UNIVERSITY OF GHANA

COLLEGE OF HEALTH SCIENCES

THE POTENTIAL ROLE OF NOCTURNAL BITING INSECTS IN THE TRANSMISSION OF *MYCOBACTERIUM ULCERANS* IN THE AKUAPEM-SOUTH DISTRICT

BY

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DECLARATION

This is to certify that this thesis is the result of research undertaken by Caitlin Selassie Naa Sarku Tetteh at the Noguchi Memorial Institute for Medical Research (NMIMR), Legon under the supervision of Dr. Anthony Ablordey (NMIMR) and Prof. Eric Sampane-Donkor (Department of Medical Microbiology, SBAHS, University of Ghana) for the award of Mphil Medical Microbiology in the Department of Medical Microbiology, School of Biomedical and Allied Health Sciences, College of Health, University of Ghana.

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ABSTRACT

Several hypotheses have been made about the possible mode of transmission of *Mycobacterium ulcerans* (*M. ulcerans*) and one of these is the role of insects as possible vectors. Although studies have been conducted extensively on aquatic insects and mosquitoes, limited work has been done on the prevalence of *M. ulcerans* in other insects. This study investigated the potential role of nocturnal biting insects as vectors of *M. ulcerans*. Nocturnal insects were sampled from Buruli Ulcer endemic and non-endemic communities in the Akuapem-South district of Ghana and identified. DNA extracts of pools of the insects captured were run against IS*2404* target to detect the presence of *M. ulcerans*. IS*2404* target positive samples were then run using other multiplex real time PCR targeting IS*2606* & KR sequences as probable confirmation of *M. ulcerans* DNA. A total of 1330 insects were captured, identified and pooled according to insect family levels. In all nine orders of insects were recorded including arthropods from the class Arachnida. The highest percentage of insects caught belonged to the order Diptera (71.44%) followed by Hymenoptera and Lepidoptera which were 13.79% and 6.33% respectively. The other orders recorded below 5%. Of the 110 pooled samples extracted which consisted of 93 pooled insect samples and 17 controls, 3 tested positive for IS*2404* target but not IS*2606* & KR targets. Samples which tested positive belonged to insects of the order Diptera and Coleoptera. Two out of the three pools which tested positive belong to families of insects with biting mouthparts. Two out of the three positive samples were from non-endemic communities and one from endemic communities. The study indicated that nocturnal biting insects may play a role in *M. ulcerans* transmission through direct contact and may be involved in the complex food web for maintenance of *M. ulcerans* in the ecosystem within the district.

DEDICATION

This thesis is dedicated to the Almighty God for being my source of inspiration throughout this work and to my one and only brother, Darrell Reginald Nii Nettey Tetteh, my earthly source of inspiration.

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LIST OF ABBREVIATIONS

ARPIS	African Regional Postgraduate study for Insect Science
BU	Buruli ulcer
Ct	Threshold cycle
DNA	Deoxyribonucleic acid
IPC	Internal positive control
IS*2404*	Insertion sequence 2404
IS*2606*	Insertion sequence 2606
KR	Ketoreductase B
LJ	Lowenstein-Jenson
MLST	Multi-locus sequence typing
M. ulcerans	*Mycobacterium ulcerans*
M. marinum	*Mycobacterium marinum*
NaOH	Sodium Hydroxide
NMIMR	Noguchi Memorial Institute for Medical Research
PCR	Polymerase chain reaction
qPCR	semi-quantitative Polymerase chain reaction
PVP	Polyvinylpyrrolidone
RNA	Ribonucleic acid
spp	species
TF	forward primer
TR	reverse primer
TP	probe
WHO	World Health Organisation
ZN	Ziehl-Neelson

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Mycobacterium ulcerans (M. ulcerans) is a slow-growing acid-fast bacilli responsible for a necrotizing cutaneous infection called Buruli ulcer (BU). The disease has been reported in over 30 countries worldwide, mainly in tropical and subtropical climates, with endemic rural communities in some West and Central African countries such as Benin, Cote d'Ivoire, Kenya, Congo and Ghana having the highest burden of the disease (Mosi *et al.,* 2008). *M. ulcerans,* the third most common among agents of mycobacterial infections, is known or believed to be associated with the environment. However the reservoir and precise mode(s) of transmission are yet to be determined. *M. ulcerans* has been detected in several environmental sources such as water, leaves, stems and roots of plants and insects, among others (Wallace *et al.,* 2010).

Although treatment of early stages of the disease is available with the use of a combination therapy of rifampicin and streptomycin, early detection is a problem especially in rural areas where the disease burden is highest (Garchitorena *et al.,* 2014). To manage the disease effectively, it is necessary to know the mode of transmission to aid in prevention strategies.

Most BU endemic areas are found close to slow flowing or stagnant water bodies. It is suspected that the aquatic ecosystem may be the likely source of *M. ulcerans* and from which it is likely to be transmitted to humans (Portaels *et al.,* 2008). Efforts to understand the ecology of *M. ulcerans* led to several attempts to cultivate the pathogen from the aquatic environment until the first isolation from an endemic area in Benin (Portaels *et al.,* 2008).

Insects have been implicated as possible vectors or hosts in the transmission cycle of *M. ulcerans* (Portaels *et al.,* 2008). Two models have been proposed on how insects might play a role. The first proposes that *M. ulcerans* may be introduced on the skin by the products of the insects contaminated with the bacilli, and upon trauma to the site of inoculation, exposure of the subcutaneous skin leads to infection. The second proposed model is by direct contact through the bite of insects (Portaels *et al.,* 1999).

Insects have been widely studied to determine how they may be involved in BU disease transmission. There have been positive results on *M. ulcerans* colonisation in insects especially in aquatic insects (family Belostomatidae and Naucoridae) with some work going further to allow infected insects to feed on mice where later, manifestations of the disease were observed (Marsollier *et al.,* 2007). The first and only isolation of *M. ulcerans* from the environment was in an aquatic insect, the water strider (family Gerridae) in Benin (Portaels *et al.,* 2008).

Recent studies from Australia suggesting mosquitoes as possible vectors has further emphasized the role of insects in BU transmission (Johnson *et al.,* 2007). In Ghana, studies by Mosi *et al.,* (2008) showed that aquatic insects (family Belostomatidae and Naucoridae) may not be vectors in the transmission of *M. ulcerans* though their work was based on a limited number of insects. Most aquatic insects only bite humans incidentally and therefore may not be real vectors for transmission of BU. Research on biting insects could therefore provide insight to their potential role in BU disease transmission.

1.2 Problem Statement

In a report by WHO in 2012, 5,076 new cases of BU were reported globally with Africa being the most affected region (WHO, 2012). In Ghana, a prevalence rate of 22.7 new cases per 100 000 individuals per annum has been reported nationally (Kenu *et al.,* 2014). However

in the Akuapem-South District, a prevalence rate of 151.4 cases per 100,000 has been recorded (Kenu *et al.,* 2014). This is of great concern since it implies that the Akuapem-South District is taking over from the Ga West District as the BU hotspot which now has a prevalence rate of 15.2 cases per 100 000 (Kenu *et al.,* 2014) as against 87.7 per 100 000 in 1999 (Amofah *et al.,* 2002).

This data indicates that the endemic nature of BU might be switching across districts which suggests that non-endemic regions where BU cases have never been reported are now at risk. To circumvent a possible epidemic it is important to manage and control the disease adequately. However, the poor understanding of the epidemiology of *M. ulcerans* makes it difficult to have primary prevention strategies for the disease.

BU in its advanced stages, causes ulcerations on the skin which can result in several complications such as osteomyelitis, contracture deformities as well as loss of organs such as the eye and in extreme cases, and amputation after surgical interventions. BU complications cause a lot of discomfort to patients who have to endure long treatment durations, which can last for two years or more. These complications usually are as a result of late reporting of cases. However it is well known that 80% of early detection results in successful treatment (WHO, 2012).

There is the need therefore to determine the means by which BU is transmitted to facilitate early case detection and implementation of appropriate prevention measures.

1.3 Justification

Most studies have detected *M. ulcerans* only in aquatic insects until its recent detection in mosquitoes (Johnson *et al.,* 2007). This raised the possibility of other invertebrates harbouring *M. ulcerans* and their role in the transmission cycle.

Investigation of aquatic insects (order Belostomatidae and Naucoridae) and mosquitoes in some endemic communities in Ghana failed to link *M. ulcerans* to aquatic insects, giving indication that, different geographic areas and epidemiological settings may have different modes of transmission (www.who.int/mediacentre/factsheets/fs199).

It has therefore become increasingly necessary to collate data on insects which harbour the bacilli within different geographic regions to provide a larger and broader scope of potential vectors.

This study which focused only on nocturnal biting insects is a contribution to a larger study researching into different categories of insects and their role in *M. ulcerans* transmission. The study would therefore provide more information on transmission studies of *M. ulcerans* by creating a database of possible vectors to aid in further research on incriminating vectors of the mycobacterium.

1.4 Aim

The aim of this study was to determine the occurrence of *M. ulcerans* in nocturnal biting insects from endemic and non-endemic communities in the Akuapem-South District in Ghana.

1.5 Specific Objectives

To achieve the aim stated above, the following objectives were undertaken;

i. To identify and catalogue the various types of nocturnal biting insects from endemic and non-endemic communities in the Akuapem-South District in Ghana.

ii. To detect *M. ulcerans* in nocturnal biting insects.

iii. To determine the prevalence of *M. ulcerans* carriage among the nocturnal biting insects.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Buruli Ulcer Disease

Buruli ulcer (BU) is a necrotizing disease of the skin, subcutaneous tissue and in some cases, bones of patients who present with the disease (Portaels *et al.,* 2008). The first clinical report of BU was made in Uganda in 1958 from an endemic region known as Buruli identified for its characteristic large ulcers which led to the reference to such ulcers as "Buruli ulcers" (Dodge, 1965). Initially the disease was not known to be caused by a bacterium until smears of three patients from Uganda were found to have acid-fast bacilli present in 1959. Culture of these bacilli revealed their resemblance to an organism previously described as *Mycobacterium ulcerans* in 1948 by an Australian research team led by MacCallum (Dodge, 1968). It is believed that the publication of confirmed cases by MacCallum and others in Australia in 1948 establishes the first account of the disease on the other hand Sir Albert Cook and Kleinschmidt are also said to have described the disease in 1897 and the 1920s in Africa (Johnson *et al.*, 2005).

According to Johnson *et al.*, (2005), before the 1980s, in Africa, BU was associated with sub-Saharan Africa. Over the years, it has been found that BU is present mostly in West African countries such as Nigeria, Ghana, Cameroon, Liberia, Benin, Côte d'Ivoire, Togo and Burkina Faso (Hospers *et al.,* 2005; Johnson *et al.,* 2005). In these countries, the disease is associated with impoverished rural communities which are humid and remote (Schunk *et al.*, 2009) with most cases found in children below 20 years (Duker *et al*., 2006). Apart from Africa, other regions in the world have been identified to be BU endemic and this includes Australia, Papua New Guinea, Mexico, Malaysia, and French Guiana (Johnson *et al.*, 2005). Buruli ulcer was identified by WHO in 1998 as a re-emerging disease and was considered as

an important public health problem with social and psychological ramifications. This led to the initiation of the Global Buruli Ulcer Initiative (GBUI) (WHO, 1998).

In Ghana, BU has been detected in all the 10 regions with the most affected being children between the ages of 2 and 15 years although other age groups are also affected (Ofori-Adjei D., 2007; Schunk *et al.*, 2009). The foci of the disease however is within the Ashanti, Western, Volta, Greater Accra and Eastern Regions (Kenu *et al.*, 2014).

The disease has a major socio-economic effect especially on the health sector. In highly endemic areas, resources are significantly drained since most often a large percentage is dedicated to patients infected with the disease. According to Asiedu *et al.*, 1998, 36 patients out of a total population of 130 000 individuals (0.028%) consumed about 83% of resources allocated to the Amansie West District of Ghana. This has led to BU being a major public health concern especially in known endemic regions in the country.

Although considered the third most common mycobacterial infection after tuberculosis and leprosy (Brou *et al.*, 2007), BU is the least understood among the three. Buruli ulcer is caused by *Mycobacterium ulcerans* which is believed to be an environmental pathogen because of its link to swamps and slow-flowing waterbodies (Fyfe *et al.*, 2007).Its mode of transmission and reservoir sources remain unknown thus there have been no well-established prevention strategies (Beissner *et al.*, 2015). Early detection of the disease is currently one of the means of reducing the burden of BU since it allows for immediate treatment to prevent progression of the disease to its advanced stages (Beissner *et al.*, 2015). Although rarely fatal, BU can lead to serious morbidity usually in the event of surgical interventions after its progression into the advanced stages (Fyfe *et al.*, 2010). Standardized treatment of BU involves combination therapy of rifampicin and streptomycin, (in some cases clarithromycin) for eight weeks (Beissner *et al.*, 2015).

2.2 Clinical manifestations of the disease

Clinically, the disease presents in two main forms; the pre-ulcerative and ulcerative forms. The pre-ulcerative form usually appears as a painless nodule or papule or as a plaque or an oedema and when these are not treated immediately they progress into the ulcerative form (Fig 2.1). These features are usually found on the extremities of patients such as the upper arm and legs however BU can be present on any part of the body. These lesions fall under three categories based on their sizes (Beissner *et al.*, 2015). Category I and II comprises of single lesions < 5cm in diameter and between 5 and 15 cm in diameter respectively while lesions classified as category III are single lesions >15cm in diameter, multiple lesions and lesions at critical sites (Beissner *et al.*, 2015).

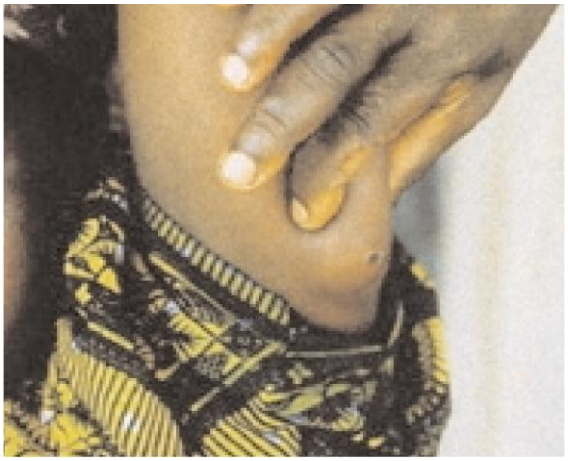
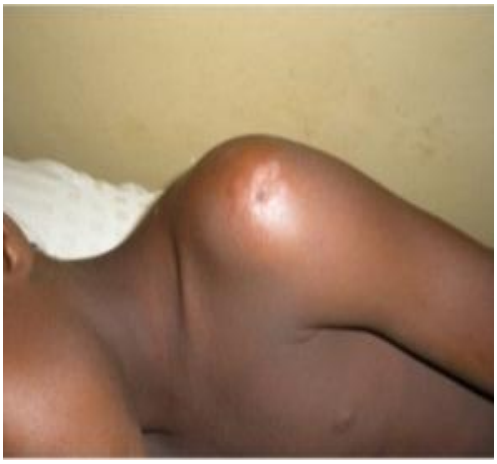
According to Walsh *et al.*, (2008), the clinical presentation of BU sometimes has several differential diagnosis such as stasis, diabetic or tropical phagedenic ulcers, leishmaniasis, noma, deep fungal or atypical mycobacterium infections, other bacterial infections (ecthyma, yaws, anthrax, tularemia, cutaneous diphtheria), arthropod bites, pyoderma gangrenosum and autoimmune disease.

Complications such as osteitis or osteomyelitis are also observed in patients especially when ulcerative BU is not reported early. In some cases, there is secondary infection with other opportunistic microbes such as *Staphylococcus aureus, Pseudomonas aeruginosa* and Aspergillus which leads to patients experiencing pain. In very critical stages, surgical interventions must be employed which result in contracture deformities or amputations.

2.3 Features of *Mycobacterium ulcerans*

M. ulcerans is seen as an acid-fast bacilli which stains pink when subjected to the Ziehl Neelson (ZN) stain when observed under the microscope. It possesses a peculiar

characteristic which is its ability to produce an exotoxin, mycolactone, with necrotizing effects (Portaels *et al.*, 2008).

a.  b. 

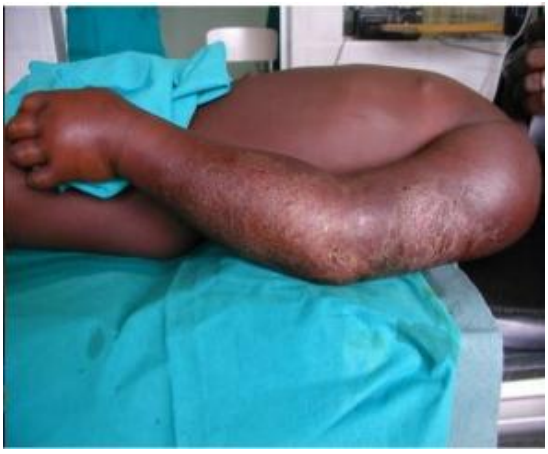
c.  d . 

Fig 2.1: Clinical representation of Buruli ulcer disease. a.) nodule on the arm of a patient b.) plaque on the arm of a patient. c.) oedema on the arm d.) ulcer on the leg

This exotoxin is responsible for the severe manifestations of the disease as it progresses to its advanced stage characterized by necrosis of subcutaneous tissue, leading to chronic, painless, and progressive ulcers (Okechukwu *et al.*, 2007). Isolation of *M. ulcerans* from culture of patients sample was first done in 1948 by an Australian, MacCallum (MacCallum *et al.*, 1948).

The optimal temperature for culturing of *M. ulcerans* is between 30 - 32°C on Lowenstein-Jensen (LJ) medium however the organism has a low doubling time thus making it a slow grower (Walsh *et al.,* 2008). Isolation of the mycobacterium from the environment for the first time was from a water strider, an aquatic insect collected in Benin (Portaels *et al.,* 2008)

The genome of *M. ulcerans* is closely related to *Mycobacterium marinum (M. marinum)* with more than 97% overall nucleotide identity (Doig *et al.,* 2012). It is believed that *M. marinum* may be the progenitor of *M. ulcerans* and other mycolactone-producing mycobacteria. The lineage of this mycobacterium is believed to have originated from *M. marinum* and has been classified as "classical" (isolates from Australia, Africa and South East Asia) or "ancestral" (isolates from Japan, China and Mexico) (Doig *et al.,* 2012).However, *M. ulcerans* and *M. marinum* differ in two main ways; the presence of the pMUM megaplasmid in *M. ulcerans* responsible for the synthesis of mycolactone synthesis as well as the presence of an insertion sequence IS*2404* present in high copy numbers of about 209 (Doig *et al.,* 2012). Complete sequencing of an isolate from Ghana by Johnson *et al.,* (2010) reveals that *M. ulcerans* is made up of 5,631,606 bp circular chromosome consisting of 4160 genes, 771 pseudogenes and the virulent 174,155 bp pMUM megaplasmid.

2.4 Pathogenesis of Buruli ulcer

The presence of the immunosuppressive polyketide, mycolactone produces an unusual pathogenesis with *M. ulcerans* (Doig *et al.,* 2012). During the early and acute phases of BU, the inflammatory response is suppressed by mycolactone through necrosis of sub-cutaneous fat which may extend beyond the infection site and may explain the rapid progression of the disease into the ulcerative forms when treatment is not immediate (Duker *et al.,* 2006). There is a down-regulation of the T–helper-1 cells (Th1) as a result of the suppressed production of interleukin 2 (IL2) and tumour necrosis factor (TNF) thus indicating that the progression of

M ulcerans infection depends on the immune response (Duker *et al.*, 2006). However it is stipulated that the toxin may not be the only virulence factor in the pathogenesis of the disease; environmental factors may also play a role (Johnson *et al.*, 1999).

According to Johnson *et al.*, 2005, it is believed that in the natural course of the disease, the host is able to regain its immune response, overcome the effects of the toxin and begin healing. This supports the suggestion by Gooding *et al.*, (2001, 2002) that *M. ulcerans* infection could be based on an individual exposure and response.

2.5 Diagnosis and Treatment

Four laboratory tests are recommended by the WHO for the diagnosis of BU. These include microscopy, histopathology, culture and Polymerase Chain Reaction (PCR) (WHO, 2012).

Microscopy is the least sensitive of the diagnostic tests. For microscopic examination, swabs obtained from undermined edges of wounds and fine needle aspirates from non-ulcerative lesions are used to prepare smears on glass slides and stained by the ZN method to observe the characteristic acid fast bacilli. Although its sensitivity ranges between 29 – 78%, microscopy is the only diagnostic method easily applicable on the field (Sizaire *et al.*, 2006, Ablordey *et al.*, 2012).

Culture of samples for the isolation of *M. ulcerans* takes about 6-8 weeks on LJ medium incubated between 30-32°C. Samples to be cultured are usually decontaminated using modified oxalic method to get rid of other bacteria as well as fungi to allow for the growth of the mycobacterium (Sizaire *et al.*, 2006). Colonies of *M. ulcerans* are usually cream or yellow and appear round. The sensitivity of the culture ranges between 20 – 60% (Sizaire *et al.*, 2006).

Both histopathology and PCR are known to have sensitivities above 90% (Sizaire *et al.*, 2006). Histopathology involves the staining of biopsy of lesions with haematoxylin and eosin

dye to observe for stained bacilli. In a study by Conner & Lunn (1966), three histological stages were identified and described. The first is a stage of necrosis where there is widespread tissue destruction with prominent numbers of mycobacteria seen. An organising stage which is composed of plasma cells, lymphocytes and large numbers of macrophages gathered around the margin of the necrotic fat which has fibroblasts which have proliferated and capillaries growing into a coagulum. The last stage is the healing stage with free lipid and necrotic cell debris which have been phagocytosed. At this stage very little numbers of mycobacteria are seen (Connor and Lunn, 1966). These stages are a reflection of the disease progression observed clinically. Observations by Haymann (1993) showed that in sections stained with Ziehl-Neelson (ZN), mycobacteria found in the necrotic subcutaneous fat region usually appear as rounded balls or spirals consisting of multitudes of organisms. On the other hand, ZN stained sections counterstained with hematoxylin usually reveal 2 components; smaller round spaces with the lipid material dissolved during processing and a larger area made up of proteinacious material which corresponds to the gelatinous substance observed clinically and macroscopically. It is believed that the ZN with hematoxylin counterstain produces a clearer view of sections of lesions (Haymann, 1993).

Polymerase Chain Reaction (PCR) is considered the gold standard for *M. ulcerans* detection (Walsh *et al.*, 2008) which explains the development of several PCR methods. These methods have been developed based on the 16S rRNA genes, hsp65 gene or the IS*2404* insertion sequence. It has been shown that IS*2404* based PCR as compared to the 16S rRNA gene-based PCR has better specificity, requires less time and is less expensive (Guimaraes-Peres *et al.*, 1999). Both conventional and real-time PCR have been employed in the detection of *M. ulcerans* however real time PCR has a sensitivity of about 10 times higher than the conventional IS2404 based PCR. (Rondini *et al.*, 2003). The conventional PCR provides qualitative results based on endpoint measurements, while real time PCR provides

quantitative results by monitoring the amount of reaction products from the starting concentration of mycobacteria DNA during the exponential phase of amplification cycles (Rondini *et al.*, 2003). Real time PCR methods used is based on development of two TaqMan Multiplex real time PCR assays which target 3 independent repeated sequences in the *M. ulcerans* genome, two multicopy insertion sequences (IS*2404*, IS*2606*) and the multicopy sequence which encodes the ketoreductase B domain (Fyfe *et al.*, 2007).

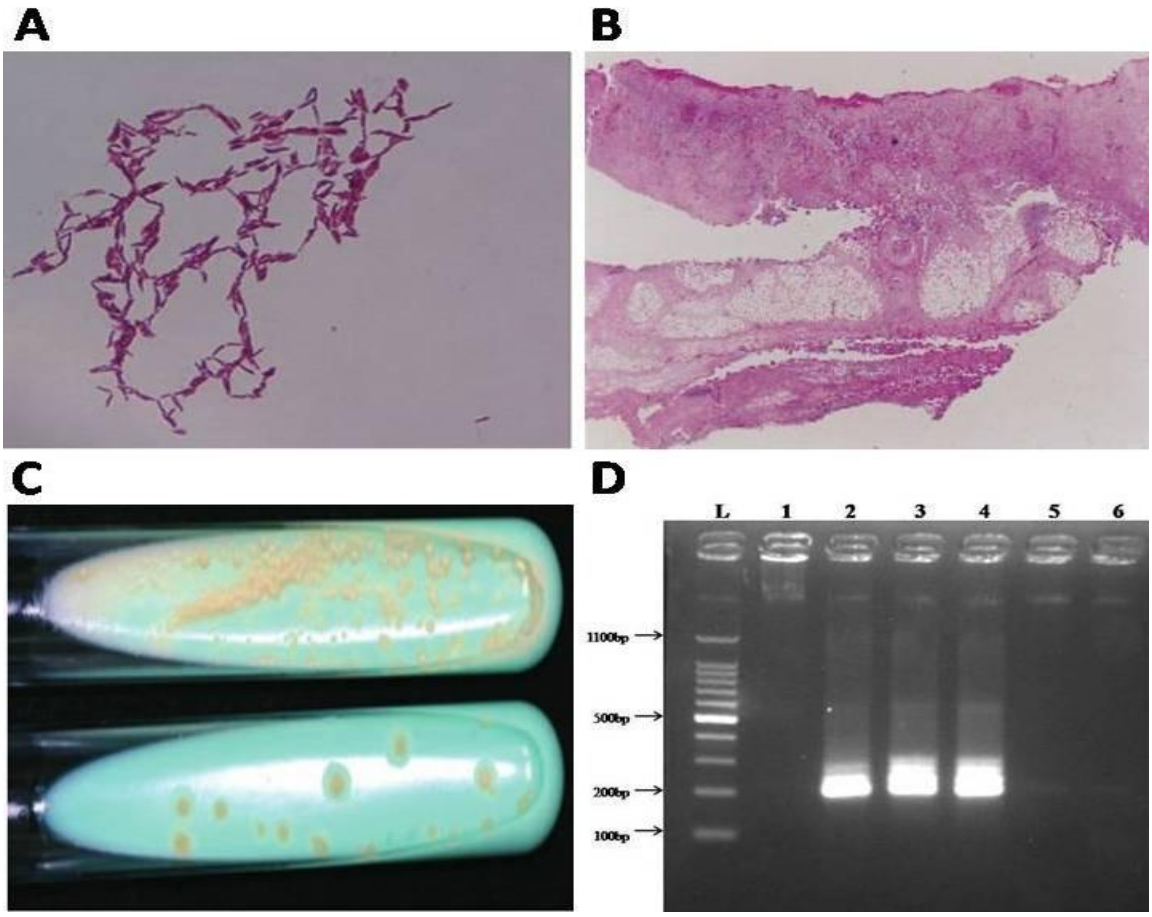


Fig 2.2: Laboratory methods of BU diagnosis. (A) Rod shaped acid fast bacilli of *M. ulcerans* (Nakanaga *et al.*, 2013). (B) A biopsy specimen of an *M. ulceran* lesion stained with haematoxylin and eosin (Meyers *et al.*, 1996). (C) Yellow colonies of *M. ulcerans* cultured on ogawa medium (Nakanaga *et al.*, 2013) (D) Gel picture of PCR products using 2% agarose gel. L= molecular weight marker; Lanes 2, 3 and 4 shows bands for *M. ulcerans* DNA; Lanes 1, 5 and 6 are negative samples.

A combination therapy of rifampicin and an aminoglycoside (streptomycin or amikacin) is given for 4 to 12 weeks for management of BU (WHO, 2004). It has been observed that antibiotic treatment reduces the surface area by more than 50% and mycobacteria are usually not observed during microscopy after treatment is completed (WHO, 2004). Healing times during treatment of BU varies according to Beissner *et al.*, (2006) with a median healing time range of 18 to 30 weeks. During the first three months of treatment, an increase in the size of lesions can be observed but it is also possible to observe new lesions appearing as much as 13 months after antibiotic treatment has been completed (Beissner *et al.*, 2006). Wound management is considered necessary in BU with a goal of minimising the healing times, optimisation of treatment outcome and minor iatrogenic damage (Velding *et al.*, 2014) and it is encouraged that wounds are dressed until healing is complete (WHO, 2004). Severe and permanent disability are usually the result of improper treatment of the disease in more than 25% of patients (Okechukwu *et al.*, 2007).

Although treatment is available by the use of rifampicin-streptomycin combination which is more effective for cases detected early, early access to diagnosis is a problem in rural areas where the disease burden is most abundant. One solution to this problem however is establishing appropriate prevention strategies in endemic communities but this is difficult since the epidemiology of *M. ulcerans* till date is still a mystery yet to be unravelled.

2.6 Epidemiology of Buruli ulcer

The epidemiology of BU is not well understood however observations made suggest that the aquatic ecosystem may be the likely niche of the mycobacterium (Ablordey *et al.*, 2005[a]). This has been cemented by the detection of *M. ulcerans* DNA in water, detritus, aquatic insects, plants and other environmental sources leading to the belief that it is an environmental pathogen (Ablordey *et al.*, 2005[a]). Despite the detection of *M. ulcerans* DNA,

low bacilli load and the difficulty attached to culturing the microorganism from the environment gives the implication that the bacilli may not multiply in these sources (Durnez *et al.,* 2010). It has been stipulated that the understandable epidemiology of the disease may be attributed to the constrained genetic diversity among *M. ulcerans* isolates with similar geographic origins (Ablordey *et al.*, 2005[b]).

The global incidence and prevalence is not also well-known because of limited data from some endemic countries (Ashford *et al.*, 2001). The limited data prompted the recommendation by WHO of the mapping of disease and prevalence and incidence estimation in endemic countries (WHO, 2012). This recommendation has had a positive effect on data generated on incidence and prevalence of the disease. Mapping of the disease in some districts in Ghana by Kenu *et al.*, (2014) determined the prevalence of BU to range from 1.6 per 100,000 populations in Ga South to 151.4 per 100, 000 in Akuapem- South. The data presented gave an indication of a change in prevalence rate in the Ga West district of 15.2 cases per 100 000 (Kenu *et al.*, 2014) as against 87.7 per 100 000 in 1999 (Amofah *et al.*, 2002). On the whole, a downward trend in case numbers has been observed (Fig 2.3) in several African countries and this has been attributed to the establishment of effective national BU control programmes in these countries (Röltgen and Pluschke, 2015).

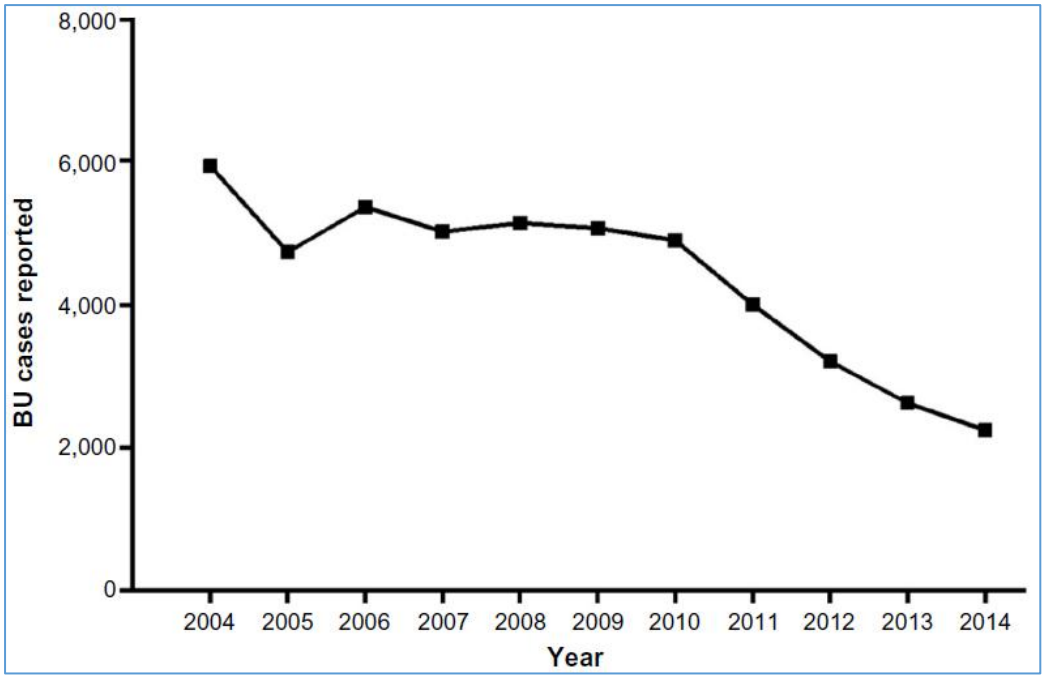


Fig 2.3: Current downward trend of BU cases in some endemic countries in Africa (Roltgen & Pluschke, 2015)

2.6.1 Geographical distribution of *M. ulcerans*

About 34 countries worldwide have reported cases of *M. ulcerans* infection (Wallace *et al.*, 2010). These countries (Angola, Australia, Benin, Brazil, Burkina Faso, Cameroon, Central African Republic, People's Republic of China, Congo, Côte d'Ivoire, Democratic Republic of the Congo, Equatorial Guinea, French Guiana, Gabon, Ghana, Guinea, Indonesia, Japan, Kenya, Republic of Kiribati, Liberia, Malawi, Malaysia, Mexico, Nigeria, Papua New Guinea [PNG], Peru, Senegal, Sierra Leone, South Sudan, Sri Lanka, Suriname, Togo, and Uganda) are known to mainly have tropical and subtropical climates however BU is mostly endemic in Africa (Fig 2.4) (Röltgen & Pluschke, 2015). However, BU may be present in other countries which have not yet made reports (2011.igem.org).

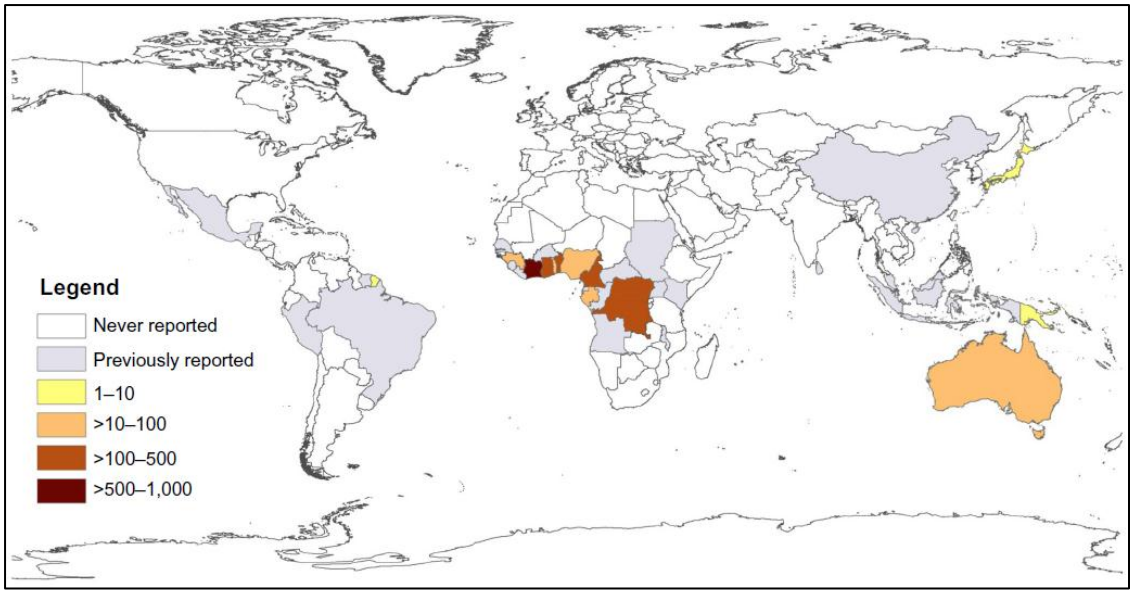


Fig 2.4: A map showing countries where BU has been reported. (Rolgen and Pluschke, 2015)

In Africa, it is reported that 21 countries in all have reported cases with 9 countries in West Africa, 4 in Eastern, 6 in Central and 2 in Southern Africa. The disease is gradually taking precedence over other mycobacterial diseases such as tuberculosis and leprosy especially in some of these areas of West Africa (Ablordey *et al.*, 2005[a]).

2.6.2 Geographical distribution in Ghana

In Ghana, the first case of BU was reported in 1971 and since then over 426 communities have reported cases nationwide (Kenu *et al.*, 2014). The disease is known to be concentrated around the southern hemisphere particularly in the Ashanti, Eastern, Western and Greater Accra Region (Fig 2.5) however a few cases have been reported in the Brong Ahafo and Northern Regions.

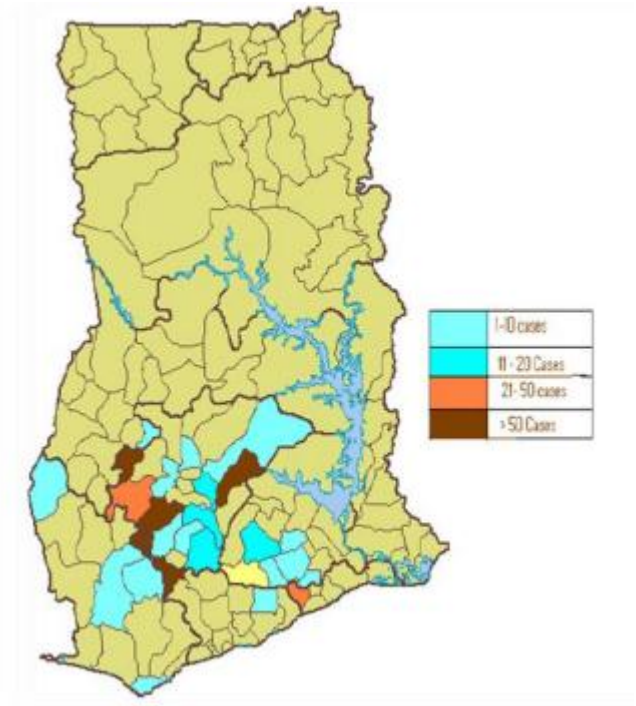


Fig 2.5: Distribution of BU cases in Ghana. Legend indicates that the pale blue regions represent 1 – 10 cases, blue shaded regions represent 11-20 cases while the orange and brown shaded regions represent 21-50 cases and >50 cases respectively (Kenu *et al.,* 2014).

2.7 Transmission of *M. ulcerans*

Knowledge on the mode of transmission of *M. ulcerans* has been accumulating from epidemiological data as well as molecular findings on the mycobacterium. Since the discovery of the IS*2404* insertion sequence specific to *M. ulcerans* in 1997 (Kotlowski *et al.*, 2004), it is possible to detect the *M. ulcerans* DNA in environmental specimens by PCR. This has led to detection of *M. ulcerans* DNA in several invertebrates, fish, water filtrate and plant materials (Wallace *et al.,* 2010). However of all the environmental specimens tested, the hypothesis of insects as possible vectors of BU is gaining more grounds in the quest to determine how humans get infected with *M. ulcerans*.

Several diseases such as malaria, leishmaniasis and trypanosomiasis are transmitted by arthropod-vectors thus it is not far-fetched that *M. ulcerans* infection may be transmitted by insects. Also, a number of studies done on environmental samples consistently show *M.*

ulcerans detection in insects (Portaels *et al.,* 2008; Merritt *et al.,* 2010; unpublished Ablordey *et al.,* 2012). Furthermore it has been observed that clinical features of the disease may support the transmission model of insect bites (Wallace *et al.,* 2010). Although these features are seen on several parts the body, most are found on the extremities, with 53% seen on the lower parts of the body, 37% on the upper parts and 10% on other parts of the body. (Portaels *et al.,* 1999; Van der Werf*et al.,* 1999).

It is believed that *M. ulcerans* may be introduced on the skin either through the products of the insects such as faeces which find their way into subcutaneous tissue upon trauma to the site of inoculation or by direct contact i.e. through the bite of an insect. (Portaels *et al.,* 1999; Van der Werf *et al.,* 1999).

The incubation period of *M. ulcerans* is known to last from 1.5 to 7 months which supports the suggested possibility of transmission through bite of insects (Lavender *et. al.,* 2011). According to Lavender *et al.,* (2011), it is believed that permanent residents of BU endemic areas or visitors to these areas may get introduced to a low inoculum of the bacilli through the bite of an insect which during its incubation period may become infectious subsequently leading to disease manifestation.

2.7.1 Review of Insects Hypothesis

The hypothesis of insects was first developed in 1999, when Portaels *et al.,* in their study of environmental samples found out that five out of the ninety-five samples that tested positive were all insects. These insects, which were found on the stem of aquatic plants collected belonged to the family Naucoridae and Belostomatidae, insects mainly associated with water bodies and predators of species of aquatic arthropods and molluscs.

Following this hypothesis, Marsollier *et al.,* (2002) in their study were able to establish the first strong evidence of insects as possible vectors. Firstly, they succeeded in concentrating

M. ulcerans from the salivary glands of insects. A non-ulcerative inflammatory lesion with oedema was also observed in mice whose tails were bitten with infected insects and *M. ulcerans* was detected in the lesion. Taken together, this indicated that insects could be a vehicle through which *M. ulcerans* might be spread. The insects studied however belonged to the family Naucoridae as well as Belostomatidae, the aquatic insects which have been implicated.

Additional progress was made in associating insects with *M. ulcerans* with the first successful cultivation of the bacilli from a water strider (Order: Hemiptera, Family: Gerridae) (Portaels *et al.*, 2008). In their study, they were able to isolate *M. ulcerans* from the insect through phenotypic characterization, identification of nucleotide sequence of 16s rRNA gene, profiling of the gene and subsequently infecting and observing disease manifestation in mice. This stretched the range of possible hemipteran transmitters since the insects of the family Naucoridae and Belostomatidae belonged to the same order. However, the significance of biting by *M. ulcerans,* that is colonization of the bacilli by aquatic insects in this order, is not known since these normally feed on other aquatic insects, fish and snails and would usually bite man incidentally. These insects may therefore not have an active role in disease transmission of the mycobacterium but may be reservoirs.

2.7.2 Insect study in Ghana

In Ghana, studies have also been done to determine the incidence of *M. ulcerans* in the suggested vectors i.e. members of the family Naucoridae and Belostomatidae. Mosi *et al.*, (2008) sought to determine whether *M. ulcerans* could colonise Naucoridae and Belostomatidae species native to the African continent as well as the distribution of the mycobacterium in the body parts of the insects. They were also able to show that insects could be infected with *M. ulcerans* through feeding. They were, however, unable to show that

aquatic insects were possible vectors although they suggested that *M. ulcerans* may have a commensal relationship with the insects.

A major shift of focus in transmission studies of *M. ulcerans* was seen following the detection of the bacilli in mosquitoes in Australia (Johnson *et al.,* 2007, Lavender *et al.,* 2010). This gave indication that other biting insects could be possible vectors (Johnson *et al.,* 2007). In the advent of an outbreak, this study showed that mosquitoes could carry *M. ulcerans* and being bitten by mosquitoes raised the odds of being diagnosed with BU. However, they established that this could be peculiar to Australia and may not necessarily be the case elsewhere. In a subsequent study, Johnson and Lavender (2009), were able to strengthen the link between *M. ulcerans* and mosquitoes by determining the correlation of the seasonal variation of mosquitoes and incidence of the disease. This study unlocked the door to the possibility of other insects being possible reservoirs or having passive or active role in the spread of the disease.

While studying the interaction of *M. ulcerans* with mosquito species, Wallace *et al.,* (2010) questioned prevalence in other invertebrates. They found out that isolates of *M. ulcerans* infected the larval forms of some species of mosquitoes and stayed through larval development but did not develop into the pupal or adult form of the mosquito which confirmed that mosquitoes may not be biological agents and proposed that other insects could be agents. They questioned whether other invertebrates within the same environment could either carry or colonise *M. ulcerans*.

Since there is limited data on prevalence of *M. ulcerans* in other invertebrates and as stated by WHO (2012) that different geographical areas and epidemiological settings may support different modes of transmission, it is necessary to have an inventory of biting insects to aid in further work on vector incrimination.

2.8 Molecular Tools for *M. ulcerans* Detection in the Environment

Detection of *M. ulcerans* from the environment was first established in Australia and West Africa with the use of PCR. Identification of the bacterium by PCR from environmental samples was made possible after insertion sequence IS*2404* for *M. ulcerans* was discovered (Kotlowski *et al.,* 2004).

Detection of *M. ulcerans* in the environment has long been found to be difficult due to the low bacilli load (Ablordey *et al.,* 2005). This has led to determination of several molecular tools for easy detection and genotyping of different strains of the mycobacterium.

Among the methods employed for easy detection of *M. ulcerans* was a study done by Ross *et al.,* in 1997 where gel fitration chromatography was used with a column made up of polyvinylpyrrolidone (PVP) to remove humic acids and phenolic substances from the soil samples to prevent inhibition during PCR. This was to help in obtaining pure DNA extracts for PCR.

Other methods which were employed included coating of magnetic beads with antibodies to *M. ulcerans* (Roberts & Hirst, 1997), a PCR procedure which captures *M. ulcerans* sequences (Stinear *et al.,* 2000) and a cell lysis and DNA extraction procedure engineered to be performed in one tube (Kotlowski *et al.,* 2004).

Following the role of tandem-repeat in molecular epidemiology studies in determining disease transmission, a study by Ablordey *et al.,* 2005 sought to investigate into variable number of tandem repeat (VNTR) loci and their possible use in distinguishing among *M. ulcerans* strains. Nine loci were identified in this study which suggested the possibility of these as markers for determining different *M. ulcerans* strains.

Currently, several types of DNA extraction kits have been developed which are able to remove humic acids among others from environmental samples. Semi-quantitative PCR

which is found to be more sensitive in detecting *M. ulcerans* in environmental samples is used and VNTR is usually done to determine the different *M. ulcerans* strains.

CHAPTER THREE

3.0 METHODOLOGY

3.1 Study area

The study was conducted in the Akwapem-South District in the Eastern region of Ghana. Akwapem-South is known to be a prominent BU endemic area in Ghana with prevalence rate of 151.4 per 100 000 population (Kenu *et al*, 2014). The Akuapem-South District is 403 sq km in size and shares boundaries with the Akuapem-North in the north, Ga District in the south, Tema Municipality in the East and Suhum Kraboa-Coaltar and West Akim in the West. The District capital, Nsawam is located along the Accra-Kumasi road and it is about 23 km from Accra. The District has a population of 116,344 with 49.3% being male and 50.7% being female. It has a population density of 289 per square km with an average household size of about 3.9 with a compound accommodating about 15.7 persons.

The major occupation of the people in the district falls within the Agricultural sector with about 40% of them being farmers while 26% are traders and 33.7% are found either in industrial or service sectors. Farming serves as a risk factor for the hypothesis of insect bites as these persons are exposed to insects at the farms. The Akuapem-South District is surrounded by two types of vegetation; the semi-deciduous forests and the coastal savannah grassland however the forests occupies about 90% of the entire District while about 10% is made up of coastal scrub and grassland vegetation. The Densu River and its tributary rivers and streams flows through the District and it is thought to contribute to the incidence of Buruli ulcer within the district. The Densu River is 115.8 km long and takes its sources from Atiwa Mountain Ranges near Kibi in the Eastern Region.

Eight communities were selected from the district namely; Otukwadwokrom, Boahenekrom, Obosono, Obotwere, Aduakrom, Okomfo, Avaga and Oparekrom. These communities were

selected and classified as endemic and non-endemic based on data from the National BU control programme. The communities were selected to be evenly distributed across the district in order to provide accurate data. Figure 3.1 shows the communities selected within the district.

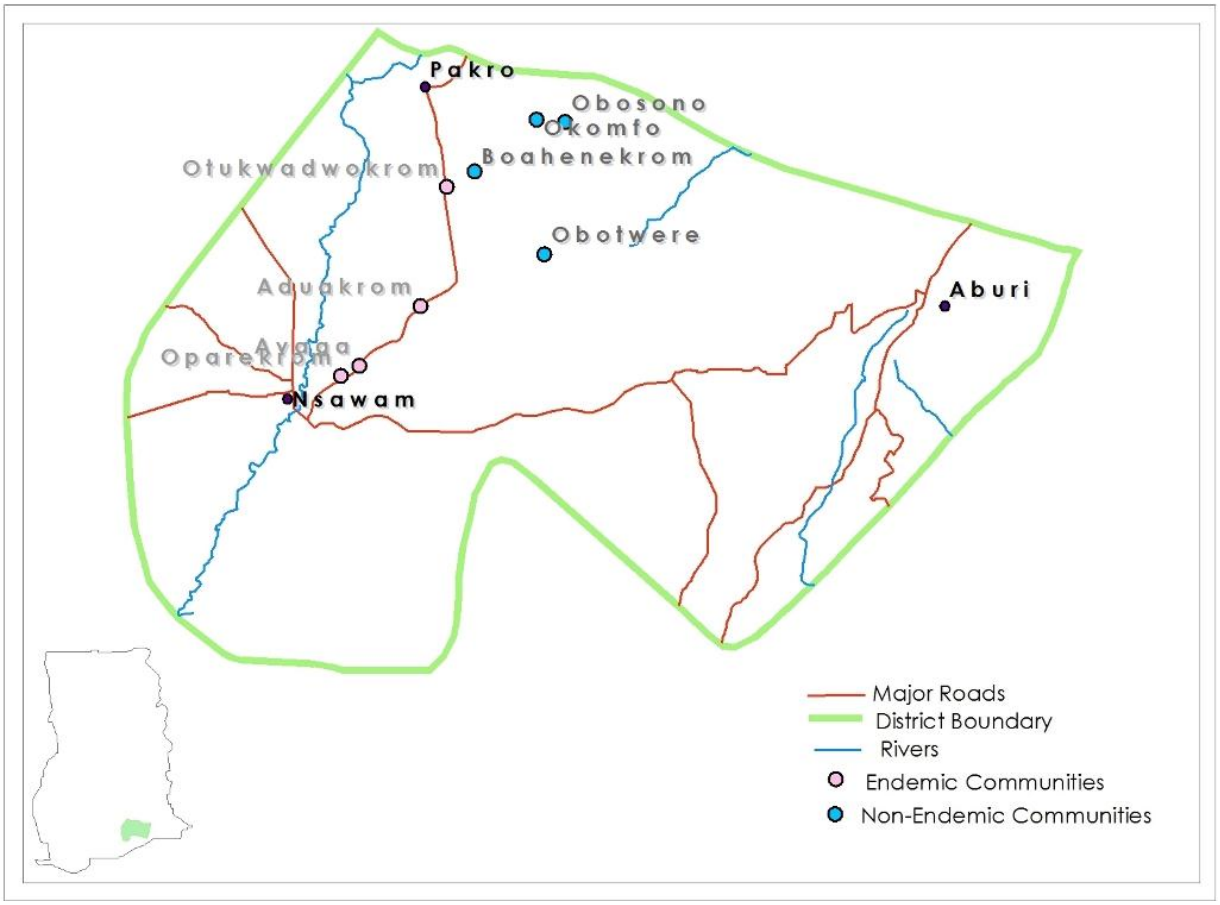


Fig 3.1 A map of the selected communities from the Akuapem South district. Endemic communities indicated pink and non-endemic indicated black.

3.2 Study design

Cross-sectional study design was used for this study

3.3 Sampling

Sampling was done over a period of six weeks with four communities visited per week. Within each community, two sites were selected by assessing the possibility of humans

passing through the sites. To capture nocturnal insects, light traps were set at each site for 12 hours (i.e. 6pm – 6am) for three consecutive days. The attraction source was a small fluorescent bulb located at the head of the trap. A fan found within the head served as a force of energy for pushing attracted insects into the net (Fig 3.2). These were both powered by a battery source containing four dry cell batteries changed every night to allow for full capacity of the traps. Every morning when the battery was disengaged, the net was tied at the neck to prevent insects captured from escaping. Each community and site were given an identification number and insects collected from the various communities were labelled accordingly. On the morning of the third day, captured insects were transported to Noguchi Memorial Institute for Medical Research (NMIMR) in the net of the trap. Using a sucking tube, the insects were transferred into universal bottles containing 70% ethanol (hitherto referred to as transport medium) and stored at 4^{o}C.

3.4 Insect classification and preparation

Insects were identified and classified to the family level by an entomologist at the African Regional for Postgraduate Studies in Insect Science (ARPIS) at University of Ghana, Legon. These were then sorted out and pooled for further work based on i) their taxonomic classification and ii) their location. They were also then divided into biting and non-biting insects based on their mouthparts. Though the study was to focus on nocturnal biting insects, non-biting insects were included to determine whether there was any significant difference between these two groups with *M. ulcerans* colonisation. Each pooled sample was collected into a 1.5mL screw cap tube containing 50mM NaOH. Mortar and pestle were sterilised for 1hr at 161^{o}C and used to completely ground together each pooled sample. Each homogenate was then stored in the 1.5mL screw cap tube at 4^{o}C.

a. **b.**

Fig 3.2: Light trap set at different sites on the field. Both images (a&b) display light traps set on the field during the day. Yellow arrow in a. shows where the power source is attached, orange arrow shows position of attraction source (fluorescent bulb), red arrow shows position of fan, blue arrow shows net which contains insects after capture & black arrow shows rope for tying the net to keep insects intact.

Fig 3.3: Identification and sorting out of insects. Insects in alcohol-based solution poured into petri-dish for identification and sorting out.

3.5 DNA preparation

After identification and preparation of the insects samples, 110 out of the 172 pooled samples were successfully sorted which was made up of 93 pooled insect samples and 17 transport medium of each site to serve as controls. The transport medium for each site was included in the study as controls to ensure that *M. ulcerans* detection in samples could be attributed solely to the insects.

3.5.1 Genomic DNA extraction using PowerSoil® DNA Isolation Kit procedure

Five hundred microliters (500ul) of the homogenised sample was added to the PowerBead Tubes provided and gently vortexed to mix after which solution C1 was added to aid in cell lysis. The PowerBead Tubes were then secured horizontally using the Fast Prep Bead Beater at maximum speed for 5 minutes. The tubes were then centrifuged at 10,000 x g for 1 minute at room temperature and 600µl of the supernatant was transferred to a clean 2 mL collection tube avoiding the pellet at the bottom of the tube as well as the foam on the surface of the solution. To remove inhibitors that may reduce DNA purity, 250µl of solution C2 was added to the sample and the mixture vortexed for 5 seconds. This was then incubated at 4°C for 5 minutes and centrifuged at room temperature at 10,000 x g for 1 minute. Avoiding the pellet, 600µl of the supernatant was transferred to a clean 2 mL collection tube and 200 µl of solution C3, a second inhibition reagent was added. Incubation was done at 4°C for 5 minutes followed by centrifugation at 10,000 x g for 1 minute at room temperature. To allow binding of the DNA to the silica in the spin filters, 1.2mL of solution C4, a high concentration salt solution, was added to 750 µl of the supernatant which was transferred into a clean 2 mL and vortexed for 5 minutes. Approximately, 675 µl of the mixture was loaded onto a spin filter and centrifuged at 10 000 x g for 1 minute at room temperature after which the flow through was discarded. The process was repeated until no supernatant remained in the tube. A total of three loads for each sample processed was done. Washing of contaminants was done using

500 µl of solution C5, an ethanol based wash solution, and centrifuged for 1 minute at 10000 x g at room temperature. The flow through was discarded from the 2mL collection tube and centrifuged at 10 000 x g for 1 minute at room temperature to remove residual of the wash solution. The spin filter was then placed in a clean 2mL collection tube while avoiding any splash of the wash solution and 100 µl of solution C6 was added for elution of the DNA. After centrifugation at 10 000 x g for 1 minute at room temperature, the spin filter was discarded and the DNA was kept at -20 C. (See Appendix 2.3 for details on solution C1-C6)

3.6 Detection of *M. ulcerans* DNA by qPCR

To determine the presence of *M. ulcerans* DNA, DNA extracts of the samples were subjected to semi-quantitative real-time PCR assay targeting the insertion sequence IS*2404* and samples which tested positive for the target were subjected to semi-quantitative real-time PCR assay targeting IS*2606* and a sequence encoding the ketoreductase B (KR) domain developed and validated for use on environmental samples by Fyfe et al (2007).

3.6.1 Detection by IS*2404* IPC multiplex

A multiplex qPCR targeting IS*2404* was performed on all the DNA extracts obtained using primers TF and TR and a probe TP (Appendix 2.4). The PCR was performed using a total reaction volume of 25 µl containing 12.5 µl of TaqMan® Universal master mix, 1.25 µl each of IS*2404* TF with total concentration of 18 µM, IS2404 TR with total concentration of 18µM and IS2404 TP with total concentration of 5µM, 0.5µl of Internal positive control (IPC) DNA (50x), 2.5µl of IPC (10x) mix (a buffer), 4.75µl nuclease free water and 1µl of DNA extract of the samples. This was then placed in Rotor gene Series® Real time PCR machine with the following conditions; 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 10 min (activation AmpliTaq Gold enzyme), and 40 cycles of 95°C for 15 s (melting) and

60°C for 1 min (annealing/extending). Threshold cycle (Ct) values of each sample was read and positive samples were confirmed using the IS*2606* KR multiplex semi-quantitative PCR.

3.6.2 Detection by IS*2606* KR multiplex semi-quantitative PCR

Samples which tested positive for IS*2404* were subjected to a multiplex qPCR targeting IS*2606* and KR genes to confirm samples as *Mycobacterium ulcerans*. The IS*2606* KR PCR mixture had a total volume of 25µl containing 1 µl of template DNA (samples), 0.9 µM concentrations of each primer (IS*2606* TF, IS*2606* TR, KR TF & KR TR), 0.25 µM concentration of the probes (IS*2606* TP & KR TP), and 12.5 µl of TaqMan® Universal PCR Master Mix. This was run in the thermocycler against the same conditions as used in the previous run (IS*2404*) and the Ct values recorded.

3.6.3 Estimation of bacterial load from Ct values

During the exponential phase of the PCR reaction, fluorescence of the relative concentration of DNA present were plotted against the cycle number on a log scale with the cycles on the X-axis and the log indication of the intensity on the Y-axis. To obtain the Ct values of the samples, a threshold of 0.5 for detection of the fluorescence was determined and the point at which a sample crossed the threshold was recorded as the Ct of the sample (Fig 3.6). Using a standard curve generated by Fyfe *et al.*, (2007) to estimate the bacterial load of the samples, IS*2404* Ct values of positive samples were compared series of DNA extracts of *M. ulcerans* spiked environmental samples to give an indication of the relative numbers present in each sample (Fyfe *et al.*, 2007) (Fig 3.7).

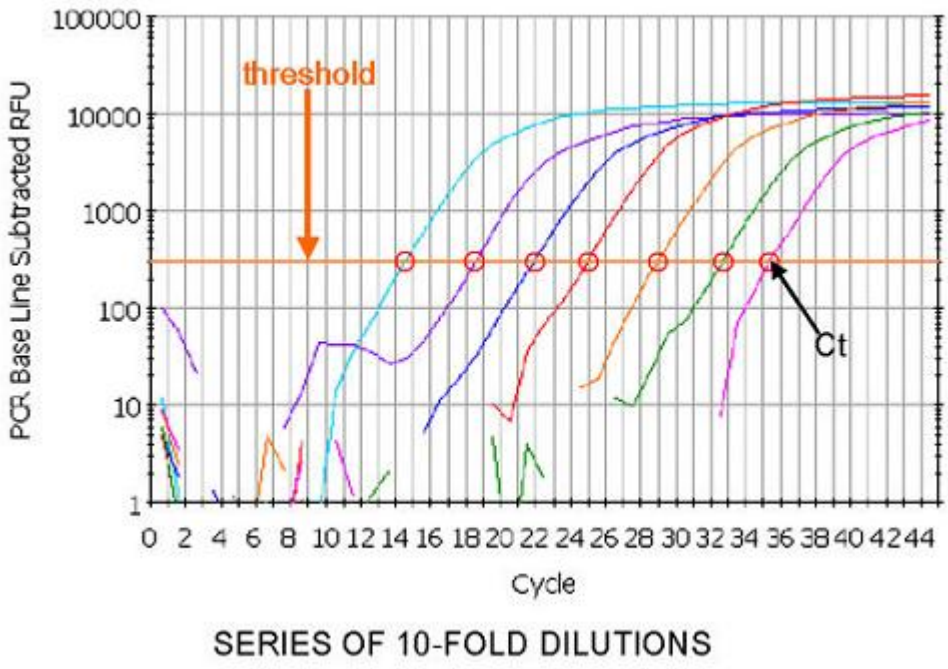


Fig 3.4: A graph printout of data using Taqman probe showing Ct values of different samples.

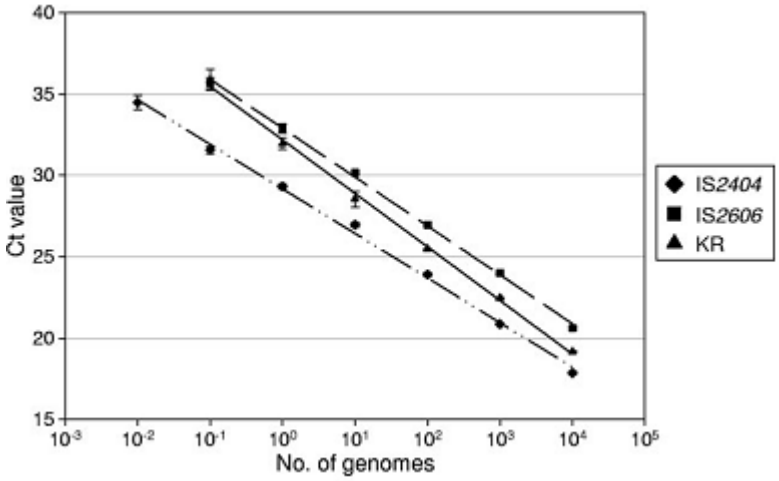


Fig 3.5 Standard curve generated using a logarithmic scale by the analysis of known amounts of genomic *M. ulcerans* DNA with the IS2404, IS2606, and KR TaqMan real-time PCR assays (Fyfe *et al.*, 2007).

3.7 Ethical issues

Ethical approval for this study was obtained from the Ethical and Research Review Committee of the University of Ghana Medical and Dental School, College of Health Sciences.

3.8 Data analysis

Data obtained at the end of the study were analysed using Microsoft Excel and IBM SPSS Statistical Software (Version 20.0).

To determine the diversity of insects identified, two diversity indices were employed; the Shannon-Weaver diversity index and the Simpson's diversity index (see Appendix 3.2 for formula). These measure of diversity were calculated for both endemic and non-endemic communities according to the families identified. A measure of the similarity (Jaccard's index) of the insects sampled between endemic and non-endemic communities was also calculated to determine the similarity of the families identified.

To determine the association between biting and non-biting insects in relation to *M. ulcerans* detection as well as association between endemicity of a community and the presence of *M. ulcerans*, chi-square test was employed with Fisher's exact test.

CHAPTER FOUR

4.0 RESULTS

4.1 Taxonomy of insects sampled

To generate a catalogue of nocturnal insects present within the Akuapem-South district, the total number of insects captured were identified and pooled according to species based on their taxonomic features. A total of 1330 insects were identified which consisted of 170 species belonging to 51 families under 9 orders of Class Insecta and 2 species under a single order of Class Arachnida under the phylum Arthropoda. The various insect orders in ascending order of species abundance are as follows; Neuroptera (2 spp.), Psocoptera (3 spp.), Hemiptera (4 spp.), Trichoptera (4 spp.), Homoptera (10 spp.), Lepidoptera (13 spp.), Coleoptera (17 spp.), Hymenoptera (26 spp.), and Diptera (91 spp.). Significant portions of three insects were damaged thus they were classified as unidentified (Table 4.1).

A summary of the families represented in the various orders is as follows; The order Diptera (commonly known as flies) recorded the highest numbers of insects captured represented by 88 different species belonging to 20 families. Although believed to be far less common in the tropics (Grootaert, 2012), the largest family represented was the Empididae which recorded a total of 336 insects of 16 different species. Its high numbers can be attributed to the mountainous nature of the Akuapem-South district (Grootaert, 2012). The least represented family was Caliphoridae which recorded 1 insect.

The order Hymenoptera was represented by 26 different species belonging to only 3 families (5 of the insects could not be identified under the family level). A large number of the species belonged to the family Formicidae (commonly known as ants) which is expected because of their dominance in tropical regions and high diversity both on ground and on vegetation (Oliveira *et al.*, 2011).

Insects of the order Coleoptera (commonly referred to as beetles) were represented by a diverse number of species belonging to 10 families (3 insects were classified on the family level as 'unidentified') with a total number of 19 insects captured. There was an even distribution of insects with 1 or 2 insects per family.

The order Lepidoptera with 13 species belonging to 4 families was highly represented by the family Tineidae (moths). The order Homoptera, although represented by only 2 families was made up of 10 species of which 9 belong to the family of Cicadellidae known to commonly infest trees with different species co-existing on the same host (Bentz and Townsend, 2005).

The order Hemiptera, which is of special interest due to the isolation of *M. ulcerans* from an insect belonging to the family Gerridae (Portaels *et al.*, 2008), was represented by 4 species belonging to 4 families (no Gerridae was represented) with most of the insects of the family Cicadellidae. The least represented orders at the family level were Neuroptera, Psocoptera and Trichoptera.

Table 4.1: Total number of insects captured and identified for each order within endemic and non-endemic communities

Order	Endemic n = 482	Non-endemic n = 845	Total number identified n = 1327
Coleoptera	8 (1.66%)	11 (1.30%)	19 (1.43%)
Diptera	363 (75.31%)	585 (69.23%)	948 (71.44%)
Hemiptera	7 (1.45%)	1 (0.12%)	8 (0.60%)
Homoptera	26 (5.39%)	27 (3.20%)	53 (3.99%)
Hymenoptera	39 (8.09%)	144 (17.04%)	183 (13.79%)
Lepidoptera	34 (7.05%)	50 (5.92%)	84 (6.33%)
Neuroptera	0 (0%)	7 (0.83%)	7 (0.53%)
Psocoptera	3 (0.62%)	5 (0.59%)	8 (0.60%)
Trichoptera	0 (0%)	15 (1.78%)	15 (1.13%)
Araneae*	2 (0.41%)	0 (0%)	2 (0.15%)

*Order of arachnids captured was included in the study to determine their possible role in harbouring the mycobacterium

4.2 Community distribution of nocturnal insects

The distribution of insects across endemic and non-endemic communities were investigated. Furthermore, the insects were sorted and pooled into families and classified as biting and non-biting based on their mouthparts. From the pooled sample of insects, 73 out of the total of 172 species were classified as biting insects of which 29 were from endemic communities and 44 from non-endemic communities (Table 4.2).

4.2.1 Entomological distribution in endemic communities

Within the endemic communities, a total of 482 insects were identified which was representing 36.3% of the total insects identified. The dipterans were the most in abundance captured (75.13%) within the endemic communities with the Pscopterans being the least

captured (0.62%). While no insects belonging to the order Tricoptera and Neuroptera were captured in the endemic communities, all the arachnids (order Araneae) were captured within these communities.

4.2.2 Entomological distribution in non-endemic communities

The total number of captured insects from non-endemic communities was 845 of which the largest order of insects the dipterans (69.23%) and the least numbers were Hemipterans (0.12%).

4.2.3 Diversity of insects identified comparing endemic and non-endemic communities

The Shannon Weaver's diversity index and Simpson's diversity index were consistent in showing that the insects captured were more diverse in the endemic communities than in the non-endemic communities (Table 4.3). However there was no significant difference in the diversity of insects captured among the communities as shown by the value of the Jaccard's index of similarity of 0.42 (Table 4.3).

Table 4.2. Comparison of biting and non-biting identified from pooled insects and total number of insects sampled (in bracket) between endemic and non-endemic communities.

Insect mouthpart	Endemic	Non-endemic	Total
Biting	29 (134)	44 (386)	73(520)
Non-biting	39 (349)	60 (463)	99 (810)
Total	68 (483)	104 (847)	172 (1330)

Table 4.3. Measure of diversity and similarity of insects between endemic and non-endemic communities.

Community	Shannon Weavers diversity index (H)[a]	Simpson's diversity index (D)[b]	Jaccard's index(J)[c]
Endemic	2.52	0.89	0.42
Non-endemic	2.36	0.84	

* a & b measure the diversity of insects per family identified within each category of communities and c measures the similarity of insects per family identified between both category of communities

4.3 Detection of *M. ulcerans* DNA in pooled samples

Of the 110 pooled samples, 39 (41.9%) were biting insects and 54 (58.1%) were non-biting (Table 4.2.) while 42 (38.2%) were from endemic communities and 68(61.8%) from non-endemic communities (Table 4.4). For the detection of the IS*2404* insertion sequence target 3 out of the 110 pooled samples tested positive while 102 were negative (Compare Tables 4.1 & 4.4). Five samples however showed inhibition when run against the target (Table 4.4) and of these, one was the control while 4 were attributed to the insect samples. For the three (3) positive samples, two (2) out of the three were non-biting insects (Order Diptera) and one (1) was a biting insect (Order Coleoptera). All the 42 (100%) pooled samples tested negative for IS*2404* from the endemic communities however for non-endemic communities 60 (88.2%) pooled samples tested negative while 3 (4.4%) tested positive and 5 (7.4%) showed inhibition (Table 4.5).

Table 4.4. Detection of IS*2404* target of *M. ulcerans* of pooled insect samples with biting and non-biting mouthparts.

Insect mouthpart	**PCR (IS*2404*)**			**Total**
	inhibition	negative	positive	
Biting	1	37	1	39
non-biting	3	49	2	54
Total	**4**	**86**	**3**	**93**

Table 4.5. Detection of IS*2404* target of *M. ulcerans* of pooled samples between endemic and non-endemic communities

Community	**PCR (IS*2404*)**			**Total**
	inhibition	negative	positive	
Endemic	0	42	0	42
non-endemic	5	60	3	68
Total	**5**	**102**	**3**	**110**

4.4 Prevalence of *M. ulcerans* carriage in nocturnal insects

The Ct values of the three (3) positive samples were determined to be 39.85, 37.63 & 37.73 for the orders Diptera and Coleoptera respectively. The estimated bacterial loads of the samples were determined to range between 10^{-2} – 10^{-3} organisms/mL (Table 4.6).

Comparative studies was done to determine whether there was an association between biting and non-biting insects to support the hypothesis that biting insects may be responsible for transmitting the mycobacterium. Results showed that the relationship between biting and non-biting insects is statistically insignificant (X^2 (1) = 0.111, p = 0.739). Comparative studies was also done between endemic and non-endemic communities and results however

indicate that the null hypothesis that there is no association between endemicity and presence of the mycobacterium should be rejected (X^2 (1) = 1.830, p = 0.176).

Table 4.6: Estimation of bacterial load using Ct values of samples positive for IS*2404*

Order	Family	Ct value	Estimated range of bacterial load (organisms/ml)
Coleoptera	Unknown	37.73	$10^{-2} – 10^{-3}$
Diptera	Calliphoridae	37.63	$10^{-2} – 10^{-3}$
Diptera	Psychodidae	39.85	$10^{-2} – 10^{-3}$

CHAPTER FIVE

5.0 DISCUSSION AND CONCLUSION

5.1 DISCUSSION

This study sought to determine the different types of nocturnal biting insects present in the Akuapem-South District and investigate whether these biting insects harboured *M. ulcerans*. The study generated nine different orders of insects including the order Hemiptera of which most detection of the mycobacterium has been observed (Portaels et al, 2001 & Mosi et al, 2008). The most numbers of insects were observed in the order Diptera which could be attributed to the type of trap used, as the light trap is more likely to capture flying insects than other types of insects. More non-biting insects were captured than biting insects which could also be based on the type of trap used.

A diverse collection of insects were captured from the district (Simpson's diversity, D = 0.89 and 0.8, for endemic and non-endemic respectively; Shannon Weaver's index, H= 2.52 & 2.36) and although more insects were captured from non-endemic communities, there were more diverse insects from the endemic communities. Simpson's diversity index usually occurs between 0 and 1 and a community is said to be more diverse when it is above 0.5 (Morris *et al.,* 2014). Two diversity indices were used in determining which category of communities was more diverse as recommended by Morris *et al.,* (2014). It is also important to note that Jaccard's index (Morris *et al.,* 2014) of 0.42 which is below 0.5 simply means there is a resemblance in the insects captured across the communities. This indicates that although some orders (or class Arachnida) of insects were not captured in some communities, there is a high possibility of sampling these orders under different circumstances. Their absence could be due to the insects not being captured during the sampling period or they might not have been present at the sampling sites. The highly diverse nature of the insects

sampled was very good for the study in determining the presence of *M. ulcerans* in other invertebrates as recommended by Merritt *et al.,* (2010) and the similarity across communities also helps in standardizing the data.

With a p value = 0.866 (p> 0.5), it showed that there was no association between endemicity and the insect mouthpart. It is necessary therefore that sampling of insects in further studies should be done in both communities to determine the possible vector of *M. ulcerans*. The study was skewed towards nocturnal biting insects however non-biting insects were also sampled to determine whether there really was an association between presence of *M. ulcerans* and insect mouthpart.

Although three out of the 110 pooled samples tested positive for IS*2404*, they tested negative for the IS*2606* and KR targets. This could be due to possible low quantities of *M. ulcerans* present in the samples which based on their Ct values may contain an estimated bacterial load of 10^{-2} – 10^{-3}. These low Ct values are consistent with Ablordey *et al.,* (2005) on the difficulty in detecting *M. ulcerans* from the environment. However the possibility of *M. ulcerans* being present in the insects especially of different orders provides evidence to support the theory by Wallace *et al.,* (2010) that other invertebrate taxa might contain the mycobacterium. This does not however dispute the possibility of other mycobacteria which possess the IS*2404* target as being the present as opposed to *M. ulcerans* since there was no confirmation of M. ulcerans b IS2606 and KR.

The study had an interesting twist with no detection of the IS*2404* target in any of the pooled insects from the endemic communities while all the three (3) positive samples for the target were found in non-endemic communities. This raises questions as to whether endemicity of an area has a factor to play in the determination of the mode of transmission of *M. ulcerans*.

The positive pooled samples were found to belong to the orders Diptera and Coleoptera. The high numbers of the order Diptera could explain why two out of the three positive pooled samples for IS*2404* target belonged to this order of insects. Despite consistent detection of *M. ulcerans* in aquatic insects of the order Hemiptera, (Mosi *et al.*, 2008) no detection was observed in nocturnal insects of the order Hemiptera. This may suggest that only families of the order Hemiptera associated with the aquatic niche (Belostomatidae & Naucoridae) may harbour the organism (Mosi *et al.,* 2008, Marsollier *et al.*, 2002). Yet detection in the other orders of insects might begin to explain why Mosi *et al.,* (2008) failed to link *M. ulcerans* to aquatic insects in Ghana.

Two out of the three positive results were found to belong to families of insects with biting mouthparts (Coleoptera: *family unknown*; Diptera: Psycodidae). Since the current study focused on the whole insect, it cannot be determined which part of the insect harboured the mycobacterium thus it is necessary that further work be done narrowing on these group of insects. For the one non-biting insect though (Diptera: Calliphoridae), detection of *M. ulcerans* could be contamination with the microorganism or could indicate that insects of this family contribute to maintenance of *M. ulcerans* in the ecosystem.

No association was found between the insect mouthpart and the presence of *M. ulcerans* ($p=0.789$, $p > 0.05$). Though this therefore refutes the hypothesis of biting insects being the mode of transmission of *M. ulcerans*, the relatively small sample size cannot totally dismiss the possibility of the hypothesis.

The findings of the study suggests that insects of the Akwapim-South district may come into contact with the other mycobacterium through interaction within a food web. Further studies though could be done to determine which mycobacterium could be present in the insects.

Limitations of the study

The study had a few limitations with sample collection. The number of insects captured from the non-endemic communities far exceeded those from endemic communities as a result of destruction of some insects during sample collection. This could account for no detection of *M. ulcerans* in insects captured from these communities.

The type of traps used during sampling also favoured some insects and might have resulted in some insects not being included in the study.

Recommendations

It is recommended that further studies be done with different sampling methods within the District to have a true reflection of the insects present within the district and their role in *M. ulcerans* ecological dynamics.

5.2 CONCLUSION

The goal of this study was to identify and provide a catalogue of nocturnal biting insects from which potential insect candidates could be selected for vector incriminating studies. Several orders of nocturnal insects were identified and after molecular analysis, nocturnal insects of the orders Coleoptera and Diptera may have a role to play in the ecological dynamics of *M. ulcerans*.

Findings in the current study are also consistent with the low detection rate of *M. ulcerans* in the environment and provides insight to the fact that environmental factors which may contribute to the transmission of *M. ulcerans* may not necessarily be confined to endemic communities within endemic districts.

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APPENDIX

Appendix 1

Materials and Instruments

- Light trap
- Sucking tube
- Mortar & pestle
- Fast Prep® Bead beater
- Centrifuge
- Rotor gene Series® Real time PCR machine
- Ethanol
- Distilled water
- Nuclease-free water
- NaOH pellets
- PowerSoil® DNA kit (MO BIO Laboratories Inc.)
- Qiagen® PCR reagents
- Taqman® MGB Probe (Applied Biosystems)
- Primers (Applied Biosystems)
- Pipettes and pipette tips
- Eppendorf tubes
- Universal bottles

Appendix 2

Reagent preparation

2.1 70% ethanol

350ml of ethanol (100%) was added to 150ml of distilled water to obtain required volume of 500ml

2.2 50mM NaOH

0.2g of NaOH pellet was added to 100ml of sterile distilled water to obtain required volume of 100ml 50mM NaOH

2.3 Reagents for DNA extraction using PowerSoil Kit

- PowerSoil® Solution C1 contains SDS and other disruption agents required for complete cell lysis.
- PowerSoil® Solution C2 is patented Inhibitor Removal Technology® (IRT) and contains a reagent to precipitate non-DNA organic and inorganic material including humic substances, cell debris, and proteins.
- PowerSoil® Solution C3 is patented Inhibitor Removal Technology® (IRT) and is a second reagent to precipitate additional non-DNA organic and inorganic material including humic acid, cell debris, and proteins.
- PowerSoil® Solution C4 is a high concentration salt solution.
- PowerSoil® Solution C5 is an ethanol based wash solution used to further clean the DNA that is bound to the silica filter membrane in the Spin Filter.
- PowerSoil® Solution C6 (10 mM Tris) contains sterile elution buffer.

2.4: Sequence of primers and probe for IS*2404* semi-quantitative PCR

Primers	**Sequence (5' – 3')**
IS*2404* TF	AAAGCACCACGCAGCATCT
IS*2404* TR	AAAGCACCACGCAGCATCT
Probe	**Sequence (5' – 3')**
IS*2404* TP	6-FAM-CGTCCAACGCGATC-MGBNFQ

2.5: Sequence of primers and probe for IS*2606* & KR semi-quantitative PCR

Primers	**Sequence (5' – 3')**
IS*2606* TF	CCGTCACAGACCAGGAAGAAG
IS*2606* TR	TGCTGACGGAGTTGAAAAACC
KRTF	TCACGGCCTGCGATATCA
KRTR	TTGTGTGGGCACTGAATTGAC
Probe	**Sequence (5' – 3')**
IS*2606* TP	VIC-TGTCGGCCACGCCG-MGBNFQ
KRTP	6-FAM-ACCCCGAAGCACTG-MGBNFQ

2.6: Reagents for IS*2404* IPC multiplex semi-quantitative PCR

Reagent (starting concentration)	**Amount to add (µL) for 1 reaction**
TaqMan® Universal PCR Master Mix (2X)	12.5
IS2404 TF (18µM)	1.25
IS2404 TR (18µM)	1.25
IS2404 TP (5µM)	1.25
Exo IPC DNA (50X)	0.5
Exo IPC Mix (10X)	2.5
Nuclease Free Water	4.75
Total	**24**

a. Stock preparation of IS*2404 primers*

18µl of 100ml reconstituted primers was added to 82µl of nuclease-free water to obtain required total volume of 100µl of 18µM primer

b. Stock preparation of IS*2404 probes*

5µl of 100ml reconstituted probe was added to 95µl of nuclease-free water to obtain required total volume of 100µl of 5µM probe

c. Master mix preparation for 40 samples

1. Pipette 500µl of TaqMan® Universal PCR Master Mix (2X)
2. Add 50µl IS2404 TF (18µM)
3. Add 50µl IS2404 TR (18µM)
4. Add 50µl IS2404 TP (5µM)
5. Add 20µl of Exo IPC DNA (50X)
6. Add 100µl of Exo IPC Mix (10X)
7. Add 190µl of nuclease-free water

8. Centrifuge and dispense 24µl into PCR tubes
9. Add 1µl of DNA extract

2.7: Reagents for IS*2606* KR multiplex semi-quantitative PCR

Reagent (starting concentration)	**Amount to add (µL) for 1 reaction**
TaqMan® Universal PCR Master Mix (2X)	12.5
IS*2606* TF (18µM)	1.25
IS*2606* TR (18µM)	1.25
IS*2606* TP (5µM)	1.25
KRTF (18µM)	1.25
KRTR (18µM)	1.25
KRTP (5µM)	1.25
Nuclease Free Water	4
Total	**24**

a. Stock preparation of IS*2606* & KR *primers*

18µl of 100ml reconstituted primers was added to 82µl of nuclease-free water to obtain required total volume of 100µl of 18µM primer

b. Stock preparation of IS*2606* & KR *probes*

5µl of 100ml reconstituted probe was added to 95µl of nuclease-free water to obtain required total volume of 100µl of 5µM probe

c. Master mix preparation for 10 samples

1. Pipette 125µl of TaqMan® Universal PCR Master Mix (2X)
2. Add 12.5µl IS*2606* TF (18µM)
3. Add 12.5µl IS*2606* TR (18µM)
4. Add 12.5µl IS*2606* TP (5µM)

5. Add 12.5µl KRTF (18µM)
6. Add 12.5µl KRTR (18µM)
7. Add 12.5µl KRTP (5µM)
8. Add 40µl of nuclease-free water
9. Centrifuge and dispense 24µl into PCR tubes
10. Add 1µl of DNA extract

Appendix 3

Calculations

1. **Determination of mass of NaOH pellet**

   NaOH has a molecular weight of 40.0g/mol. To obtain a final volume of 100ml with concentration of 50mM, the mass was calculated using the formula below;

   $$\text{Mass} = n \times M$$

   where n is the amount of substance and M is the molecular weight.

   To determine the amount of substance (n), the formula below is used;

   $$n = C \times V,$$

   where C is the concentration and V is the volume. Given that the concentration is 50mM and the volume is 100ml, the amount of substance is calculated as;

   $$n = 50\text{mM} \times 100\text{ml}$$

   $$= 5\text{mmol}$$

   Thus the mass of NaOH required is calculated as;

   $$m = 5\text{mmol} \times 40.0\text{g/mol}$$

   $$= 0.2\text{g}$$

2. Determination of diversity indices for endemic and non-endemic communities

Simpson's diversity index (D) represents the probability that two randomly chosen individuals belong to different species/families and is calculated using the formula;

$$D=1 - \sum P^2$$

where p is the proportion of individuals found in a particular specie/family.

Shannon-Weaver's diversity index (H) represents the uncertainty about the identity of an unknown individual. According to Morris *et al.*, (2014), predicting the identity of an unknown individual of a particular specie/family is highly uncertain in a more diverse community. This index is calculated using the formula;

$$\text{Shannon's diversity (H)} = -\sum P_i \ln(P_i)$$

where P_i is the proportion of individuals of the nth family.

Jaccard's similarity index (J) is used to determine how the families of each community varies and is calculated using the formula;

$$J= \frac{Sc}{Sa + Sb + Sc}$$

where Sa and Sb are the number of families unique to community A and B and Sc is the number of families common to the two communities.

Diversity data of insects captured and identified

Diversity table for communities					
Endemic					
Family	Sum of qty identified(n)	Relative abundance (p)	In(p)	p(ln(p))	n(n-1)
Aderidae	2	0.004158004	-5.48272009	-0.022797173	2
Agelenidae	2	0.004158004	-5.48272009	-0.022797173	2
Alydidae	1	0.002079002	-6.17586727	-0.012839641	0
Asilidae	5	0.01039501	-4.566429358	-0.047468081	20
Bibionidae	50	0.103950104	-2.263844265	-0.235326847	2450
Braconidae	1	0.002079002	-6.17586727	-0.012839641	0
Ceratopogonidae	20	0.041580042	-3.180134997	-0.132230145	380
Chaoboridae	10	0.020790021	-3.873282177	-0.080525617	90
Chironomidae	15	0.031185031	-3.467817069	-0.108143983	210
Cicadellidae	31	0.064449064	-2.741880066	-0.176711605	930
Corculionidae	1	0.002079002	-6.17586727	-0.012839641	0
Empididae	94	0.195426195	-1.632572488	-0.31904743	8742
Formicidae	42	0.087318087	-2.438197652	-0.212898755	1722
Gelechidae	1	0.002079002	-6.17586727	-0.012839641	0
Largridae	1	0.002079002	-6.17586727	-0.012839641	0
Lycidae	1	0.002079002	-6.17586727	-0.012839641	0
Lygaeidae	1	0.002079002	-6.17586727	-0.012839641	0
Mordellidae	1	0.002079002	-6.17586727	-0.012839641	0
Myceptophilidae	3	0.006237006	-5.077254981	-0.031666871	6
Phoridae	2	0.004158004	-5.48272009	-0.022797173	2
Pipunculidae	26	0.054054054	-2.917770732	-0.157717337	650
Psopsidae	3	0.006237006	-5.077254981	-0.031666871	6
Psycodidae	59	0.122661123	-2.098329826	-0.257383492	3422
Pterophoridae	6	0.012474012	-4.384107801	-0.054687415	30
Sciaridae	71	0.147609148	-1.913187393	-0.28240396	4970
Simulidae	2	0.004158004	-5.48272009	-0.022797173	2
Sphecidae	1	0.002079002	-6.17586727	-0.012839641	0
Staphylinidae	2	0.004158004	-5.48272009	-0.022797173	2
Tineidae	27	0.056133056	-2.880030404	-0.161664908	702
				-2.519085951	24340
Total(N)	481				
Shannon Weavers diversity index (H)	2.519085951	12.41724151			
Simpson's diversity index (D)	0.89457727				

Non-endemic communities					
Family	**Sum of qty identified(n)**	**Relative abundance (p)**	**In(p)**	**p(ln(p))**	**n(n-1)**
Asilidae	7	0.008363202	-4.783913921	-0.040008838	42
Braconidae	3	0.003584229	-5.631211782	-0.020183555	6
Caliphoridae	1	0.001194743	-6.72982407	-0.008040411	0
Cecidomyidae	3	0.003584229	-5.631211782	-0.020183555	6
Ceratopogonidae	38	0.045400239	-3.092237911	-0.14038834	1406
Cercopidae	4	0.004778973	-5.343529709	-0.025536582	12
Chironomidae	10	0.011947431	-4.427238977	-0.052894134	90
Chrysopidae	4	0.004778973	-5.343529709	-0.025536582	12
Cicadellidae	23	0.027479092	-3.594329855	-0.098768921	506
Dixidae	12	0.014336918	-4.244917421	-0.060859031	132
Dolichopodidae	4	0.004778973	-5.343529709	-0.025536582	12
Empididae	242	0.289127838	-1.240886344	-0.358774785	58322
Formicidae	141	0.168458781	-1.78106418	-0.300035901	19740
Hydroptilidae	11	0.013142174	-4.331928798	-0.056930964	110
Lampyidae	1	0.001194743	-6.72982407	-0.008040411	0
Lauxanidae	3	0.003584229	-5.631211782	-0.020183555	6
Lycidae	1	0.001194743	-6.72982407	-0.008040411	0
Mantispidae	3	0.003584229	-5.631211782	-0.020183555	6
Mordellidae	1	0.001194743	-6.72982407	-0.008040411	0
Mycetophilidae	11	0.013142174	-4.331928798	-0.056930964	110
Phoridae	24	0.028673835	-3.55177024	-0.101842874	552
Phryganeidae	4	0.004778973	-5.343529709	-0.025536582	12
Pipunculidae	2	0.002389486	-6.03667689	-0.014424556	2
Pseudocaecillidae	2	0.002389486	-6.03667689	-0.014424556	2
Psopsidae	3	0.003584229	-5.631211782	-0.020183555	6
Psycodidae	172	0.205495818	-1.582329594	-0.325162115	29412
Pterophoridae	14	0.016726404	-4.090766741	-0.068423816	182
Ptinidae	1	0.001194743	-6.72982407	-0.008040411	0
Pyralidae	7	0.008363202	-4.783913921	-0.040008838	42
Reduvidae	1	0.001194743	-6.72982407	-0.008040411	0
Sciaridae	35	0.04181601	-3.174476009	-0.132743919	1190
Sciomyzidae	5	0.005973716	-5.120386158	-0.030587731	20
Scotylidae	2	0.002389486	-6.03667689	-0.014424556	2
Sepsidae	3	0.003584229	-5.631211782	-0.020183555	6
Silphidae	1	0.001194743	-6.72982407	-0.008040411	0
Simulidae	1	0.001194743	-6.72982407	-0.008040411	0
Tenebrionidae	1	0.001194743	-6.72982407	-0.008040411	0
Tineidae	29	0.034647551	-3.362528241	-0.116503368	812
Trichoceridae	7	0.008363202	-4.783913921	-0.040008838	42
				-2.359758402	112800
Total(N)	837				

Shannon Weavers diversity index (H)	2.359758402	10.58839301			
Simpson's diversity index (D)	0.838795425				
Jaccard's index					
S_a	11				
S_b	18				
S_c	21				
	0.42				