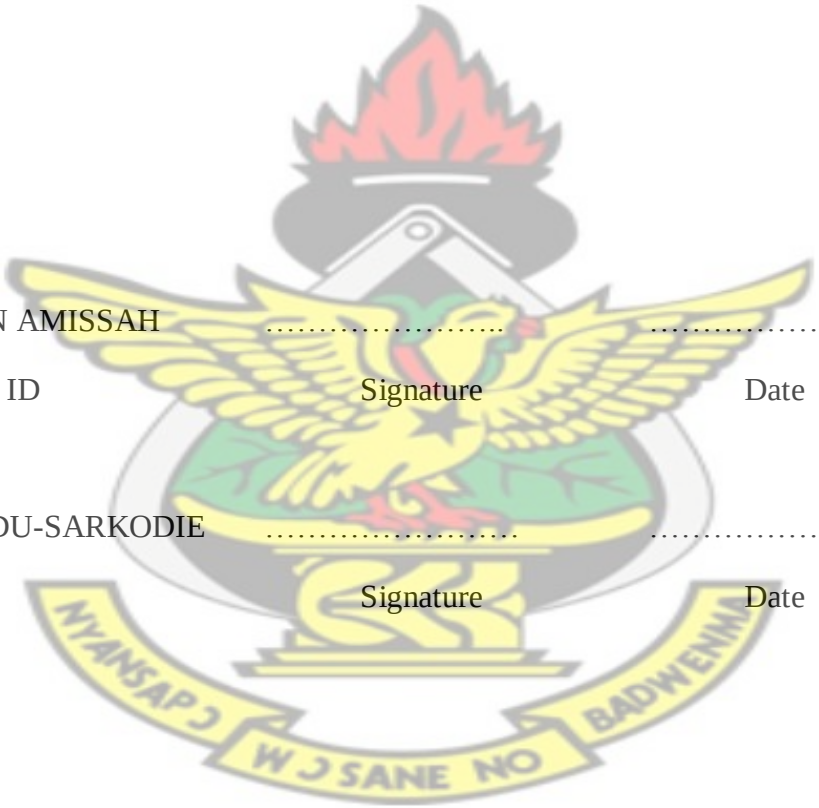
DECLARATION

I hereby declare that this submission is my own work towards the MPhil and that, to the best of my knowledge, it contains no material previously published by another person nor material which has been accepted for the award of any other degree of the University, except where due acknowledgement has been made in the text.



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ABSTRACT

Mycobacterium ulcerans causes Buruli ulcer disease. The bacterium can be found predominantly in swamps and slow-flowing water areas. Understanding the ecology of *M. ulcerans* has been severely hampered due to a knowledge gap of the reservoir and precise mode of transmission although aquatic insects and mosquitoes have been implicated in its transmission. Free-living amoebae (FLA) are common inhabitants of the aquatic ecosystems and have been shown to be naturally infected with environmental mycobacteria that may cause human infections. This study investigated the hypothesis that FLA could serve as reservoirs of *M. ulcerans* and aid in its transmission to humans. Five hundred and thirty nine environmental specimens were collected from water bodies in five endemic and two non endemic communities for ten months; 13 aerosols, 223 biofilms from plant, 205 biofilms from trunks, 45 detritus, 53 water filtrates from five endemic and two non endemic communities. FLA were isolated from 369 (68.5%) environmental specimens and three genera were identified by PCR; *Acanthamoeba* 157 (29.1%), *Vahlkamfiidae* 306 (56.8%) and *Naegleria* 118 (21.9%). The effect of pH, temperature and bacterial load on the isolation of amoebae were investigated. While isolation of *Vahlkampfiidae* was independent of physical parameters (pH and temperature), an increase in these parameters increased the isolation *Naegleria* and *Acanthamoeba* species. FLA were not dependent on bacterial load specifically *E. coli* as source of nutrient since they may have had alternative sources (yeast, fungi and other protozoa) for food. One hundred and thirty one intracellular mycobacteria were isolated and identified. Intracellular mycobacteria were often isolated from specimens positive for isolation of amoebae and positive for 16S rDNA PCR from amoebae cultures. Culture of *M. ulcerans* from FLA and environmental specimens was attempted. One

isolate was positive for IS*2404* PCR but negative upon confirmation by Variable-Number Tandem Repeat typing. *M. ulcerans* DNA was detected from a water filtrate and IS*2404* target was detected from 2 environmental specimens and 25 amoebae cultures. The study showed no association between *M. ulcerans* and FLA, however, results indicated a significant association between FLA (*Acanthamoeba* and *Vahlkampfiidae* genera) and IS*2404* target. These data may have implications for the reservoir and transmission of environmental mycobacteria that habour IS*2404* target. These findings may provide important information for understanding the ecology of *M. ulcerans*.

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6 FAM	6-carboxyfluorescein dye
AFB	Acid-fast bacilli
BU	Buruli ulcer
CT	Threshold cycle
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
FLA	Free-living amoebae
IPC	Internal positive control
IS	Insertion sequence
ITS	Internal transcribed spacer
IWGMT	International working group on mycobacterial taxonomy
KR	Ketoreductase
LJ	Löwenstein-Jensen
MGBNFQ	Minor groove binder non-fluorescent quencher
MPM	Mycolactone producing mycobacteria
MU	*Mycobacterium ulcerans*
NMIMR	Noguchi Memorial Institute for Medical Research
NNA	Non nutrient agar
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pH	Negative logarithm of hydrogen ion activity
rRNA	Ribosomal ribonucleic
R-T	Real-time
RT	Room temperature

TE	Tris-EDTA
TF	Forward primer
TP	Probe
TR	Reverse primer
VIC	5´ reporter dye
VNTR	Variable-Number Tandem Repeat
WHO	World Health Organization
ZN	Ziehl-Neelsen

CHAPTER ONE

1.0 Introduction

1.1 Background to the study

Mycobacterium ulcerans (MU) disease/Buruli ulcer (BU) is a disease that affects the skin, subcutaneous tissue and occasionally the bones. The disease has been reported in over 30 countries worldwide, however, endemic rural communities in some West and Central African Countries have the highest burden of BU (Janssens *et al.*, 2005).

Epidemiological data link the disease with stagnant or slow flowing water. However, the ecological niches and modes of transmission still remain unclear (Portaels, 1995; Debacker *et al.*, 2006). Previous studies have implicated aquatic insects; Naucoridae and Belostomatidae (Portaels *et al.*, 2008; Marsollier *et al.*, 2002) as likely vectors of MU, however, their significance in the transmission of the pathogen to humans remain unknown.

Mycobacteria and other micro-organisms are known to interact with free-living amoeba (FLA), (Baker and Brown, 1994). The first report of mycobacteria surviving in amoeba was suggested by Jadin, (1975) who demonstrated the uptake of *M. leprae* by *Acanthamoeba castellanii*. Strong association between FLA and other mycobacteria has been shown (*M. gordonae, M. kansasii* and *M. xenopi*) in biofilms sampled from hospital tap water (Thomas *et al.*, 2006).There are a variety of situations where human and mycobacterial geographic and environmental distributions overlap and lead to exposure of humans as well as impacting mycobacterial ecology. A major overlap occurs with water through drinking, routine leisure interests, farming activities and

fouling by wild animals (Primm *et al*., 2004). Aerosols generated during these activities can lead to human exposure.

A number of *Mycobacterium* species, including *M. avium*, *M. marinum*, *M. simiae*, *M. phlei*, *M. smegmatis* and *M. fortuitum*, live intracellularly in amoebae (Krishna-Prasad *et al*., 1978). Adekambi *et al*., (2006) has demonstrated the intracellular uptake and survival of; *M. peregrinum, M. terrae, M. intracellulare, M. lentiflavum, M. septicum, M. porcinum* and other mycobacteria in trophozoites and cyst acanthamoebae species. *Mycobacterium* species demonstrated to grow within FLA include *M. bovis* (Taylor *et al*., 2003), *M. xenopi* (Drancourt *et al*., 2007), *M. marinum* (Solomon *et al*., 2003), *M. massiliense* (Adekambi *et al*., 2004), *M. smegmatis* (Sharbati-Tehrani *et al*., 2005) and *M. avium* (Mura *et al*., 2006; Whan *et al*., 2006).

It has been proposed that bacterial virulence for mammalian cells may be a consequence of adaptations associated with intra-amoebal survival (Cirillo *et al*., 1997; Molmeret *et al*., 2005) and these adaptive changes; evolution by duplication of non-essential genes (Hughes, 2002) and by the lateral acquisition of genes (Ochman *et al*., 2000) may have led to the expression of symbiotic or pathogenic phenotypes according to their impact on the host cell. Thus, FLA represent a potent evolutionary crib and an important genetic reservoir for its internalized microbes (Goebel and Gross, 2001).

M. avium infected in *A. castellanii* was deomonstrated to be protected from antibiotics like rifabutin, clarithromycin and azithromycin in comparison with when infected in macrophages (Miltner and Bermudez, 2000). This may have implications on the use of drugs like clarithromycin for the treatment of BU. FLA may be involved in transmission by (i) acting as a vehicle carrying huge numbers of microorganisms (Danes and Cerva, 1981), (ii) acting as "Trojan horses" for their host (Baker and Brown, 1994), thus, protecting it from the first line of

human defenses and (iii) producing vesicles filled with bacteria (Anand *et al*., 1983; Berk *et al*., 1998; Greub and Raoult, 2002; Marolda *et al*., 1999; Rowbotham, 1986).

1.2 Statement of problem

Presently, very little is known about the ecology of *M. ulcerans* as well as the epidemiology of BU. Reasons for the poor understanding of the disease include the difficulty in isolating *M. ulcerans* from the environment by culture despite compelling data linking the disease to aquatic ecosystems (Portaels *et al*., 2001). The detection of *M. ulcerans* in the environment is based on demonstrating the presence of *M. ulcerans* specific DNA, insertion sequence IS*2404* (Ross *et al*., 1997). This sequence has been identified in water, fish, aquatic insects, detritus, leeches, soil, biofilms, frogs, snails, other invertebrates and mollusks (Benbow *et al*., 2008; Eddyani *et al*., 2004; Fyfe *et al*., 2007; Johnson *et al*., 2007; Kotlowski *et al*., 2004; Marsollier *et al*., 2002; 2004a; 2004b; Portaels *et al*., 1999; 2001; 2008; Stinear *et al*., 2000; Trott *et al*., 2004; Williamson *et al*., 2008). Aquatic insects have been increasingly suspected to play a role in the transmission of *M. ulcerans*. The hypothesis that predatory aquatic insects were transmitters of *M. ulcerans* was advanced in 1999 (Portaels *et al*., 1999). The hypothesis was later reinforced by Marsollier and colleagues (Marsollier *et al*., 2002; 2007) on the basis that; (1) the salivary glands of *Naucoris cimicoides* are colonised with *M. ulcerans* upon feeding on grubs containing the pathogen; (2) *M. ulcerans*-infected *N. cimicoides* transmit the pathogen to mice upon biting; and (3) *N. cimicoides* in BU endemic areas can be naturally colonised by *M. ulcerans*. This colonization may occur through feeding on aquatic snails and fish, which take up *M. ulcerans* from water, mud, and aquatic plants (Duker *et al*., 2006; Merrit *et al*., 2005).

The successful cultivation of *M. ulcerans* from another family of aquatic Hemiptera (Gerridae) extends the range of hypothetical hemipterans as transmitters (Portaels *et al*., 2008). However, there are no reports of Gerridae biting humans (Portaels *et al*., 2008).These insects may be only passive, incidental and transient reservoirs of *M. ulcerans* without an obvious role in the transmission of BU to humans or other mammals. Benbow *et al*., (2008) assessed possible transmission of *M. ulcerans* by aquatic biting insects, where field examination of biting water bugs (Hemiptera: Naucoridae, Belostomatidae, Nepidae) in 15 disease endemic and 12 non disease endemic areas of Ghana were conducted. From collections of 22, 832 invertebrates, they compared composition, abundance and associated *M. ulcerans* positivity among sites. Biting hemipterans were rare and represented a small percentage (usually <2%) of invertebrate communities. No significant differences were found in hemipterans abundance or pathogen positivity between disease endemic and non disease endemic sites, and between abundance of biting hemipterans and *M. ulcerans* positivity. Therefore, although infection through insect bites is possible, little field evidence support the assumption that biting hemipterans are primary vectors of *M. ulcerans*. This hypothesis was also disputed by Williamson *et al*., (2008) whose work showed that, even where large numbers of Belostomatidae were collected the rate of *M. ulcerans* infection was very low and despite repeated seasonal sampling the numbers of Naucorids found were also very low.

As a result of poor understanding of BU epidemiology, it is particularly difficult at the moment to formulate primary preventive measures for the control of the disease.

1.3 Justification of the study

It has been suggested that *M. ulcerans* will be difficult to persist in the environment as free living organism due to its natural fragility (Portaels *et al*., 2001). *M. ulcerans* may be maintained in a

commensal relationship in hosts that protect the bacilli against unfavourable physical parameters of the environment and changes in these parameters. Plankton such as protozoa may act as filters for *M. ulcerans* present in water (Portaels *et al*., 2001) or permit the multiplication of the bacteria. Protozoa may protect the bacilli against adverse environmental conditions (Miltner *et al*., 2001) and may inhibit *M. ulcerans* from growing in-vitro. *M. ulcerans* might be in an unculturable or difficult to culture state in the environment.

A role of reservoir of FLA for mycobacteria potentially explains the discrepancy between the fastidious nature of the bacteria and their widespread presence in the environment. If FLA would truly be the environmental hosts of *M. ulcerans* this would be very important since amoebae are common inhabitants of natural aquatic environments and water systems and they have been found to be resistant to extreme temperature, pH and osmolarity while encysted. This might explain the difficulty in isolating the bacteria from environmental specimens.

The aim of the study was to gain knowledge about the ecology of the environmental pathogen, *M. ulcerans*. It is hypothesized that one of the actual hosts of *M. ulcerans* could be a protozoon that may be involved in the transmission of the bacterium (Portaels *et al*., 2001). Internalization of *M. ulcerans* in FLA or the ability of the pathogen to form a biofilm (Marsollier *et al*., 2004b) may partly explain the difficulty in cultivating the bacterium from the environment.

Genomic analysis indicates that *M. ulcerans* is unlikely to be free-living in the environment but is instead undergoing adaptation to a specific ecological niche in which the product of ancestral genes are no longer essential (Stinear *et al*., 2007). This suggests that *M. ulcerans* may be host restricted. The demonstration of intracellular survival and/or multiplication of *M. ulcerans* and other mycobacteria in protozoal hosts (Jadin, 1975; Adekambi *et al*., 2006; Eddyani *et al*., in preparation) lend credence to this hypothesis.

Water bodies in areas of high BU endemicity are reported to significantly contain FLA than in low endemic areas (Eddyani *et al*, 2008). Recent studies also demonstrated that *M. ulcerans* has an intracellular phase in mammalian macrophages (Schutte *et al*., 2009; Torrado *et al*., 2007) although *M. ulcerans* infection is often characterized as extracellular. (Adusumilli *et al*., 2005; Cosma *et al*., 2003; Dobos *et al*., 1999; George *et al*., 1999; Hayman, 1993; van der Werf *et al*., 2005). Ingestion or inhalation of aerosols from contaminated water has been proposed by Hayman (1991) as a possible transmission route of *M. ulcerans*.

1.4 Study objective

To acquire knowledge about the reservoir and possible modes of transmission of *M. ulcerans*.

1.5 Specific objectives

1. *To* investigate whether *M. ulcerans* and other *mycobacteria* reside in FLA in nature.
2. To determine if there is an association between *M. ulcerans* and particular genus of FLA.
3. To determine the effect of temperature and pH in the isolation of FLA.
4. To determine whether the presence of coliforms affect the isolation of FLA.

CHAPTER TWO

2.0 Literature Review

2.1 Buruli ulcer

Mycobacterium ulcerans disease or BU is an indolent, necrotizing infection of the skin, subcutaneous tissue and occasionally bones (Portaels and Meyers, 2006). It is the third most common human mycobacteriosis worldwide after tuberculosis and leprosy. BU has emerged as an important health problem in over 30 countries worldwide; however, the major burden of the disease is borne by several rural communities in West and Central Africa (WHO, 2000). *M. ulcerans* is a slow growing pathogen thought to be transmitted to humans from aquatic environments. The reservoirs as well as the route of transmission of BU are yet to be determined.

2.2 History

The first report of BU is believed to date back to 1897, when Sir Albert Cook described ulcerative lesions consistent with BU in Uganda. In 1940, acid fast bacilli (AFB) were detected in a biopsy from ulcerative lesion of a patient in Bairnsdale, Australia. This was the first evidence that the disease, then known as Bairnsdale ulcer was caused by a *Mycobacterium*. The first definitive description of *M. ulcerans* was, however, published in 1948 when MacCallum and others in Australia reported lesions in different stages of BU disease in two Australian children and four adults in a riverine area in Bairnsdale, Victoria (MacCallum *et al*., 1948).

A group of experts, The Uganda Buruli Group investigated a large outbreak of BU near the river Kyoga in the Buruli County in Uganda and named the disease Buruli ulcer to commemorate the

first detection of this outbreak (Uganda Buruli Group, 1971). Since 1980, dramatic increases in the incidence of BU have been reported in the West African countries of Benin (Aguiar and Stenou, 1997), Côte d'Ivoire (Marston *et al*., 1995) and Ghana (Amofah, 1995). New foci have been also discovered in Togo (Meyers *et al*., 1996) and Angola (Bär *et al*., 1998).

The first probable case of BU in Ghana was reported in the Greater Accra Region in 1971 (Bayley, 1971). van der Werf *et al*., (1989) described 96 cases in the Asante Akim North District of Ashanti Region. This report was followed by the description of a major endemic focus in Amansie West District in the same region (Amofah *et al*., 1993).

In view of the growing spread and impact of the disease, the World Health Organization (WHO) in collaboration with the heads of state of Benin, Ghana and Cote d'Ivoire signed the Yamoussoukro Declaration on BU in 1998 to stimulate interest in BU research and also call on the international community to assist endemic countries to deal with the disease. This declaration led to the establishment of the Global Buruli Ulcer Initiative (GBUI), which is dedicated to raising awareness about BU, improving access to early diagnosis, treatment and prevention of disability, strengthening BU surveillance systems and promoting and supporting priority research in diagnosis, treatment and prevention of BU.

2.3 Aetiology

M. ulcerans, the causative agent of BU belongs to the genus *Mycobacterium* which is the only genus in the family Mycobacteriaceae in the order Actinomycetales. *M. ulcerans* produces a toxin, a polyketide-derived macrolide called mycolactone (George *et al*., 1999). Mycolactone is thought to be the major factor responsible for tissue necrosis in BU disease. *M. ulcerans* can be

cultured from human lesions on Löwenstein-Jensen (LJ) medium (or other media used for cultivation of mycobacteria) at an incubation temperature and oxygen concentration of 30–32 °C and 2.5% respectively. Colonies of *M. ulcerans* on LJ medium are non-chromogenic or slightly yellow and visible after 6-8 weeks of incubation at 30-32°C. *M. ulcerans* thrives under micro-aerophilic conditions (Palomino *et al*., 1998) and within a pH range of 5.4-7.4. Growth of *M. ulcerans* is impaired at 37°C or higher (Meyers *et al*., 1974; Eddyani *et al*., 2007).

2.4 *M. ulcerans* genome and evolution

Whole genome sequence of the epidemic strain Agy99 from Ghana consists of two replicons, a chromosome of 5632 kb and a 174,155 bp plasmid pMUM001 which contain 4160 and 771 genes and pseudogenes respectively (Stinear *et al*., 2007). There are 302 insertion sequence elements, comprising 213 copies of IS*2404* and 91 copies of IS*2606* in the genome. More than 60% of the pMUM001 contains six genes coding for proteins involved in mycolactone synthesis. The presence of numbers of insertion sequence elements, genome downsizing and accumulation of pseudogenes, together with a high degree of clonality (Stinear *et al*., 2007) gives indication that *M. ulcerans* has recently emerged. The *M. ulcerans* genome shows 98% nucleotide sequence identity and genome wide synteny with the genome of *M. marinum* which is thought to be the progenitor species from which *M. ulcerans* emerged.

2.5 Clinical presentation of BU

Two clinical forms of the disease are recognized; an active form which is an ongoing infection and an inactive form which is a depressed stellate scar with or without sequelae due to previous

infection (WHO, 2001). The active form is either ulcerative or non ulcerative (includes papule, nodule, plaque and oedema) and is characteristically painless, usually on the limbs.

A papule is a raised skin lesion less than 1 cm in diameter (figure 2.1). This form is usually found in Australian patients (WHO, 2000).

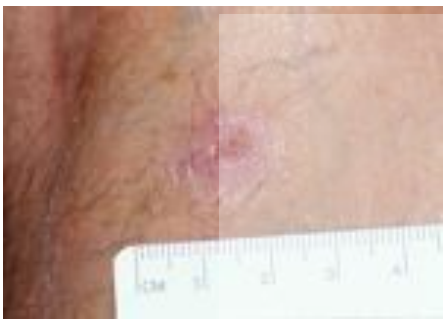

Figure 2.1 Papule (Photo; John Hayman, Australia)

A nodule is a palpable, often pruritic, firm lesion, 1-2 cm in diameter, situated in subcutaneous tissue and usually attached to the skin (figure 2.2). This form is usually seen in African patients.

Figure 2.2 Nodule (Photo; S. Etuaful, Ghana)

A plaque is a well demarcated, elevated, firm, indurated lesion more than 2 cm in diameter with irregular edges (figure 2.3). The skin around a plaque is often hypo pigmented in dark-skin people.

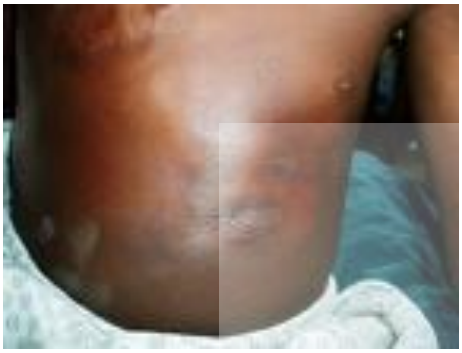

Figure 2.3 Plaque (Photo; A. Paintsil, Ghana)

An oedematous lesion is a diffuse, extensive, firm non-pitting swelling with ill-defined margins, may slightly be painful with or without colour changes over the affected skin and sometimes associated with fever (figure 2.4).

Figure 2.4 Oedema (Photo; K. Asiedu, Ghana)

A characteristics BU has scalloped edges and a base covered with necrotic slough (figure 2.5). Although the mycobacteria are concentrated in the center of the lesion, the ulcer is widely undermined, with vast extension of necrosis into the subcutaneous fat.

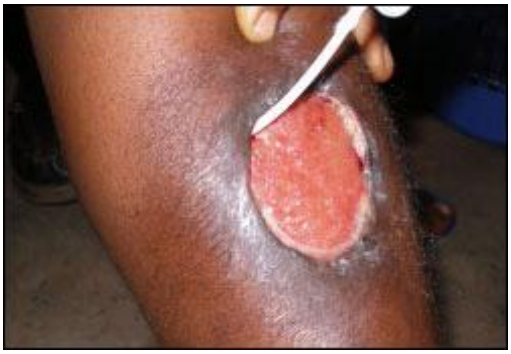

Figure 2.5 Ulcer (Photo; A. Tiendrebeogo, Nigeria)

2.6 Diagnosis of Buruli ulcer

2.6.1 Clinical diagnosis

The characteristic undermined edges, presence of necrotic slough and oedema surrounding the ulcer often make clinical diagnosis straightforward.

2.6.2 Laboratory diagnosis

The WHO has recommended four methods for the laboratory diagnosis of BU (WHO, 2001). These are microscopy, culture, PCR and histopathology. Specimens for diagnosis are tissue fragments, fine needle aspirates or swabs obtained from the necrotic base or undermined edges of ulcers.

2.6.2.1 Culture

M. ulcerans can be cultivated on mycobacterium isolation media (eg LJ, Ogawa and Middlesbrook media, however, LJ is most commonly used) following prior decontamination of diagnostic specimens. Cultures are visible after 6-8 weeks of incubation at 30-32 °C and appear

as slightly yellow or off-white colonies (figure 2.6). *M. ulcerans* can be identified using biochemical tests; however, due to lack of laboratory facilities, culture is not routinely done in most endemic countries. Sensitivity of culture is approximately 35 to 50% (Meyers *et al*., 1974; Portaels *et al*., 1997).

Figure 2.6 Colonies of *M. ulcerans* on LJ media (Photo; F. Portaels, Belgium)

2.6.2.2 Microscopy

Smears are made with swabs taken from BU lesions and stained using the Ziehl-Neelsen (ZN) method. Microscopic examination of positive smears reveals the presence of AFB (figure 2.7). Microscopy is simple to perform and a cost effective diagnostic method available at district health care centres in BU endemic countries. A major drawback of microscopy is its low sensitivity and specificity. The sensitivity of this method is about 40% because *M. ulcerans* bacilli are not uniformly located within tissue and their numbers tend to decrease over time (Portaels *et al*., 1997).

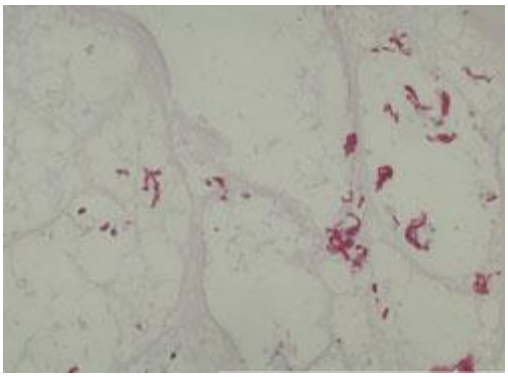

Figure 2.7 AFB in ZN stained specimen (W. Meyers, USA)

2.6.2.3 PCR

PCR diagnosis is based on demonstrating the presence of the insertion sequence IS*2404* by amplification with specific primers. IS*2404* PCR is sensitive due to the high copy number of IS*2404* and therefore makes this method highly specific for the diagnosis of BU; sensitivity is around 98% (Ross *et al*., 1997). Although PCR offers a rapid means of diagnosis, this method is unavailable in district hospitals in developing countries due to lack of trained laboratory personnel and high cost in running it routinely.

2.6.2.4 Histopathology

Haematoxylin and eosin stained sections of tissue fragments are examined microscopically for characteristic histopathologial changes such as coagulation necrosis, presence of ghost cells, extracellular AFBs and vasculitis; sensitivity is about 63 to 90% (Guarner *et al*., 2003; Portaels *et al*., 2001).

2.7 Treatment and prevention

Treatment aims at halting progression of the infection and repair of damaged tissue. Surgery has been the main stay of therapy in the past and patients presenting with pre-ulcerative lesions usually have good treatment outcome when their lesions are surgically excised although it is sometimes beset with recurrence of lesion (Amofah *et al*., 1998; Kanga *et al*., 2003; Teelken *et al*., 2003). However, for several reasons most patients report to hospitals with advanced ulcers requiring radical surgery and skin grafting. The ulcers are painless and the victims often live in remote communities, most patients do not receive medical attention until the damage is extensive.

In 2004, WHO published provisional guidelines recommending treatment with a combination of rifampicin and streptomycin (WHO, 2004) based on results of a small randomised controlled clinical trial (Etuaful *et al*., 2005). The study showed for the first time that antibiotic treatment of patients with nodules or plaques of early MU disease can render the diseased tissue culture negative within 4 weeks. It demonstrated clearly that antibiotics can penetrate the necrotic subcutaneous fatty tissue in which *M. ulcerans* organisms are seen on tissue sections (Etuaful *et al*., 2005). Patients in this study did not have ulcers, but results of a subsequent prospective study in Benin showed that small ulcers reduced in size during treatment for 4 weeks with rifampin and streptomycin and many were healed after 8 weeks (Chauty *et al*., 2005).

Physiotherapy is important for all BU patients to prevent contractures since lesions are mostly at the joints (WHO, 2001).

Prevention of *M. ulcerans* infection would best be accomplished by avoidance of exposure to the pathogen, unfortunately, such a strategy must wait until the reservoir is identified.

2.8 Complications and socioeconomic impact of BU

Mortality from BU is low, however, serious morbidity caused by the disease includes functional disabilities that result in permanent social, economic and developmental problems (WHO, 2000).The social and economic impact of BU is enormous and includes (i) Prolonged hospitalization (patients are hospitalized for more than three months and this represents a huge loss in productivity for adult patients and family caregivers), (ii) High treatment cost, (it is estimated that the average treatment cost per patient is US$ 780, an amount which far exceeded per capital government spending on health) and (iii) Loss of educational opportunities for children (high rate of school drop outs among children and stigmatization) (Asiedu and Etuaful, 1998). Due to the ugly appearance of such deformities, patients are socially alienated from their communities (WHO, 2000).

2.9 Epidemiology

2.9.1 Reservoir and Transmission of *M. ulcerans*

BU commonly occurs in swampy areas or areas in close proximity to slow-moving rivers (Portaels, 1978). Due to the difficulty in culturing *M. ulcerans*, molecular assays have been relied upon for the detection of the bacterium in environmental specimens. Previously IS*2404* PCR was used for the detection of *M. ulcerans* in environmental specimens. IS*2404* was detected in water (Ross *et al*., 1997), detritus (Stinear *et al*., 2000), aquatic insects (Portaels *et al*., 1999; Marsollier *et al*., 2002; Roberts and Hirst, 1997; Stinear *et al*., 2000), fish (Eddyani *et al*., 2004) and aquatic snails (Kotlowski *et al*., 2004; Marsollier *et al*., 2004). The presence of other mycolactone-producing mycobacteria (MPM) (Mve-Obiang *et al*., 2005; Ranger *et al*., 2006;

Rhodes *et al*., 2003, 2005; Yip *et al*., 2007) has made the interpretation of IS*2404* positive environmental specimens unreliable.

Fyfe *et al*., (2007) developed two multiplex real-time PCR assays that target IS*2404*, IS*2606* and ketoreductase B domain (KR) for detection and confirmation of the identity of *M. ulcerans* in environmental specimens. The change in cycle threshold (ΔC_T) value for the detection of IS*2404* and IS*2606* was used in distinguishing *M. ulcerans* from other MPM. *M. ulcerans* DNA has since been detected in mosquitoes (Fyfe *et al*., 2007; Johnson *et al*., 2007), soil and earthworm (Fyfe *et al*., 2007). Variable-number tandem repeat (VNTR) (Ablordey *et al*., 2005) typing further distinguishes *M. ulcerans* from other MPM in environmental specimens (Fyfe *et al*., 2007). It is therefore suspected that *M. ulcerans* is an environmental pathogen. However, the role of PCR positive environmental specimens as reservoir as well as the exact route of transmission of *M. ulcerans* remains to be determined.

These knowledge gaps, together with the absence of an effective vaccine and chemotherapy for all stages of the disease, make formulation of effective primary prevention strategies particularly difficult to achieve. BU therefore presents an enormous epidemiological challenge.

Following the detection of *M. ulcerans* sequences by PCR in aquatic insects, Portaels *et al*., (1999) hypothesized that *M. ulcerans* was directly inoculated into hosts through breaks in the skin from the contaminated environment such as water, soil, vegetation or aquatic insects.

Aquatic insects have increasingly been suspected to harbour and transmit *M. ulcerans*. Hemipterans of the family Naucoridae and Belostomatidae have tested positive for *M. ulcerans* in a number of studies (Marsollier *et al*., 2003; Portaels, 1995; Portaels *et al*., 1999). Also

experimentally infected *Naucoris cimicoides* have been demonstrated to transmit *M. ulcerans* to mice through biting (Marsollier *et al*., 2002).

For over half a century several attempts have been made at cultivating *M. ulcerans* from the environment with no success until Portaels *et al*., (2008) reported the first isolation and characterization of the pathogen from an aquatic insect (Gerris species). These notwithstanding, the role of insects as vectors of *M. ulcerans* have not been firmly established and a number of observations may not be consistent with the involvement of insects in transmission.

In the paper that reported the first isolation of *M. ulcerans*, the authors noted that *Gerris sp.* from which the bacterium was isolated do not bite humans and may therefore be a passive reservoir of *M. ulcerans* without an obvious role in transmission to humans (Portaels *et al*., 2008). Also Belostomatids and Naucorids rarely bite humans (only when they are disturbed) and their bite cause severe pain. However, most BU patients hardly remember painful bites from insects (Benbow *et al*., 2008). Their potential role as vectors has been questioned by studies done in Ghana which showed that the distribution of BU disease does not mirror the distribution of these aquatic insects (Benbow *et al*., 2008; Williamson *et al*., 2008).

Vertebrate mammals other than humans reported to be infected with *M. ulcerans* include Koalas (*Phascularta cinereus*), ringtail possum (*Pseudocheirus peregrinus*), alpacas (*Lamas pacos*), cat ((*Felis sylvestris catus*), dog (*Canis lupus familiaris*) and horses (*Equus ferus caballus*) (Elsner *et al*., 2008; Portaels *et al*., 2001).

Although epidemiological data do not support human to human transmission, a few reports suggest that this may be possible (Pettit *et al*., 1966; Radford *et al*., 1975; Smith, 1970).

The availability of whole genome sequence of *M. ulcerans* provides insights into the nature of this pathogen. Functional and comparative genomic analysis indicate that *M. ulcerans* has recently evolved from a generalist bacterium *M. marinum* to become a host adapted species (Stinear *et al*., 2007). Important genomic changes that marked this evolution include the accumulation of pseudogenes, proliferation of insertion sequence elements and acquisition via lateral transfer of a toxin encoding plasmid. These genomic markers suggest that *M. ulcerans* is unlikely to be free-living in the environment but is instead undergoing adaptation to a specific ecological niche in which the product of ancestral genes are no longer essential (Stinear *et al*., 2007).

Internalization of etiologic agents in other parasites is a recurring theme in biology and represents an evolutionary strategy for survival that may sometimes enhance pathogenesis or transmissibility.

2.10 FLA

FLA are a heterogenous group inhabiting the same biotopes containing distantly related organisms, which have been selected based on morphological and behavioral resemblance rather than on phylogenetic relationship. Therefore the term "FLA" does not represent a taxonomic category (Winiecka-Krusnell *et al*., 2001). *Acanthamoeba, Neagleria* and *Balamuthia* are known genera of potential human pathogens (Martinez and Visvesvara, 1997).

FLA are found worldwide (Rodriguez-Zaragoza, 1994) and have been isolated from soil (Anderson 2000, 2002; Nacapunchai *et al*., 2001), water (Arias Fernandez *et al*., 1989; Grimm *et al*., 2001; Hauer *et al*., 2001; Hoffmann and Michel, 2001; Kyle and Noblet, 1986, 1987), air

(Kingston and Warhurst, 1969; Rivera *et al*., 1987, 1991; Rodriguez-Zaragoza, 1994; Rodriguez-Zaragoza *et al*., 1993) and the nasal mucosa of human volunteers (Amann *et al*., 1997; Michel *et al*., 1994). FLA live at interfaces: water-soil, water-animal, water-plant, water-air etc., where they feed on bacteria, fungi, yeasts, algae and protozoa including other FLA (Rodriguez-Zaragoza, 1994). Their abundance and diversity in the environment are strongly dependent on season, temperature, moisture, precipitation, pH, and nutrient availability (Anderson, 2000; Bass and Bischoffl, 2001; Rodriguez-Zaragoza, 1994). FLA are more abundant at pH 7.6; their numbers are lower at pHs of 7.3 to 7.5 or 7.7 to 7.8, and are even lower at pH 7.0 to 7.2 and 7.9 to 8.0. Some amoebae, however, can tolerate pH of 8.6 or higher. FLA species normally tolerate temperatures between 10 and 30°C (Rodriguez-Zaragoza, 1994). Thermotolerants such as *Naegleri fowleri* grow very we11 above 37°C, and some strains of *Hartmannella* can even tolerate 48°C (Griffin, 1983). FLA have at least two developmental stages: the trophozoite, a vegetative feeding form, and the cyst, a resting form. Its most recognizable features include one or more nuclei and a simple contractile vacuole to maintain osmotic equilibrium. The trophozoite, the metabolically active stage, feeds on bacteria and multiplies by binary fission. Cysts generally have two layers, the ectocyst and the endocyst (Greub and Raoult, 2004).

2.11 Environmental mycobacteria

Environmental mycobacteria are normal inhabitants of a wide variety of environmental reservoirs (Primm *et al*., 2004) (including natural and municipal water, soil, aerosols, protozoans, animals and humans) which are also found to be colonized by FLA (Rodriguez-Zaragoza 1994; Nwachuku and Gerba, 2004). Environmental mycobacteria exhibit great variation in growth rates (2- to 48-h doubling times), colony morphologies (Cooper *et al*., 1998; Wright *et al*., 1996), antibiotic and biocide sensitivities (Cangelosi *et al*., 1999), plasmid carriage (Dale, 1995; Kirby

et al., 2002; Picardeau *et al*., 1998), and virulence (Cooper *et al*., 1998). Environmental mycobacteria include both slow-growing (i.e., colony formation requires 7 days or more) and rapidly growing (i.e., colony formation in less than 7 days) species. Slow growth of mycobacteria is due to the possession of either one (slow growers) or two (rapid growers, except *M. chelonae* and *M. abscessus*, which have only one) 16S rRNA cistrons (*E. coli* has seven operons) (Prammananan *et al*., 1998), impermeability of the lipid-rich cell wall and the synthetic energy cost of the long-chain mycolic acids (e.g., C60 to C90). Shared characteristics of environmental mycobacteria (along with the *M. tuberculosis* complex) are great hardiness, an acid-fast cell wall containing mycolates and intracellular pathogenicity (Primm *et al*., 2004). The same physiological factors which slow the growth and restrict the nutrient access of mycobacteria also grant tremendous compound and stress tolerance and provide favorable hydrophobic interactions facilitating nutrient acquisition, biofilm formation, and spread by aerosolization (Primm *et al*., 2004). Phylogenetic analysis of ribosomal 16S sequences suggests that slow growth is of recent evolution in mycobacteria and possibly of great adaptive value (Pitulle *et al*., 1992).

Dust can be rich sources of environmental mycobacteria, especially dust rich in peat (Primm *et al*., 2004). Environmental mycobacteria are found at air-water interfaces where complex hydrophobic hydrocarbons are found and enriched relative to bulk water (Primm *et al*., 2004). Biofilms from artificial water systems can be colonized by mycobacteria (September *et al*., 2004) and FLA (Barbeau and Buhler, 2001), suggesting possible close interactions. A strong association was shown between FLA and mycobacteria (*M. gordonae*, *M. kansasii* and *M. xenopi*) in biofilms sampled from hospital tap water (Thomas *et al*., 2006).

2.12 Mycobacteria within FLA

M. leprae was the first *Mycobacterium* sp. shown to survive in FLA (*Naegleria* and *Acanthamoebae)* forming globi of AFB (Jadin, 1975; Lahiri *et al*., 2008). The ability of *M. ulcerans* to enter FLA was demonstrated in-vitro (Krishna-Prasad *et al*., 1978). Other *Mycobacterium* species demonstrated to grow in FLA include *M. bovis* (Taylor *et al*., 2003), *M. xenopi* (Drancourt *et al*., 2007), *M. marinum* (Solomon *et al*., 2003), *M. massiliense* (Adekambi *et al*., 2004), *M. smegmatis* (Sharbati-Tehrani *et al*., 2005) and *M. avium* (Mura *et al*., 2006; Whan *et al*., 2006). *M. avium*, *M. fortuitum* and *M. marinum* all invaded and replicated inside *Acanthamoeba*, while the soil-dwelling *M. smegmatis* was killed (Mura *et al*., 2006). There is evidence that some mycobacterial species can persist for years within amoebal cytoplasm without detrimental effects (Yu *et al*., 2007). The interaction of the slow-growing pathogenic species *M. avium* has been reported with *A. castellanii* and *A. polyphaga* (Cirillo *et al*., 1997; Steinert *et al*., 1998; Whan *et al*., 2006), and also with *Tetrahymena pyriformis* and *Dictyostelium discoideum* (Strahl *et al*., 2001; Skriwan *et al*., 2002). Growth was observed within trophozoites, and saprophytically on secreted products (Steinert *et al*., 1998). A study demonstrated that 26 mycobacterial species tested were able to survive within *A. polyphaga* trophozoites and cysts (Adekambi *et al*., 2006). Among these, 20 have been reported to be present in water distribution systems (Vaerewijck *et al*., 2005).

2.13 Pathogenicity of amoebae-borne mycobacteria on mammalian cells

It has been proposed that bacterial virulence for mammalian cells may be a consequence of adaptations associated with intra-amoebal survival (Molmeret *et al*., 2005). Cirillo *et al*., (1997), showed that *M. avium* was able to inhibit phago-lysosomal fusion and grow within *A. castellanii*,

unlike nonpathogenic *M. smegmatis*. *M. avium* grown within *A. castellanii* were more efficient in both entry and intracellular replication within epithelial cells and macrophages compared to when grown in 7H9 broth (Cirillo *et al*., 1997).

These results suggested common mechanisms of mycobacterial virulence in *A. castellanii* and mammalian cells. Solomon *et al*., (2003) demonstrated that a *M. marinum* mutant with reduced growth in macrophages also had reduced growth in *D. discoideum*. Other studies further support the connection between virulence in FLA and mammalian cells in growth/cytotoxicity tests with *M. kansasii* strains in *A. castellanii* (Goy *et al*., 2007). Pathogenic subtype 1 *M. kansasii* strains grew better in *A. castellanii* than nonpathogenic subtype 3 strains, both in the number of bacteria per amoeba and the percentage of infected amoebae. Moreover, a *M. kansasii* subtype 3 strain isolated from blood culture, thus considered pathogenic, was also shown to grow similar to the pathogenic subtype 1 strains in *A. castellanii*. *M. avium* grown in *T. pyriformis* are more virulent in chickens than those grown in laboratory medium (Falkinham, 2002). *M. avium* can also grow on compounds released by *A. polyphaga* (Steinert *et al*., 1998) and *T. pyriformis* cells infected with *M. avium* grew more rapidly than uninfected *T. pyriformis* (J. Falkinham, unpublished data), suggesting an exchange of compounds during co growth.

These genetic adaptative changes include evolution by duplication of nonessential genes (Hughes, 2002) and by the lateral acquisition of genes (Ochman *et al*., 2000) that has led to the expression of symbiotic or pathogenic phenotypes according to their impact on the host cell. Thus, amoebae represent a potent evolutionary crib and an important genetic reservoir for its internalized microbes (Goebel and Gross, 2001).

2.14 FLA as Trojan horses of the microbial world

Aerosolized water is probably one of the predominant vehicles for transmission of internalized mycobacteria. It is only when aerosolized water was produced by new devices such as air-conditioning system, showers, clinical respiration devices, and whirlpool baths that *Legionella* became a recognized human pathogen during large outbreaks. However, protozoa had probably hosted *Legionella* spp. for millions of years (Dondero *et al*., 1980; Muder *et al*., 1986).

FLA may increase transmission by acting as a vehicle carrying huge numbers of microorganisms. This carrier role is particularly obvious for enteroviruses, which although not entering the amoebal cells, may persist by adsorption onto FLA and spread by these vehicles (Danes and Cerva, 1981). FLA may be more than simple vehicles; in addition, they may be "Trojan horses" for their host (Baker and Brown, 1994). Thus, the protozoal "horse" may bring a hidden amoeba-resistant microorganism within the human "Troy," protecting it from the first line of human defenses. Cirillo *et al*., (1997) definitively confirmed this hypothesis by demonstrating an increased colonization of the intestines and replication in the liver and spleen of mice when viable amoebae were inoculated with *M. avium* suggesting that the ability of *M. avium* to cross the intestinal epithelium was increased in presence of amoebae. The "Trojan horse" is also thought to protect the internalized bacteria from the first line of cell defenses in the respiratory tract (Danes and Cerva, 1981). FLA may also increase the transmission of amoebae-resistant bacteria by producing vesicles filled with bacteria (Anand *et al*., 1983; Berk *et al*., 1998; Greub and Raoult, 2002; Marolda *et al*., 1999; Rowbotham, 1986.). Vesicles have also been reported to contain *Burkholderia cepacia* (causes pneumonia in immunocompromised individuals with underlying lung disease especially cystic fibrosis patients), (Marolda *et al*.,

1999) and *Parachlamydia acanthamoebae* (causes pneumonia in humans) (Greub and Raoult, 2002).

2.15 Biofilms

Besides the different ways that individual microorganisms can evolve to resist protozoan predation, there is cumulative evidence for the existence of more complex antipredator mechanisms involving cell consortia. Biofilms are structured communities of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface (Costerton *et al*., 1999). Biofilms mostly occur at solid-liquid interface between a surface and aqueous medium providing an ideal environment for the attachment and growth of microorganisms (Donlan, 2002). Biofilms grow slowly in one or more locations and biofilm infections are often slow to produce overt symptoms (Ward *et al*., 1992). Biofilms have been identified as the predominant life-style of many bacteria, accompanied by an increasing awareness of their medical and ecological significance (Hall-Stoodley *et al*., 2004). The complexity of biofilm structure and metabolism has led to the analogy of biofilms to tissues of higher organisms (Costerton *et al*., 1995). From predator–prey studies of higher organisms (Duffy and Hay, 1990; Strathmann, 1986), sessile prey communities such as biofilms – face intense grazing pressure due to the lack of avoidance or escape options. However, the close proximity enables cells to interact and cooperate (e.g. by horizontal gene transfer (Hausner and Wuertz, 1999) and signaling (De Kievit *et al*., 2001)) and might have led to a more complex antipredator adaptations in bacterial biofilms compared with planktonic bacteria.

CHAPTER THREE

3.0 Materials and method

3.1 Study area

The study was carried out in surrounding communities of Agogo in the Asante Akim North district (population 149,491), the second most endemic BU district in Ghana (Amofah *et al*., 2002). There are 28 endemic communities in this district, however, sampling was done in the five most endemic communities: Ananekrom, Nshyieso, Serebouso, Dukusen and Bebuso (averagely 18 km apart) and two non endemic communities: Mageda and Pataban. These communities were selected based on patients' records available at the Agogo Presbyterian Hospital (APH), the district health facility serving all the endemic areas. The study could not be carried out in three other non endemic communities due to the absence of water bodies frequently sourced by the inhabitants for domestic activities (most communities use borehole water for domestic activities). The various activities carried out in the water bodies are shown in table 3.1. The APH is a designated training centre for BU management by both the WHO and the Ministry of Health of Ghana. Since 2005, three BU training workshops have been held in the hospital to train surgical teams from the various endemic regions in Ghana. Ananekrom is the most endemic community with three water bodies namely site 1, 2-upper and lower and site 3 which are about 3 km apart. Sampling was mainly done in site 1 since it was frequently used by inhabitants than the other two. Nshyieso and Bebuso has two water bodies each, however, we stopped sampling in site 1 of Nshyieso after the first month because it only served farming activites unlike the other site (2) which served domestic activities. All the other sampling sites have one water body each about 3-5 km apart from the residence of the inhabitants. All

communities except Nshyieso and Bebuso (water bodies are not surrounded by tree, looks more swampy) are served by gentle flowing stream/ river and villagers usually have a common point at which water is sourced. The locations of the sampling sites were determined using Garmin eTrex Venture HC GPS (figure 3.1). The study was approved by the ethics review board of Noguchi Memorial Institute for Medical Research.

Table 3.1 Information on sampling sites

				Clinically diagnosed positive cases by PCR	
Communities	**Water body**	**Activities**	**Population size**	**2008**	**2009**
Ananekrom	Egyaah river	Fishing, recreation, pathway for humans and animals, *domestic activities	1951	28	32
Nshyieso	Esuo-Efi river	*Domestic activities	1429	8	10
Serebouso	Onwamtifi river	*Domestic activities	1275	7	16
Dukusen	Onwam river	*Domestic activities	675	4	2
Bebuso	Pupunasoe river	*Domestic activities, human and animal crossing, fishing	966	6	4
Mageda	Abena Supuni river	Market traders drink from it	773	0	0
Pataban	Pataban river	drinking	1421	0	0

*Domestic activities- cooking, washing, bathing, drinking. Bu cases were confirmed by IS*2404* PCR by Kumasi Centre for Collaborative Research. The population size of the various communities was provided by census taken by the APH in 2009.

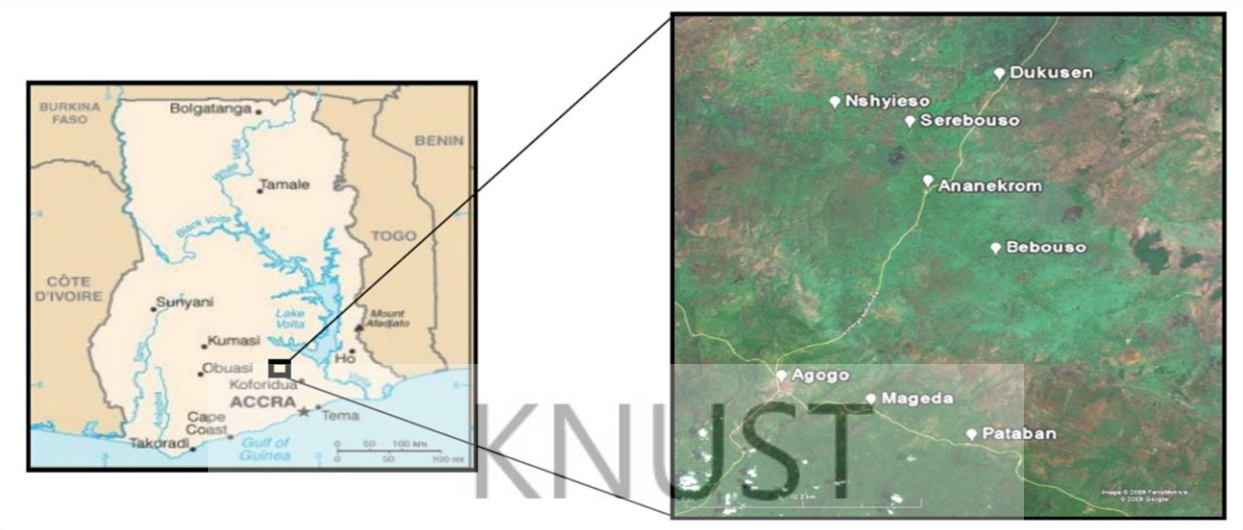

Figure 3.1 Map of sampling sites around Agogo

3.2 Collection and transport of specimen

Week long field visits were made monthly for ten months to randomly collect environmental specimens; water, biofilm from plants and tree trunk, detritus and aerosols (figure 3.2). The specimens were sampled around 6:00 am to 8:00 am; this is the peak period of human contact activities with water bodies. The pH and temperature of the water around the area from which specimens were taken was measured using water proof pH meter (Wagtech International, United Kingdom). Biofilms from tree trunks and plants in and around the water bodies were sampled using sterile cotton swabs and scalpels into 50 ml sterile falcon tubes. Detritus samples were taken from water bodies by hand into 50 ml sterile falcon tubes for the first two months as well as sampling from non endemic communities. During the last two months of sampling, non nutrient agar (NNA) plates seeded with *E. coli* were exposed around the water bodies for 30 minutes for isolation of amoebae from aerosols generated as they frequent the water bodies. Twelve litres of water were taken from mid column with buckets of which three to ten litres were concentrated via 0.45 µm membrane filters (Sartorius stedim, Germany) depending on the

turbidity of water. All specimens collected at the end of the field trip were transported in a cool box to the Bacteriology Department of Noguchi Memorial Institute for Medical Research (NMIMR) for microbiological and molecular analysis.

Figure 3.2 Environmental specimens; A-water, B-biofilms from submerged plant, C-biofilms from submerged tree trunk, D-aerosols generated as children walk through the waterbody.

3.3 Effect of bacterial load on the isolation of FLA

The bacterial load (total coliforms and *Escherichia coli*) of the water bodies was measured by dipping a commercially available kit; Envirocheck® Contact C (Germany) slide (coated with Chromocult Coliform agar) for about 5-10 seconds into the water body. The slide was incubated in an upright position for 48 hours at 37°C in the laboratory of APH. The bacterial load of the

water bodies was measured to give indications as to whether the FLA depended on bacteria as a source of nutrient (this was done after two months of sampling).

3.4 Specimen processing

The used membrane filters above were cut into pieces using sterile scissors and a piece of the filter was inoculated at the centers of 1.5% NNA plates seeded with *E. coli* for cultivation of FLA (Page, 1976) at 28.5ºC. Fifteen milliliters phosphate buffered saline (PBS) was added to the pieces of membrane filters, swabs and scalpels contained in 50 ml sterile falcon tubes and shaken vigorously to dislodge the substrate and biofilms from the surface. For detritus, 50 g was placed in stomacher bags and mixed with 100 ml 0.2% Tween 80 in PBS. The suspensions were transferred into 50 ml falcon tubes and allowed to settle for 1 minute. Two to three drops of the suspension of the specimens (biofilms and detritus) was inoculated on NNA for isolation of FLA. Two, five milliliters aliquot of each suspension were processed for isolation of intracellular (inside amoeba) and extracellular mycobacteria. The remaining suspensions were kept in 2 ml microfuge tubes and stored at -20°C as back up.

3.5 Isolation of FLA

The amoebae cultured plates were examined daily for the presence of trophozoites and cysts using the 10× objective of a bright field microscope. When trophozoites (with no fungal contamination) were observed growing away from the centre of inoculation, they were subcultured by lifting a small portion of the agar and inoculating it upside down on a new agar plate with a 10 µm inoculation loop (Ash and Orihel, 1987). Subculturing was repeated thrice to get rid of fungal contamination. Amoebae suspensions were made from the subcultures by

addition of 1.5 ml sterile distilled water to the plates and the surface scraped with an inoculating loop.

3.6 Isolation of extracellular mycobacteria from specimens

Five milliliters suspension of specimens (water, biofilms and detritus) were centrifuged at 1700×g for 5 minutes. The sediments were decontaminated using the oxalic acid method (Portaels *et al.*, 1989). Five milliliters of 0.2% malachite green, 5 ml of 1N sodium hydroxide and 1 ml of 0.8% cycloheximide were added to the suspensions. After shaking gently, the tubes were left to stand for 40 minutes at room temperature (RT) and centrifuged at 3000 ×g for 20 minutes. The sediments were resuspended in 10 ml 5% oxalic acid and left to stand for 30 minutes at RT after which they were centrifuged at 3000×g for 20 minutes. The sediments were resuspended in 0.5 ml PBS and inoculated on LJ media. The tubes were left slanted at room temperature overnight and then incubated upright at 28.5°C. The tubes were examined weekly for one year.

3.7 Isolation of intracellular mycobacteria from specimens

Five hundred microliters of kanamycin (200 µg/ml) was added to 5 ml of suspension (water, biofilms and detritus) and incubated overnight at 4°C to kill extracellular mycobacteria. Suspensions were centrifuged at 1700 x g for 5 minutes. Sediments were resuspended in 4.5 ml PBS and 0.5 ml of 5% SDS was added to lyse amoebae cells. Suspensions were centrifuged at 3000 x g for 20 minutes. The sediments were decontaminated using the oxalic acid method (Portaels *et al.*, 1989) and inoculated on LJ medium as described previously.

3.8 Isolation of intracellular mycobacteria from FLA

Fifty microliters of kanamycin (200 µg/ml) was added to 500 µl of amoebae suspension and left to stand overnight at 4°C to kill extracellular mycobacteria. Suspensions were centrifuged at

1700 rpm for 10 minutes. Sediments were resuspended in 450 µl PBS and 50 µl of 5% SDS was added to lyse amoebae cells. The suspensions were centrifuged at 14000 rpm for 10 minutes. Sediments were washed twice in 1000 µl PBS and centrifuged at 14000 rpm for 5 minutes. Sediments were resuspended in 0.5 ml PBS and inoculated on LJ media for a year.

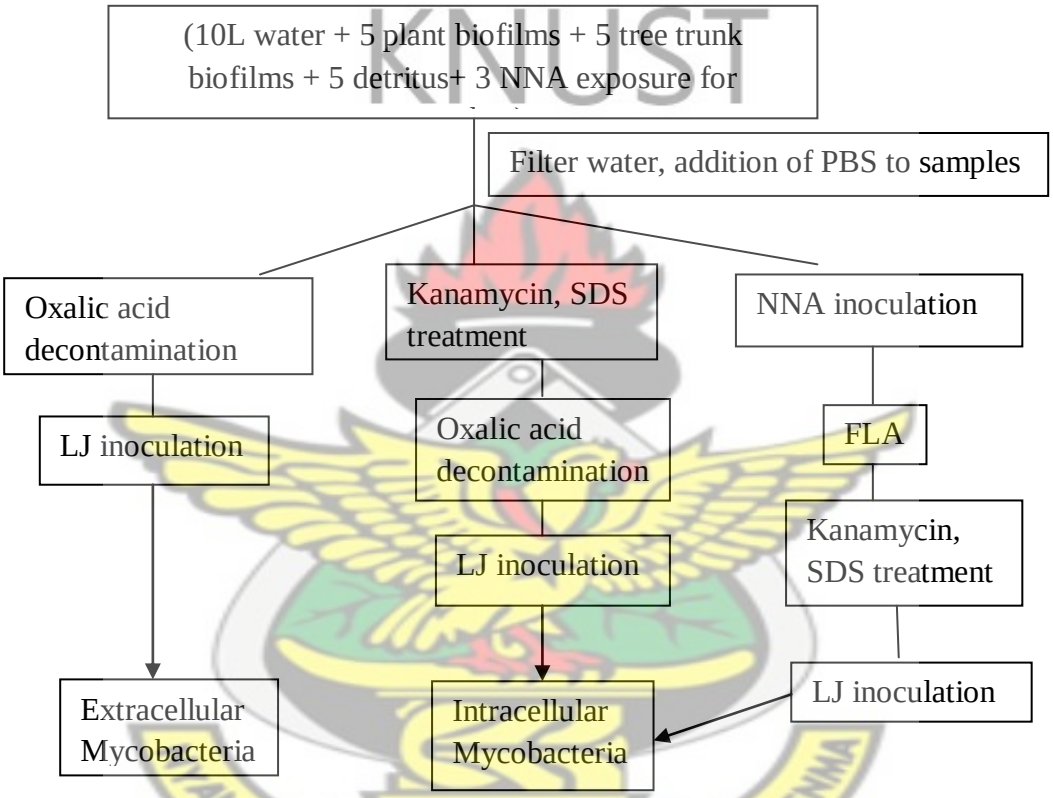

Figure 3.3 Flow chart for the cultivation of mycobacteria and FLA

3.9 Detection of genera of FLA

3.9.1 DNA extraction of FLA

Five hundred microliters of amoebae suspension was aliquoted into 1.5 ml microfuge tube and centrifuged at 4500 rpm for 10 minutes. DNA was extracted from the amoebae sediments using the UNSET method (Hugo *et al*., 1992). The cells were lysed with 500 µl UNSET lysis buffer

and equal volume of phenol: chloroform: isoamyl alcohol (25:24:1). Suspensions were centrifuged at 8000 rpm for 10 minutes and the aqueous phase was transferred to a new microfuge tube and treated with 500 µl of phenol: chloroform: isoamyl alcohol. To capture the DNA, 10 µl of 3 M NaCl was added to the aqueous solution (290 µl) after which 1000 µl absolute ethanol was added and mixed gently. The suspensions were kept at -20°C overnight and centrifuged at 15000 rpm for 30 minutes. The sediments were washed with 500 µl of 70% ethanol and centrifuged at 15000 rpm for 30 minutes. The pellets were dried in a heat block at 55°C and 100 µl TE was added. The DNA extracts were stored at -20°C until PCR analysis.

3.9.2 PCR for detection of the genera of FLA

3.9.2.1 PCR primers

Genus specific primers from the nucleotide sequence of 18S rDNA and internal transcribed spacers (ITS) including 5.8S rDNA were amplified for the identification of FLA (table 3. 2). For *Acanthamoeba,* Acanthamoeba-specific amplimer ASA.S1 was amplified using the primer set JDP1 and JDP2 derived from 18S rDNA (Dykova *et al*., 1999). For *Vahlkampfiidae* of the genus *Naegleria*, the ITS including 5.8S rDNA were amplified with the primer set ITS (De Jonckheere, 1998, 2004) and the DNA of other genera of *Vahlkampfiidae* was amplified using the more general primer set JITS (De Jonckheere and Brown, 2005).

Table 3.2 Primer for PCR identification of the genera of amoebae

Primer	**Sequence (5´-3´)**	**Genus of FLA**	**Expected amplicon size**
JDP1	GGGCCCAGATCGTTTACCGTGAA		423-551 bp
JDP2	CTCACAAGCTGCTAGGGGAGTCA	*Acanthamoeba*	
JITS*TF*	GTCTTCGTAGGTGAACCTGC	*Vahlkampfiidae*	350-800 bp
JITS*TR*	CCGCTTACTGATATGCTTAA		
ITS*TF*	AACCTGCGTAGGGATCATTT	*Naegleria*	300-750 bp
ITS*TR*	TTTCCTCCCCTTATTAATAT		

JDP1, JITS*TF* and ITS*TF*-forward primers, JDP2, JITS*TR* and ITS*TR*-reverse primers

3.9.2.2 PCR

PCR was performed in 25 µl reaction volume containing 1 µl DNA, 25 pmol/µl of each primer, 2.5 µl of 10× PCR buffer (containing 1.5 mM magnesium chloride), 5.0 µl Q-solution, 0.2 mM deoxynucleotide triphosphates (dNTPs) and 1.0 U HotStar *Taq* polymerase (QIAGEN). Amplification was carried out with a thermal cycler (Eppendorf mastercycler) as follows: denaturation at 95°C for 15 minutes, amplification for 40 cycles of 94°C for 45 seconds, 52°C for 45 seconds, 72°C for 45 seconds and a final extension at 72°C for 10 minutes.

3.9.2.3 Amplicon detection on agarose gel

A mixture of 4 µl of amplicon with 2 µl loading buffer was electrophoretically separated using 2% agarose in 1×TAE (0.04 M Tris-acetate and 0.001 M EDTA pH 8.0) buffer with ethidium bromide at 100V. The size of amplicons was estimated by comparison with 100 bp plus DNA ladder (Fermentas Life Sciences, EU) and visualized using Kodak Gel logic 100 Molecular Imaging System (USA).

3.10 Identification of mycobacteria

Direct smear examination (DSE) test using the ZN method was performed on single colonies from the LJ cultures for AFB. Cultures positive for AFB were subjected to 16S rRNA nested PCR using specific primers (table 3.4) after DNA extraction by boiling mycobacterial suspensions in TE for 10 minutes. Mycobacterial 16S-rRNA-DNA was also detected from amoebae cultures after extraction using the modified Boom method as described (Boom *et al*., 1990; Durnez *et al*., 2009) for detection of mycobacteria using the specified primer set (table 3.3) (Böddinghaus *et al*., 1990; Kirschner *et al*., 1993).

Table 3.3 Primers for 16S rRNA PCR

Primers	Sequence (5'- 3')	Nested PCR	Expected amplicon size
P1	TGCTTAACACATGCAAGTCG		
P2 new	TCTCTAGACGCGTCCTGTGC	first run	band of +/- 900 bp
P7	CATGCAAGTCGAACGGAAAGG		
P16new	AAGCCGTGAGATTTCACGAACA	second run	band of +/- 500 bp

P1 and P7- forward primers, P2new and P16new-reverse primers

3.10.1 PCR

First run amplification was performed in a 30 µl reaction volume containing 2 µl DNA, 25 pmol/µl of each primer, 5× Green GoTaq Flexi buffer, 25 mM magnesium chloride solution 10 mM dNTPs, and 5.0 U GoTaq DNA polymerase (Promega). Amplification: 94°C for 5 minutes followed by 40 cycles of 94°C for 45 seconds, 56°C for 45 seconds, 72°C for 45 seconds with final extension at 72°C for 10 minutes.

For the second PCR run, 1 µl of the first run product was added to 24.0 µl reaction mixture containing 25 pmol/µl of each primer, 5× Green GoTaq Flexi buffer, 25 mM magnesium chloride solution, 10 mM dNTPs, and 5.0 U GoTaq DNA polymerase (Promega). Amplification:

94°C for 5 minutes followed by 25 cycles of 94°C for 45 seconds, 58°C for 45 seconds, 72°C for 45 seconds with final extension at 72°C for 10 minutes. Electrophoresis was carried out as previously described.

Amplicons of 16S rRNA were sent to the VIB Genetic Service Facility (Antwerp, Belgium) for sequencing (figure 3.4). The sequenced data were compared to known sequences in the GenBank database and interpreted using the BlastN algorithm (available on http://www.ncbi.nlm.nih.gov/BLAST/). The 16S rRNA sequences were also mached against entries in the RIDOM (Ribosomal Differentiation of Medical Microorganisms) database (http://www.ridom-rdna.de/) by online analysis.

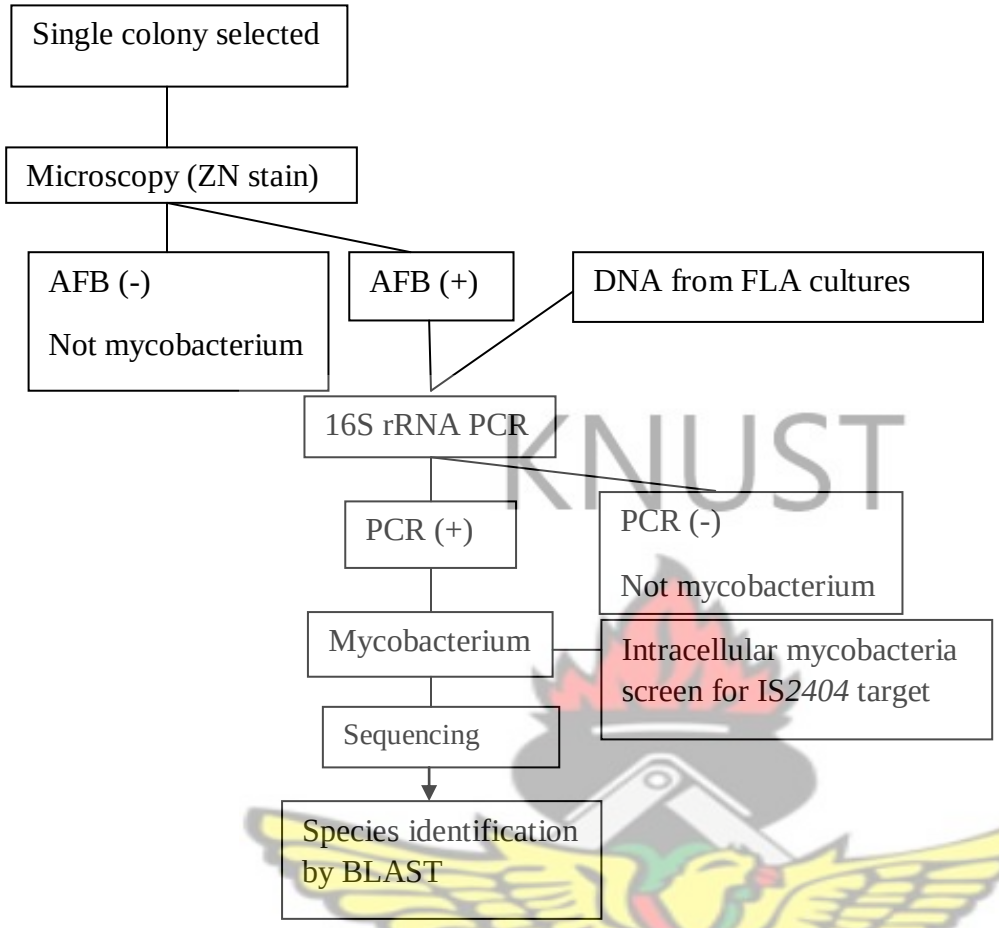

Figure 3.4 Flow chart for the detection of mycobacteria following culture isolation

3.11 Multilocus Variable-Number Tandem Repeat typing

Variable-Number Tandem Repeat (VNTR) typing was done on intracellular mycobacteria that tested positive for IS*2404* target to confirm whether it was similar to the strain causing disease in humans in that region (Lavender *et al.*, 2008). PCRs for VNTR loci 1, 4, 6, 8, 9, 14, 15, 18 and 19 (table 3.4) were performed using the following conditions as described by Ablordey *et al.*, (2005). The PCR was carried out using a Hotstar Taq DNA polymerase kit (Qiagen). DNA from mycobacterium (3 µl) was added to 27 µl of PCR mix containing 15.5 µl of water, 3 µl of 10× PCR buffer (containing 1.5 mM $MgCl_2$ at the final concentration), 0.4 mM of each primer, 0.2 mM of each dNTPs (Roche), 1× Q-solution and 0.1 µl of Hotstar Taq DNA polymerase (0.5 U).

Amplification was carried out with a thermal cycler (Eppendorf mastercycler) as follows: denaturation at 95°C for 15 minutes, amplification for 40 cycles of 94°C for 30 seconds, 59°C for 1 minute, 72°C for 1 minute 30 seconds and a final extension at 72°C for 10 minutes.

Electrophoresis was carried out as previously described.

Table 3.4 Primers for VNTR PCR

Locus	**Primers sequence (5´ to 3´)**
1	F GGCAGTGGGTGACGTCTCAGT
	R TCGAGGCGATCTACACCAAGGATTA
4	F GCCTTGCTTACCGTCGTGCCAA
	R CGAGCCAAGTTGGACCGTCAACACAT
6	F GACCGTCATGTCGTTCGATCCTAGT
	R GACATCGAAGAGGTGTGCCGTCT
8	F CGGATGACGTCGGAACTCTGA
	R GGACGCGGTAGCACGTTTTGT
9	F GGTGGATCTCCGCGTCATTTG
	R CGACCGCCCTCGAGACAG
14	F CCTTGTATCCGAGTTTCAGTT
	R GTCGACCAGATATGAGCAAT
15	F GCCACCGGTCAGGTCAGGTT
	R TCACCAACTACGACGGCGTTC
18	F CCCGGAATTGCTGATCGTGTA
	R GGTGCGCAGACTGGGTCTTA
19	F CCGACGGATGAATCTGTAGGT
	R TGGCGACGATCGAGTCTC

F- forward primer, R- reverse primer

3.12 Detection of *M. ulcerans* DNA

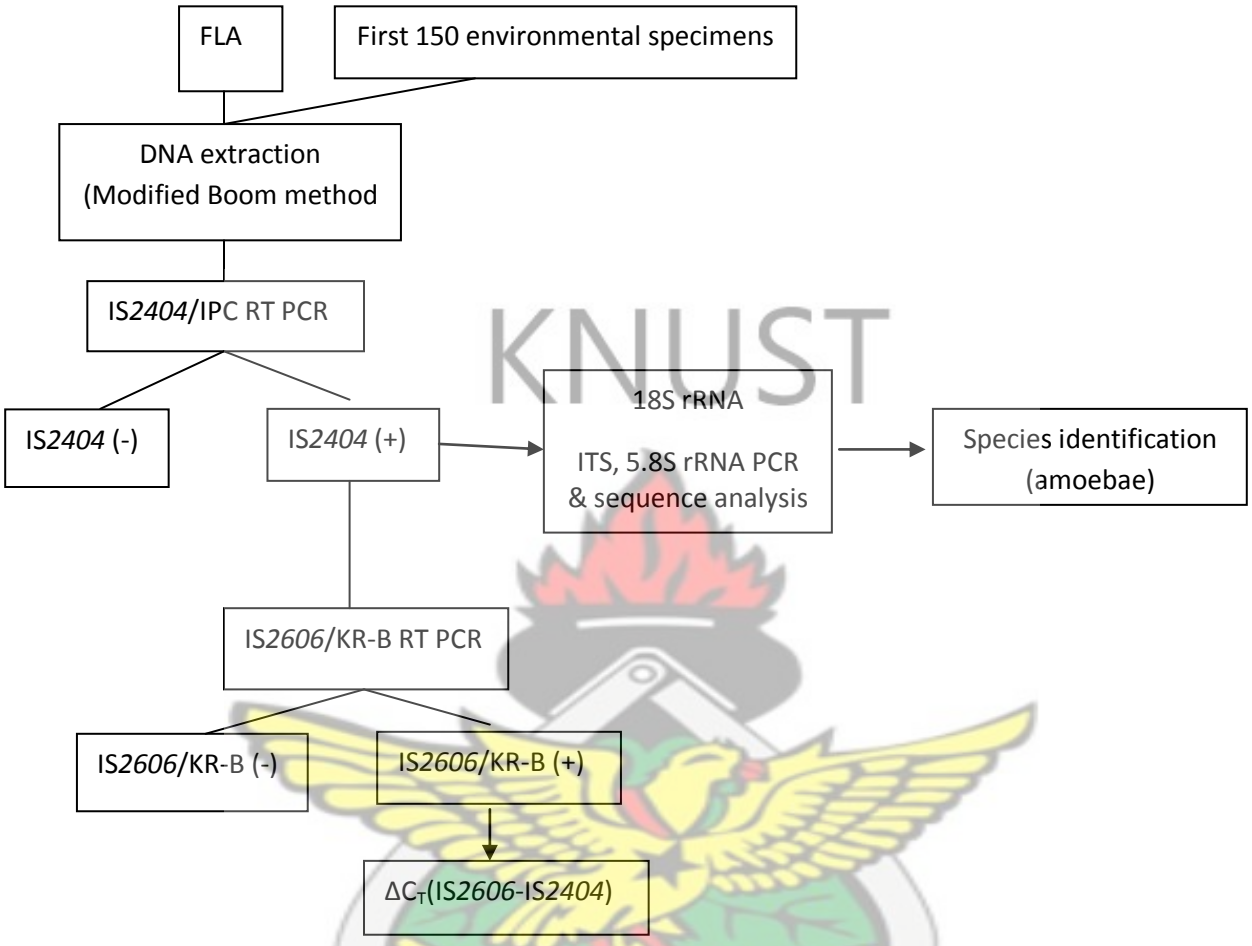

Figure 3.5 Flow chart of the detection of *M. ulcerans* and identification of FLA

3.12.1 DNA extraction of *M. ulcerans*

The modified Boom method (Boom *et al.*, 1990; Portaels *et al.*, 2008; Durnez *et al.*, 2009) was used for the extraction of DNA from the first 150 environmental specimens and of internalized *M. ulcerans* in FLA (figure 3.5). Volumes of 250 µl 2 x lysis buffer, 50 µl Proteïnase-K and 500 µl glassbeads were pipetted into a 1.5 ml microcentrifuge tube. Two hundred and fifty microliters of amoebae suspension was added together with positive (a suspension of heat

inactivated bacilli in TE) and negative (250 µl milli Q water) controls. The mixtures were incubated horizontally in a shaker (200 rpm) at 60°C overnight. To capture the DNA, 40 µl of diatomaceous earth solution was added to the suspensions and incubated between 60-120 minutes at 37°C (200 rpm). The mixtures were centrifuged at 14000 rpm for 10 seconds. The pellets were washed twice with 900 µl of 70% ethanol (2-8°C) followed by 900 µl of acetone and dried at 50°C for 20 minutes. The dried pellets were resuspended in 100 µl TE and centrifuged at 14000 rpm for 10 seconds.The DNA extracts were stored at -20°C.

3.12.2 Real-time (R-T) PCR for detection of *M. ulcerans*

Table 3.5 Primers and probes for R-T PCR

Primers and probes	**Sequence (5´-3´)**
IS*2404TF*	AAAGCACCACGCAGCATCT
IS*2404TR*	AGCGACCCCAGTGGATTG
IS*2404TP*	6 FAM-CGTCCAACGCGATC-MGBNFQ
IS*2606TF*	CCGTCACAGACCAGGAAGAAG
IS*2606TR*	TGCTGACGGAGTTGAAAAACC
IS*2606TP*	VIC-TGTCGGCCACGCCG-MGBNFQ
KR*TF*	TCACGGCCTGCGATATCA
KR*TR*	TTGTGTGGGCACTGAATTGAC
KR*TP*	6 FAM-CCCCGAAGCACTG-MGBNFQ

TF- forward primer, *TR*- reverse primer, *TP*- probe

Two multiplex R-T PCR was performed targeting three distinct sequences IS*2404*, IS*2606* and KR enzyme in the *M. ulcerans* genome with primers and probes (table 3.5) as described by Fyfe *et al*., (2007). The R-T PCR assays detected 0.01 genomes equivalent to two copies of IS*2404*, 0.1 genomes equivalent to 9 copies of IS*2606* and 1.5 to 3 copies of KR-B. The IS*2404* and KR were detected using 6 FAM labeled probes and IS*2606* was detected using the VIC labeled

probes at the 5´ end of the probe (reporter dye) and a minor groove binding non-fluorescent quencher (MGBNFQ) at the 3´end of all the probes (table 3.3).

All template DNAs were first screened for IS*2404* target and internal positive control (IPC) to check for inhibitors such as humic and fulvic acids (commonly found in environmental specimens) in PCR (figure 3.5). For the first run of R-T PCR (IS*2404* IPC multiplex), each well of a MicroAmp™ Optical 96-Well Reaction Plate contained 1 µl of the template DNA or 1 µl TE (no template control, NTC) or 2.5 µl Exo IPC Block (10X) (no amplification control, NAC), TaqMan® Universal PCR Master Mix (2X) - 12.5 µl, IS*2404TF* (0.9 µM) - 1.25 µl, IS*2404TR* (0.9 µM) - 1.25µl, IS*2404TP* (0.25 µM) -1.25 µl, Exo IPC DNA (50X) - 0.5 µl, Exo IPC Mix (10X) - 2.5 µl and Nuclease Free Water- 4.75 µl in a total volume of 25 µl. The plate was sealed with MicroAmp™ Optical Adhesive Film using the MicroAmp™ Adhesive Film Applicator.

The second run of R-T PCR (IS*2606* KR multiplex) was done on samples that turned out to be positive for the IS*2404* by detecting the presence of IS*2606* and KR. One microliter of the DNA extract was added to 24 µl PCR reaction mixture containing TaqMan® Universal PCR Master Mix (2X) - 12.5 µl, IS*2606TF* (0.9 µM) - 1.25 µl, IS*2606TR* (0.9 µM) - 1.25 µl, IS*2606 TP* (0.25 µM) - 1.25 µl, KR*TF* (0.9 µM) - 1.25 µl, KR*TR* (0.9 µM) - 1.25 µl, KR*TP* (0.25 µM) - 1.25 µl and Nuclease Free Water- 4 µl. Amplification and detection was carried out using the 7500 R-T PCR system (Applied Biosystems) with programmed conditions: 1cycle of 50°C for 2 minutes, 1 cycle of 95°C for 10 minutes (activation AmpliTag Gold enzyme), 40 cycles of 95°C for 15 seconds (melting) and 60°C for 1 minute (annealing/extending).

Control measures were taken in the processing of environmental specimens for extraction of DNA, PCR and post-PCR activities. These include space and time separation of pre and post-PCR activities, use of ultraviolet (UV) light before and after work, use of aliquoted PCR

reagents, addition of positive and negative controls (during DNA extraction, PCR (TE substituted for DNA template) and use of one or more contamination control methods that use chemical (eg. Bleach) reactions. Barrier-filter tips were used for all activities especially with post-PCR analysis to prevent contamination through generation of aerosols. Pre-PCR (DNA extraction and PCR preparation) and post-PCR (PCR execution and analysis) activities were performed in four separate rooms with dedicated equipments (pipettors, reagents, pipettor tips, racks, centrifuge etc.). DNA extraction and PCR preparation were done in safety cabinet with laminar flow and UV light. DNA extraction and PCR preparation were scheduled for the morning and PCR and post-PCR analysis in the afternoon.

3.13 Statistical analysis

Statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL) software. Logistic regression model was used to determine the effect of pH, temperature and coliform load in isolation of the genus of FLA. Chi-square was used to determine the association of the presence of *M. ulcerans* targets (IS*2404*, IS*2606* and KR-B) and FLA and p values < 0.05 were considered significant.

CHAPTER FOUR

4.0 Results

4.1 Isolation of FLA

Five hundred and thirty nine environmental specimens were collected from October 2008- July 2009. The specimens collected were; 13 aerosols, 223 biofilms from plant, 205 biofilms from trunks, 45 detritus, 53 water from five endemic and two non endemic communities. Amoebae were cultured from 405 (75%) specimens, however, the limited primers set used in the study permitted the classification into three genera. FLA cultures were confirmed by PCR from 369 (68.5%) specimens (table 4.1); *Acanthamoeba* 157 (29.1%), *Vahlkamfiidae* 306 (56.8%) and 118 (21.9%) *Naegleria*, some cultures had more than one genus of FLA. Further experiments were made only on the amoebae confirmed by PCR. FLA were isolated more frequently from aerosols and detritus (table 4.1) and from Nshyieso site 2 and Bebuso site 2 (table 4.2). The isolation frequency of FLA was significant with respect to the type of specimen and sampling site (tables 4.1 and 4.2) ($p= 0.001$ and $p= 0.027$).

Table 4.1 Isolation of FLA from environmental specimens

Type of sample	Amoebae		Total
	Negative	Positive (%)	
Aerosols	1	12 (92.31)	13
Biofilm plant	84	139 (62.33)	223
Biofilm trunk	63	142 (69.27)	205
Detritus	8	37 (82.22)	45
Water	14	39 (73.58)	53
Total	170	369	539

Table 4.2 Type of specimens collected and isolation of amoebae per sampling site

Community	Aerosols	Biofilm plant	Biofilm trunk	Detritus	Water	Total	Amoebae present (%)
Ananekrom site 1	1	38	32	4	11	86	55 (63.95)
Ananekrom site 2-upper	0	1	1	1	0	3	2 (66.67)
Ananekrom site 2-lower	0	1	1	1	0	3	1 (33.33)
Ananekrom site 3	0	2	2	2	1	7	5 (71.43)
Bebuso site 1	1	26	28	3	7	65	41 (63.08)
Bebuso site 2	2	15	9	0	2	28	23 (82.14)
Dukusen	4	49	39	8	10	110	66 (60.0)
Mageda	0	5	5	5	1	16	13 (81.25)
Nshyieso site 1	0	3	0	2	1	6	4 (66.67)
Nshyieso site 2	1	38	39	6	9	93	78 (83.87)
Pataban	0	5	5	5	1	16	11 (68.75)
Serebouso	4	40	44	8	10	106	70 (66.04)

NNA plates exposed to the atmosphere (aerosols) were discarded if the surface of the agar was touched by an insect, animal and human.

Table 4.3 Co-detection of mycobacteria and amoebae cultures

		Amoebae cultures		16S rDNA analysis on amoebae cultures	
		Negative	**positive**	**Negative**	**positive**
Intracellular mycobacteria	Negative	1	2	3	0
	Positive	35	109	66	78
Extracellular mycobacteria	Negative	0	3	2	1
	Positive	47	112	84	75

4.2 Effect of pH and temperature on isolation of FLA

The mean pH and temperature recorded were 6.41(median; 6.5) and 24.99°C (median; 25.9°C) respectively. We sought to ascertain whether there was any significant effect of pH and temperature in the isolation of FLA using a logistic regression model. Changes in pH levels did not affect the isolation of *Acanthamoeba* and *Vahlkampfiidae*, however, a rise in pH by one unit increased the isolation of *Naegleria* (by 0.399×). Temperature changes had no effect in the isolation of *Vahlkampfiidae,* however, it was observed that a rise in temperature by 1°C increased the isolation of *Acanthamoeba* (by 0.116×) and *Naegleria* (by 0.155×).

4.3 Effect of bacterial load in the isolation of FLA

The readings from the Envirocheck kit after incubation at 37°C after 48 hours were represented by pink dots for total coliforms and purple dots for *E. coli*. Total coliforms recorded were classified as follows (positive cultures/total number of water specimens examined), (figure 4.1):

10^3(very slight) - 15/45, 10^4(slight) - 23/45, 10^5(moderate) - 4/45, and 10^6(heavy) - 2/45 and 10^7(very heavy) - 1/45. Few water specimens were quantified as: moderate (4), heavy (2) and very heavy (1) with higher coliform load. A different classification was used for the identification of *E. coli* (not quantified): cultures were either positive (9/45) or negative (36/45) for the presence or absence of *E. coli* from the sites sampled (table 4.4). The presence or absence of *E. coli* in the water bodies had no effect in the isolation of the three genera of FLA identified (*Acanthamoeba*: p= 0.441, *Vahlkampfiidae*: p= 0.119 and *Naegleria*: p= 0.121). The coliform load did not have any significant effect in the isolation of the three genera of FLA (*Acanthamoeba*: p= 0.995, *Vahlkampfiidae*: p= 0.970 and *Naegleria*: p= 0.908).

Figure 4.1 Envirocheck slide showing classification of coliforms

Table 4.4 Frequency of the presence and absence of *E. coli* per sampling site

Communities	***E. coli***		**Total**
	Negative	**Positive**	
Ananekrom site 1	7	2	9
Bebuso site 1	7	0	7
Bebuso site 2	2	0	2
Dukusen	8	1	9
Mageda	1	4	5
Nshyieso site 2	4	4	8
Pataban	1	0	1
Serebouso	6	2	8
Total	36	9	45

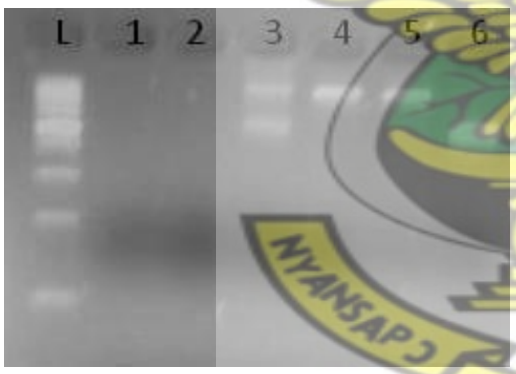

Figure 4.2 PCR analysis of intracellular mycobacteria isolates from specimens.

Lanes: L-100 bp ladder; 1 and 2-negative controls for PCR preparation and DNA extraction respectively; 3 to 5-positive 16S rRNA PCR results for mycobacteria (500 bp to 900 bp); 6-*M. ulcerans* positive control

4.4 Identification of environmental mycobacteria

Extracellular and intracellular mycobacterial isolates were cultured from 159 (29.5%) and 162 (30.8%) specimens respectively. All isolates were confirmed by DSE test for AFB and 16S rRNA PCR (figures 2.7 and 3.4). Mycobacterial DNA were also detected by 16S rRNA PCR in 159 (43.09 %) of 369 amoebae cultures. Intracellular mycobacteria were often isolated from specimens positive for isolation of amoebae (table 4.3). One hundred and thirty one intracellular mycobacteria were identified after sequence comparison to available database (NCBI and RIDOM 16S rDNA) using BLASTN (Basic Local Alignment Search Tool) (table 4.5). Eight sequences of the intracellular isolates were too short to be identified and 23 isolates had mixed growth which made identification impossible. One intracellular mycobacterium isolate was positive for IS*2404* target but negative upon repetition of R-T PCR for IS*2404*, IS*2606* and KR targets and also negative for the nine VNTR loci tested. This intracellular mycobacterium could not be identified after sequence comparison with available database as described previously. One intracellular mycobacterium was recovered in culture from directly lysed amoebae cultures after five months incubation but this isolate could not be identified using the 16S rRNA analysis. *M. ulcerans* was not isolated intracellularly from all methods explored. Mycobacteria (DNA) detected directly from amoebae cultures and isolated extracellularly were not identified to the species level since with those detected from amoebae cultures it might contain mixed mycobacteria making interpretation of results and identification difficult. Identification of extracellular mycobacteria was later found not to be of interest in addressing the present needs of the study.

Table 4.5 Identification of intracellular mycobacteria isolates from specimens

Intracellular mycobacterium	**Aerosols T.N=13 (%)**	**Biofilm plant T.N=223 (%)**	**Biofilm trunk T.N=205 (%)**	**Detritus T.N=45 (%)**	**Water T.N=53 (%)**
M. IWGMT (Internation Working Group on Mycobacterial Taxonomy)	0(0)	1 (0.45)	0 (0)	0(0)	1 (1.89)
M. alvei	0 (0)	1 (0.45)	1 (0.49)	0(0)	0 (0)
M. arupense	0(0)	11 (4.93)	19 (9.27)	7 (15.56)	5 (9.43)
M. arupense-like	0 (0)	5 (2.24)	2 (0.98)	2 (4.44)	1 (1.89)
M. arupense/arupense-like	0 (0)	3 (1.35)	0 (0)	1 (2.22)	0 (0)
M. asiaticum	0 (0)	1 (0.45)	0 (0)	0 (0)	1 (1.89)
M. branderi	0 (0)	1 (0.45)	2 (0.98)	0 (0)	0 (0)
M. branderi/simiae	0 (0)	0 (0)	0 (0)	0 (0)	1 (1.89)
M. celatum	0 (0)	2 (0.90)	0 (0)	0 (0)	0 (0)
M. cookii	0 (0)	1 (0.45)	2 (0.98)	0 (0)	0 (0)
M. fortuitum	0 (0)	4 (1.79)	4 (1.95)	1 (2.22)	1 (1.89)
M. fortuitum/arupense	0 (0)	1 (0.45)	0 (0)	0 (0)	0 (0)
M. gilvum	0 (0)	0 (0)	1 (0.49)	0 (0)	0 (0)
M. gordonae	0 (0)	2 (0.90)	1 (0.49)	0 (0)	0 (0)
M. gordonae/fortuitum	0 (0)	1 (0.45)	0 (0)	0 (0)	0 (0)
M. interjectum	0 (0)	1 (0.45)	0 (0)	0 (0)	0 (0)
M. interjectum/conspicuum	0 (0)	0 (0)	1 (0.49)	0 (0)	0 (0)
M. intracellulare	0 (0)	2 (0.90)	1 (0.49)	0 (0)	0 (0)

Table 4.5 (continued)

Intracellular mycobacterium	Aerosols T.N=13 (%)	Biofilm plant T.N=223 (%)	Biofilm trunk T.N=205 (%)	Detritus T.N=45 (%)	Water T.N=53 (%)
M. intracellulare/simiae	0 (0)	0 (0)	1 (0.49)	0 (0)	0 (0)
M. lentiflavum	0 (0)	3 (1.35)	1 (0.49)	0 (0)	2 (3.77)
M. nebraskense	0 (0)	1 (0.45)	0 (0)	0 (0)	1 (1.89)
M. nonchromogenicum	0 (0)	0 (0)	3 (1.46)	0 (0)	0 (0)
M. paraffinicum	0 (0)	2 (0.90)	0 (0)	0 (0)	0 (0)
M. paraffinicum/scrofulaceum	0 (0)	1 (0.45)	0 (0)	0 (0)	0 (0)
M. peregrinum	0 (0)	1 (0.45)	0 (0)	0 (0)	0 (0)
M. peregrium/septicum	0 (0)	2 (0.90)	0 (0)	2 (4.44)	0 (0)
M. scrofulaceum	0 (0)	3 (1.35)	0 (0)	0 (0)	1 (1.89)
M. simiae	0 (0)	3 (1.35)	0 (0)	0 (0)	0 (0)
M. simiae/arupense	0 (0)	0 (0)	1 (0.49)	0 (0)	0 (0)
M. terrae	0 (0)	2 (0.90)	4 (1.95)	0 (0)	1 (1.89)
M. terrae-like	0 (0)	0 (0)	1 (0.49)	1 (2.22)	0 (0)
M. terrae/terrae-like	0 (0)	0 (0)	1 (0.49)	0 (0)	0 (0)
Mycobacterium sp.	0 (0)	1 (0.45)	0 (0)	0 (0)	0 (0)

T.N is total number of specimens. *Mycobacterium sp.-like* refer to nucleotides with mismatches between 1-8 after blasting and sequence comparison with the reference from NCBI database. When mismatches exceed 8, it was identified as *Mycobacterium sp.* Some specimens contained more than one mycobacterium.

4.5 Detection of *M. ulcerans* DNA

The insertion sequence *2404* was detected from DNA in environmental specimens as well as in amoebae cultures. Three specimens were positive for IS*2404* target and one of the three specimens was further positive for the other two targets; IS*2606* and KR-B (table 4.6). The change in ΔC_T (IS*2606*-IS*2404*) value was 1.96 suggesting that *M. ulcerans* DNA was detected and not DNA from other MPM (Fyfe *et al*., 2007). Twenty five (4.6%) amoebae cultures tested positive for IS*2404* target (table 4.6) and all but one of the positive IS*2404* amoebae culture were positive for 16S rDNA analysis for detection of mycobacteria. The C_T values ranged from 29.46 to 38.05 with an average of 35.91. None of the IS*2404* positive amoeba cultures tested positive for IS*2606* and KR-B targets. The IS*2404* positive amoebae cultures were isolated from endemic as well as non endemic communities and often from biofilms (figure 4.3 and table 4.6). Amoebae cultures derived from the two specimens that tested positive for IS*2404* but negative for the other two targets, were negative for all three targets; IS*2404*, IS*2606* and KR-B. We could not isolate amoebae (we rather observed cyst which did not grow when subcultured) from the specimen that tested positive for *M. ulcerans* although an amoebal DNA (*Vahlkampfiidae*) was detected by PCR in the specimen. Identification to the species level of the IS*2404* positive amoebae isolates is ongoing. The distribution of IS*2404* positive amoebae cultures over the sampling period as shown in figure 4.4 peaked in the ninth month (June). No association was found between *M. ulcerans* and FLA, however, the study indicated a significant association between FLA and one of the DNA targets assayed; IS*2404* [25 (4.6%) χ_1^2 = 9.144 p = 0.002] but not with the other two targets; IS*2606* and KR-B. *Acanthamoeba* and *Vahlkampfiidae* genera showed significant association with IS*2404* target (χ_1^2 = 6.643 p = 0.010; χ_1^2 = 5.764 p = 0.016, 95% confidence level respectively). The water specimen from which all three targets were detected was sourced from

Nshyieso site 2 which has a small but deep pool of water surrounded by tall shaded trees about 2-3 km from the nearest house unlike the other water bodies that are gentle flowing streams and rivers. Ananekrom, which reports the highest numbers of cases in the country, recorded six IS*2404* positive amoebae. This community has three water bodies two of which are surrounded by tall trees, and are sourced for domestic use. The water bodies serves as pathways for humans and animals (table 3.1).Farming is the major activity of the people.

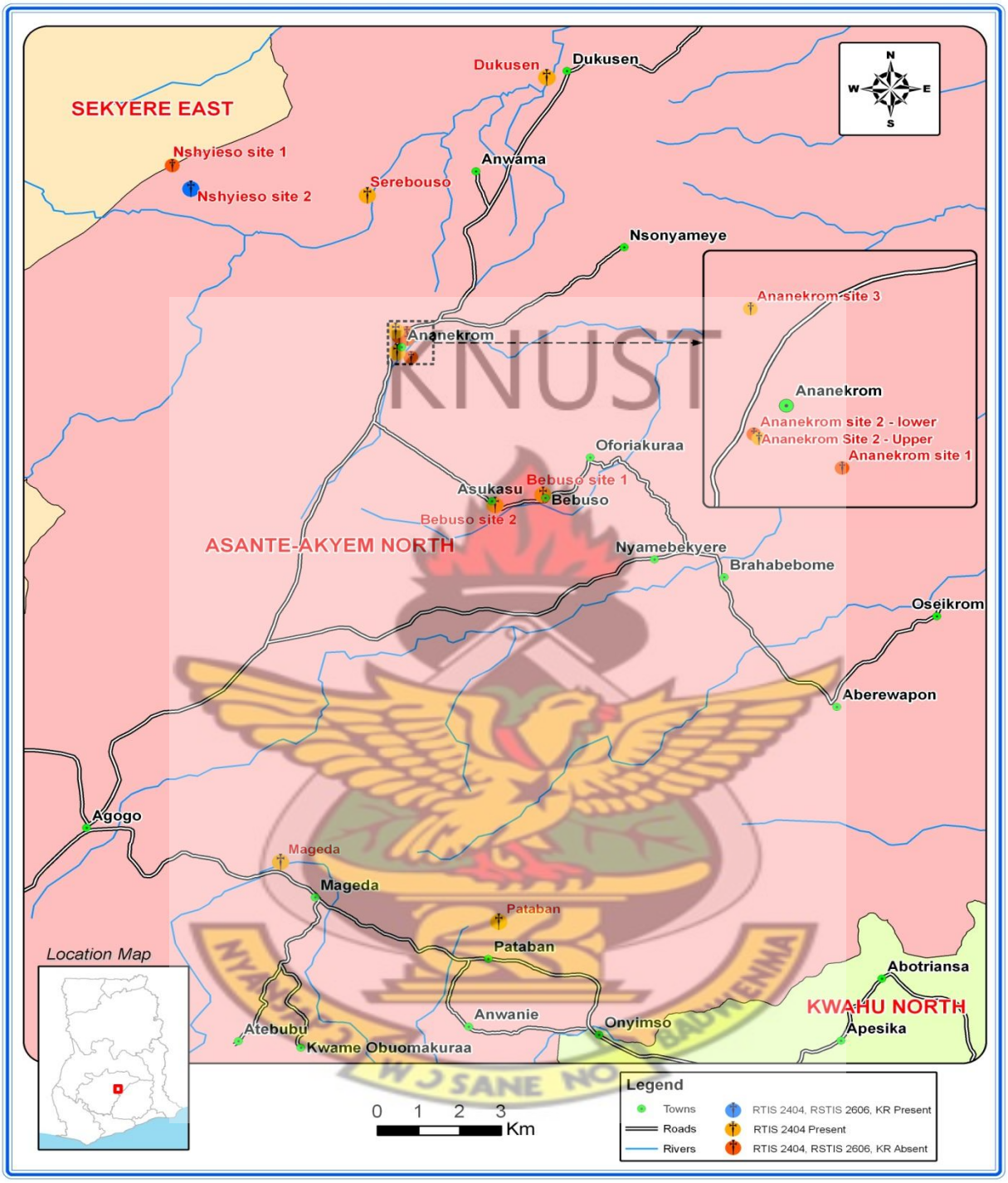

Figure 4.3 Map of sampling sites and distribution of the presence and absence of IS*2404*, IS*2606* and KR-B targets for *M. ulcerans*

Table 4.6 R-T PCR C_T values of specimens and amoebae cultures

DNA extract	**Community**	**Habitat**	**C_T IS*2404***	**C_T IS*2606***	**C_TKR-B**
Specimen	Nshyieso site 2	Water	36.31	38.27	37.6
Specimen	Serebouso	Biofilm trunk	38.45	ND	ND
Specimen	Pataban	Biofilm plant	37.95	ND	ND
Amoebae	Ananekrom site 2-upper	Biofilm plant	37.37	ND	ND
Amoebae	Ananekrom site 3	Detritus	30.96	ND	ND
Amoebae	Serebouso	Biofilm plant	36.78	ND	ND
Amoebae	Dukusen	Detritus	35.73	ND	ND
Amoebae	Nshyieso site 2	Biofilm trunk	37.14	ND	ND
Amoebae	Mageda	Detritus	37.48	ND	ND
Amoebae	Pataban	Detritus	35.94	ND	ND
Amoebae	Ananekrom site 1	Biofilm trunk	30.22	ND	ND
Amoebae	Serebouso	Biofilm plant	29.72	ND	ND
Amoebae	Bebuso site 1	Biofilm plant	29.46	ND	ND
Amoebae	Serebouso	Aerosols	36.28	ND	ND
Amoebae	Serebouso	Aerosols	37.05	ND	ND
Amoebae	Dukusen	Biofilm trunk	35.83	ND	ND
Amoebae	Bebuso site 2	Water	36.83	ND	ND
Amoebae	Bebuso site 2	Biofilm plant	35.96	ND	ND
Amoebae	Bebuso site 1	Biofilm trunk	36.84	ND	ND
Amoebae	Bebuso site 1	Biofilm trunk	36.27	ND	ND
Amoebae	Ananekrom site 1	Water	37.49	ND	ND

Table 4.6 (continued)					
DNA extract	**Community**	**Habitat**	**C_T IS*2404***	**C_T IS*2606***	**C_TKR-B**
Amoebae	Ananekrom site 1	Biofilm plant	35.99	ND	ND
Amoebae	Ananekrom site 1	Biofilm plant	38.05	ND	ND
Amoebae	Nshyieso site 2	Biofilm plant	36.77	ND	ND
Amoebae	Nshyieso site 2	Biofilm plant	37.24	ND	ND
Amoebae	Nshyieso site 2	Biofilm trunk	37.10	ND	ND
Amoebae	Nshyieso site 2	Biofilm trunk	37.07	ND	ND
Amoebae	Nshyieso site 2	Biofilm trunk	37.28	ND	ND

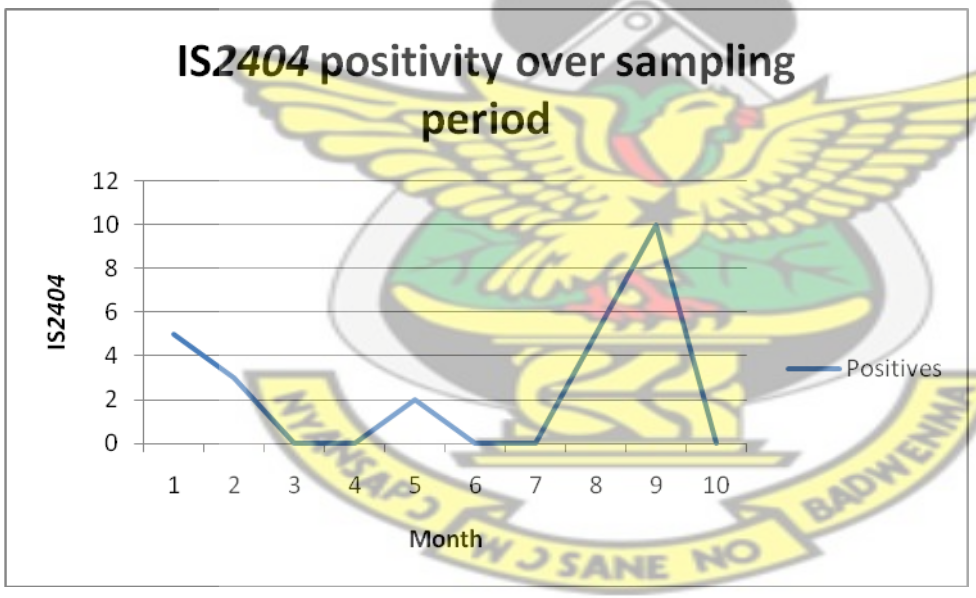

Figure 4.4 Distribution of IS*2404* positive amoebae cultures over the sampling period (October 2008-July 2009)

CHAPTER FIVE

5.0 Discussion

Mycobacteria and other micro-organisms are known to interact with FLA, which act as 'Trojan Horses' in microbial ecology (Barker and Brown, 1994). Mycobacteria are widely distributed in the environment and can be recovered from various sources (Primm *et al*., 2004), which are also found to be colonized by FLA (Rodriguez-Zaragoza 1994; Nwachuku and Gerba 2004). These include natural and municipal water, soil, aerosols, protozoans, animals and humans. Biofilms from artificial water systems can be colonized by mycobacteria (September *et al*., 2004) and amoebae (Barbeau and Buhler 2001), suggesting possible close interactions. A strong association has been shown between amoebae and mycobacteria (*M. gordonae*, *M. kansasii* and *M. xenopi*) in biofilms sampled from hospital tap water (Thomas *et al*., 2006).

It has been suggested that *M. ulcerans* will be difficult to persist in the environment as a free living organism due to its natural fragility (Portaels *et al*., 2001) and may be maintained in a commensal relationship in hosts that protect the bacilli against unfavourable physical parameters of the environment. Reductive evolution and niche adaptation inferred from the genome of *M. ulcerans* implicates the inability of *M. ulcerans* to withstand exposure to direct sunlight and diminished capacity of free-living (Stinear *et al*., 2007). Internalization of *M. ulcerans* in FLA or the ability of the pathogen to form a biofilm (Marsollier *et al*., 2004) may partly explain the difficulty in cultivating the bacterium from the environment. In accordance with this assumption the study investigated the hypothesis that FLA could serve as reservoir in the transmission of *M. ulcerans*.

FLA cultures confirmed to the genus level were obtained from 68.6% of specimens sampled from environment. FLA were isolated frequently from aerosols and detritus although these specimen types were not collected throughout the sampling period (table 4.1). The isolation of amoebae was as frequent in endemic as in non endemic communities (table 4.2) and around water bodies that were surrounded by tall trees as in swampy areas.

FLA were isolated from the laboratory at a temperature of 28.5ºC (average of the temperatures recorded after sampling for two months) to mimic temperatures from the water bodies. Analysis of the recorded pH and temperature indicates that a rise in pH by one unit increases the isolation of *Naegleria* and an increase in temperature of 1°C increases the isolation of *Acanthamoeba* and *Naegleria* (above the average pH and temperature recorded). *Vahlkampfiidae* was the most isolated genus and was independent of changes in these recorded parameters. The recorded temperature (median; 25.9°C) was within the tolerable temperature range of 10-30°C for amoebae but a pH of 6.5 (median) differed with that of a previous study (pH of 7.6) (Rodriguez-Zaragoza, 1994). This could be due to adaptation of FLA to different pH ranges in different geographical aquatic habitats.

Isolation of FLA was independent of bacterial load, specifically the presence of *E. coli* (faecal contamination) (table 4.4) detected from the water bodies sampled. This could imply their dependence on other sources of nutrients; fungi, yeasts, blue-green algae and protozoa (Rodriguez-Zaragoza, 1994).

Our data showed that FLA and other protozoa may be part of the reservoir of the mycobacteria species; *M. IWGMT, M. alvei, M. arupense, M. asiaticum, M. branderi, M. celatum, M. cookii, M. fortuitum, M. gilvum, M. gordonae, M. interjectum, M. intracellulare, M. simiae, M. lentiflavum, M. nebraskense, M. nonchromogenicum, M. paraffinicum, M. scrofulaceum, M.*

peregrinum, *M. septicum* and *M. terrae* (table 4.5). A number of *Mycobacterium sp*. have been reported to live/survive intracellularly in amoeba; *M. simiae*, *M. fortuitum*, *M. septicum*, *M. peregrinum*, *M. terrae*, *M. gordonae*, *M. intracellulare* and *M. lentiflavum* (Krishna-Prasad *et al*., 1978, Adekambi *et al*., 2006). The possibility that the intracellular (mycobacteria inside amoebae) mycobacteria might have been contaminated with extracellular mycobacteria during processing of specimens was well considered but unlikely since 500 µl of kanamycin (20 µg/ml) was added to each (5 ml) specimen (water, biofilms and detritus) and incubated overnight at 4°C to kill extracellular mycobacteria with 0.5 ml of 5% SDS added to lyse amoebae cells. Although mycobacteria have been reported to be intrinsically resistant to disinfection due to their complex cell wall structure in comparison to other vegetative bacteria (McDonnell, 2007), the survival of extracellular mycobacteria for 20-24 hours in 20 µg/ml concentration of kanamycin and 5% SDS was doubted. Rather, we know intracellular mycobacteria will be protected from antibiotics. Report from a previous study showed that *M. avium* strains living in *A. castellanii* had increased resistance to antibiotics in comparison with macrophages (Miltner and Bermudez, 2000). Resistance may have been because of limited penetration (e.g. related to decreased uptake or inactivation within amoebae), or changes in the phenotype of the bacteria once internalized within protozoa. Some of the mycobacteria species isolated have been recovered from patients (*M. septicum*, *M. fortuitum* and *M. gordonae*) (Martin-Casabona *et al*., 2004; Adekambi and Drancourt, 2006) and associated with a devastating outbreak in fish colony (*M. septicum*) (Kent *et al*., 2004). This demonstrates the potential significance of the association between FLA and mycobacterial species involved or potentially associated with human and animal infections.

Not all specimens positive for isolation of intracellular mycobacteria were confirmed to contain amoebae. This could be due to the limited number of genera of amoebae identified since some

amoebae cultures identified morphologically were negative by PCR for the genera of amoebae assayed. Probably some of these mycobacteria may have been internalized in other protozoa.

We did not pursue further investigation due to time constrains into the isolate with discordant results (initially tested positive but subsequently negative for IS*2404*).

Although the study showed no association between the type of specimen or isolation of FLA and isolation of intracellular/extracellular mycobacteria, intracellular mycobacteria were often isolated from specimens positive for isolation of amoebae (table 4.3). Also the study showed a link between the detection of mycobacteria from amoebae cultures and the isolation of intracellular mycobacteria. Intracellular mycobacteria were frequently isolated from specimens positive for the isolation of amoebae and positive for detection of mycobacteria from amoebae cultures. The low isolation by culture, however, could be due to slow growth rate or viable-but-non-culturable state of internalized mycobacteria in planktonic or adherent biofilms as stated to exist *insitu* in natural, industrial, hospital and domestic environments of microorganisms (Costerton *et al*., 1995; 1999; Brown *et al*., 1995). Supporting this statement is the evidence of growth of 1/369 intracellular mycobacterium from lysed amoebae suspension after five months. Isolation of environmental mycobacteria in rich media naturally non representative of a nutrient-depleted, starved and stressed environment could account for the low isolation rate (Angenent *et al*., 2005; Brown and Williams, 1985; Brown and Barker, 1999). FLA can act as important microbial reservoir in these nutrient deprived environments. Also effects of pH, temperature, aeration, osmolarity and starvation by different nutrients resulting in diversity of bacterial phenotypes (planktonic and surface adherent biofilms) in the environment may not be the same as laboratory growth conditions and therefore affect isolation rate. Contamination of some cultures with fungi may also have accounted for the low rate of isolation.

In 2008, Eddyani *et al*. reported a link between the occurrence of FLA and BU endemicity. *A. polyphaga* were experimentally infected with *M. ulcerans* and was shown to persist and survive for 14 days (Eddyani *et al*., unpublished data). Based on the above information it was reasoned that the intracellular survival or replication of *M. ulcerans* in FLA may provide the necessary mechanisms for their persistence in the environment. Data analysis of DNA from environmental specimens and amoebae cultures from the R-T PCR (assays targeting IS*2404*, IS*2606* and KR-B) showed that there is no association between *M. ulcerans* and FLA. IS*2404* target was detected in endemic as well as non endemic communities. Only one specimen (water) tested positive for all the three targets assayed (table 4.6). Although the change in ΔC_T (IS*2606*-IS*2404*) value suggested that *M. ulcerans* DNA was detected (Fyfe *et al*., 2007), we were unable to isolate but rather detected amoebal DNA (*Vahlkampfiidae*) from the specimen. We focused mainly on trying to detect *M. ulcerans* DNA from amoebae cultures and not from environmental specimens also positive for amoebae. Twenty five amoebae cultures were positive for IS*2404* target but not for the other two targets suggesting the detection of IS*2404* mycobacteria. The absence of the other two targets (few copy numbers) was not surprising since the C_T values (with the exception of two amoebae cultures) of the IS*2404* target recorded were high indicative of low DNA concentration. Growth of *M. ulcerans* and other IS*2404* mycobacteria are slow on LJ media (it takes 8-12 weeks for growth of *M. ulcerans* from clinical specimens), we doubt their growth on NNA plates within 2-7 days of culture and subculture unless probably through co-cultivation with amoeba. The distribution of IS*2404* positive amoebae cultures over the sampling period peaked in the ninth month (June). Much cannot be discussed on the seasonal variation of IS*2404* positive amoebae cultures although detection was observed to be high in the rainy season. *Acanthamoeba* and *Vahlkampfiidae* genera showed significant association with the detection of

IS*2404* target (χ_1^2 = 6.643 p = 0.010; χ_1^2 = 5.764 p = 0.016, 95% confidence level respectively). Possible reasons for the lack of detection of these two targets could include (i) very low numbers of bacilli colonizing FLA hence low sensitivity of fewer target copies per genome, (ii) other closely related environmental mycobacteria within the *M. ulcerans* complex that also harbor IS*2404* such as *M. pseudoshottsii* and *M. liflandii* may have been detected. *Acanthamoeba* and *Vahlkampfiidae* may be implicated in the reservoir and transmission of IS*2404* mycobacteria. The possibility that the detection of IS*2404* target must have been contaminated by mycobacteria from an external source was considered unlikely. Negative controls and internal positive control (to test for inhibitions) were used for each batch of samples processed; PCR mix were prepared in facilities where no other DNA was present and DNA from amoebae and specimens were added in facilities dedicated for the purpose in a carefully, controlled manner to avoid aerosols and mistakes.

Detection of IS*2404* was highest in Nshyieso followed by two other endemic communities; Ananekrom and Bebuso. It was anticipated that we would detect more specimens colonized by FLA containing *M. ulcerans* from Ananekrom which reports the highest number of clinical cases in Ghana annually and has three water bodies than from Nshyieso which has one stagnant pool and reports fewer number of clinical cases. Reasons could be that although Nshyieso reports fewer cases, the site of its stagnant pool might provide IS*2404* mycobacteria with a suitable habitat; very tall trees with shaded branches which minimize sunlight penetration and an undisturbed quiet environment about 2 km from the nearest settlement. With Ananekrom, the water bodies serve as leisure and domestic activites, pathways for humans and animals and farming. This could probably generate aerosolized FLA colonized with IS*2404* mycobacteria and serve as vector for transmission. FLA isolated from two exposed plates (aerosols) from

Serebouso, were positive for IS*2404* target. FLA from submerged plant biofilms gave the highest frequency for IS*2404* detection.

CHAPTER SIX

6.0 Conclusion

FLA were frequently isolated from aerosols and detritus as well as from aquatic habitat (swamps, rivers, stagnant pools). While isolation of some genera of amoebae (*Vahlkampfiidae*) were independent of physical parameters (pH and temperature) others were dependent (*Naegleria* and *Acanthamoeba*). FLA were also not dependent on bacterial load specifically *E. coli* as source of nutrient since they must have had alternative sources (yeast, fungi and other protozoa). Twenty intracellular mycobacteria species were isolated and identified. Intracellular mycobacteria were often isolated from specimens positive for isolation of amoebae and positive for detection of mycobacteria DNA from amoebae by 16S rRNA PCR. *M. ulcerans* could not be cultured from FLA. One intracellular isolate was positive for IS*2404* PCR but negative upon confirmation by VNTR typing. This could be due to the low cell density of *M. ulcerans* amongst other contaminating mycobacteria isolated, hence the indeterminate 16S rRNA analysis and untypeable multilocus VNTR results. *M. ulcerans* DNA was detected from a water specimen. IS*2404* DNA target was detected in two environmental specimens and twenty five amoebae cultures. High C_T values obtained indicated low load of IS*2404* mycobacteria in FLA. The results showed no association between *M. ulcerans* and FLA, however, the data showed that *Acanthamoeba* and *Vahlkampfiidae* genera can be inhabited by environmental mycobacteria carrying the insertion sequence IS*2404*. Detection of IS*2404* DNA target in aerosolized amoebae could give indication of how IS*2404* bearing mycobacteria may be dispersed in nature. The study was not conclusive on the role of FLA as reservoir of *M. ulcerans* it however, showed

that several *Mycobacterium sp*. and possibly *M. ulcerans* could be found intracellularly in FLA. These findings may provide important information for understanding the ecology of *M. ulcerans*.

6.1 Recommendations

Further research into FLA colonised with *M. ulcerans* in biofilms and aerosolized amoebae from contaminated water bodies in most endemic communities are needed to acquire knowledge about the reservoirs and mode of transmission to aid in the development of preventive strategies. Research on co-cultivation of amoeba with environmental specimens may improve isolation rate of *M. ulcerans* from the environment. This could be important in improving our understanding or gaining further insights into the ecology of *M. ulcerans*.

6.2 Study limitations

Although the study isolated about 75% of FLA from 539 environmental specimens only 68.6% were confirmed by PCR. This could be due to the limited number of available primer sets for the identification of the genera of amoebae (the study was limited to three genera of amoeba). Most often subculture of environmental isolates did not grow, this made it difficult to store isolates for future work. Inhibitions of the R-T PCR due to concentrated environmental contaminants from specimens were encountered but were later rectified by using powersoil DNA isolation kit (USA) for the DNA extraction of inhibited specimens. One intracellular isolate tested positive for IS*2404* PCR but upon repetitions of the PCR tested negative. This was said to often happen with environment isolates that tested positive from other researchers in BU disease with no known reasons.

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APPENDUS

Preparations of reagents for processing and decontamination of environmental specimens

- PBS
- 0.5%SDS
- Kanamycin:

  Basic solution (10000 µg/ml): dissolve 259 mg Kanamycin powder in 20 ml of Mili-Q water. Divide this in 40, 1.5 ml microfuge tubes. Keep in freezer at -18°C.

  Work solution (200 µg/ml): add 9.8 ml Mili-Q water to 0.2 ml basic solution.

- For decontamination:

  Digester (1N NaOH)

  - Dissolve 20 g of NaOH pellets in 500 ml distilled water.
  - Autoclave with a loosened screw cap and fasten cap after cooling.
  - Store at 2-8°C for a maximum of 6 months.

  0.2 % malachite green

  - Dissolve 200 mg of malachite green in 100 ml distilled water.
  - Autoclave with a loosened screw cap and fasten cap after cooling.
  - Store at 2-8°C for a maximum of 1 year.

  5 % oxalic acid

  - Dissolve 5 g of oxalic acid in 100 ml distilled water.
  - Autoclave with a loosened screw cap and fasten cap after cooling.
  - Store at 2-8°C for a maximum of 1 year.

0.8 % actidion

- Dissolve 0.4 g of actidion in 50 ml RO-Di water.
- Autoclave with a loosened screw cap and fasten cap after cooling.
- Store at 2-8°C for a maximum of 1 year.

Preparation of 2× lysis buffer for DNA extraction of *M. ulcerans*

- Proteïnase K: 20 mg/ml
  - Dissolve 1 g Proteinase K (stock solution 1 g/ bottle) in 50 ml milli-Q water (UV-treated).
  - Aliquot approximately 1000 µl in eppendorf tubes (1.5 ml) and keep at <-10°C (+/- 2°C) for maximum 1 year.
- 2 x lysis buffer: Composition
  - 1,6 M GuHCl
  - 60 mM Tris PH 7,4
  - 1% Triton X 100
  - 60 mM EDTA
  - 10% Tween 20
  - Store at 2-8°C for maximum 1 year

Preparation scheme

Tris-HCl 1M pH7,4	3 ml	12 ml	24 ml
EDTA 0,5M pH 8	6 ml	24 ml	48 ml
GuHCl 6M **	13,5 ml	54 ml	108 ml
Triton X100	0,5 ml	2 ml	4 ml
Tween 20	5 ml	20 ml	40 ml
milli-Q	22 ml	88 ml	176 ml
Total volume	50 ml	200 ml	400 ml

- GU-HCl consists of: 6M Guanidine-25 mM HCl (pH7,5) and cannot undergo UV-treatment. Therefore the lysis buffer is treated with diatomeceous earth powder.
- Add approximately 5 g Diatomeceous earth to freshly prepared lysis buffer and shake it at 37°C (+/- 2°C) for 2 hours. Filtrate the solution in a 0.2 µM filter and repeat the filtration. After filtration the lysis buffer is ready to use.

- Diatomeceous earth (with HCl)
  - Add 10 g diatomaceous earth to 50 ml sterile milli-Q water
  - Add 500 µl HCl 37%
  - Store at 2-8°C indefinitely.
- 70% ethanol (cooled at 4°C)
- Acetone

Preparation of UNSET lysis buffer for DNA extraction of free-living amoebae

Preparation scheme

UNSET lysis buffer	**100 ml**	**200 ml**
8 M urea	48.05 g	96.10 g
2% SDS (heat to dissolve)	2 g	4 g
Add distilled water until	84.5 ml	170 ml
0.15 M NaCl	5 ml	10 ml
0.001 M EDTA	0.05 ml	0.1 ml
0.1 M Tris pH 7.5	10 ml	20 ml

- Phenol:chloroform:isoamyl alcohol (25:24:1)
  - 50 ml phenol
  - 48 ml chloroform
  - 2 ml isoamyl alcohol
- 3 M NaCl
  - 17.53 g / 100 ml (solution precipitates)
  - Dissolve in 80 ml distilled water and adjust to 100 ml
  - Autoclave
- 0.5 M EDTA
- 1 M Tris pH 7.5
  - 12.11g / 100 ml (pH 7.5)
  - Dissolve in 80 ml AD, set pH using HCl and adjust to 100 ml

- Autoclave

- 100% ethanol
- 70% ethanol
- TE buffer

Table 3.2 Primer sets for PCR identification of the genera of amoebae

Primer	**Sequence (5´-3´)**	**Genus of FLA**	**Expected amplicon size**
JDP1	GGGCCCAGATCGTTTACCGTGAA		423-551 bp
JDP2	CTCACAAGCTGCTAGGGGAGTCA	*Acanthamoeba*	
JITS*TF*	GTCTTCGTAGGTGAACCTGC	*Vahlkampfiidae*	
JITS*TR*	CCGCTTACTGATATGCTTAA		
ITS*TF*	AACCTGCGTAGGGATCATTT	*Naegleria*	300-450 bp
ITS*TR*	TTTCCTCCCCTTATTAATAT		

JDP1, JITS*TF* and ITS*TF*-forward primers, JDP2, JITS*TR* and ITS*TR*-reverse primers

Table 3.3 Primers for 16S rDNA PCR

Primers	**Sequence (5'- 3')**	**Nested PCR**	**Expected amplicon size**
P1	TGCTTAACACATGCAAGTCG		
P2 new	TCTCTAGACGCGTCCTGTGC	first run	band of +/- 900 bp
P7	CATGCAAGTCGAACGGAAAGG		
P16new	AAGCCGTGAGATTTCACGAACA	second run	band of +/- 500 bp

P1 and P7- forward primers, P2new and P16new-reverse primers

Table 3.4 Primers for VNTR PCR

Locus	Primers sequence (5´ to 3´)
1	F GGCAGTGGGTGACGTCTCAGT
	R TCGAGGCGATCTACACCAAGGATTA
4	F GCCTTGCTTACCGTCGTGCCAA
	R CGAGCCAAGTTGGACCGTCAACACAT
6	F GACCGTCATGTCGTTCGATCCTAGT
	R GACATCGAAGAGGTGTGCCGTCT
8	F CGGATGACGTCGGAACTCTGA
	R GGACGCGGTAGCACGTTTTGT
9	F GGTGGATCTCCGCGTCATTTG
	R CGACCGCCCTCGAGACAG
14	F CCTTGTATCCGAGTTTCAGTT
	R GTCGACCAGATATGAGCAAT
15	F GCCACCGGTCAGGTCAGGTT
	R TCACCAACTACGACGGCGTTC
18	F CCCGGAATTGCTGATCGTGTA
	R GGTGCGCAGACTGGGTCTTA
19	F CCGACGGATGAATCTGTAGGT
	R TGGCGACGATCGAGTCTC

F- forward primer, R- reverse primer

Table 3.5 Primers and probes for R-T PCR

Primers and probes	**Sequence (5´-3´)**
IS*2404TF*	AAAGCACCACGCAGCATCT
IS*2404TR*	AGCGACCCCAGTGGATTG
IS*2404TP*	6 FAM-CGTCCAACGCGATC-MGBNFQ
IS*2606TF*	CCGTCACAGACCAGGAAGAAG
IS*2606TR*	TGCTGACGGAGTTGAAAAACC
IS*2606TP*	VIC-TGTCGGCCACGCCG-MGBNFQ
KR*TF*	TCACGGCCTGCGATATCA
KR*TR*	TTGTGTGGGCACTGAATTGAC
KR*TP*	6 FAM-CCCCGAAGCACTG-MGBNFQ

TF- forward primer, *TR*- reverse primer, *TP*- probe

Amplicon detection on agarose gel

- Molecular Biology Grade agarose
- 1 x TAE buffer: composition
  - 0,04 M Tris-acetate
  - 0,001 M EDTA pH 8,0
  - Preparation of *stock solutions* 50 x TAE
  - Dissolve 242 g Tris base in 700 ml AD.
  - Add 57.1 ml acetic acid (100%).
  - Add 100 ml 0.5 M EDTA pH 8
  - Adjust to 1 litre
  - Autoclave and store at RT for a maximum of 1 year
- DNA-marker: 100 bp
- Ethidium bromide