THE DYNAMICS OF *MYCOBACTERIUM ULCERANS* TRANSMISSION BY NAUCORID SPECIES (HEMIPTERA: NAUCORIDAE) IN PARTS OF GREATER ACCRA REGION OF GHANA.

BY

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DECLARATION

I do here by declare that the experimental work described in this thesis was carried out by me and that all cited references have been duly acknowledged. This thesis, either in whole or in part has not been submitted for any other degree in any institution or organization elsewhere.

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DEDICATION

To my beloved family

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$AgNO_3$	silver nitrate
bp	base pair
BOD	biological oxygen demand
$CaCO_3$	Calcium carbonate
CSIR	Council for Scientific and Industrial Research.
DNA	deoxyribonucleic acid
DO	dissolved oxygen
EDTA	disodium ethylene diamine tetra acetate.
EtBr	ethidium bromide
EtOH	ethanol
$K2CrO_4$	Potassium chromate
KOH	potassium hydroxide
$MnSO_4$	manganese sulphate
LSD	least significance difference
M	molar
MU	*Mycobacterium ulcerans*
Mw	molecular weight
NH4	ammonium
NO2	nitrite
NO3	nitrate
PCR	polymerase chain reaction

pH	-log10[H+]
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolution per minute
sddw	sterile double distilled water
S.E.M.	standard error of the mean.
TAE	Tris acetate EDTA
T_m	melting temperature
Tris	2-amino-2-(hydroxymethyl)-1,3 propanediol
µl	micro liter
µM	micro molar

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ABSTRACT

Mycobacterium ulcerans disease (Buruli ulcer) is increasing worldwide but there is currently no effective chemotherapeutic treatment. Prevention and control has also not been possible because the mode of transmission remains illusive and at best speculative. Recent investigation however points to the possible involvement of aquatic bugs belonging to the familily Naucoridae in the transmission process. In the laboratory, infected Naucorid species have been shown to transmit the bacterium through their bites. However, to establish that insects of this group are the vectors of Buruli ulcer disease pathogen, it is more important to understand if the insects are naturally infected in their natural environment in the wild. Although considered of much importance for prevention and control, the distribution and ecological determinants of the distribution of these insects are lacking. Naucorid species were collected in three endemic and three non-endemic areas of Buruli ulcer in the Ga District using a D- net and coordinate of collection points were recorded using GPS. Eighteen water bodies were surveyed in this District to study the distribution of Naucorid species. 15 physico-chemical parameters of water habitats were analyzed in water samples where Naucorid species were found and where there were not found.

A total of 2181 insects were identified as Naucorid species. It was observed that Naucorid species were widespread across all the study areas. Mean distributions were not significantly different between endemic and non-endemic areas of Buruli ulcer. Statistical analysis using binary logistic regression revealed dissolved oxygen, PH and turbidity to be the most significant water parameters that influences the distribution of Naucorid species in a given habitat.

The prevalence of infection of the bugs was detected using polymerase chain reaction (PCR) with already published oligonucleotide primers MU1, MU2, PGP3 and PGP4. Out of a total of 1513 of the insects examined by PCR to detect the presence of *M. ulcerans*, (4.4%) were positive for the bacterium. The infection rates of Naucorid species between water bodies ranged from 2-7%. This result indicated that there were natural infections in the insects in the wild at all the study areas where the insects were examined. The prevalence of infections of Naucorids between endemic and non- endemic areas was not significantly different $P > 0.05$. Whereas, the prevalence of infection of the bugs between dry and wet season showed a significant difference $P < 0.05$. The occurrence of infection in these bugs and their ubiquitous nature suggest that they may play an important role in the transmission of *M. ulcerans* than presently understood.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Introduction and Rationale

Buruli ulcer (BU) is a disfiguring condition caused by the bacterium *Mycobacterium ulcerans*. The disease is estimated to be the third most common *Mycobacterium* infection in immunocompetent human in the world (Portaels, 1995; Portaels *et al.*1999). In some parts of West Africa where the incidence of *M. ulcerans* disease appears to be increasing, there are more cases of BU than of tuberculosis and leprosy (Harry *et al.*, 1999). This disease is of considerable public health importance in parts of Africa, central and southeastern Asia and to a lesser extent, in the Americas. The disease occurs in close proximity to slow moving or stagnant water suggesting that the bacterium may be multiplying in the aquatic environment (Brenda and Hirst, 1997; Asiedu, 2002). Some aquatic organisms such as snails, insects, fish and other animals such as koalas and opossum have been found to be infected with *M. ulcerans* (Mitchell *et al.*, 1984). The mode of transmission of the disease is yet to be established (Harry *et al.*, 1999). Initial epidemiological studies suggested that trauma and aerosol could be the most frequent means by which the *M. ulcerans* is introduced into the skin from surface contamination (Harry *et al.*, 1999). Recently, Marsollier *et al.*, (2002) experimentally infected some Naucorids (aquatic insects) in the lab and fed them on the laboratory mice. Subsequently the mice developed ulcer similar to *M. ulcerans* disease after been bitten by the bugs suggesting the possibility of transmission by these bug species.

Drug treatment for this disease is disappointing (Dariel *et al.*, 1993) and therefore surgical excision of necrotizic and infected skin is the current treatment of choice. However, surgery when performed, it leads to prolonged hospitalization, which may be as long as eighteen months or more (Dariel *et al.*, 1993). Furthermore, surgery may lead to significant morbidity and permanent deformity (Asiedu and Etuaful, 1998). In most hospitals in the developing countries, hospitalization of patient requires the concomitant "hospitalization" of a healthy relative to provide indirect care for the patient. This becomes a huge burden on the patient and their families. In countries without social programmes in the rural areas to take care of the disabled, members of the extended family constitute the social safety net and, in the event of illness and disability, the burden falls on the family. Thus the long-term care of people disabled by the disease could lead to considerable loss of productivity and greater poverty. The direct consequences of the sickness could be serious disruption of school due to prolong morbidity or permanent disabilities since about 70% of those affected by BU are children under 15 years (Harry *et al.*, 1999). In adults, BU with its physical and cosmetic problems could have similar enormous social stigma such as those of leprosy and tuberculosis (TB) in addition to the socio- economic burden involved. The social stigmas associated with these diseases in Ghana include loss of work, divorce, ostracism by family members and the local community, and loss of housing, resulting from the fears of co-tenants and landlords (Stephen, 2000).

The socio-economic burdens of BU have not been adequately studied in the endemic areas. However, one recent study on the socio- economic cost of the disease in Ghana shows that within the average hospitalization of 130 days, the total treatment cost per person was

estimated at US$783 (Asideu and Etuaful. 1998). This comprised of direct costs. that is the cost of services provided during period of hospitalization and indirect costs (the productivity losses incurred by the patient and the attending relative). With an escalating number of cases (Marston *et al.*, 1995; Muelder. 1998; Johnson *et al.*, 1996) and associated complications, the socio-economic impact of BU on the rural economic is becoming devastating at an alarming rate requiring a serious attention on prevention and control. The World Health Organization (WHO) established the Global Buruli Ulcer Initiative (GBUI) in 1998 with the aim to control the disease through research efforts as an acknowledgement of this fact. Recent research effort towards the control of BU has concentrated on chemotherapy and transmission. The WHO meeting on transmission (WHO, 1998) recommended studies on Naucorid species and other aquatic bugs such to elucidate the part they may play in the transmission of *M. ulcerans*. This project investigated the possibility of Naucorid species in the transmission of *M. ulcerans* in the wild. It also intends looked at the distribution and ecological determinants of the distribution of these insect species in their natural environment.

1.2 The main objectives

The main objectives of the proposed study was to investigate by PCR the prevalence of infection of *Naucorid species* in the wild and to compare the level of infection between endemic and non-endemic areas of BU in the Ga district of the Greater Accra region of Ghana.

1.2.1 Specific Objectives;

The specific objectives were as follows:

1. To collect and identify Naucorid species from water bodies in the study areas.
2. To study the distribution of the Naucorid species in the study sites.
3. To measure the physical and chemical properties of water samples collected at the study sites.
4. To use PCR to detect *M. ulcerans* in the bugs.
5. To determine the prevalence of *M. ulcerans* infection rates in the insects.
6. To use statistical methods to identify the physico-chemical parameters that most influence the distribution of *Naucorid* species.

CHAPTER 2

Literature Review

2.1 History and Global distribution of Buruli ulcer

MacCallum *et al.* (1948) first described BU in Australian patients who had an ulcerative lesion with undermined edges on the arms and legs. However, before then, there was evidence that the disease had been in existence in some other parts of African countries such as Uganda, and the Democratic Republic of Congo among others (Meyers, 1995; Van Oye *et al.*, 1950; Clancey *et al.*, 1962).

Presently BU has been reported from some 27 countries around the World mostly in tropical areas (Hayman and Asiedu, 2000). Figure 1a shows the global distribution of BU. The number of countries mentioned may be an under estimation since cases may not have been reported to health centers. In Africa, the history of the distribution of BU is classified into two main periods; i.e. foci that emerged before and after 1980 (Fig. 1b). There were many reported foci on the disease before 1980 in some African countries including Cameroon, Democratic republic of Congo (DRC), Gabon, Nigeria and Uganda. After 1980, new foci of BU emerged in West Africa with dramatic increase in the incidence in several West African countries, especially in Benin and Côted'Ivoire. New foci were also recently discovered in Angola (Bar *et al.*, 1998), Burkina Faso (Ouoba *et al.*, 1998), Guinea and Togo (*Meyers et al.*, 1996) and Ghana (Amofah *et al.*, 2002).

The first recorded case of BU in Ghana was in 1971 when one patient from Amasaman, Greater Accra region. reported at Korle Bu Teaching Hospital in Accra (Bayley, 1971). The author also reported three other probable cases that were all living in villages along tributaries of river Densu in Ga district. Van der Werf *et al.* (1989) reported a series of 96 cases from an endemic focus in the Asante Akim North district of the Ashanti region. This report was followed by the description of a major endemic focus in Amansie West district in the same region in which a lot of cases were found (Amofah *et al.*, 1993). A recent national case search for BU (Amofah *et al.*, 2002) reported a total case of 5,619 from ten regions in Ghana with overall crude national prevalence rate of active lesions of 20.7 per 100,000, but the rate was 150.8 per 100,000 in the most disease-endemic district. The overall crude prevalence rate exceeded that of leprosy (9 per 100,000) in 1999, making BU the second most prevalent mycobacterial disease in Ghana after tuberculosis (prevalence 66 per 100,000).

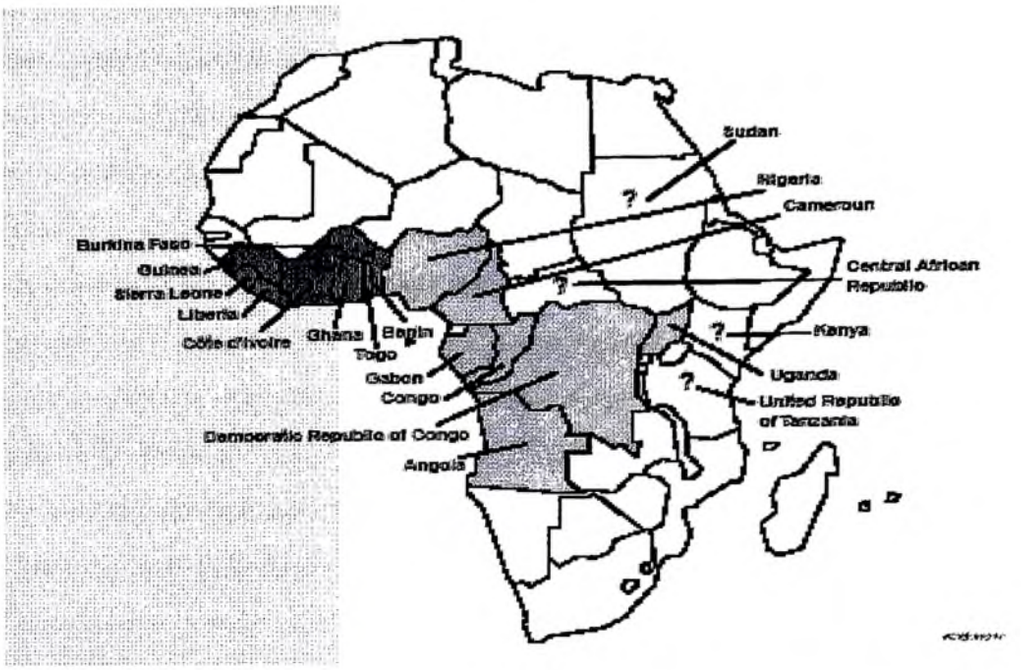

Fig. 2.1 Map of Africa showing the distribution of BU foci before 1980 (dark blue shaded) and after 1980 (light blue shaded) (W.H.O, 2002).

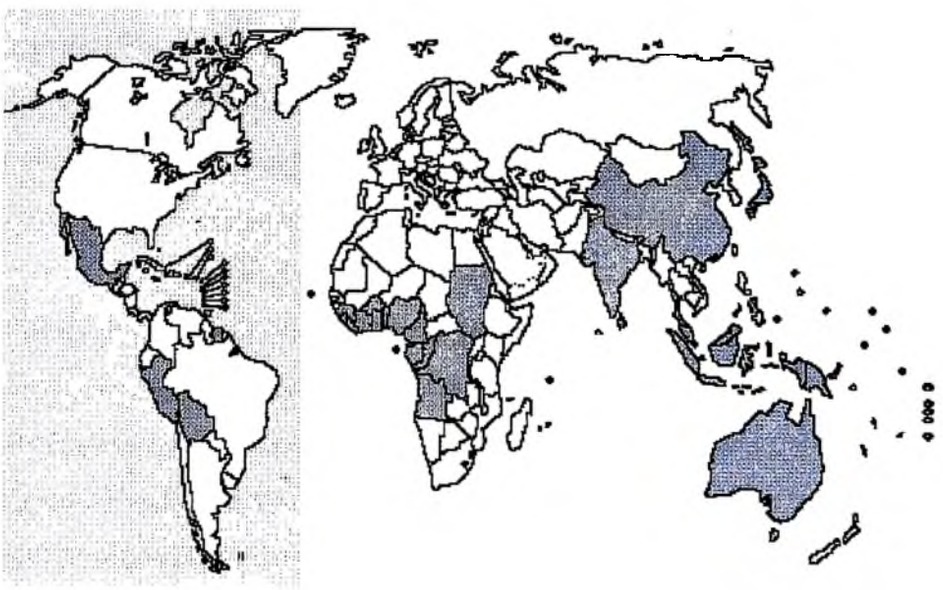

Fig. 2.2 Map of the world showing the geographical distribution of BU (shaded) (W.H.O, 2002).

2.2 Clinical and Histopathological aspect of BU

The name Buruli ulcer was derived from a district in Uganda where large numbers of cases were seen (Uganda Buruli Group, 1971). MacCallum and colleagues first established the association between *M. ulcerans* infection and the disease called *Bairnsdale ulcer* when the ulcers were found to occur in farmers in Australia (MacCallum *et al.*, 1948). *Mycobacterium ulcerans* is a slow-growing Mycobacterium often taking 6-9 months to form visible colonies on solid medium following primary isolation and it has higher affinity to skin than deeper tissue in patients (Mitchell *et al.*, 1984) The bacterium grows best in culture with optimum temperature below 34°C. It is characterized by its thermo-sensitivity, growing at 31-33°C but not at 37°C. which might explain its affinity for the skin rather than deeper tissues (Mitchell *et al.*, 1984). The initial lesion is a painless nodule or skin papule and leads to an oedematous swelling sometimes-producing diffuse oedema of a limb as a result of necrotizing panniculitis. Finally an ulcer is formed with undermined edges lined by necrotic tissue. Ulceration can be extensive and disfiguring, often affecting 50% or more of a limb (Harry *et al.*, 1999) (Figures 2.3 -2.6).

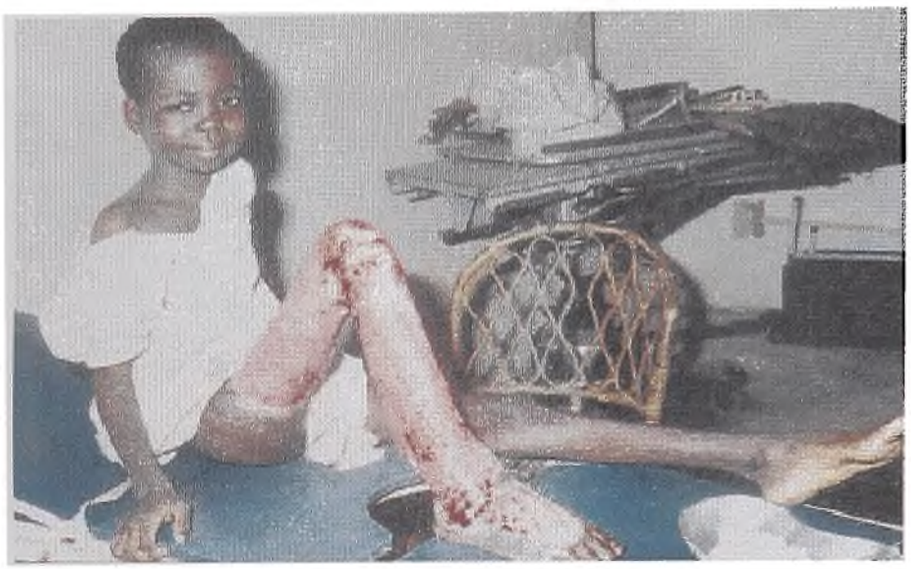

Fig. 2.3. Ulcer of the limbs (Asiedu, 2002)

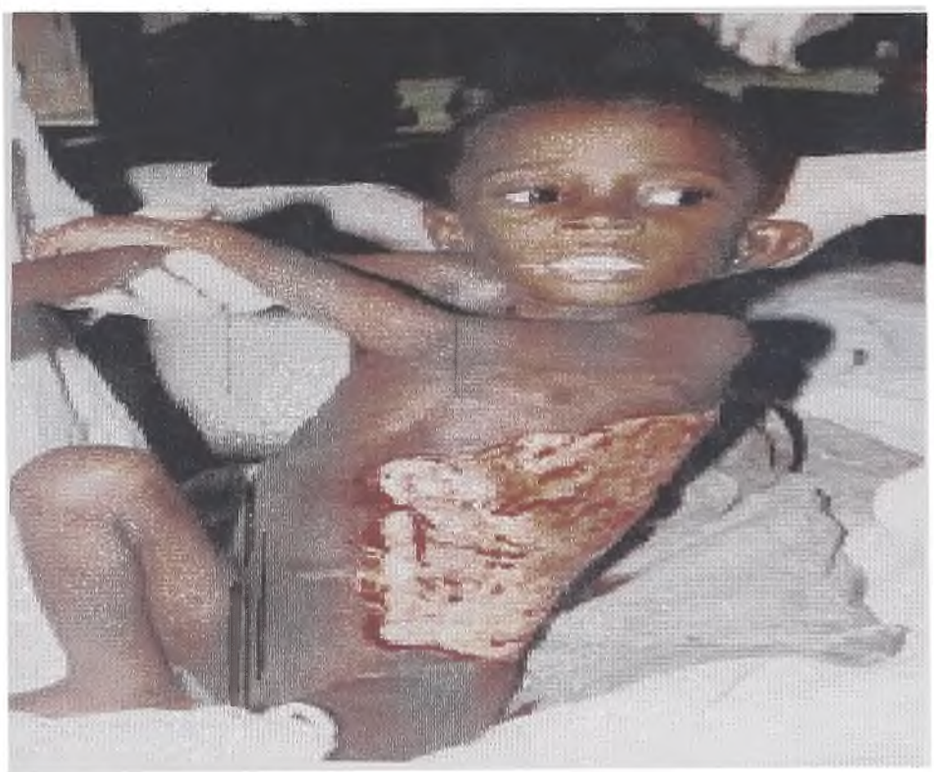

Fig. 2.4. Limb and rib ulcer Asiedu (Asiedu, 2002).

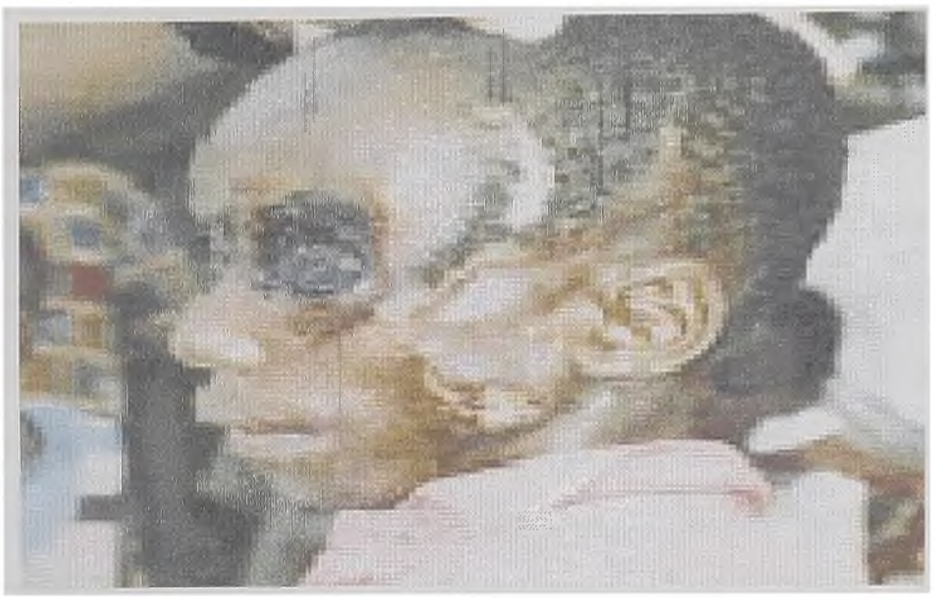

Fig. 2. 5. Blindness resulted from lesions close to the eyes (W. H. O., 2002 unpublished documents)

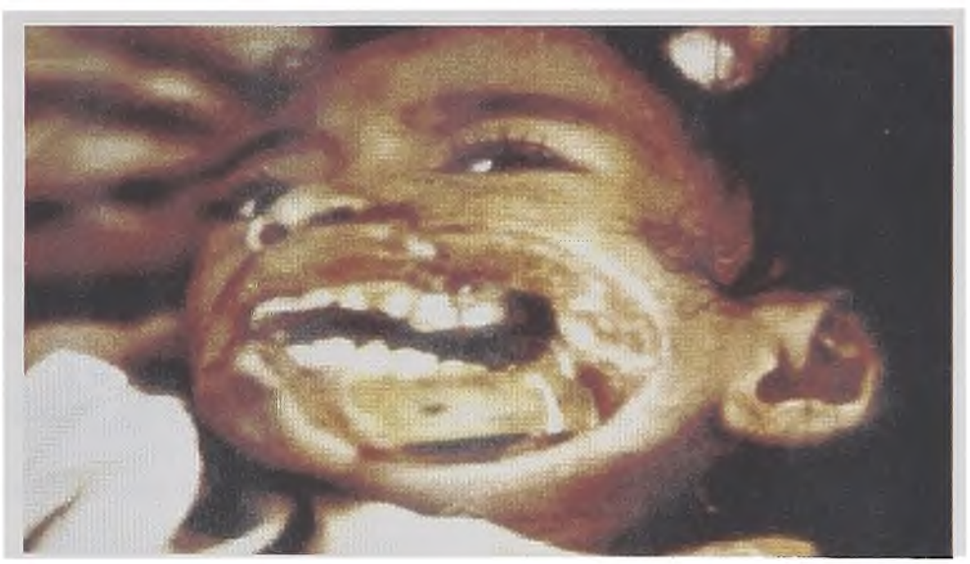

Fig. 2. 5. Mouth skin damage by lesions (W. H. O., 2002 unpublished documents)

It is thought that smaller lesions are self-healing over the course of many months but it is not known how often this occurs. Patients usually report to hospital when large ulcers are established or when secondary infection causes new symptoms of pain and fever, but sometimes the oedematous form of disease progresses rapidly and this associated with a worse prognosis. Rarely, in advanced lesions with extensive ulceration, the infection spreads beneath the deep fascia, affecting the bone. Recently it has been suggested that some new lesions arise from haematogenous spread (Portaels *et al.*, 1998). Long-term complications and contractures depend on the site and extent of the lesion. Contractures are common and also up to 10% of cases admitted to hospital require amputation of a limb (Asiedu & Etuaful, 1998). Furthermore lesions close to the eye may lead to blindness and those mouth parts could completely damage the mouth skin (Figs. 2. 5 and 2. 6 respectively.

The tissue changes produced by progressive *M.ulcerans* infection are different from those caused by other mycobacterial infection. Initially there is coagulating necrosis of the lower dermis and subcutaneous calcification. This is unlike the gaseous necrosis seen in other mycobacterioses. The necrosis does not appear to be due to infarction, as patient blood vessels are present. The lesions are highly bacilliferous with the organisms present in clumps or in smaller units in the center of the lesion. Unlike lepromatous leprosy, where clumps of *M. leprae* are present inside macrophages, *M. ulcerans* organisms are very rarely intracellular (Lucas. 1989). In these early lesions there is little or no evidence of an inflammatory response and no granulomas. As the disease progresses, all elements of the skin are affected including the nerves, and blood vessels and the epidermis is ultimately undermined forming an ulcer. Healing occurs through the formation of granulation tissue at the edges of the ulcer, laying down of fibrous tissue, and inward growth of epidermis. During this stage the number

of bacilli decreases and granulomas containing epithelia cells and Langhan's giant cells may be seen indicating that host cell-mediated immunity is important in the elimination of the organisms (Lucas, 1989).

2.3 The toxin of *M. ulcerans*

Mycobacterium ulcerans disease is characterized by tissue necrosis caused by a diffusible exotoxin (Krieg *et al.*, 1974). In human lesions, the area of necrosis extends well beyond the presence of mycobacterium clumps. In early necrosis lesions there is no evidence of inflammation or cellular response (Krieg *et al.*, 1974), suggesting that the tissue damage is not host mediated and is more likely to be due to a bacterial product. Sterile filtered supernatants of *M. ulcerans* have markedly cytotoxic activity *in vitro* and cause skin ulceration similar to human lesions when injected into the skin of mouse or guinea pig (Krieg *et al.*, 1974; Read *et al.*, 1974). Such necrosis was also observed after inoculation with live organisms. The cytotoxic and immunosuppressive effects of whole-culture filtrates and purified fractions subjected to intensive study indicated that culture filtrates produce cytopathic effects on the L-929 mouse fibroblast cell-line characterized by rounding-up and lifting-off of adherent (Hockmeyer *et al.*, 1978; George *et al* 1998). Culture filtrates also have marked immunosuppressive activity *in vitro*, suppressing T-cell responses and inhibiting phagocytes of latex beads by macrophages (Pimsler *et al.*, 1988).

Using flow cytometric analysis, George *et al.* (1998) demonstrated that the cytopathic effect of the toxin on L 929 cells is due to cell-cycle arrest in the G1 phase of cell cycle. This was accompanied by fiber rearrangement in the actins cytoskeleton. George *et al.*, (1999) further

separated the lipids by chromatography and found that this activity was within 2 of 11 spots. A major break through has been achieved by the recent report that the cytotoxic molecule is a polypeptide-derived macrolide (George *et al.*, 1999). A number of macrolide toxins that depolymerize actins have previously been described (Saito & Karaki, 1996).

The relatively benign cytotoxic effect on cultured cells is reversible *in vitro* and does not mirror the extensive necrosis seen in human or in experimental animal lesions. These differences suggest the possibility that the toxin may accumulate in far greater concentrations in the skin than it does *in vitro*. It is also possible that induction *in vivo* of genes involved in the pathways responsible for toxin production may play a contributory role. Human macrophages when grown *in vitro* harbor *M.ulcerans* for up to 7 days (Sharif, 1991) yet intracellular organisms are seldom seen in human lesions (Krieg *et al.*, 1974). Thus it is possible that macrophages may initially engulf the organisms after entry through the skin. The subsequent accumulation (and/or induction) of toxin may be of a sufficient concentration to cause lyses of the macrophage host and to paralyze the cellular functions of infiltrating lymphocytes or macrophages. Toxin from *M. ulcerans* exerts local immunonosupressive and /or antiphagocytic effects hence the absence of fever, malaise and regional lymphadenopathy in patients. In turn, this localized immunosuppressant may contribute to a delay in an early systemic immune response to mycobacterial antigen. This event may account for the observation that patients with active lesions are often unresponsive to *M. ulcerans* derived antigen (burulin) on skin testing (Stanford, 1983). Later during the healing phase characterized by the appearance of granulomas, there is conversion to a positive burulin test (Harry *et al.*, 1999) unlike that seen in tuberculosis where patients are tuberculin positive

regardless of the stage of the disease. Buruli ulcer patients may not necessarily be due to the ability of the granulomas which could converts to burulin.

2.4 Possible mode of transmission of BU

Epidemiological data have not clearly supported person-to –person transmission of BU indicating that it is not contagious (Portaels, 1989) and despite recent advances the mode of transmission remains unclear. Several hypotheses have been proposed with regard to transmission. For example it has been suggested that aerosols from the environment may carry *M. ulcerans* and infect the host via the respiratory tract or contaminate the skin surface (Hayman, 1991). Another hypothesis is that trauma or an insect bite are probably the most frequent means by which *M. ulcerans* is introduced deep into the skin or subcutaneous tissue from the contaminated surface of the skin or from contaminated insects (Meyers *et al.*, 1974; Portaels *et al.*, 2001). Some patients, albeit a few, have reported that the ulcer had started after an abrasion of some sort (Hayman, 1991; Amofah *et al.*, 1993) but patients may have forgotten such injuries by the time they develop overt disease.

Hayman (1991) postulated that *M. ulcerans* is normally a harmless environmental microorganism that are present in the soil, and lives in symbiosis with the roots of certain plants in tropical rain forests or warm temperate environments. Following environmental disturbance the mycobacterium is washed from its normal habitat into draining rivers, lakes, dams, ponds, swamps or irrigated areas in greater numbers than would normally occur. Within this lacustrine system, given favourable conditions, there may be proliferation of the

organism similar to the "bloom" of algae, which occurs over the surface of small lakes or ponds during months of years rather than days, depending on daily water temperatures.

The author furthermore postulated that, from this water environment, naturally generated aerosols might disperse *M. ulcerans*. Organic materials and bacteria may be propelled into the air, where they may be carried upward by convection and wind currents and transported long distances. This could explain why some patients without history of direct contact with water got infected with BU. Recently, Portaels *et al.* (1999) proposed a hypothesis that environmental mycobacteria are present in water and mud at the bottom of swamps. Small water-filtering organisms such as microphagous fish, mosquito larvae, small crustacean or mollusc, or even some protozoa such as amoeba may mechanically concentrate these mycobacteria. These water-filtering organisms could concentrate environmental mycobacteria, including *M. ulcerans*, and subsequently be ingested by water-dwelling predators or even by some non-microphagous fish. These water-dwelling predators thereby become passive reservoirs of environmental mycobacteria. Some of these aquatic predators may bite animals or humans and mechanically introduce environmental mycobacteria (including *M.ulcerans*) into the skin or deposit the mycobacteria on the surface of the skin.

2.5 The epidemiology of BU

The epidemiology of BU is poorly understood mostly due to the poor system of reporting cases in most endemic countries hence it is difficult to establish whether the incidence is changing. Reporting has also being influenced by lack of awareness of the disease, interest shown by outside groups and by political factors among other things (Asiedu and Etuaful,

1998) leading to unnoticed increase of cases in endemic countries of west Africa. In Ghana for instance the bed occupancy by patients with BU increased from about 35% to 70% in some hospitals between 1989 to 2002 (Asiedu and Etuaful, 1998). In an endemic area in Cote d'Ivoire, new cases increased more than 3-fold between 1987 and 1991 (Marston *et al.*, 1995). In addition to a large increase in new cases documented in Benin between 1986 and 1996 there has been an outward spread of the disease from initially restricted foci (Aguiar *et al. 1997*). In areas of high incidence up to 22% of the population may be affected (Asiedu & Etuaful, 1998). There is recent evidence for a zoonotic spread involving water insects, which may bite humans (Portaels *et al.*, 1999). Previously, Barker (1973) had advocated the hypothesis that *M. ulcerans* is able to survive on certain kinds of vegetation in swamps, and could enter the skin via sites of trauma. This has not been substantiated because until recently, all attempts to culture the organism from the environment have been unsuccessful (Ross *et al.* 1997b). Portaels (1998) reported initially the detection of *M. ulcerans* in roots of aquatic plants growing in swamps of endemic areas of Benin using PCR. However, further analysis showed that this DNA was related to water insects in the roots rather than in the vegetation itself. Portaels *et al.* (1996) have identified a region in the 3^1 end of the 16SrRNA sequence of *M. ulcerans*, which allows classification of strains. The variation in the sequence corresponds to the geographical origin of the isolates and these new molecular methods promise a better understanding of the epidemiology of the disease.

2.6 Treatments of BU

The mainstay of treatment is surgical excision of early lesions, which is often curative. Unfortunately many patients do not report their cases to hospital until there is extensive and

with positive tuberculin tests were better protected than tuberculin-negative individuals. These findings indicated considerable antigenic overlap of *M ulcerans* with *M. tuberculosis* and BCG. Though the incidences of the disease have been reported to have fallen, the efficacy of protection appeared to wane after the first 6 months (Uganda Buruli Group, 1969). These and occasional unpublished attempts at chemotherapy have led to the assumption that antibiotics are not of any use because of poor perfusion into diseased tissue. Antibiotics may be useful in particular situations such as after debridement, to prevent later recurrence of new lesions or to prevent haematogenous spread. However, work done by (Palomino *et al.*, 1998) showed the bacteria to be present in bone marrow, liver, and spleen even after amputation of infected limb in mice. This shows a possibility of resurgence at any trauma site even if the patient does not come into contact with *M. ulcerans* from the environment.

Treatment of BU by the use of Bio-oxidative medicine particularly Hydrogen peroxide have been suggested (Palomino *et al.*, 1998) but not proven to be effective without surgery (Adjei *et al.* 1998) Report by (Adjei *et al.* 1998) suggested that phenytoin an anti-convulsant, when applied topically to ulcers of laboratory mice promoted healing without the scaring and contractures that are normally associated with the disease. This treatment would be relatively simple to administer. However, the results need to be substantiated on human beings.

Taking advantage of the temperature sensitivity of the organism, localized application of heat to lesions has been reported to help healing with or without surgery in a few cases (Glynn, 1972). However, others have found this treatment ineffective and the sophisticated nature of

the devices and dressing will prevent it from being used routinely in a tropical setting making surgery as the ultimate means of treatment. The cost of surgery is high and requires a long time hospitalization making it difficult for ordinary patients to afford the cost of treatment. Treatment costs are high because of the need for prolonged hospital stay, often exceeding 100 days per patient. Recurrence after surgeries have been reported (Harry *et al.*, 1999) which may further complicate the management of cases suggesting the serious need of prevention and control rather than treatment. However, because there is increasing evidence that human infection is derived from *M. ulcerans* in the environment, prevention will not be simple. An understanding of the nature of the environment in which *M. ulcerans* resides may help in the modification of aquatic reservoirs in endemic areas and in areas where there are fresh outbreaks. More research is needed for environmental remedial actions (Harry *et al.*, 1999).

2.7. Polymerase Chain Reaction (PCR)

The PCR is an *in vitro* technique for synthesizing specific DNA sequences. It is an enzymatic catalyzed biochemical reaction in which small amounts of a specific DNA segments are amplified using two oligonucleotide primers into large amounts of linear double stranded DNA. The principle was first described in detail by Khorana and Colleagues (Kleppe *et al.* 1971; Panet and Khorana, 1974), but was revised and named a decade later by K. Mullis (Saiki *et al.*, 1985; Mullis *et al* 1986).

The fundamental idea of PCR technology is the principle of DNA amplification using DNA polymerase (Jackson *et al.* 1995). It involves the use of pairs of synthetic oligonucleotide

primers chosen to flank the region of DNA that is to be amplified.The most widely used polymerase is the Taq DNA derived from the bacterium *Thermophilus aquatious* which inhabits hot springs due to its unique properties of being stable at high temperature of 95°C and an optimum working temperature of 76°C (Jackson *et al*, 1995). Following cycles of DNA denaturing by heat primer annealing by cooling and strand extension with thermo stable polymerase enzyme such as Taq polymerase, microgram quantities of double stranded DNA (dsDNA) can be synthesized from nanogram amounts of template.

As the amplification process is repeated several times, the DNA products continue to be produced from the original template during each PCR cycle, and they increase in a linear fashion. Enhancements, such as the use of thermo stable DNA polymerase and automation, have fostered the development of numerous and diverse PCR applications throughout the scientific world. The technique has also revolutionized the way some genetic diagnosis are performed, and it is now possible in many cases to diagnose inherited disorders such as α-1-antitrypsin. β-thalassaemia, fragile X-syndromes, haemophilia A and B phenylketonuria and sickle cell anemia among others using PCR in less than ten hours.

PCR like other methods used in scientific studies also has its own limitation in that some sequence information must be available to enable synthesis of the specific primers, which delimit the ends of the PCR fragment (Pattyn *et al.*, 1992). There is also risk of contamination of samples with materials from other source which if not properly handled gives a misleading result and information. Since the PCR amplification reaction utilizes

products from one cycle as a template in the next cycle, misincorporation could accumulate during the course of amplification (Innis *et al.*, 1990)

2.8 The genus Mycobacterium

The genus mycobacterium is the only genus in the family of *Mycobacteriaceae*. Currently there are more than 85 recognized or proposed species (Rastogi *et al.*, 2001). The genus includes obligate pathogens, opportunistic pathogens and saprophytes (Portaels *et al.*, 1988; Iiwanainen *et al.*, 1993). Almost all mycobacterial species can be classified as environmental, because only members of the *M. tuberculosis* complex, *M. leprae, M. haemophilum*, and *M. ulcerans* and a few others have not been isolated from the environment. Most of species live in water and soil although some species have been found only in human and animals in the wild (Mitchell *et al.*, 1984). The author described mycobacteria as aerobic, non-spore forming, non-motile, and mainly slightly curved or straight rods. When stained with basic fuchsin or a fluorochrome, they are resistant to decolourisation with acid alcohol, and are thus termed acid-fast (Portaels *et al.*, 1988). A few species seem to be rare in the environment, and others are isolated from the environment only after human or animals pollution.

2.9 Identification of *M. ulcerans*

Various methods have been used to detect the bacterium in clinical samples and the environment. In most countries where the disease is endemic the only routinely available test is detection of acid-fast bacilli and the treatment is determined largely on the basis of clinical

criteria. The two clinical methods commonly used in the detection and identification of *M. ulcerans* are culture and PCR.

M. ulcerans is best cultured in Löwenstein-Jensen medium at 32°C.The optimal pH for the growth of *M.ulcerans* lies between 5.4 and 7.4 (Portaels and Pattyn, 1982). Reduced oxygen concentration enhances the growth of *M.ulcerans*, suggesting a preference of this organism for microaerophilic environments (Palomino *et al.*, 1998). The culture technique could have been the gold standard for the detection and identification of *M. ulcerans*, however *M. ulcerans* is a slowly growing mycobacterium. Its generation time is about 23 hours (Portaels *et al.*, 2001). Primary cultures generally take between 7 and 8 weeks but subcultures are generally positive within two weeks depending on the number of acid-fast bacilli (AFB) in the inoculums. Attempts to culture the microorganism from clinical specimen fail in many cases due to contamination by organisms that grow faster. Standard techniques require a lot of time for isolation and identification. If carbon-14 is added to the culture medium, a radiometric assay (BACTEC system) may detect growth much more quickly (Palomino *et al.*, 1998).

The limitations of culture methods due to contaminations can be overcome by molecular amplification techniques such as the PCR. DNA sequences specific for *M. ulcerans* have been amplified by means of PCR (Ross *et al.*, 1997a; Portaels *et al.*, 1997; Robert & Hirst, 1997). Several target sequences have been used for the detection of *M.ulcerans* in clinical isolates and in environmental samples. For example the gene coding for 16S ribosomal RNA has been used by Portaels *et al.* (1997), the repetitive DNA insertion sequences IS2404 Ross

et al. (1997a) and IS2606 (Stinear *et al.* 1999 have been used). The first of the three method targets a gene with a single copy and high sequence conservation among all mycobacteria (Stinear *et al.*, 1999). Insertion sequences are mobile genetic elements, which have the ability to modify gene expression, sequester genes and promote genetic rearrangements. They possess terminal direct and inverted repeats. For this reason they are used to develop sensitive targets for PCR amplification. Primers based on this repetitive DNA sequence allow detection of *M.ulcerans* in clinical specimens with high sensitivity and specificity. Restriction fragment length polymorphism (RFLP) analysis has also been used to "fingerprint" the different geographic strains of *M.ulcerans* (Jackson *et al.*, 1995).

2.10 *M. ulcerans* association with water environment

In many countries *M. ulcerans* infection has occurred only after significant environmental disturbances. In the original paper describing the disease, the first patient from the Bairnsdale district in Australia (MacCallum, 1948) became infected after a heavy flood. It was the worst flood recorded in the district, when all road and rail links were cut and much property destroyed. In Uganda, Barker examined cases of *M.ulcerans* in the Busoga district on the east side of the Victoria Nile, north of Lake Victoria (Barker, 1971). Although cases were known in other parts of the country, there had been no known cases in that district before flooding of the Lake Uganda. Barker postulated that the outbreak of the disease in 1965 was related to unprecedented flooding of the lakes of Uganda as a result of heavy rainfall.

In Nigeria, infections occurred among Caucasians living on the campus of the University of Ibadan (Oluwasan *et al.*, 1975), after a small stream flowing through the campus was dammed to make an artificial lake. The first case reported in Cote d'Ivoire was a 7-year-old

boy who lived with his parents beside Lake Kossu (Perraudin *et al.*, 1980). In Liberia, cases have been reported in the north of the country following the introduction of a swamp rice field to replace an upland one (Ziefer *et al.*, 1981). This agricultural change was accompanied by the construction of dams on the major river to extend the wetlands. In Papua New Guinea, the infection occurs mainly near the Sepik and Kumusi River, hence the disease is known as the "Kumusi ulcer" (Hayman and Asiedu, 2000). The disease in Papua New Guinea spread after the flooding and devastation that followed the eruption of Mount Lamington (Raford, 1974).

The recent outbreak of the disease on Phillip Island, Victoria, Australia was found to be associated with the formation of a small swamp that backed up behind a newly constructed vehicle track (Veitch *et al.*, 1997). Improved drainage of this area was followed by a cessation of cases in the immediate vicinity of the marsh. These observations suggested that *M. ulcerans* might be present in groundwater, that a nutrient-rich environment may favour its survival and growth, that *M. ulcerans* is able to colonize man-made reticulation systems and that it is likely to be spread by aerosol.

All major endemic foci are in wetlands of tropical or subtropical countries; environmental factors must play an essential role in the survival of the etiological agent. Focal outbreaks have followed flooding, human migration and man-made topographical modifications such as dams and resorts (Uganda Buruli Group 1971). Deforestation and increased basic agricultural activities may have significantly contributed to the recent marked increases in the incidence of *M. ulcerans* infections, especially in West Africa, where the disease is rapidly

emerging. In Benin, for example, the disease prevalence in areas with environmental changes is about 180 per 100,000 population, whereas in those without environmental changes it is about 20 per 100,000 (Hayman and Asiedu, 2000). Despite the rapid increase in the incidence of *M. ulcerans* infection and its association with the water environment, the mode of transmission is still unknown. However, recent researches have shown that water bugs belonging to the family Naucridae and Belostomatidae could transmit the infection (Portaels *et al.*, 1999; Marsioller *et al.*, 2002). In the laboratory, when the insects belonging to these families were infected with *M. ulcerans* , it was found the bacterium replicated in the salivary gland of these insects species.

2.11 Naucorid species the possible vector of BU.

The family Naucoridae *(sensu lato)*, commonly known as creeping water bugs or saucer bugs, is represented worldwide by 413 described extant species in 39 genera and 7 subfamilies (Sites, 1990). Most species occur in the New and Old world tropics with moderate representation in temperate regions. These predaceous, aquatic insects are the predominant members of macro invertebrate communities in tropical streams in which they are found (Stout, 1982) and are keystone consumers (Sites and Willig, 1991). Naucorid species probably greatly influence the biota of tropical streams; ecological parameters associated with the distribution of these insects are poorly known (Stout, 1981; Sites and Willig, 1991). The family occupies a variety of both lotic and lentic mesohabitat, and species are often specific in microhabitat association (Nieser, 1975).

Dispersion patterns have been reported for some species to be contagious (Lopez Ruf and Kehr, 1995), whereas in others males and females are distributed randomly more often under the influence associated with floating vegetation and physico-chemical characteristics of the water habitat in which they are found. For example, certain genera of naucorids have been reported to flourish only in tropical streams where there are lots of water plants water lilies (Polhemus and Polhemus, 1988).

In others it was recently salinity of the water affects the distribution of Naucori*d* species. Sites and Bowles, (1995) reported the Naucorid species prefers water with pH above 6.1 to water with pH below. In others, passive transport by water was suggested to be the dispersal mechanism (Larsen, 1970). Usinger (1956) reported that some Naucorid species are attracted to light but rarely do they fly to light at night for dispersal. It is probable that flight occurs because some species occur in habitats that can be reached only by flight (Polhemus, 1979). Other species crawl upstream in compensation for downstream displacement (Stout, 1982). Views of dispersal for some Naucorid species are contradictory because different morphs occur with and without fully developed flight musculature and associated structures (Stout, 1982).

2.12 Life cycle of Naucorid species

Naucorid species group generally have three distinctive stages in their life cycle- the egg, the instars and the adult. All life stages are passed through water. They have a wide range of breeding sites and the species occupies a variety of both lotic and lentic mesohabitat of tropical streams, ponds, dams and other water bodies (Site *et al.*, 1997). Several species are

able to inhabit brackish or saline waters (Nieser, 1975). It has been reported that courtship is not involved in the mating behaviour of Naucorids (Sites and Nichols, 1990). Typically, the male moves around and upon contacting the female, positions himself above the female by grasping the notum with his prothoracic legs and mating takes place.

After mating, the female lays eggs in 15 $\pm$ 2 days but this can vary with species (Sites, 1990). In most species in which oviposition is known the eggs are laid singly and adhere either to plants or any hard substrata in the water (Hinton, 1981; Sites and Nichols, 1993; Labrun, 1960). Some species insert eggs in plant tissues under water (Cobben, 1968) whilst in other species eggs are cemented at the back of the male and are carried till hatching takes place. Each female lays between 16-32 eggs depending on the species and the incubation period ranges between 15 and 18 days during which they hatch into the first instars. Naucorids eggs cannot withstand desiccation and in tropical environment eggs hatch in relatively a shorter period compare to temperate climatic environment. Naucorids passes through six developmental stages to reach adult. The first stage is the egg stages followed by the first instars larvae and subsequently second, third, fourth and the fifth stages of instars respectively. Figure 3 shows a typical life cycle of Naucorids. The development time from oviposition to adult has been estimated to be between 29 and 114 days depending on the species (Sites and Nichols, 1990). The first instars begin feeding within one day after hatching. This suggests that all the stages of development of Naucorid species could be a mechanical vector. The first through fifth stadia averaged 11, 12, 19, 21 and 33.3 days interval respectively between each stage of development but could vary with species (Sites, 1990). Except for the small size of the nymphs and absence of the wings, Naucorid sp. instars

resemble the adult. The overall survival rate based on instars-specific survivorship is estimated to be around 15%, with the greatest mortality occurring in the later instars (LopezRuf and Kehr, 1994).

The number of generations per year appears to increase with milder seasonal conditions. The temperate species, for instance *Naucoris cimicoides* is univoltine (McPherson *et al.,* 1987). In transitional temperate regions with mild winter conditions, some Naucorid species have been reported to be bivoltine. Although studies on voltinism is lacking in the tropical species, reports have shown that in milder climate such as that which occurs in Texas, (USA), the development from third instars through to adult of some species over winter (Sites and Nichols, 1990, 1993). Unusual cases of over- wintering have been reported for an unspecified Naucorid specie (Kramer, 1938) in which adult over winters in hibernation near water but not in the water. Adults of some Naucorids have been reported to over winter in hibernation at the bottom of ponds and pools in muck and detritus and survived until the following autumn (Polhemus, 1979).

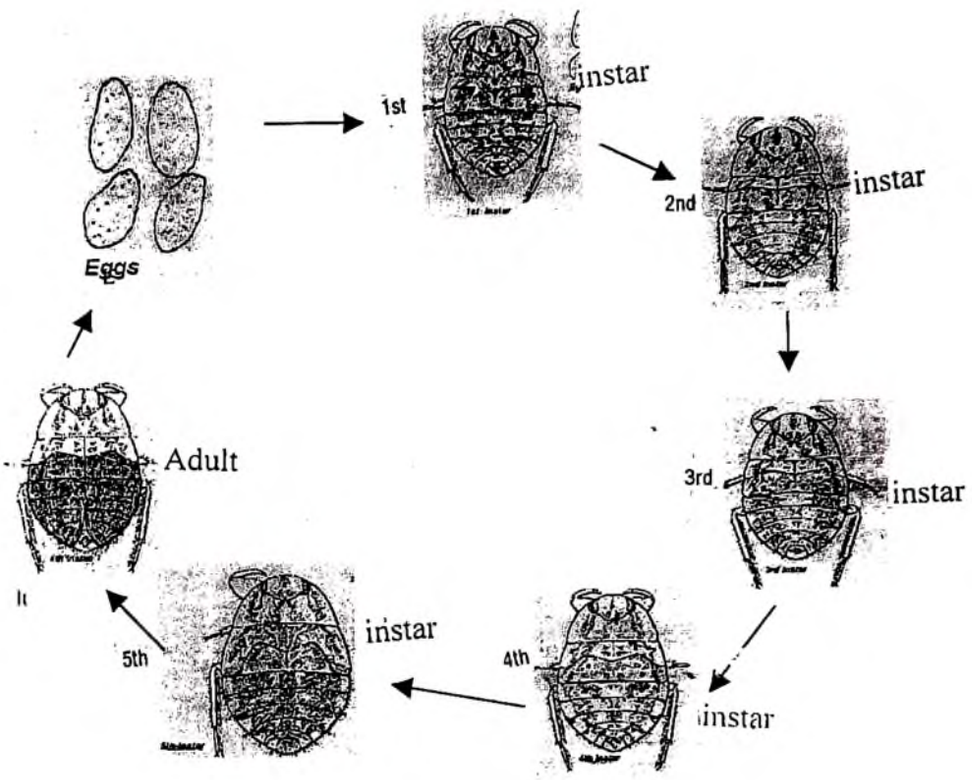

Fig. 2 3 Life cycles of Naucorid species (Sites and Nichols, 1990).

2.13 Medical importance of Naucorid species

Little is known about the medical importance of Naucorid species. No evolutionary history has been published between water bugs and bacteria infection (Portaels *et al.*, 1999). However, reports available have shown that members of the family Naucoridae whether found in temperate or tropical climatic environment, all exhibit the same way of life preying according to their size, on mollusc, snails, larvae and adult insects and other small aquatic animals such as leeches and fish fingerlings. It has also been reported that these insects bite villagers upon encounter in water (Marsollier *et al.*, 2002). Despite their painful bites inflicted upon aquatic animals (both vertebrate and invertebrate), these bugs are considered beneficial because they often feed on immature stages of several families of noxious Diptera larvae and pupae that are vectors of other disease pathogens. For instance, Sites and Nichols (1990) reared some Naucorid *sp.* on larvae of *Prosimulium sp.* (Simuliidae). Clarke and Baroudy (1990) showed that members of the genus Naucoris feed on midge larvae. Several papers cited Naucorids *sp.* to feed on mosquito larvae and pupae including Anopheles *sp.* (Federici, 1920). This implies that the naucorid species help in the control of some disease vectors population especially those associated with water. Recently, it has been demonstrated experimentally that Naucorids could serve as a mechanical vector of *M. ulcerans* (Portaels *et al.* 1999).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study sites.

The study was conducted in six villages in the Ga District, Greater Accra Region of Ghana. The villages were Amasaman, Doblo-Gono, Dome, Hobour, Obom and Weija. Buruli ulcer cases have been reported from the first three of the villages but none from the other three (Amasaman Health Center Medical record report, 2000 unpublished data). Thus for the purpose of this study the first three villages were considered to be endemic and the other three non- endemic. The Greater Accra region is located in the Southern part of Ghana and within the coastal savanna ecological zone with annual rainfall ranging between 740 and 890 mm annually (Dickson and Benneh, 1988). The mean monthly temperature is about 25°C with the highest temperature (about 30°C) between March and April and the mean relative humidity is about 64% (Dickson and Benneh, 1988). The dry season is from November to March. The inhabitants of these villages are mainly peasant farmers who grow corn, cassava, rice, yam, plantain and other agricultural crops. Insects' samples were collected from a variety of water habitats, including fishponds, dams and rivers within the six villages. Figures 3.1- 3.6 show some sample from each village. A global positioning system (GARMIN GPS 40^{TM}, VBA) was used to determine the geographical coordinates of each site where samples were collected.

Fig. 3.1 A sample site at Amasaman village.

Fig. 3.2 A sample site at Doblo-Gono village

Fig.3.3 A sample site at Hobour village

Fig 3.4 A sample site at Obom village.

Fig. 3.5 A sample site at the Weija village

3.2 Sampling regime

Samples were taken twice a month from September 2002 through to April 2003. Three water bodies were chosen in each in each study area for sampling. Sampling time was 2 hours per site. An aquatic D – net (width 0.32m) (Fitter and Manuel 1994) was held by hand and swept through submergent and emergent vegetation within the measured area. Small samples of rooted vegetation and bottoms sediments were dredged up and examined. Dense weed beds were worked through with a sawing motion to dislodge inhabitants. Net content were emptied into a white plastic pan so that macro invertebrates that at once swam or crawled out from the trash were easily seen against the white background and were then removed with fine forceps. Samples collected were placed in 70% isoproponol until brought to the laboratory for identification. Water samples were collected thereafter into 1.5 liter plastic bottles for physico-chemical analysis.

3.3 Study of physico-chemical parameters of water samples

All analysis for chemical parameters was done at the Water Research Institute, Council for Scientific and Industrial Research (CSIR), Ghana.

Measurements of physical parameters (temperature, turbidity and pH) were taken right in the field at the time of sampling. The water temperature was directly measured using a glass thermometer each time samples were collected. The turbidity was measured using turbidity a meter and the hydrogen ion concentration (pH) was measured using pH meter. For the estimation of dissolved oxygen (DO), a 300ml glass stoppered bottle covered with aluminium foil to shield light was filled with the water sample and 2ml of manganese

sulphate solution, and then 2ml of alkali iodide – azide solution was added to fix the oxygen (O_2) until the sample was brought to the laboratory for analysis. Water samples for chemical analysis were taken there after into 1.5l and brought to the laboratory for analysis.

3.3.1 Analysis of water samples

Calcium ion (Ca^{2+}) concentration was determined using EDTA titrimetric method. A mixture of 50 ml of water sample, 2.0 ml of 1M NaOH solution and a few crystals of Murexide indicator were titrated with 0.01 M of EDTA until the colour changed from salmon to orchid purple. Calcium ion concentration was determined by the equation (1) below.

$$Ca^{2+}\,(mg/l) = \frac{A \times B \times 400.8}{\text{Volume of EDTA}} \quad \text{(Equation 1)}$$

Where A = volume of EDTA,

B (mg equivalent of $CaCO_3$ to 1.0 ml of EDTA titrant at the calcium indicator end point), which is related to the volume of standardized EDTA titrant was calculated using the equation below B = C/ EDTA

Where, C = Volume of standard calcium solution used to standardized the EDTA

Chloride ion (Cl^-) concentration was determined using the argentometric method. The pH of 100 ml of the water sample was first adjusted to between 7 and 10 using conc. H_2SO_4, then 1ml of potassium chromate (K_2CrO_4) indicator solution was added followed by titration with standard $AgNO_3$ until a pinkish yellow colour endpoint was reached. A blank value was also determined by titration method.

The chloride ion (Cl^-) concentration was determined using equation 2 below.

$$Cl^- \text{ (mg/l)} = \frac{(A-B) \times M \times 35\,450}{\text{Volume of sample}} \quad \text{(Equation 2)}$$

Where: A = Volume of $AgNO_3$ used for sample titration,

B = Volume of $AgNO_3$ used for blank titration,

M = Molarity of $AgNO_3$

Potassium (K^+) and Na^+ concentrations (mg/l) were determined using the flame photometric method. Water samples were introduced in a flame analyzer (Gallenkamp model FGA – 350L, UK). The calculation of potassium or sodium was given directly by the flame photometer at wavelengths of 768. In instances where the potassium concentration was very high the water samples were diluted and the concentration was calculated as follows:

K^+ or Na^+ = (K^+ /Na^+ in aliquot) × D

Where, D = dilution factor, which is determined using equation (3)

$$D = \frac{\text{Volume of sample + distilled water used for dilution}}{\text{Volume of sample}} \quad \text{(Equation 3)}$$

Ammonia-nitrogen (NH_4-N) amounts (mg/l) were determined by direct Nesslerization. For the calibration curve, 10ml of the stock ammonium chloride solution was diluted to 100ml with distilled water. From this intermediate solution, each of 1, 2, 3, 4 and 5 ml was diluted to 100 ml in a conical flask, mixed well, followed by the addition of 2 ml Nessler's reagent. The solution was then allowed to stand for 10 minutes for colour development. The water

sample was left to settle and an aliquot of 50 ml of the supernatant was pipette into a fresh 100 ml conical flask. Then 1 to 5 ml of sample was pipette into a fresh flask and ammonia-free water added until it reached 50 ml mark. For very turbid samples, the water was filtered and the filtrate was used for the analyses. Two drops of Rochelle salt were added to the diluted sample or 5 drops in the case undiluted sample, mixed well and 2 ml of Nessler's reagent added. They were then allowed to stand for 10 minutes for colour development and the absorbance for samples and blank determine using a UV/VIS spectrophotometer (Philips model PU 8625B, the Netherlands) at a wavelength of 410nm using a 1 cm light path cuvette. The concentration of ammonia-nitrogen in the unknown sample was determined by extrapolating from a calibration curve.

To determine the concentration of nitrite (NO_2), the diazotization method was used. A series of standard working nitrite solution was diluted to 50 ml with distilled water. Then 2 ml of buffer-colour reagent were added to 50 ml of sample in a Nessler tube, mixed well and left for at least 15 minutes to allow colour to develop. The absorbance was measured in the UV/VIS spectrophotometer at 540nm. The amount of nitrite (mg/l) was determined by extrapolating from the calibration curve.

To determine the concentration (mg/l) of nitrate (NO_3), the hydrazine reduction method was used. NO_3 calibration standards were prepared by diluting to 10 ml with distilled water the following intermediate nitrate solutions; 0, 2, 4, 6, 8, and 10 ml. In a test-tube, 1 ml of 0.3M NaOH was added to 10ml of the water sample and mixed gently. The mixture was heated at 60°C for 10 minutes in water bath, cooled to room temperature and 1 ml of colour developing

reagent was added, and mixed well by shaking. The absorbance at 520nm was read and the nitrate concentration was directly computed from a calibration curve. The value obtained was due to both NO_3-N and NO_2-N. The value of NO_3-N was obtained by subtracting the value of NO_2 from the value of the calibration curve.

Sulphate (SO_4) concentration (mg/l) was determined using a turbidimetric method. From the sulphate solution, each of 5, 10, 15, 20, 25, and 30 ml was diluted to 100 ml with distilled water and used as standard solution in calibration. 5 ml conditioning reagent were added to 100 ml sample in a 250 ml. Erlenmeyer flask and mixed by stirring. Thereafter a spoonful of barium chloride crystals was added still stirring at a constant speed for 60 seconds. After stirring and within 5 minutes the absorbance at 420nm was measured. The concentration of sulphate was extrapolated from the calibration curve standard concentration.

Phosphate (PO_4) concentration (mg/l) was determined using the stannous chloride method. Standard phosphate solution of known concentrations ranging from 0.1 mg/l to 1.0 mg/l were prepared and treated as samples. To a 100 ml sample free from colour and turbidity was added 0.05 ml (approximately 1 drop) of phenolphthalein indicator till the sample turned pink. A strong acid solution was added drop wise to discharge the colour. Where more than 0.25 ml (5 drops) was required, a small volume of sample was diluted to 100 ml with de-ionized water then a drop of phenolphthalein indicator was added, discharged if the sample turned the pink colour with the acid. A volume of 0.4 ml molybdate reagent was added with thorough mixing after each addition and was followed by 0.5ml (10 drops) of stannous chloride reagent. The absorbance at 690nm was measured after 10 minutes using a UV/VIS

spectrophotometer. The phosphate concentrations in the samples were determined from the calibration curve.

Determination of dissolved oxygen (DO) was done using azide modification of Winkler's method. 2 ml conc. H_2SO_4 was added to 250 – 300 ml of sample in a BOD bottle, shaken till dissolution was complete. 100 ml of this solution were taken and titrated with M/80 sodium thiosulphate solution to a straw yellow colour, followed by addition of 1 – 2 ml starch solution and titration continued until the blue colour turned to colourless. Dissolved oxygen as mg/l O_2 was calculated using equation below.

$$O_2\,(mg/l) = \frac{\text{Volume of M/80 thiosulphate} \times 101.6}{\text{Volume of water sample}}$$

Where: M = molarity of thiosulphate

Biochemical oxygen demand (BOD) was determined by dilution method. 2 ml of $MnSO_4$ were added to the diluted 250 – 300 ml sample in a BOD bottle followed by 2 ml of alkaline-iodide-azide and corked carefully to exclude bubbles. After the precipitate had settled, 2 ml of conc. H_2SO_4 were added, corked and the bottle inverted several times to dissolve the precipitate which gave an intense yellow colour. The solution was then titrated with M/80 sodium thiosulphate until a pale yellow colour was obtained. Then 1 ml of starch as indicator was added and the titration was continued until the appearance of blue colour. The rest of the water sample was incubated at 20°C for 5 days after which it was treated likewise. The

difference in dissolved oxygen (DO) of days 1 and 5 was used to calculate the BOD using equation below.

$$BOD = (D1 - D2) \times P$$

Where: D1 (mg/l) = DO of diluted sample immediately after preparation

D2 (mg/l) = DO of diluted sample after 5 days incubation at 20°C

P = decimal volumetric fraction of sample used.

3.4 Identification of Naucorid species

Naucorids belong to the order Hemiptera and the family Naucoridae. Generally members of Naucorid species have been distinguished from other water bugs species using morphological key (Paul, 1964; Robert *et al.*, 1997; Michael, 2000). Briefly, the body of the Naucorid species are broadly oval flatten bugs ranges between 11-13mm for length for adult. They have large broad and retentorial prothoracic legs for holding preys (Site & Nichols, 1990) and natatorial meso-and meta-thoracic legs. The hind legs are fringed with swimming hairs. The rostrums of Naucorid species do not reach behind the base of the legs. The antennae of the members of these species are short embedded in the head and are frequently hidden in grooves beneath the eyes which are not visible from above. The front wing membranes are without veins and ocelli are absent. Some members of Naucorid species are structurally similar to back-swimming Notonectidae, however, Naucorid species are distinguished by the deeply concave anterior prenatal margin (Menke, 1979; Carver *et al.*, 1991). Coloration is predominantly dull medium brown sometimes with scattered yellowish markings on the head

FIG. 3.6 A picture of an adult Naucorid species (Genus Ambrysus)

and thorax. All identified insects used for this study were individually placed in a labelled specimen tubes and were stored frozen in a freezer until used for molecular studies.

3.5 Molecular study of *M. ulcerans* in the insects

The DNAeasy Tissue Kit (Qiagen Company Inc., USA) was used to isolate genomic DNA of *M. ulcerans* from the insects. DNA was mostly extracted from individual insects but in a few cases it was extracted from pools of five or ten insects. The DNA was used as template for PCR to diagnose the presence of *M. ulcerans* in the insects. The various buffers used for the molecular studies were either supplied with the DNA extraction kit or were prepared as outlined in Appendix 1

3.5.1 DNA Extraction

Genomic DNA was extracted from Naucorid species following the manufacturer's protocol with little modification. Briefly, for individual extractions, the head of each specimen was placed in a 1.5 ml micro centrifuge tube and ground with a sterilized disposable micro tube pestle in 180ml lyses buffer until completely homogenized. 25µl of proteinase K and 200µl Buffer AL were added to the homogenate. This was then thoroughly mixed by vortexing and incubated at 70°C for 15mins. 200µl ethanol (96 – 100%) was added to the mixture and re-mixed thoroughly by vortexing. The mixture was then pipetted into the DNeasy® spin column in a 2 – ml collection tube and then centrifuged at 1000 rpm for 1min. The flow through and collection tube discarded. The DNA spin column was then placed in a new 2-ml collection tube and 500ml Buffer AW1 was added centrifuged for 1min and the tube and flow through again discarded. The DNeasy spin column was then placed into another new 2-

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ml collection tube, 500µl Buffer AW2 was added and centrifuged for 3mins at 14000rpm to dry the DNeasy membrane. After this step the DNA spin column was placed in a clean 1.5 ml micro centrifuge tube and 200µl Buffer AE pipetted directly unto the DNAeasy membrane.to elute the DNA until ready for amplification by PCR.

3.5.2 PCR Product Amplification

A nested PCR that amplified a repetitive insertion sequence in the *M. ulcerans* genome (Ross *et al.*, 1997a) was used for this study. Four Primers MU1, MU2, PGP3 and PGP4 (Guimaraes – Peres *et al.*, 1999) were used in the amplification of the bacteria DNA segments. The first round PCR was with the primers MU1 and MU2 and the second with PGP3 and PGP4.

For each first round reaction, the volume of each reagent in the PCR mix consisted of 15µl of double distilled water, 2.5µl 10X thermobuffer, 2.5µl $MgCl_2$, 0.8µl of deoxyribonucleotide triphosphate (dNTPs), 0.5µl each of MU1 and MU2 primers and 0.5µl of 5 units/ML Taq polymerase enzyme (Sigma , USA). Together total volumes of 25µl of PCR mix was obtained for one reaction. A Master mix containing multiples of the initial volumes was made for each set of PCR reactions depending on the number of PCR reactions and then aliquoted into individual tubes according to number of template DNAs reactions to be amplified at a particular time. Into each PCR tube containing 22µl aliquot of master mix was added 2µl of extracted genomic DNA. The total volume for each reaction mix was therefore 24µl. The reaction mix was span down briefly at 14,000 rpm and overlaid with mineral oil to

avoid evaporation and reflux during thermo cycling. For each PCR run, a positive control (DNA extracted from *M. ulcerans*) and a negative control (distilled water) were included.

The amplification of PCR products were carried out using an automated PTC 100 thermal cycler (MJ Research Inc., USA) and the cycling protocols were as follows; 94°C for 4min (initial denaturing), followed by 40 cycles of 94°C for 40 sec, 60°C for 40 sec, 72°C for 40 sec. The reaction ended with a single cycle of 94°C for 40 sec, 66°C for 40 sec and 72°C for 40 sec and a final extension step at 72°C for 10 minutes. Samples were held at 4°C until ready for analysis on agarose gel for the first PCR round. For the second PCR round, two microliters of the first round PCR products were used as templates with primers PGP3 and PGP4. The second round PCR mix had similar concentrations and volume of reagents as the first. Also, the cycling conditions were similar except the annealing temperature, which was increased to 64°C. 3µl of each amplified DNA was analyzed using electrophoresis through an ethidium bromide stained (0.5mg/ml) 2% agarose gel.

3.5.3 Agarose gel Electrophoresis and Visualization of PCR Product

3µl of each PCR product, mixed with 1µl of orange G [5X] gel loading dye, was electrophoresed through 2% agarose gel containing 0.5µg/ml ethidiumbromide to detect the presence of amplified DNA fragments. The gel was ran in 1X TAE buffer (40mM Tris acetate, 1mM EDTA) using a midi gel system (BIORAD, USA) at 100 volts for 40-50 mins and photographed over a UV transilluminator (UPC, USA) at short wave length using a type 667 film (Polaroid USA). For the first PCR, samples were considered positive if it yielded a product size between 515-540bp that lined up exactly with positive control and all negative

control should not band (Marsioller *et al.*, 2002). For the nested (second) PCR using PGP3 and PGP4 primers, samples were considered positive if they yielded a product size between 215 and 240bp that line exactly with the positive control and all negative control should not band

3.6 Data analysis

All statistical analysis was performed using SPSS 10.0 software (SPSS Inc., USA) with the exception of the estimation of the rate of infection in insects examined in pools. The estimated infection rate for insects examined in pools was performed using the program Poolscreen™ (Katholi *et al.*, 1995). Water parameters were initially subjected to a *t* test to determine the equality of means between two groups of water samples i.e. those with and without Naucorids. The two-sample t-test is based on the assumption that each set of observations is sampled from a population with a normal distribution and that the variants of the two populations are the same. Water parameters whose means were different were submitted to binary logistics analysis to identify the best discriminatory parameters between the two groups. A total of 15 parameters were investigated. These include temperature, turbidity, pH, electrical conductivity, calcium, magnesium, ammonium, chloride, sulphate, nitrite, nitrate, phosphate, total hardness, biological oxygen demand (BOD) and dissolved oxygen (DO). Water samples collected were grouped into two: Group one sample where Naucorids were found and group two samples where Naucorids were absent. Water parameters of these two groups of observations were compared using a t-test. Water parameters whose means were significantly different were submitted to binary regression test to identify the best discriminatory parameters for the presence or absence of Naucorid species between the two groups.

CHAPTER FOUR

Results

4.1 Effects of physico- chemical parameters on the distribution of Naucorid species

The comparison of means values of the parameters of habitats with Naucorids and those without Naucorids revealed that only 4 parameters pH, dissolved oxygen, turbidity, and total hardness significantly differed between the two groups $P < 0.05$ (see Appendix V). The P value for the significant parameters is shown in Table 4.1

Table 4. 1 Comparison of water parameters of habitats with Naucorids and those without.

Water parameters	Habitats				P. value
	Naucorids present		Naucorids absent		
	Mean	S.E.M	Mean	S.E.M	
pH	7.60	0.11	6.81	0.19	0.000
Turbidity	532.56	30.48	395.08	22.87	0.016
Total hardness	171.75	15.35	274.33	24.05	0.001
Dissolved oxygen	5.82	0.35	3.18	0.52	0.000

4.2 Parameters predicting the presence or absence of Naucorid species.

The result of the stepwise discriminant function analysis revealed that dissolved oxygen, pH and turbidity were the best water parameters that influence the distribution of Naucorid species. Dissolved oxygen was first selected followed by pH and then turbidity in that order Table4.

Table 4. 2 Parameters that most predict the presence or absence of Naucorid species in a given aquatic habitats.

Steps	Number of variables	Lambda	Exact F statistics	P value
1	1	0.755	14.922	0.000[a]
2	2	0.595	15.284	0.000[b]
3	3	0.452	17.791	0.000[c]

At each step, the variable that minimizes the overall Wilks' Lambda was entered.

a. Dissolved oxygen.

b. Dissolved oxygen and pH

c. Dissolved oxygen, pH and turbidity

To determine which of the non-selected parameters was associated with each of the predicting variables, multiple regression analyses were performed using each of the predicting variables as a dependent variable and all the measured parameters as independent variables. The result obtained revealed that only total hardness, dissolved oxygen (DO) and ammonium were associated with pH. When total hardness was entered as a dependent variable against all the other measured parameters as independent variables, pH, magnesium and calcium were the variables associated with total hardness. However, when dissolved oxygen (DO) was entered as dependent variables against all the measured parameters as independent variables, only pH was picked in the model to be associated with DO. Biological oxygen demand, ammonium, sulphate and phosphate were observed to be associated with turbidity Tables 4.3 and 4. 4.

Table 4.4 P Values of parameters associated with pH.

Model	Correlation	R. square	Adjusted R.square	Std error of estimate	F	P. value
1	0.473	0.224	0.207	0.6658	13.254	0.001a
2	0.541	0.292	0.261	0.6427	9.295	0.000b
3	0.591	0.302	0.283	0.6528	9.318	0.000c

a. Total hardness

b. Ammonium

c. Dissolved oxygen

Table 4. 4 P Values of parameters associated with total hardness.

Model	Correlation	R. square	Adjusted R. square	Std error of estimate	F	P. value
1	0.538	0.290	0.274	84.9914	18.752	0.00[a]
2	0.705	0.496	0.474	72.3510	22.177	0.000[b]
3	0.621	0.503	0.342	37.493	19.743	0.000[c]

a. pH

b. Magnesium

c. Calcium

4.3 Seasonal distribution of Naucorid species.

The total number of aquatic insects identified as Naucorid species in both endemic and non-endemic areas of BU was 2181.Collections for wet season were done for three months in both areas while dry season collections were done for five months and the results presented as below:

Statistical analysis on the data revealed a significant variation between the mean number of Naucorids between wet and dry season and between endemic and non-endemic areas of BU $P < 0.05$. However, no significant variation were observed between water bodies (sites) in which collection were made $P > 0.05$ (see Table 4.5 for ANOVA)

Comparison of the interaction effects of season, area and sites revealed that there were significant interaction between season and area and between area and site $P < 0.05$ (Table 4.5). On the contrary, the interaction between seasons and sites did not show any significant difference in the mean number of bugs $P > 0.05$ (Table 4.5).

Combine interaction between seasons, sites and area show a significant effect on the mean number of the bugs $P < 0.05$. Within wet season mean number of Naucorids significantly differed between area $P < 0.05$. However, no difference was observed between sites $P > 0.05$.

The interaction between areas and sites within wet season showed a significant effect on the mean number of Naucorids $P < 0.05$. On the contrary, comparison within dry season

did not show any significant difference between mean numbers of Naucorids between areas as well as between sites $P > 0.05$. Interaction between these factors did not show any effect on the mean number of Naucorids within dry season $P < 0.05$ (Table 4.5).

Table 4.5 Analysis of Variance of numbers of Naucorids (per season per area per site) to examine the effects of site and season on the distribution of the bugs in endemic and non-endemic areas of Buruli ulcer in Greater Accra Region of Ghana 2002.

Season	Source of Variation	DF	SS	MS	F	P
	Season	1	2416.192	1208. 096	36.005	0.000
	Area	4	2367.881	591.970	17.643	0.000
S1+S2	Site	2	8.745	4.373	0.130	0.878
(Both season	Season* Area	4	989.323	247.331	7.371	0.000
combine)	Season* Site	2	55.323	27.662	0.824	0.441
	Area * Site	8	724.794	90.599	2.700	0.010
	Season*Site* Area	8	1096.661	137.083	4.086	0.000
	Error	107	3590.200	33.553		
	Total	144	43759.000			
	Area	4	2589.630	647.407	13.035	0.000
S1	Site	2	33.926	16.963	0.342	0.713
(Wet season)	Area * Site	8	1313.704	164.213	3.306	0.006
	Error	36	49.66	49.667		
	Total	54	28225.000			
	Area	4	236.502	59.125	2.329	0.064
S2	Site	2	28.600	14.300	0.563	0.572
(Dry season)	Area * Site	8	247.320	30.915	1.218	0.301
	Error	71	1802.200	25.383		
	Total	90	15534.000			

4.3.1 Distribution of Naucorid species across the studied villages

The percentage distribution of the Naucorid species by village were as follows: Weija village had the highest percentage with a proportion of 24.98% followed by Amasaman with 17.61% while Obom had the lowest proportion of 12.76% (Fig. 4.1). However, the result of ANOVA test revealed a significant difference in the mean distribution of Naucorid species between the villages ($P < 0.05$) (Appendix II).

4.3.2 Distribution of Naucorid species across the water bodies (sites) in each village.

The mean distribution of Naucorid species did not significantly differ across the water bodies for Amasaman, Doblo-Gono, Hobour and Obom ($P > 0.05$) (Appendox iv) However, significant differences were observed across the sites in Dome and Weija ($P < 0.05$) (Table 5

Tables 4. 6 Mean (±S.E.M) distributions of *Naucorid* species per site within each village (values of insect numbers were transformed using square root (x+1).

Site	Amasaman	Doblo-Gono	Dome	Hobour	Obom	Weija
1	18.90 ± 2.3a	14.60 ± 1.3a	17.37 ± 1.4a	13.25 ± 2.6a	13.12 ± 1.4a	26.80 ± 5.3a
2	19.50 ± 1.7a	18.30 ± 2.0a	17.87 ± 1.5a	16.75 ± 2.1a	15.62 ± 1.4a	13.00 ± 1.4b
3	14.40 ± 2.0a	17.00 ± 2.1a	12.50 ± 1.1b	16.62 ± 1.4a	17.75 ± 2.1a	22.60±4.8a

Means followed by the same letters vertically are not significantly different from each other $P = 0.05$ by (LSD).

4.3.3 Distribution of Naucorid species between the months for each village

During the 8 months of collection significant differences were observed in the mean number of Naucorid species between the months in Amasama, Doblo-Gono and Weija (see Table 4.7). In Amasama and Doblo-Gono a across the months, the highest collection were recorded in November, followed by October and September, in that order and the least collections were in April. In Weija, the highest collection was recorded in September followed by October and the least collection was in February and March. For each of Dome, Hobour and Obom, collections across the month were closely similar with little difference in population abundance.

Statistical analysis revealed significant differences in the number of Naucorid species across the month in Amasama, Doblo-Gono and Weija ($P < 0.05$) (see Appendix II). However, no significant differences were seen across the months in Dome, Hobour and Obom.

Table 4.7 Mean (± S.E.M) distributions of Naucorid species from September 2002 – April 2003

	Village					
Month	Amasaman	Doblo	Dome	Hobour	Obom	Weija
Sept.	23.3 ±3.3a	16.0 ± 2.6a	21.3 ± 2.9a	1 6.0 ± 1.2a	14.7 ± 2.4a	32.7± 13.2
Oct.	25.0 ± 2.3a	19.3 ± 3.0a	18.3 ± 0.3a	1 7.3± 1.8a	21.0 ± 3.8a	22.7 ± 5.9
Nov.	22.7 ± 1.3a	21.3± 5.7a	15.3 ± 2.7a	13.3± 2.0a	15.3 ± 1.5a	17.0 ± 3.9
Dec.	13.0 ± 1.0c	14.0 ± 0.6ab	1 6.3 ± 1.2a	22. ± 4.7a	1 7.7 ± 4.1a	1 6.3 ± 3
Jan.	15.3 ± 3.2bc	19.7 ± 1.2a	1 6.0 ± 3.2a	16.7± 1.2a	1 2.0 ± 1.2a	1 4.3 ± 0
Feb.	16.3 ± 12.2abc	14.7 ± 2.4a	1 4.0 ± 2.0a	1 4.3 ± 1.5a	1 4.3 ± 3.0a	1 3.7 ± 0
Mar.	12.7 ± 1.5bc	16.0 ± 2.6a	1 6. ± 2. 0a	12.7 ± 1.3a	1 6.7 ± 0.6a	1 3 .0 ± 3
April	13.2 ± 1.9c	13.3 ± 2.4b	1 6.7 ± 5.2a	1 2.0 ± 1.0a	1 2.3 ± 2.3a	1 6.7 ± 5

Means followed by the same letters vertically are not significantly different from each other by (LSD)

(P = 0.05).

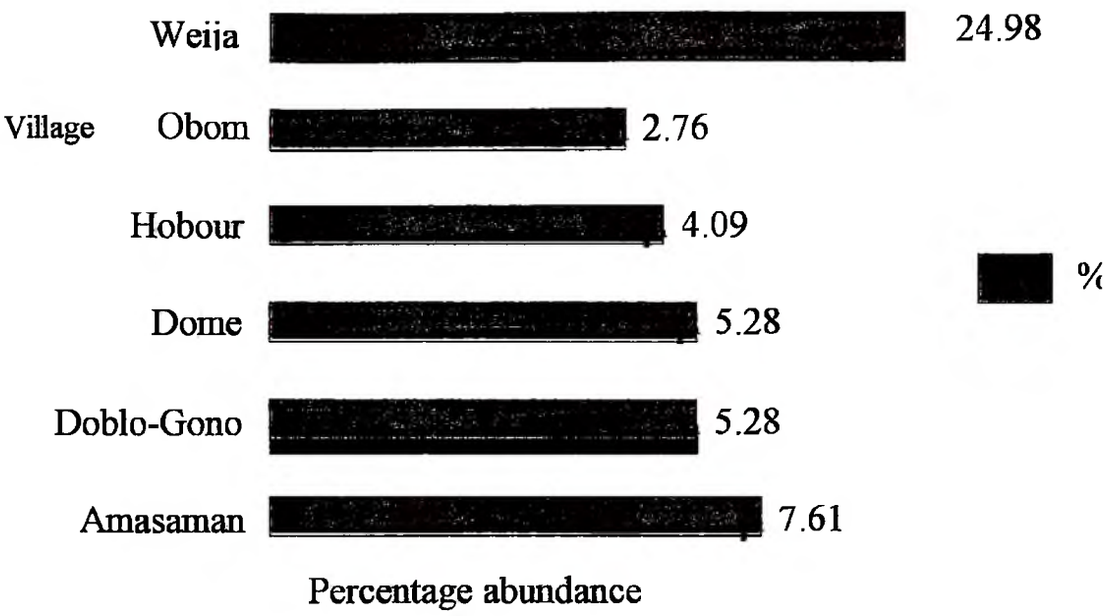

Fig. 4.1 Percentage distribution of Naucorid species across the studied villages. From top to bottom the first-three villages have no reported case of BU whilst the last three are considered endemic of BU.

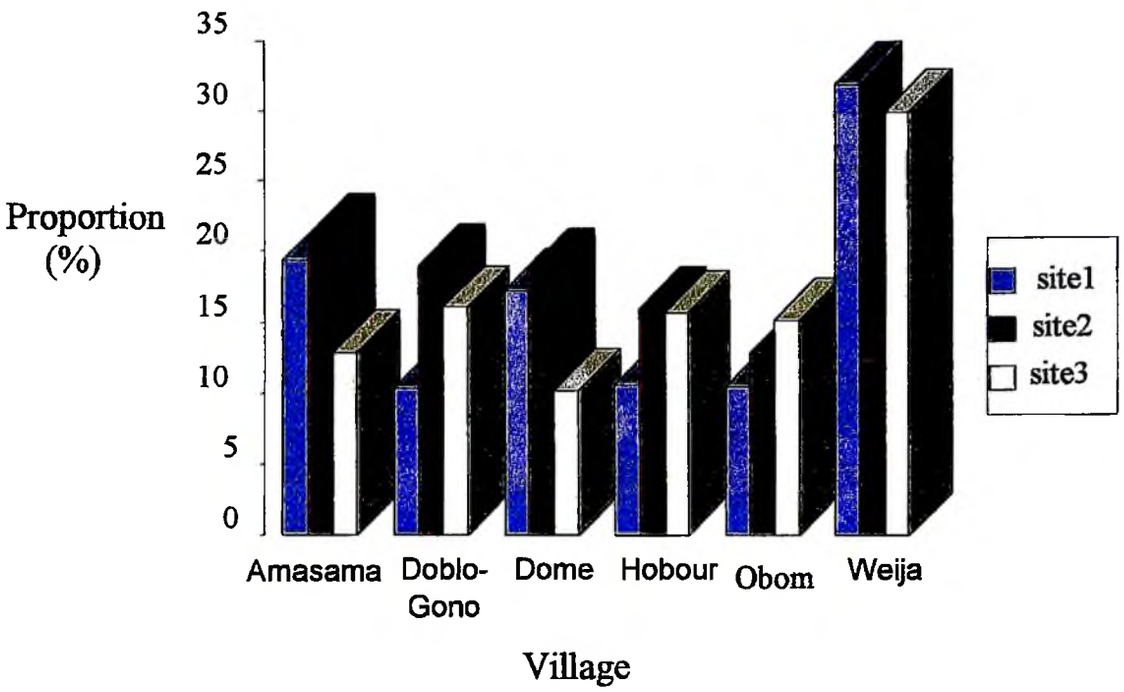

Fig.4.2 Distribution of Naucorid species between water bodies within each village

4. 4 Prevalence of infection of Naucorid species by *M. ulcerans*

Out of a total of 2181 samples collected, (72.8%) samples were successfully examined for the detection of *M. ulcerans* using PCR out of which 4.4% were found infected with the bacterium.

4.4.1 Seasonal infections

Of the total number of the infected Naucorids, the mean prevalence of infections was significantly different between wet and dry seasons $P < 0.05$. More than 50% of the infected bugs came from the wet season collections (Fig. 4.5). However, no significant difference was observed between endemic and non- areas as well as between various water bodies (sites) $P > 0.05$. The interaction effect between site, season and area did not significantly affect the mean number of infections of Naucorids between wet and dry season $P > 0.05$

Comparison of infection of bugs within wet season did not show significant difference of infection of bugs between endemic and non-endemic areas and between various water bodies $P > 0.05$. Within dry season no significance difference was found in the mean number of Naucorid species between endemic and non-endemic areas neither was there any significant difference between the various water bodies.

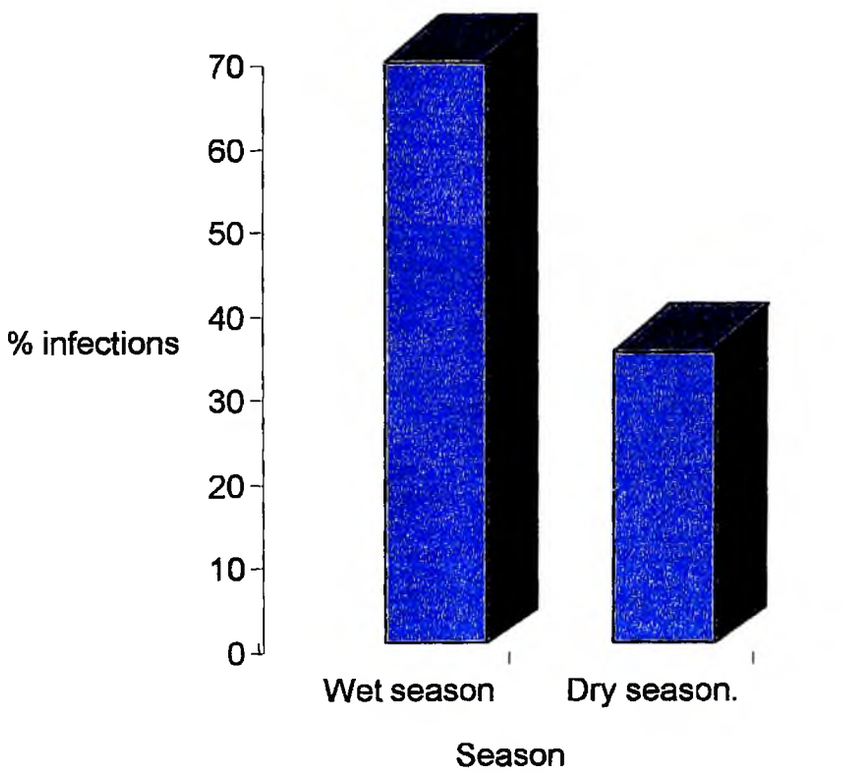

Fig. 4.3 The distribution of infected Naucorids between the seasons.

4.4.2 Prevalence of infections of Naucorid species across villages

Out of a total number of 317 PCR samples from Amasaman 4.1% were positive of *M. ulcerans*, 5.9% of 268 samples from Doblo-Gono were positive while 3.7% of 244 samples from Dome were as well positive of the bacterium. In the non-endemic villages of BU, out a total number of 191 samples from Hobour, 3.7% were found infected with the bacterium while 4.6% of 392 and 3.9% of 180 samples from Weija and Obom respectively were found infected with *M. ulcerans* too (see Fig. 4.6).

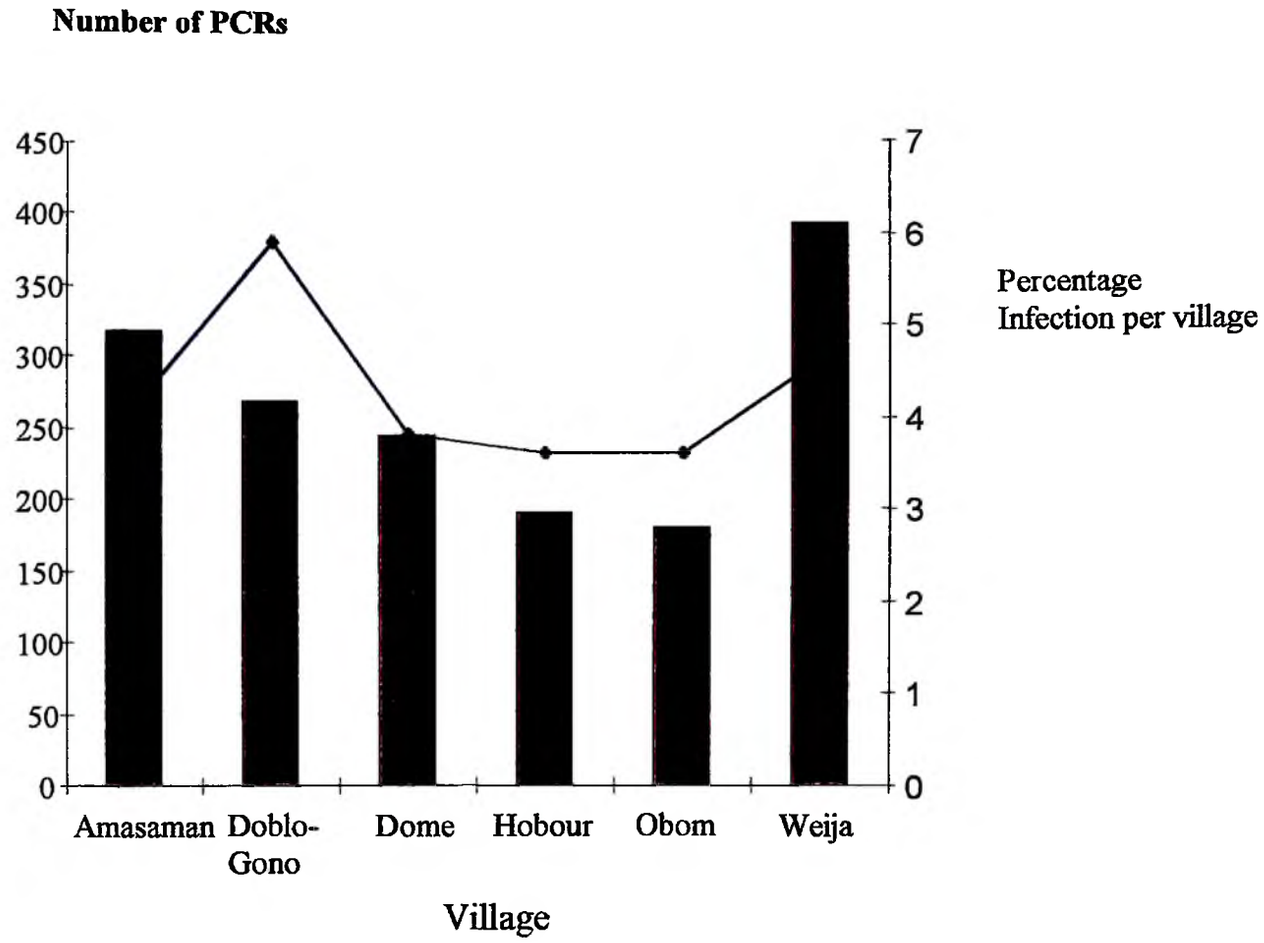

Fig. 4.4. Graph showing the number of PCR per village and the proportion of infection of Naucorid species. The bar represents the number of PCR per village, whilst, the line represent the percentage of insects found infected with *M. ulcerans*

4.4.3 Prevalence of infection between sites

The distribution of infection among the 18 various sites were as follows: Weija site 1 and Doblo-Gono site 2 constituted the highest infection rate of 12.9% and 11.4% respectively, followed by Amasaman site 2 and Weija site 3 with an equal infection rate of 8.6%. Similarly, Amasaman site 1, Doblo-Gono site 3 and Hobour site 2 had equal percentage of infection rate of (7.1%) while Dome site1 had 5.7%. Five of the remaining sites also had equal positivity rate of 4.3% each include: Amasaman site3, Doblo-Gono site1, Dome site 2, Obom site 3 and Weija site 2. No insect samples were found infected with *M. ulcerans* at Hobour site 1. The least infection was observed in Obom sites1and 2 which had the same infection rate with Dome site3 and Hobour site 3, each with infection rate of 2.9%. Figure 4.4 shows the band size of amplified DNA of *M. ulcerans* from insects samples.

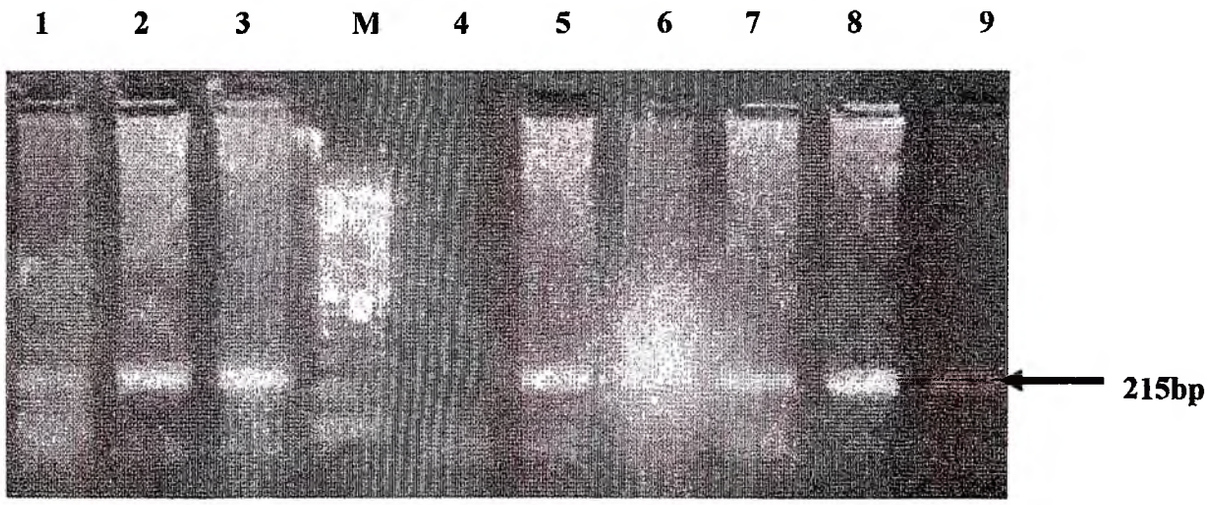

Fig. 4.4. An example of electrophoregram obtained for the detection of *M. ulcerans* in insect samples. Lane M =100bp molecular weight marker. Lanes 1 and 9 positive control (DNA extract of *M. ulcerans*), lane 4 negative control (water), lanes 2, 3, 5, 6, 7 and 8 samples positive for *M.ulcerans* from Amasaman, Doblo-Gono, Dome, Hobour, Obom and Weija respectively.

CHAPTER FIVE

Discussion

Buruli ulcer disease is assuming a public health importance in many countries, prompting the establishment of a Global Buruli Ulcer Initiative by the World Health Organization (WHO) recently. Ever since *Mycobacterium ulcerans* infection was first described, cases have been reported throughout the tropical and subtropical world including Africa especially West Africa (WHO, 2000). One characteristics of the disease is its apparent association worldwide with bodies of water, (Muelder, 1992; Oluwasanmi *et al.*, 1975) but the mode of transmission is not known.

The PCR results of this study have confirmed the presence of *M. ulcerans* DNA in Naucorid species in the wild at the rates of 4.4%. The interpretation of this finding is in agreement with that of Marsollier *et al.*, (2002) and Portaels *et al.*, (1999) who also found these water bugs of these species to be naturally infected in the endemic area of Buruli ulcer in Cote d'Ivoire. For the first time a human disease causing Mycobacterium was found in insect vector compared to other disease causing pathogens such as viruses, protozoa and other parasites. Very few insects- born bacteria are known to cause illness in humans (Marsollier *et al.*, 2002). Insects, ticks and other arthropods vectors transmit many other devastating human diseases mainly due the physiological adaptation of these niches

for the survival of these parasites. For example, the saliva of ticks and mosquitoes is an important factor in the transmission of diseases such as arboviruses (Jones *et al.*, 1992) and *Leishmania* protozoa by sand flies (Titus and Ribeiro, 1988).

Interestingly the local physiological conditions of the salivary glands of Naucorids have been reported to be suitable fit for the survival and replication of *M. ulcerans* (Marsollier *et al.*, 2002). Most insect vectors transmit disease(s) when they feed on the host. For example, the oriental rat flea, which is the primary vector of *Yersinia pestis* in the transmission of black plague, the Anopheles mosquitoes the vector of plasmodium parasites that causes malaria infection, the black flies which are the vectors of onchorcerciases and many others all transmit disease because they are hematophagous insects. Naucorid species unlike the other vectors do not have a distinctive feature of being heamatophagous but it has been reported that they are predators suggesting that the insects through the bites could achieve transmission. The presence of the DNA of *M. ulcerans* in the head of these bugs provides an evidence to suggest the implication of these bugs in the transmission of Buruli ulcer. However, the finding of specific DNA of *M. ulcerans* alone in these insects does not establish that these bugs contain live bacteria. The DNA could as well have come from dead bacteria. Since these bugs are predacious in their mode of life spending most of their time at the bottom of water (Stout, 1982), this could lead to the possibility of then having being contaminated by either dead or living *M. ulcerans* which also prefers muddy bottoms of water with low oxygen concentration (Portaels and Pattyn, 1982; Portaels *et al.*, 1999). To establish that there are living bacteria in these insects, it will be essential to cultivate *M. ulcerans* from these insects. This is yet to be studied

Comparison of seasonal rate of infection of these insects showed a higher rate of infection in the wet season as compare to dry season periods $X^2 = 26.33$; $P < 0.05$. This correlates with the observation of (Amofah *et al.*, 2002), who reported that the prevalence of infections by BU were mostly in the rainy season. It could be that the bacterium prefers wet to dry season for survival and multiplication there by resulting to higher infection or contamination from environmental reservoirs. About 75% of total sample were collected in the two months of rainy season (September and October). With the high number of the Naucorids in the wet season, it is not, surprised if more of the bugs have been contaminated or infected with the bacterium.

One interesting finding from this study was the fact that there was no significant difference between the mean number of infection of Naucorid species between areas endemic and non- endemic of Buruli ulcer in Greater Accra region yet cases of Buruli ulcer have not been reported from non- endemic areas with the same ecological set up. Possible explanations could be that people are ignorant of the disease and do not report cases to Amasaman Health Center where source of information for this study was obtained and where Buruli ulcer cases from most parts of Greater Accra receive treatment. It could also be that peoples contact with stagnant or slow moving water differs from one area to another or other social factors that need to be investigated.

The variation shown in the prevalence of infection between wet and dry season could be attributed to the variation in the ecological habitats of *M. ulcerans* or Naucorid species. Unfortunately previous survey of Naucorids distribution has not been carried out in Ghana.

This present study may be the first in an attempt to map the distribution of the bugs in both endemic and non-endemic areas of BU in Ghana. These studies have shown that these insects are widely distributed in all the study sites in both BU endemic and non-endemic areas. This observation agrees with that of Site, (1990) who also reported that the bugs are cosmopolic insects found throughout temperate and tropical regions especially rich in fresh water and that they constitute about 10% of all species of Hemiptera associated with water. Also with Dejoux *et al.*, (1982) who reported that in Cote d'Ivoire, an endemic country for Buruli ulcer, insects of the family Naucoridae and Belostomatidae are the predominant families of Hemiptera and that the bugs are present in swamps, ponds, dams or river localizations where a lot of human activities such as farming, fishing or bathing are intensively practiced.

The observations of the presence of these insects from all the collection points in all the villages might suggest a countrywide distribution of these insect species and *M. ulcerans* as well. This may be the same situation in other Buruli ulcer endemic countries especially Africa and in particular West African countries. The family Naucoridae spend most of their lifetime under water and whether found in temperate or tropical countries like Ghana, they all exhibit the same way of life preying upon other aquatic organisms (Portael, *et al.* 1999), which strongly suggest evidence of the possibility of a mechanical vector.

Naucorid species are adapted to a wide variety of micro and macro environmental conditions, as evidenced by their wide geographical distribution. This study identified certain water parameters that influence the distribution of Naucorid species. Of the 15

physico-chemical parameters studied, dissolved oxygen, pH and turbidity were the most significant discriminatory parameters for the presence or absence of Naucorid *s*pecies in a given habitat. Dissolved oxygen was found to be the strongest followed by pH and turbidity in that order. This observation is similar to that of Rivers (1953) and Mosi (2003) who also reported that water bugs preferred a habitat with a pH range between 7.3 – 7.5 and that the higher the dissolved oxygen in water the greater the number of water bugs.

Turbidity affects aquatic life in so many ways. The particles of clay, silt, and organic matter which constitute turbidity settle to the bottom especially in slow moving stretches of rivers and stand still water bodies. These settled particles can smoother the eggs of aquatic insects or suffocates insect larvae. Turbidity reduce growth rate and prevent eggs and larval /nymphal development of aquatic insects (Siboman *et al.*, 2002). Several physical and biological factors including nutrients from fertilizers, pesticides, organic matter, bacterial/fungal/algal contamination and aquatic weeds all contribute to turbidity (Bos, 1991). The present study also found that turbidity was associated with biological oxygen demand, ammonium and sulphate concentrations. In addition to being more turbid, the habitats of Naucorids were also found to have higher pH value than those without. The pH of water is dependent on the concentration of anions, cations, salts and synthetic compounds, which indicates its acidity or basic character (Bos. 1991). Therefore, pH may directly or indirectly affect the life of any aquatic organisms, including Naucorid species. Apart from the direct effect of physical and chemical properties of water on Naucorid species, water quality may also indirectly affect Naucorids breeding by favoring certain

aquatic vegetations or organisms on which Naucorids feed or affect the potential biological control agent within the environment.

The finding of dissolved oxygen to be the best discriminatory parameter for the presence or absence of Naucorid species in this study is not surprising because most if not all aquatic insects depend on dissolve oxygen for survival (Chapman, 1992). However, surprisingly, none of the parameters measured were picked in the model to be associated with dissolved oxygen. The reason(s) for this cannot be explained in this study. Studies by (Sibomana, 2002) showed that pH, total dissolved solids, silica and calcium were found to be associated with dissolved oxygen. Reduced oxygen concentration (Palomino *et al.*, 2001) and low pH (Portaels and Pattynes, 1982) enhance the growth of *M. ulcerans* suggesting a preference of this organism to microaerophilic environment. Dissolved oxygen, pH and turbidity, which act, as the major determinant of the distribution of Naucorid species could as well be the same factors that determine the presence or absence of *M. ulcerans* in the water environment.

In conclusion this study has provided evidence to suggest that Naucorid species could transmit *M. ulcerans* in the wild. The wide distribution of these bugs and the presence of *M. ulcerans* DNA found in these insects in both endemic and non-endemic villages of Buruli ulcer in Ga district have raised several important challenges. Firstly, isolation of living *M. ulcerans* bacterium in these insect species as well as whether there is any correlation between the infection of the bugs and the prevalence of Buruli ulcer in human population is imperative for making a meaningful conclusion as to whether these insects

transmit the bacterium or not. Secondly, the detail biology and systematic of these insects have not been studied. It is therefore of paramount importance to study the biology and detail identification to a level of species as a taxon for better understanding of the relationship between *M. ulcerans* and these bugs and thirdly, apart from Naucorids, quite a number of other water bugs were found in association with Naucorids. It is therefore of utmost importance to cover other members of the families such as Pleidae, Napidae, Notonectidae and Corixidae across endemic regions of Buruli ulcer in Ghana.

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APPENDICES

Appendix 1:

STANDARD SOLUTION

The following standard solutions were prepared using sterile double distilled water (sddw). Where appropriate the solutions were autoclaved at 121lb/sq for 15mins in an Eyela autoclave (Rikakki Tokyo).

DNA Extraction

Bender buffer

0.1M NaCl, 0.2M, sucrose 0.1M Tris HCl, pH 7.5,

0.05M EDTA PH 9.1, 0.5% SDS stored at4°C.

0.5 MEDTA (PH 8.0)

186.1g/l in water, pH adjusted with NaOH pellets,

and stored at room temperature.

Agarose Gels

10X TAE buffer

142g Tris base, 57ml glacial acetic acid,

100ml 0.5 MEDTA pH adjusted to 7.7 and the volume

made to 1000ml with sddw.

0.5 MEDTA (PH 8.0).

186G of EDTA, dissolved in 800ml sddw, PH adjusted with NaOH pellets, the volume made to 1000ml with sddw and stored at room temperature.

Gel loading buffers

5X orange G

20% w/v Ficoll, 2.5m MEDTA 2.5% (w/v) orange G stored at room temperature.

6X Bromophenol blue

0.25% bromophenol blue was added to 40% sucrose in sddw and stored at 4°C

DNA molecular weight size marker.

A 100bp ladder molecular weight size marker was used throughout the study. The first band size is 100bp the subsequent ones measured 200, 300, 400 . . .1000bp

Appendix II:

***Analysis of variance carried on the distribution of Naucorid species across villages ***

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Villages	5	489.78	97.96	2.04	0.077
Residual	138	6621.21	47.98		
Total	143	7110.99			

Appendix III:

Analysis of variance carried on the distribution of Naucorid across months for each village

1. Amasaman

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Months	7	579.83	82.83	5.62	0.002
Residual	16	236.00	14.75		
Total	23	815.83			

2. Dolo-Gono

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Months	7	193.62	27.66	1.08	0.420
Residual	16	410.00	25.62		
Total	23	603.62			

3. Dome

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Months	7	223.17	31.88	2.29	0.080
Residual	16	222.67	13.92		
Total	23	445.83			

4. Hobour

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Months	7	220.62	31.52	2.26	0.084
Residual	16	223.33	13.96		
Total	23	443.96			

5. Obom

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Months	7	182.00	26.00	1.13	0.392
Residual	16	368.00	23.00		
Total	23	550.00			

6. Weija

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Months	7	2132.0	304.6	2.99	0.033
Residual	16	1630.0	101.9		
Total	23	3762.0			

Appendix IV:

*** Analysis of variance performed on the distribution of Naucorid species across the sites of each village *****

Amasaman

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Site	2	125.08	62.54	1.90	0.174
Residual	21	690.75	32.89		
Total	23	815.83			

Doblo-Gono

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Site	2	54.25	27.12	1.04	0.372
Residual	21	549.37	26.16		
Total	23	603.62			

Dome

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Site	2	141.08	70.54	4.86	0.018
Residual	21	304.75	14.51		
Total	23	445.83			

Hobour

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Site	2	63.08	31.54	1.74	0.200
Residual	21	380.87	18.14		
Total	23	443.96			

Obom

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Site	2	85.75	42.88	1.94	0.169
Residual	21	464.25	22.11		
Total	23	550.00			

Weija

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Site	2	796.6	398.3	2.82	0.08
Residual	21	2965.4	141.2		
Total	23	3762.0			

Appendix V:

Physico-chemical parameters measured with P.values obtained on the test of equalities of means between habitats with Naucorids and those without but with other species

Parameters	Formula	Units	P. value
Temperature		°C	0.210
pH		pH unit	0.000
Turbidity		NTU	0.0I6
Conductivity		µS/cm	0.515
Total hardness		mg/l	0.001
Calcium	Ca	mg/l	0.539
Magnesium	Mg	mg/l	0.421
Chlorine	Cl	mg/l	0.447
Ammonium	NH_4-N	mg/l	0.346
Nitrites	NO2-N	mg/l	0.787
Nitrates	NO3-N	mg/l	0.622
Phosphates	PO_4-P	mg/l	0.403
Sulphates	SO_4	mg/l	0.578
Bio-oxygen demand		mg/l	0.329
Dissolved oxygen		mg/l	0.000