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# Laboratory Models of Infection and Transmission of Mycobacterium ulcerans; causative agent of Buruli ulcer disease

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*University of Tennessee - Knoxville*

To the Graduate Council:

I am submitting herewith a dissertation written by Lydia Mosi entitled "Laboratory Models of Infection and Transmission of Mycobacterium ulcerans; causative agent of Buruli ulcer disease." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

Pamela L. C. Small, Major Professor

We have read this dissertation and recommend its acceptance:

Tim Sparer, Chunlei Su, Erik Zinser, Juan Luis Jurat-Fuentes

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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# Laboratory Models of Infection and Transmission of *Mycobacterium ulcerans*, Causative Agent of Buruli Ulcer Disease

**A Dissertation Presented for**

**the Doctor of Philosophy**

**Degree in Microbiology**

**The University of Tennessee, Knoxville**

**Lydia Mosi**

**December 2009**

**Dedication**

He makes all things beautiful in His time!

I would like to dedicate this work to the memory of my parents especially my mom, Mrs. Comfort Dede Mosi. You did not get to see me through this, but you certainly sowed the right seed and paved the way for a bright futre!

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**Abstract**

Identification of the environmental reservoir of *Mycobacterium ulcerans*, the etiological agent of Buruli ulcer, within the aquatic ecosystem has been a salient research area within the last five years. Based on extensive environmental sampling and elegant laboratory models, associations have been made between the bacterial DNA and aquatic invertebrates, biofilms, plants, fish and detritus material captured on 0.2µm pore filters. These studies have suggested that *M. ulcerans* is widely distributed within many functional feeding groups and may be concentrated through different trophic links; however, the specific route of transmission to humans remains a mystery. In this study we have used laboratory models of infection to ascertain the role of aquatic invertebrates and fish in *M. ulcerans* transmission. A biologically relevant infection model in which *M. ulcerans*-infected mosquito larvae were fed to a species of predaceous hemiptera (African Belostomatidae) was used to demonstrate the persistent colonization of *M. ulcerans* and subsequent transmission of bacteria to naïve prey. The association of *M. ulcerans* with specific anatomical compartments showed that *M. ulcerans* accumulates preferentially on the exoskeleton. No difference was found between the ability of wild-type *M. ulcerans* and an *M. ulcerans* isogenic mycolactone-negative mutant to colonize belostomatids. These data show that African belostomatids can successfully be colonized by *M. ulcerans* and support the trophic transfer of *M. ulcerans* within the environment. We have shown that *M. ulcerans* with or without the toxin is not lethal to fish (Medaka) even at high doses following direct inoculation. Over time (23wks), infected Medaka do not exhibit any visible signs of infection or toxicity and histopathological sections do not reveal significant gross pathogenesis. *M. ulcerans* also appears not to replicate in infected Medaka. We also show that fish monocytes are susceptible to nanogram amounts of purified mycolactone. This is the first study to demonstrate the possibility of fish as a reservoir for *M. ulcerans* within the aquatic environment.

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# Chapter 1: Background and significance

### *1.1 Buruli ulcer disease*

During the last two decades, there has been a re-emergence of the debilitating skin disease Buruli ulcer across diverse regions of the world. The disease was named for its initial onset in the Buruli district in Uganda, Central Africa (10) and is also known as Bairnsdale ulcer in Australia, where the first clinical case was published (32, 47). Until recently, much of the public and the medical community were unaware of the disease, which has now been reported in over 30 countries worldwide (Fig. 1.1). West and Central Africa, especially, Ghana (3, 4, 9), Cote d'Ivoire (31, 36), Nigeria (39) and Congo (54), and some parts of Australia are the most affected regions (33, 38, 56). Since 1980, significant increases in the incidence of the disease have been reported in these areas with new foci also developing in previously non-endemic countries like Togo (37) and Angola (7). Other endemic areas of the world include Papua New Guinea (14), Malaysia (41), Mexico (3) and French Guiana (20).More than 20,000 cases have been reported in West Africa during the last decade (69).

All age groups are affected by Buruli ulcer, but children under 15 years represent the largest disease burden (12). There does not appear to be a sex difference in disease incidence among both adults and children, and this is somewhat puzzling since men and women have very different roles in the poor rural areas where the disease occurs (12). Again there might be a bias in case reporting because of the lack of immediate health care facilities and the preference of treatment by herbalists compared to health centers (65, 66). Buruli ulcer disease is a significant cause of morbidity in affected individuals but lethal infections are extremely rare.

The disease is focal in nature and has been associated with slow moving and stagnant water bodies within endemic areas. There may be pockets of cases within the same geographic area that are endemic for the disease within neighboring areas that do not have any disease at all. In view of this, the disease burden is difficult to obtain, but it is has been estimated that 25% of people in West Africa are affected by the disease (28). Some seasonal variation has been thought to exist with the disease. In Australia for instance, it was noted that the disease appeared at the end of the autumn or winter (21, 23). In Uganda, two previous studies have reported a peak of incidence in the low rainfall

months between May and September (1, 10). In Ghana and Cote d'Ivoire, the disease is at its highest during the months of September and October, which is also the onset of the dry season (4, 36). Thus, this seemingly temporal dry period onset of the disease cuts across geographical boundaries and could be indicative of a potential risk factor. Common to all these areas is the correlation between disease prevalence and environmental disturbance, both natural and man made including flooding, sand winning, deforestation, and urbanization of previously rural areas (28).

### *1.2 Causative organism and pathology*

Buruli ulcer is caused by an environmental member of the Mycobacteria family, *Mycobacterium ulcerans. M. ulcerans* is a slow growing bacterium with a generation time of about 72 hours. It has a restricted growth temperature range of 28°C to 34°C. It stains acid-fast positive with the Ziehl Neelsen stain and typically tends to grow in clumps and cords. Molecular analysis shows that *M. ulcerans* is closely related to *M. marinum*, which causes disease in fish. In addition to its chromosome, *M. ulcerans* has a 174-kilobase plasmid, pMUM001, which produces a virulent macrolide toxin, mycolactone (17, 58). Mycolactone is composed of an invariant core comprising a 12-membered macrolactone that is esterified to a highly unsaturated acyl side chain (26). It is interesting to note that different congeners of mycolactone are made by the different geographical isolates and this directly corresponds to their degree of toxicity. The most potent congener, mycolactone A/B, is made by the African and Malaysian isolates. The Australian isolates make less toxic mycolactone C whilst the Chinese isolates make mycolactone D. Differences in structure are confined to the mycolactone side chain (Fig 1.2). The core lactone structure is conserved in all mycolactone congeners.

Recently a group of slow growing mycolactone procuding mycobacteria (MPM) that make unique molecules have been identified (Table 1.1) (48, 49, 50). *M.liflandii*, isolated from Xenopus tropicalus and Xenopus laevis frogs, makes mycolactone E (49). *M. pseudoshottsii* and *M. marinum DL*, isolated from fish in the Chesapeake Bay and Red and Mediterranean Sea respectively, make mycolactone F (48, 50). Both forms of

mycolactone are cytotoxic to L929 mouse fibroblasts, but are less potent than mycolactones produced by *M. ulcerans* (48). To date, there have been no reported cases of human infection by any of these MPMs. Mycolactone producing mycobacteria are all though to have evolved directly from *M. marinum* (42)

The gross pathology associated with the disease has been attributed to mycolactone (16). In addition to this, the slow growth rate directly translates to the slow progression of the disease and the restricted low growth temperature makes the skin a good target. The disease is characterized by severe subcutaneous necrotic lesions that lead to chronic open sores and ulcerations, ultimately affecting bone in extreme cases (Fig 1.3). It starts as a painless nodule localized mainly on the extremities and cooler parts of affected individuals (21), with exceptional cases on the torso and buttocks (2). As the disease slowly progresses, affected areas may progress to the ulcerative stage with development of large ulcers with undermined edges (21, 22, 66). Oedema can be extensive in some cases and in severe cases there may be bone involvement (osteomyelitis) in some cases (62, 66). Bacteria can be identified in all forms of the disease. Systemic infection is rarely encountered presumably because the bacteria can notgrow at 37°C.

Unlike *M. marinum* that produces primarily intracellular infection in humans triggering inflammatory responses, cell-mediated immunity (CMI) and delayed type hypersensivity (DTH), *M. ulcerans* is mostly extracellular in acute disease (8). It is thought that there might be a transient intracellular state during infection in which *M. ulcerans* is taken up by macrophages and disseminated beyond the site of infection followed by subsequent lysis of the immune cells to release extracellular bacteria (53, 63).

The difference in the pathology of *M. ulcerans* compared to other environmental mycobacteria, especially its closest relative *M. marinum*, has been attributed to its ongoing reductive evolution. *M. marinum* and *M. ulcerans* share 98% nucleotide sequence identity, but over the course of evolution *M. ulcerans* has lost some of its genome (51). There are over 700 pseudogenes in *M. ulcerans* which are intact in *M. marinum.* Over 300 of these pseudogenes have been created by the insertion of two IS

elements, IS2404 and IS2606 which are highly represented in *M. ulcerans* but absent from *M. marinum.* In the most virulent strains of *M. ulcerans*, the region of difference 1 (RDI) is absent (63). The RD1 locus encodes a secretory apparatus responsible for exporting two antigenic immunomodulatory proteins ESAT-6 and CFP10 (60). In other mycobacteria these proteins are crucial in enhancing virulence, and promoting expansion and dissemination of the infection (8). Another major event in the evolution of *M. ulcerans* was the acquisition of a large plasmid which produces mycolactone. Mycolactone has also been shown to alter the immune system by suppressing the production of interleukin-2 (IL-2) and tumor necrosis factor (TNF) thus down-regulating T-helper-1 (Th1) responses (19, 40). This leads to a limited inflammatory response during the acute and early stages of infection and explains why early detection of cases is hampered (18). These events could contribute significantly to the induction and regulation of immune responses in hosts (27).

Other factors may also contribute to the observed host response to infection such as genetics of the affected individuals, previous mycobacterial infection and the extent of an individual's exposure to the bacteria in nature. For instance, it has been found that individuals with prior BCG vaccination show reduced rates of ulcers upon infection (1, 61). However, there is not enough information to accurately outline which of these could be determining risk factors for infectivity or variation in the virulence observed.

### *1.3 Diagnosis, Treatment and Control*

Due to the painless nature of the disease and the lack of epidemiological data on the incubation period, early stages of the diease are often ignored. Thus most affected individuals report to health centers only after development of a large persistent ulcer. The current diagnosis requires biopsies of affected tissue and analysis by Polymerase Chain Reaction (PCR). Treatment in Buruli ulcer infections hence seeks to curtail the spread of ulcers and repair existing tissue damage to affected areas. In early and intermediate stages of the disease, anti-mycobacterial drugs including rifampicin and streptomycin or amikacin are highly effective (53, 62). These drugs are used in combination and require

administration up to 8 weeks. In more advanced cases where larger lesions are involved, complete surgical excision of the affected area remains the most effective treatment option (5, 24). Surgery is typically followed by skin grafting and anti-mycrobacterials are still administered to prevent relapses and accelerate the healing process. Traditional treatments have also been used, and although some may be efficacious they leave behind patients with severe scaring and disability. In some cases, in the natural history of the disease, the immunosuppressive effect of the toxin is overcome by the host with subsequent healing of ulcers (53). Deformities and scarring associated with healing are common and this ultimately has a negative effect on the productivity of affected individuals. There is a significant amount of stigma also associated with the disease (6, 55, 56).

Treatment in hospitals often requires long stays, up to 3 months, during which serial surgeries and wound dressings are performed. The average cost of treating a Buruli ulcer case was estimated to be US$ 780 per patient in 1994–1996 (68). For example, in Ghana in 2001–2003, the median annual total costs of BU to a household by stage of disease ranged from US$ 76.20 (16% of a work-year) per patient with a nodule to US$ 428 (89% of a work-year) per patient who had undergone amputation (65).

There is no vaccine against Buruli ulcer disease. Current prospects include generation of a live attenuated *M.ulcerans*, sub-unit based vaccines and improvement of the BCG vaccine (26). There is evidence that the BCG vaccine may offer a cross-reactive protective role, but this has not thoroughly been investigated (1, 26, 61))

### *1.4 Transmission*

The mode of transmission of Buruli ulcer remains elusive. Person-to-person transmission is rare and there has only been one reported case (11). For over 50 years, all the regions of the world that have reported cases of Buruli ulcer have associated the disease with proximity to rivers, swamps and watercourses in general. It is of importance to note here that even though water bodies have been associated with the disease, the clustering of cases does not necessarily correlate with the population distribution along

the watercourse. With the ongoing genome reduction of *M. ulcerans*, it is believed that the bacterium is undergoing niche specialization, diverging from being a generalist environmental bacterium to a more host specific bacterium. Despite intense investigation with transmission , an amplifying reservoir has not been identified.

*M. ulcerans* DNA was first detected in the environment in the 1990s by PCR in Africa by Franscoise Portaels in Naucorids and Belostomatids (45). *M. ulcerans* DNA has subsequently been detected in a variety of aquatic organisms including Belostomatids, Naucorids, Odonates, Coleopterans, mosquitoes, snails and small fish (15, 29, 34, 45, 51, 57). The DNA has also been detected in biofilm collected from the surface of leaves and other inanimate objects and materials captured on 0.2µm pore filters (35. 67). Animals such koalas and possums have also been found to be naturally infected with *M. ulcerans* (30, 43). Most of the DNA evidence has been based on the detection of the insertion elements IS2404 and IS2606. Recently however, it has been shown that these sequences are not unique to *M. ulcerans* and are present in other novel mycobacterial species that also contain the mycolactone producing plasmid (26, 48) (Table 1.1 and Fig. 1.2).

Various attempts have been made to culture the organism from water, soil, insects and the environment at large.These efforts are, however, thwarted by the overgrowth of faster growing bacteria in the environment. There was one reported successful culture from a Naucorid collected in the Ivory Coast (34). The culture however, could not be propagated successfully in the lab and has since died out. More importantly, *M. ulcerans* has recently been isolated from a *Gerridae* after serial passage of the insect homogenate through mice (44). This isolate has been successfully been propagated and characterized.

Despite the significant efforts made toward identification of potential vectors and reservoir species in the environment, the exact mechanism by which the bacterium is introduced into unsuspecting hosts is still an enigma. There are three main hypotheses involved in the transmission of the bacterium. *M. ulcerans* has been thought to be introduced to persons through a preexisting wound; however this hypothesis has not been tested. In 1997, Ross et. *Al*. published a report indicating that *M. ulcerans* can be aerosolized from suspensions of tap water then be inhaled or ingested by otherwise

healthy individuals and subsequently reactivated in areas of the body where the temperature is lower or where there has been some recent trauma (23).

Another hypothesis suggests that humans are primarily infected by traumatic introduction of *M. ulcerans* into the skin through contact with a contaminated environment (45). This could occur through injuries from sharp edges of leaves or twigs or even insect vectors. The latter hypothesis has been supported with substantial research by Marsollier et. *Al*. In a publication in 2002, Marsollier et. *Al*. carried out studies on naïve Naucorids collected in France, where he infected these insects in the lab with *M. ulcerans* and followed the infection over a period of 90 days. He was able to show that the bacteria successfully colonized the insects, causing no growth impairment throughout this period. He showed that the insects could transmit the bacteria to mice at the sites of trauma when allowed to feed on their tails. In a later experiment, he showed that the bacteria were localized in the salivary glands of infected insects. Although these studies were of significance, it is difficult to determine their relevance to transmission of Buruli ulcer in Africa for a number of reasons; (i) the insect species used were not African species, (ii) the primary *M. ulcerans* used was not representative of the classical *M. ulcerans* from Africa, and (iii) none of the studies have provided comprehensive quantitative data on the location of the bacterium within the insect.

Considering the wide range of organisms that have been found positive for *M. ulcerans* DNA within endemic aquatic water bodies, there is the possibility of a trophic relationship between these organisms that could lead to a concentration of the bacteria along the food chain and eventually introduction into a likely host. Naucorids and Belostomatids are aggressive predaceous hemiptera, known to attack and immobilize a wide range of prey even prey larger than themselves (59). Both insect families consume small fish. Taking into account the fact that fish in endemic water bodies have been found positive for potential *M. ulcerans* DNA based on detection of the IS2404 insertion sequence, it is possible that there may be a transfer of the bacteria via this route.

Mycobacteriosis in fish has been well documented over the years (13, 32, 49,46,64). The three most important pathogenic species are *M. abscessus, M. fortuitum* and *M. marinum*. More recently however, outbreaks of mycobacteriosis have been

reported within the Mediterranean and Red Sea and the Chesapeake Bay. These infections have been attributed to newly recognized mycobacterial strains that are closely related to *M. ulcerans* including *M. marinum DL, M. pseudoshottsii, M. chesapeake* and *M. shottsii* (48, 49, 50) (Table 1.1). One striking revelation is that most of these novel strains possess a plasmid that encodes for variants of the virulent macrolide toxin, mycolactone, similar to the mycolactone produced by *M. ulcerans* (27, 42) (Fig 1.2). Despite the historic association between mycobacteria and fish, no study has been done to determine whether *M. ulcerans* can infect fish, thus being a possible link in the transmission process.

In order to investigate potential reservoirs and vectors of Buruli ulcer, it is important to obtain experimental data for their ability to colonize or cause disease in a host. The key issues are to determine whether the bacterium colonizes and replicates within these organisms. This can best be answered experimentally using laboratory models. In this work, we have used an African insect species that has been found to have positive PCR results for *M. ulcerans* in nature and a classical lineage strain of *M. ulcerans* to determine the following: (i) whether *M.ulcerans* persistently colonizes or grows within African predaceous water bugs, (ii) how the bacterium is partitioned within external and internal body parts, (iii) whether mycolactone plays a role in insect infections, and (iv) whether *M. ulcerans* can be transmitted by water bugs to prey within a food chain. We have also developed an *in vivo* and *in vitro* model for understanding *M. ulcerans* pathogenesis in fish by the following; (i) artificially infecting Japanese medaka with *M. ulcerans*, (ii) determining the pathogenicity of *M. ulcerans* in medaka, (iii) determining the role of mycolcatone in medaka infection (iv) determining whether *M. ulcerans* can actively colonize and replicate in Medaka and (v) determining the cytotoxicity of mycolatones to fish macrophages. This is the first study to address the possibility that *M. ulcerans* survives and replicates in fish.

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### *1.6. Appendix*

**Table 1.1.Mycolactone producing Mycobacterium isolates** (Ranger *et al*., 2006)

| Strain | Species | Host | Geographic origin | Reference or source |
|---|---|---|---|---|
| Agy99 | *M. ulcerans* | Human | Ghana | 29 |
| 1615 | *M. ulcerans* | Human | Malaysia | ATCC[a] 35840 |
| V2 | *M. ulcerans* | Human | Australia | 23[b] |
| 753 | *M. shinshuense* | Human | Japan | 18 |
| XL5 | *M. liflandii* | Frog (*Xenopus laevis*) | University of Virginia | 24 |
| L6 | *M. pseudoshottsii* | Striped bass (*Morone saxatilis*) | Chesapeake Bay | 25 |
| L15 | *M. pseudoshottsii* | Striped bass (*Morone saxatilis*) | Chesapeake Bay | 25 |
| L21 | *M. pseudoshottsii* | Striped bass (*Morone saxatilis*) | Chesapeake Bay | 25 |
| L23 | *M. pseudoshottsii* | Striped bass (*Morone saxatilis*) | Chesapeake Bay | 25 |
| L50 | *M. pseudoshottsii* | Striped bass (*Morone saxatilis*) | Chesapeake Bay | 25 |
| 1218 | *M. marinum*[c] | Salt water fish | Philadelphia, Pa. | ATCC 927 |
| M | *M. marinum*[c] | Human | San Francisco, Calif. | ATCC BAA-535 |
| DL/DK1 | *M. marinum*[c] | Sea bass (*Dicentrarchus labrax*) | Denmark | 33 |
| DL240490 | *M. marinum*[d] | Sea bass (*Dicentrarchus labrax*) | Red Sea, Israel | 33 |
| DL150991 | *M. marinum*[d] | Sea bass (*Dicentrarchus labrax*) | Mediterranean Sea, Israel | 33 |
| DL180892 | *M. marinum*[d] | Sea bass (*Dicentrarchus labrax*) | Ein Yahav, Israel | 34 |
| 045 Thalassa | *M. marinum*[d] | Sea bass (*Dicentrarchus labrax*) | Mediterranean Sea, Greece | 33 |
| DL241200 | *M. marinum*[d] | Sea bass (*Dicentrarchus labrax*) | Mediterranean Sea, Greece | This work |
| DL300/04 | *M. marinum*[d] | Sea bass (*Dicentrarchus labrax*) | Mediterranean Sea, Italy | This work |
| DL272/05 | *M. marinum*[d] | Sea bass (*Dicentrarchus labrax*) | Mediterranean Sea, Italy | This work |
| CF030494 | *M. marinum*[d] | Butterflyfish (*Chaetodon fasciatus*) | Red Sea, Israel | 33 |
| SA200695 | *M. marinum*[d] | Sea bream (*Sparus aurata*) | Red Sea, Israel | 33 |
| Hybrid270995 | *M. marinum*[d] | Red sea bream (*Pagrus major* [female] × *Sparus aurata* [male]) | Red Sea, Israel | 33 |
| SV300500 | *M. marinum*[d] | Lizard fish (*Synodus variegatus*) | Red Sea, Israel | 33 |
| SV061004 | *M. marinum*[d] | Lizard fish (*Synodus variegatus*) | Red Sea, Israel | This work |
| SR030597 | *M. marinum*[d] | Rabbitfish (*Siganus rivulatus*) | Red Sea, Israel | 33 |
| CC240299 | *M. marinum*[d] | Koi (*Cyprinus carpio*) | Ma'agan Michael, Israel | 33 |
| BB170200 | *M. marinum*[d] | Silver perch (*Bidyanus bidyanus*) | Dor-Ma'agan Michael, Israel | 32 |

[a] ATTC, American Type Culture Collection.
[b] The strain is named TS-2 in reference 23.
[c] *M. marinum* isolate displaying light-induced pigment production (photochromogenic).
[d] *M. marinum* isolate displaying constitutive pigment production (scotochromogenic).

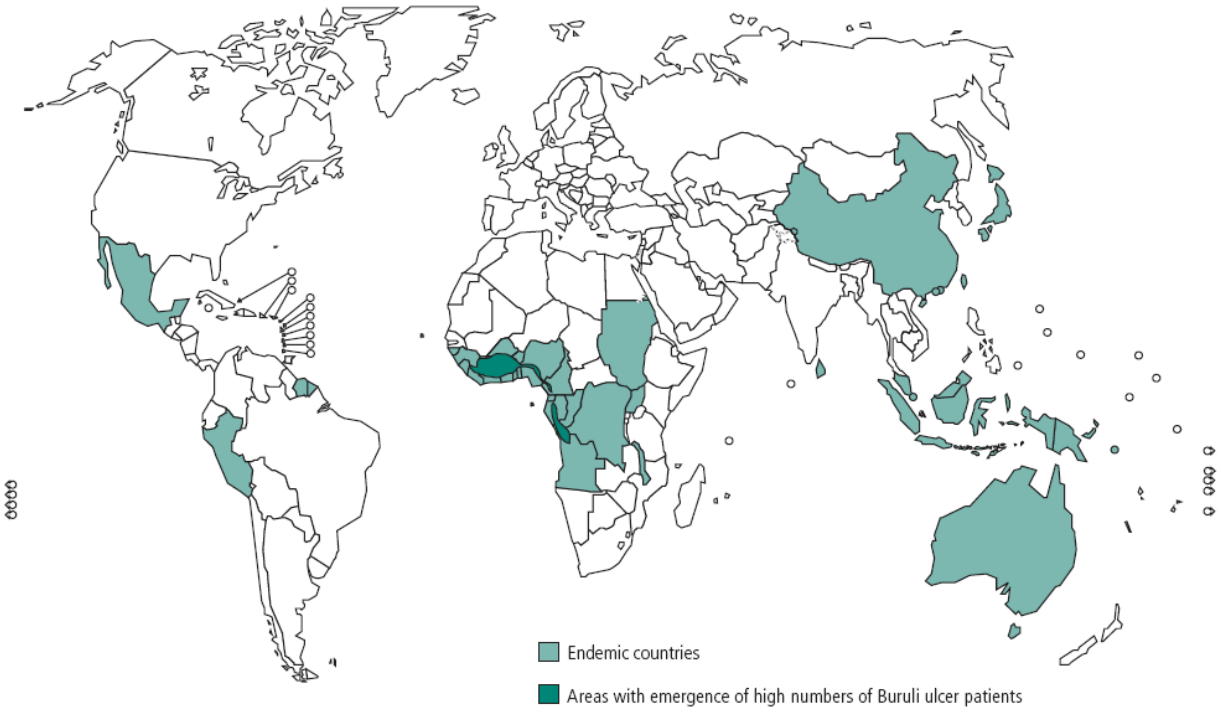


**Figure 1.1: Geographic distribution of Buruli ulcer disease (**Johnson *et al.*, 2005**).**

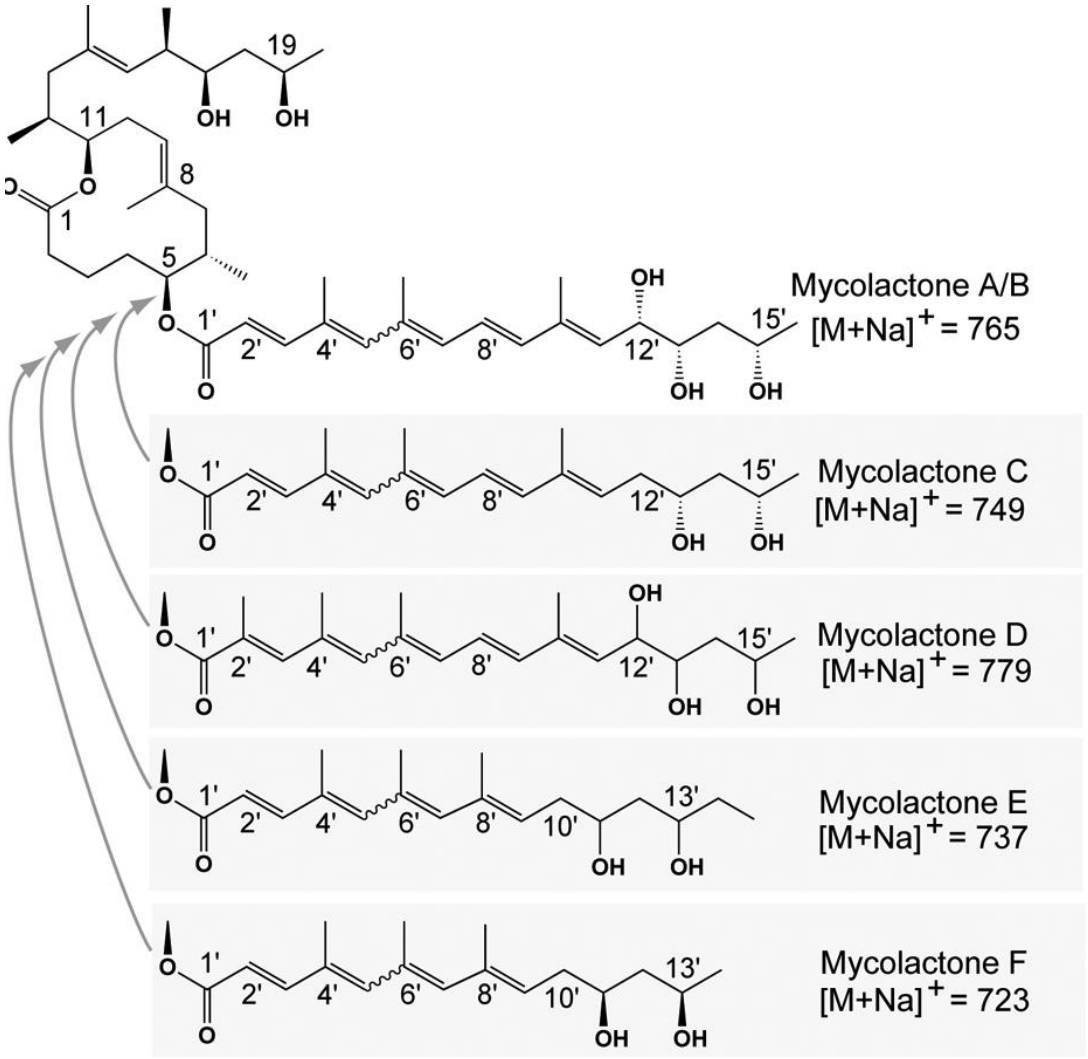


**Figure 1.2: Mycolactone variations represented in naturally occurring isolates.** The core is the same in all these molecules with variations occurring in the side chains. (Pidot *et al.*, 2008)

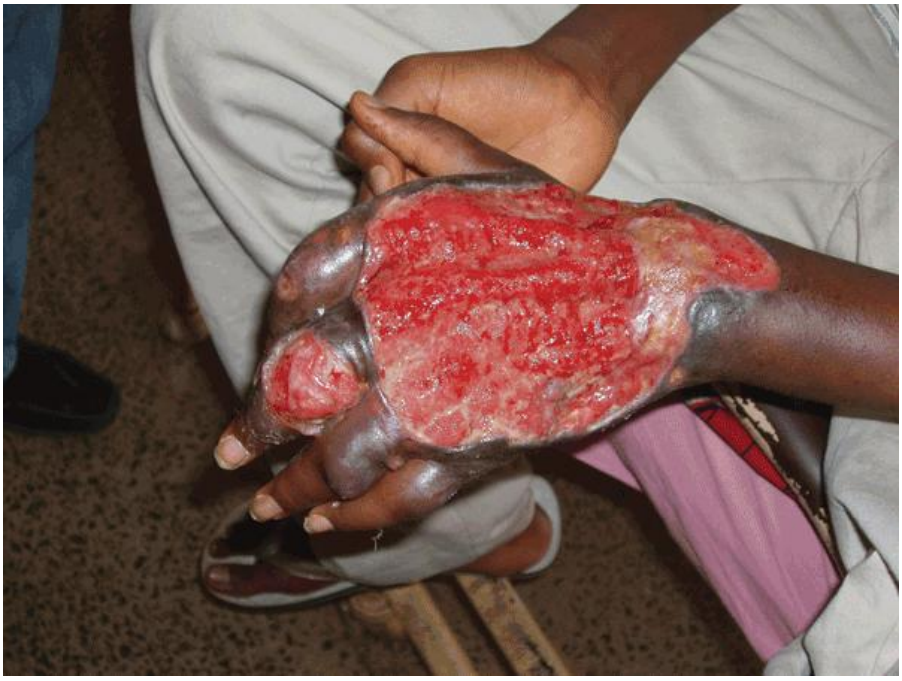

**Figure 1.3. Buruli ulcer disease (**WHO, 2004**)**

# Chapter 2: Materials and Methods

### *2.1 Insect colonization studies*

#### *2.1.1. Bacterial strains and growth conditions*

The strains used in this study and their sources are shown in Table 2.1. The MU1615 strain is a well-characterized Malaysian human isolate with physical and biochemical properties very similar to the genome strain Agy99 from Ghana and makes mycolactone A/B. Transposon mutagenesis (12) was used to generate the mycolactone negative mutant MU1615::Tn118 with an insertion in the FABH gene (mup045). MU1615g and Mu1615::Tn118g express a green fluorescent protein via an integrating vector *psm5* (13). By using this method, the GFP gene is inserted into the chromosome of MU1615 in the phage attachment site (*att*) and has no effect on the virulence of the bacterium. All strains were grown to mid-log phase in Middlebrook 7H9 (M7H9) media supplemented with 10% oleic acid-albumin-dextrose enrichment (OADC) {DIFCO}. *M. ulcerans* and *M. marinum* 1218 strains were incubated at 32°C the MMDL strain was incubated at room temperature and the XL5 strain was incubated at 28°C with 5% $CO_2$.

#### *2.1.2. Inoculum preparation*

The total number of bacteria used for the infections was determined via colony forming units (cfu). One loop-full of bacteria growing at exponential phase was emulsified in 0.01% sodium dodecyl sulfate, and clumps were broken by being passaged through a 25-gauge needle 15 times. One hundred microliters of the resulting suspension was plated on M7H9 agar medium supplemented with 10% OADC (Difco) and incubated at 32°C for 6 weeks to determine cfu.

#### *2.1.3. Aquatic insects*

Adult belostomatids (*Appasus* sp. [*Diplonychus* sp.]), 1- to 3-cm long were collected from aquatic sampling sites in Ga district, Ghana. Insects were housed individually in deep petri dishes filled with double-distilled water and maintained under a 12-h light and dark photoperiod at 28°C. Insects were fed either midge larvae (Chironomidae) or blowfly larvae (Diptera: Calliphoridae) (*Phormia regina*) every other day, and the housing water was changed at the same time. Mosquito (*Ochlerotatus*

*triseriatus*) egg rafts were obtained from Michigan State University. The egg rafts were submerged in double-distilled water so that they could hatch into larvae in about 3 days. Larvae were maintained on powdered fish food each day until they developed into third instars, when they were fed bacteria and used for infection. Because some belostomatids were collected from areas endemic for Buruli ulcer and *M. ulcerans* DNA has been detected in a small number of belostomatids from West Africa, 110 belostomatids were analyzed by using microscopy and PCR for the presence of *M. ulcerans* DNA. All insects tested were PCR negative for *M. ulcerans* and therefore were used as negative controls.

*2.1.4. Experimental infection of insects*

Mosquito larvae infected with MU1615g via feeding were used as the primary prey for adult belostomatids in these studies (Fig 2.1) . Infected prey were prepared by starving the naïve larvae (*Ochlerotatus triseriatus*) for 24 h and then transferring them to a fresh container containing $10^6$/ml *M. ulcerans* bacteria in 10 ml of double-distilled water, where larvae were allowed to feed on fluorescently tagged *M. ulcerans* for 24 h. Five representative larvae were removed and analyzed for the presence of *M. ulcerans* by using light (acid-fast stain) and fluorescent microscopy. At this time point, the guts of virtually all larvae were packed with *M. ulcerans*. Three infected larvae were then fed to each belostomatid that had previously been starved for 7 days. There were 36 adult belostomatids per bacterial strain used. Twenty-four hours after infection, each insect was transferred to a new petri dish and maintained on chironomid (Diptera: Chironomidae) midge larvae for the duration of the study period. The water was changed each time insects were fed.

*2.1.5. Detection of M. ulcerans in insect tissues*

For infection studies, 36 insects were used for each *M. ulcerans* strain tested. At 1 day, 30 days, and 60 days post infection (p.i.), 12 insects were sacrificed for analysis. The 24-h time point was chosen to determine the rate of infection, whereas the later time points were chosen to detect viability, colonization, and multiplication of the bacteria within the insects. At each time point, individual belostomatids' internal organs were carefully removed, and the salivary gland, gut, head, thorax, and forearms (Fig 2.2) were homogenized in 200 µl of 1 M Tris-HCl buffer [pH 7.5]. For quantification of the

bacteria, four 10-fold dilutions were made of each anatomical section, and smears were made for acid-fast staining and fluorescent microscopy. Acid-fast bacilli (AFB) were viewed with a light microscope (Olympus BX51/BX52). Wet mounts of each section were viewed using a fluorescent microscope (Nikon Eclipse E400) equipped with a standard epifluorescent attachment filter set for the detection of the fluorescently labeled bacteria. Although AFB microscopy provided better visualization of *M. ulcerans* morphology, the presence of fluorescently labeled *M. ulcerans* was required for scoring a belostomatid positive for *M. ulcerans* as determined by microscopy. For scanning electron microscopy, infected insects were vacuum dried, sputter coated with gold using a SPI-Module sputter coater for 10 s, and mounted on carbon-coated metal stubs. Imaging was performed on a Zeiss 1525 field emission scanning electron microscope equipped with a GEMINI field emission column.

For recovery of viable bacteria from the infected insects, 100 µl of each insect section homogenate was decontaminated via the modified Petroff's method (15). Briefly, 150 µl of 4% NaOH was incubated with 100 µl of insect homogenate for 15 min, followed by a 15-min incubation with 800 µl of sterile saline of the recovered pellet. The resulting pellet, after centrifugation at 3,000 µ*g*, was resuspended in 100 µl sterile saline and plated on M7H9 agar plates supplemented with 10% OADC supplement (Difco), chloramphenicol (20 mg/ml), and cycloheximide (20 mg/ml).

*2.1.6. Transmission of M. ulcerans infection to blowfly larvae via feeding*

Twelve infected belostomatids removed at 1 day, 30 days, and 60 days p.i. were allowed to feed individually on a single blowfly larva (*Phormia regina*). Larval exuviae were collected immediately, homogenized as described above, and analyzed by using microscopy and PCR for the presence of *M. ulcerans*. As controls, uninfected belostomatids were also allowed to feed on larvae and were analyzed for the presence of *M. ulcerans*.

*2.1.7. DNA extraction and PCR analysis*

DNA was extracted from insect and larval homogenates with the UltraClean soil DNA extraction kit (Mo Bio Laboratories) according to the manufacturer's instruction. The enoyl reductase (*mlsA*) gene was chosen to determine the presence of mycobacterial

DNA in insect tissues as previously described (14). Five microliters of each DNA sample was amplified with the *mlsA* primer pair 5'-GAGATCGGTCCCGACGTCTAC-3' and 5'-GGCTTGACTCATGTCACGTAAG-3' in 50-µl PCR mixtures using the GoTaq polymerase buffer system (Promega). Each reaction mixture contained 36.7 µl double-distilled water, 5 µl GoTaq green master mix (400 µl of each deoxynucleoside triphosphate, 3 mM $MgCl_2$, blue and yellow dyes), 1 µM of forward and reverse primers, 1.5 U of GoTaq polymerase, and 5 µl of DNA template. Cycling was performed in a Mastercycler gradient thermal cycler (Eppendorf) as follows: 95°C for 5 min; 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and 72°C for 10 min. Nine microliters of each reaction mixture was analyzed on 1.5% agarose gels in 1µM Tris-acetate-EDTA stained with 1 µg/ml ethidium bromide for visualization of amplicons.

*2.1.8. Statistical analysis*

Statistics were calculated using STATA version 10.0. For the analysis of numbers of anatomical sections positive for microscopy and PCR, the Mann Whitney rank sum test was used. For the analysis of differences between MU1615g and MU1615::Tn118g infected belostomatids, P values were calculated for the number of insects positive for bacteria as determined by either microscopy or PCR using the Mann-Whitney test for comparison of two groups. Z values correspond with the strains used. A positive value indicates greater significance with MU1615g than MU1615::Tn118g. A negative value means greater significance with MU1615::Tn118g than MU1615g.

***2.2. Insect microflora studies***

*2.2.1. Aquatic insects*

Adult belostomatids (*Appasus* sp. [*Diplonychus* sp.]), 1 - 3cm long were collected from aquatic sampling sites in Ga district, Ghana. Adult naucorids (Naucoris sp.), 0.5 - 1 cm long were also collected from aquatic sampling site in Ga district, Ghana. Insects were housed individually in deep petri dishes filled with double-distilled water and maintained under a 12-h light and dark photoperiod at 28°C. Insects were fed either midge larvae (Chironomidae) or blowfly larvae (Diptera: Calliphoridae) (*Phormia regina*) every other day, and the housing water was changed at the same time.

*2.2.2. Culture-dependent methods of isolating bacteria*

Ten uninfected belostomatids and naucorids were used for this study. The external parts of the insect were surface sterilized with 70% ethanol prior to dissection of salivary glands, head, thorax and gut. The sections were homogenized in 500 µl of sterile saline with a mortar. One hundred microliters of the resulting homogenate was aliquoted out and serially diluted 10-fold up to $10^{-5}$. Fifty microlitres of the resulting solutions was plated directly onto Middlebrook 7H9 (M7H9) media supplemented with 10% oleic acid-albumin-dextrose enrichment (OADC) {DIFCO} and LB agar plates (DIFCO). The plates were incubated at 37°C for 24 – 48 h. The M7H9 mycobacterial media was used to isolate bacteria that were likely to compete with *M.ulcerans* recovery from infected insects. The nutrient rich LB media was used to isolate dominating and supporting microflora from the insects.

Plates were screened for bacterial growth. Forty colonies from each media type were selected for sub-culture and further screening. After three sub-culture passages, 47 colonies were randomly selected based on colony morphology, color, elevation, size, margin and frequency of occurrence. The morphology of the bacterial isolates was also determined by gram staining. The selected bacterial isolates were further characterized based on their distinctive metabolic properties. The isolates were plated individually onto selective media including gelatin, urea slants, triple sugar iron slants, MacConkey agar and Simmons citrate agar to identify their ability to produce gelatinase and urease, ferment sugars and lactose and utilize citrate as the sole carbon source respectively.

*2.2.3. Antibiotic susceptibility testing*

A Kirby Bauer test (2) was also conducted to identify susceptibility of bacteria to various antibiotics. Each bacterial isolate was cultured in LB broth medium for 24 hours. One hundred microliters of the resulting broth cultures was spread plated onto a Mueller Hinton agar plate and tested against 12 different antibiotic discs: Ampicillin 25 µg, Carbenicillin 100 µg, Streptomycin 10 µg, Tetracyclin 10 µg Chloramphenicol 10 µg, Gentamycin 10 µg, Ciprofloxacin 30 µg, Clindamycin 30 µg, Penicillin 10 units, Amikacin 100µg and Sulphonamides10 µg were equidistantly dispensed on the M-H

plates. The plates were incubated for 24 h at 37°C and the zones of inhibition of bacterial growth were measured and recorded as described.

*2.2.4. Culture independent methods of bacterial identification*

Bacterial DNA was isolated as described above from the insect homogenates. The 16S ribosomal RNA gene was amplified using universal bacterial primers (9). The PCR reactions were performed according to the following; 5µl of each DNA sample was amplified with the forward primer UnivBacF 5'-AGGAGGTGATCCAACCGCA-3' and reverse primer UnivBacR 5'-GAGGAAGGTGGGGAT-3' in 50-µl PCR mixtures using the GoTaq polymerase buffer system (Promega). Each reaction mixture contained 36.7 µl double-distilled water, 5 µl GoTaq green master mix (400 µl of each deoxynucleoside triphosphate, 3 mM $MgCl_2$, blue and yellow dyes), 1 µM of forward and reverse primers, 1.5 U of GoTaq polymerase, and 5 µl of DNA template. Cycling was performed in a Mastercycler gradient thermal cycler (Eppendorf) as follows: 95°C for 5 min; 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and 72°C for 10 min. Nine microliters of each reaction mixture was analyzed on 1.5% agarose gels in 1µM Tris-acetate-EDTA stained with 1 µg/ml ethidium bromide for visualization of amplicons.

*2.2.5. Cloning and sequencing*

The amplified partial 16S rRNA gene PCR products were cloned into vectors by ligating them into PCR 2.1 TOPO vector system (Invitrogen) according to the manufacturer's instructions. The ligated products were transformed into competent *E. coli* DH5α vectors and resulting transformants were grown on LB plates supplemented with100 µg mL-1 each of ampicillin, X-gal and Isopropyl β-D-1-thiogalactopyranoside. Isolated white colonies that grew upon 24 h of incubation were selected for plasmid DNA extraction (Qiagen mini prep) and subsequent sequencing. Sequencing reactions were performed using the Big Dye terminator v3.1 kit (Applied biosystems). Purified DNA was sequenced using the M13 forward (5'-GTAAAACGACGGCCAG-3') and reverse primers (5'-CAGGAAACAGCTATGAC-3') which flank the inserted partial 16SrRNA DNA sequence. Sequence analysis was performed by using the BLASTn search software (GenBank).

### *2.3 Fish In vitro Studies*

#### *2.3.1. Tissue culture*

The adherent carp monocyte cell line CLC (European Collection of Cell Cultures no. 95070628) was a kind gift from Jeffery Cirillo (Texas A&M Health Science Center). Cells were maintained at 28°C and 5% $CO_2$ using high glucose MEM (Gibco) supplemented with 10% essential amino acids (Gibco), 10% heat-inactivated fetal bovine serum (Gibco) and 2mM L-Glutamine as previously described in (4). Cytopathicity assays were performed in 24-well tissue culture plates.

#### *2.3.2. Mycolactone sensitivity assay*

Mycolactones from MU1615, MMDL, and XL5 were extracted as described previously in (6, 10). Briefly, lipids were extracted with chloroform –methanol (2:1, vol/vol), and phospholipids were removed by precipitation with ice-cold acetone to obtain acetone-soluble lipids (ASLs). Acetone soluble lipids were serially diluted in tissue culture medium and added to cells in a 24-well tissue culture plate. Cytopathicity was defined as the minimal concentration of mycolactone necessary to produce cell rounding in 24 h and loss of the monolayer by 48 h (1).

#### *2.3.3. LDH release and apoptosis*

Fish monocytes (CLC cells) were assayed for cell death via apoptosis and necrosis. Apoptosis and necrosis were measured using a colorimetric kit from Promega as previously described (1). Briefly, cells were suspended in culture media and seeded in a 96 – well tissue culture plate. The release of cytoplasmic lactate dehydrogenase from mycolactone treated and permeabilized cells was measured at 24 h p.i. using the colorimetric kit following the manufacturer's instructions. Background release of LDH was determined from lyses of ethanol treated cells according to the manufacturer's protocol. The percentage of LDH release was then computed using the following calculation: [(release of LDH from mycolactone treated cells – background release from ethanol-treated cells)/ (maximum release of LDH by cell lysis – background release)] × 100.

Apoptosis of mycolactone treated CLC cells was measured at 24 h p.i. by using the Cell Death Detection Plus enzyme-linked immunosorbent assy (Roche, Indianapolis, IN) as described previously in (1). Apoptosis was determined as fold enrichment of nucleosomes [(measurement of DNA-histone complex from treated cells/background measurement of untreated cells)].

### *2.4 Fish In vivo Studies*

#### *2.4.1. Medaka aquaculture*

Japanese Medeka used in this study were obtained from Don Ennis (University of Louisiana, Lafayette, LA, USA). Medaka were maintained in the laboratory in aquaria at 28°C, as described in (3). Fish were infected with mycobacteria and maintained post infection in a BSL-2 laboratory at 28°C.

#### *2.4.2. Experimental design*

Two infection experiments were performed in this study as outlined in Figure 2.4A and B. Bacterial inocula was prepared as described above and diluted in PBS to obtain a concentration range of $10^2$-$10^8$. Medaka were anaesthetized with tricaine methanesulphonate (MS-222) (0.0175%) and injected with 30µl of bacterial suspension at respective doses. Sham infections were also performed where medaka were inoculated with 30µl of sterile PBS. All fish were maintained separately under similar environmental conditions and monitored for survival, mortality, gross behavioral changes and gross morphological pathology.

#### *2.4.3. Histopathology*

At the set time points (Figure 2.4A and B), 1-3 infected fish were euthanized using an overdose of tricaine methanesulphonate (MS-222) (0.1%). Fishes were processed whole in 10% neutral-buffered formalin followed by embedding in paraffin wax. Thin sections of the paraffin embedded fish were made and stained with hematoxylin and eosin (H&E) and Ziehl-Neelsen acid fast stain. The morphological pathology and presence of acid fast bacilli (AFB) present in the fish tissues was scored

between bacterial strains used and within doses of inoculum delivered respectively. "Sham" control infected fish were sacrificed and subjected to similar treatments for comparison.

*2.4.4. Microscopic evidence of microbial colonization*

Between 4 to 9 bacteria-infected Medaka were euthanized as described above at the time points shown in figure 2.4A and B. Each fish was dissected by making a single anterior to posterior incision along the abdomen followed by removal of the kidney, liver, spleen, gut and heart. Whole organs of fish infected with fluorescently labeled bacteria were inspected microscopically as described in (3). Briefly, fish organs were placed in a petri dish and observed for fluorescence using a Nikon SMZ800 (Nikon, Tokyo, Japan) stereomicroscope equipped with X-cite TM 120 for fluorescence. The organs as well as the remaining fish carcass were kept separate and homogenized in 500µl of M7H9 broth media supplemented with 100µg/ul cyloheximide, 20µg/ul chloramphenicol and 25µg/ul ampicillin. For detection of AFB in the dissected organs, smears were made from the homogenized suspensions and stained using the Zeihl-Neelsen technique. AFBs were viewed light microscopy using an Olympus BX51 microscope (USA). Wet mounts of representative organs were viewed using a Nikon ECLIPSE E400 fluorescent microscope for the detection of the fluorescently labeled bacteria.

*2.4.5. PCR analysis of infected Tissue*

DNA was extracted from organ homogenates using a protocol adapted from Lamour and Finely (8). Amplification of the enoyl reductase (*mlsA* module of the mycolactone plasmid) gene was chosen to determine the presence of MU1615g and MU1615::Tn118g DNA in fish tissues. The early secreted antigen protein (ESAT-6) gene was chosen to determine presence of MMDL240490 and MM1218 in fish tissues. Two and a half microlitres of each respective DNA sample was amplified with the *mlsA* primer pair; 5'– GAGATCGGTCCCGACGTCTAC-3' and 5'-GGCTTGACTCATGTCACGTAAG-3' or the ESAT-6 primer pair; 5' – GACAGCAGCAGTGGAATTTCG – 3 and 5' – CTTCTGCTGCACACCCTGGTA – 3 in 25µl polymerase chain reaction mixtures using the GoTaq polymerase-buffer system (Promega). Each reaction contained 18.3µl double-distilled water, 2.5µl GoTaq green

master mix (400µl of each deoxynucleoside triphosphate, 3mM $MgCl_2$, blue and yellow dyes), 0.5µM of forward and reverse primers, 0.75U GoTaq polymerase each and 5µl of DNA template. Cycling was performed in a Matercycler gradient thermal cycler (Eppendorf) as follows: 95°C for 5 min; 35 cycles of for 95°C for 1 min, 55°C for 1 min 72°C for1 min; and 72°C for 10 min. Seven microlitres of each reaction mixture was analyzed on 1.5% agarose gels in 1X Tris-acetate-EDTA stained with 1µg/ml ethidium bromide for visualization of amplicons.

For quantitative PCR, DNA was extracted as described before. Genome forming units of MU1615g and MU1615::Tn118g in fish tissue were quantified by the exponential detection of expression levels of an internal probe designed for the enoyl reductase gene. Five microlitres of representative DNA was amplifed with the *mlsA* primer pair; 5'- CGCCTACATCGCTTTGG -3' and 5'- ATTGAATCGCAGCCATACC -3' and an internal probe; 5' -TET CTGATCCATGCCGGCA MGBNFQ -3' in 25µl polymerase chain reaction mixtures using the fluorescent Taqman PCR system. Each reaction mixture contained 3µl double-distilled water, 12.5µl environmental mastermix, 1µl each of forward and revers primers and 2.5µl probe. Cycling and detection of the Taqman fluorophore was performed in an Applied Biosystems Division 7700 thermocycler and sequence detector.

*2.4.6. Statistical analysis*

Statistics were calculated using SPSS version 17 and GraphPad Prism version 4 software. For the analysis of cytotoxicity via apoptosis and LDH release, the Students t-test was used to determine significant differences between the congeners of mycolactone used. For the analysis of percent survival of Medaka post infection with different mycobacteria, standard deviations were computed to determine significance. For analysis of numbers of infected organs that were AFB and ER-PCR positive within and between strains, the Mann-Whitney test for comparison of two groups was used to determine significance.

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### *2.6 Appendix*

**Table 2.1. Mycobacterial strains used for this study**

| Strain | Species | Host | Geographic origin | Charateristics | Reference or source |
|---|---|---|---|---|---|
| MU1615 | *M. ulcerans* | Human | Malaysia | Wild-type strain. Makes Mycolactone A/B | ATCC[a] |
| MU1615g | *M. ulcerans* | Human | Malaysia | Wild-type strain. Makes Mycolactone A/B. Intrinsically expresses green fluorescent protein (gfp) | ATCC[a] |
| MU1615::Tn118 | *M. ulcerans* | Human | Malaysia | Mycolactone negative mutant due to insertion in FabH gene via transposon mutagenesis | ATCC[a] |
| MU1615::Tn118g | *M.ulcerans* | Human | Malaysia | Mycolactone negative mutant due to insertion in FabH gene via transposon mutagenesis. Intrinsically expresses green fluorescent protein (gfp) | ATCC[a] |
| MU01G897 | *M. ulcerans* | Human | French Guyana | Wild – type. Makes mycolactone A/B | 4 |
| 1218 | *M. marinum* | Salt water fish | United States marine captive | Wild-type strain. | ATCC[b] |
| MMDL | *M. Marinum* DL240490 | Sea Bass (Dicentrarchus labrax) | Red Sea Israel | Wild-type strain. Makes mycolactone F | 11 |
| XL5 | *M. liflandii* | Frog (Xenopus laevis) | University of Virginia | Wild – type strain. Makes mycolactone E. | 10 |

[a]ATCC[a] – American Type Cell Culture 35840

[b]ATCC[b] – American Type Cell Culture 927

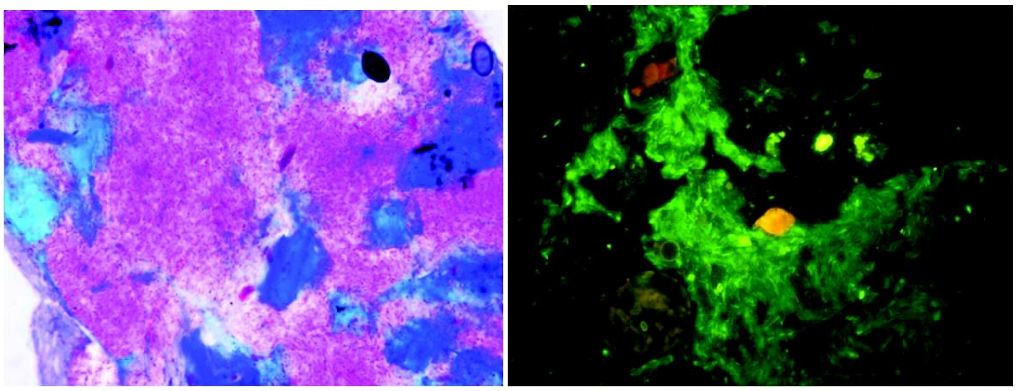

**Figure 2.1: Location of acid-fast (bright-field microscopy) and GFP-labeled (epiflourescence microscopy) MU 1615g in mosquito (*Ochlerotatus triseriatus*) larva gut.**

Total magnification = 1000X

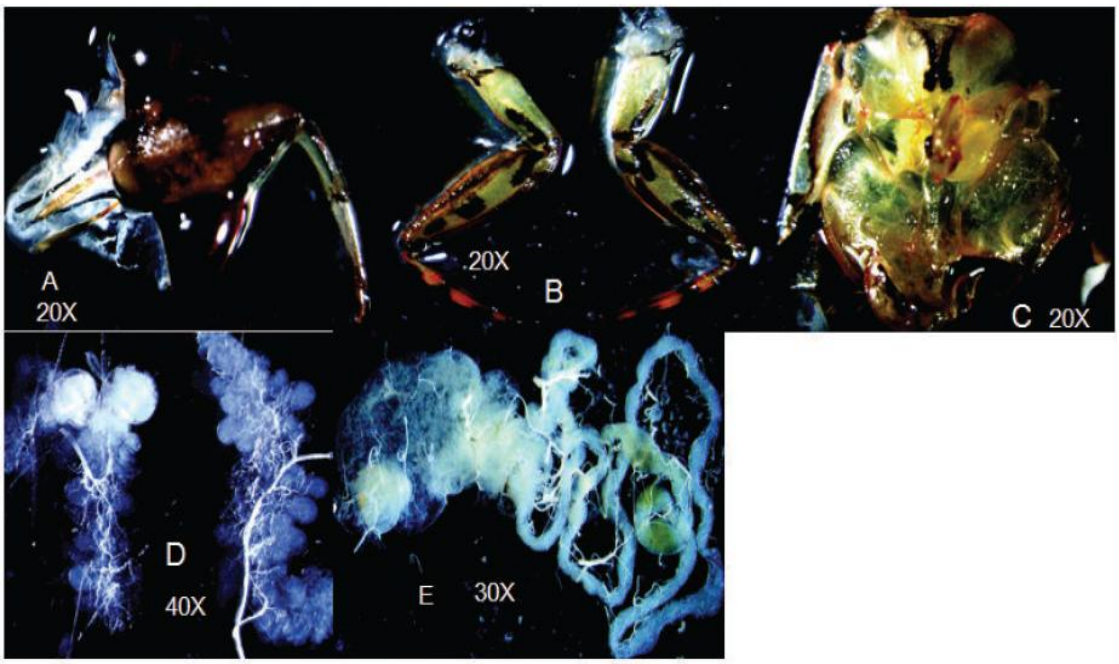


**Figure 2.2: Images of dissected belostomatid, showing the relative sizes of the following insect compartments assayed in this study***:* head (A), raptorial arms (B), thorax (C), salivary glands (D), and gut (E).

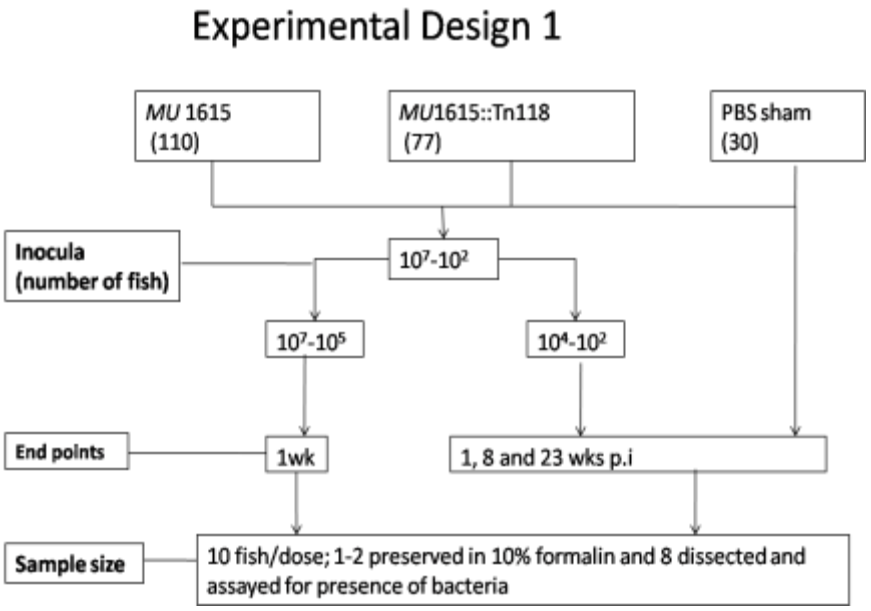


**Figure 2.4A: Experimental design 1 for fish *in vivo* study**

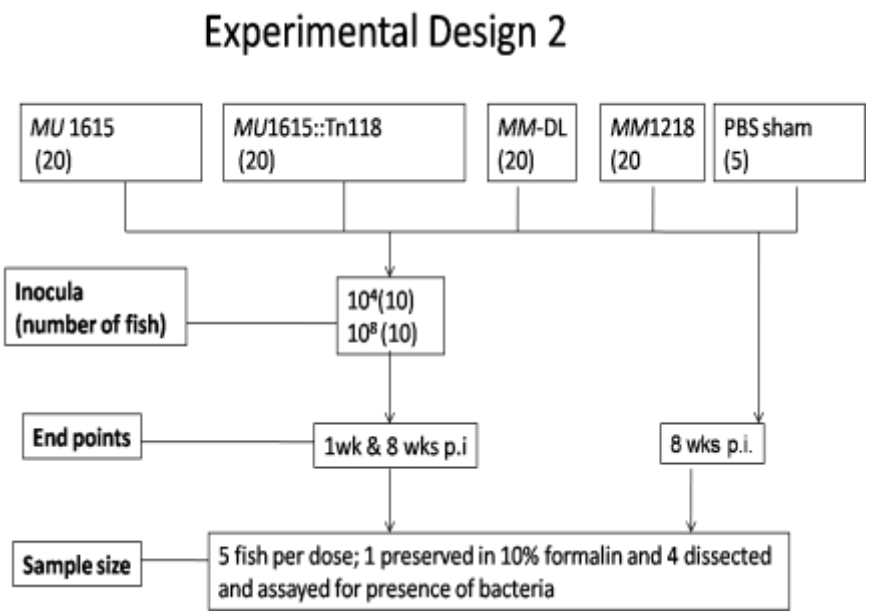


**Figure 2.4B: Experimental design 2 for fish *in vivo* study**

# Chapter 3: Persistent Association of *Mycobacterium ulcerans* with West African Predaceous Insects of the Family Belostomatidae

This chapter is a lightly revised version of a paper by the same name published in the journal Applied and Environmental Microbiology in 2008 by Lydia Mosi, Heather Williamson, John R. Wallace, Richard Merrit and Pamela L. C. Small:

Lydia Mosi, Heather Williamson, John R. Wallace, Richard W. Merritt, and P. L. C. Small. Persistent Association of *Mycobacterium ulcerans* with West African Predaceous Insects of the Family Belostomatidae. *Applied and Environmental Microbiology* Vol. 74, No. 22. 7036-7042

My use of "we"in this chapter refers to my co-authors and myself. My primary contributions to this paper include (1) selection of the topic and development of the experiments, (2) Most of the gathering and interpretation of literature, (3) statistical analysis of the data, (4) pulling the various contributions into a single paper, and (5) most of the writing.

3.1 Introduction

*Mycobacterium ulcerans* disease (Buruli ulcer) continues to be one of the most debilitating cutaneous diseases in West Africa. Although the distribution of the disease is global and affects people of all ages, the burden of disease is most severe in West Africa, where Buruli ulcer is an emerging disease. In West Africa, cases typically occur among rural, economically deprived populations (11, 31). *M. ulcerans* is an environmental pathogen; however, the method of transmission from the environment to humans remains elusive (5, 11, 32). Person-to person transmission of Buruli ulcer is extremely rare, and a large body of evidence implicates exposure to slow moving or stagnant water as the most universally defined risk factor for infection (23, 25). A striking characteristic of the disease in all regions is its discontinuous focal distribution. Villages where the disease is endemic and those where it is not endemic may be found within a few kilometers of each other along a waterway (23, 25, 26).The absence of the disease in arid parts of the world strongly suggests that environmental constraints may limit the distribution of disease. In addition, there is a strong association of the bacterium within aquatic ecosystems.

The possibility of the bacterium being concentrated through trophic links and ultimately delivered to an unsuspecting host, via a vector or some other unknown route, has been suggested by several investigators (6, 14). A major advance in understanding transmission occurred with the detection of *M. ulcerans* DNA in predaceous insects (Naucoridae and Belostomatidae), leading to the hypothesis that insects may be involved in the transmission (20). Naucorids and belostomatids are aquatic hemiptera that exploit a wide range of prey, including snails, fish, anuran larvae, and other terrestrial and aquatic insects (29). Both insect groups are found worldwide near vegetative areas of stagnant water bodies. Although they do not feed on humans, they can bite if they are disturbed and for this reason are called “toe biters.” Subsequent work from Ghana and Cote d’Ivoire has confirmed the presence of *M. ulcerans* DNA in a large number of aquatic invertebrate and vertebrate taxa (6, 18, 20, 21). None of the predaceous hempitera that were found to have positive PCR results for *M. ulcerans* DNA in Africa are hematophagous, and the percentage of biting hemiptera is often quite low in areas where the disease is endemic (2).There have been numerous unsuccessful attempts to culture the bacterium from the environment due to competition from other, faster-growing

environmental bacteria. A major breakthrough occurred with the successful culture of *M. ulcerans* from a water strider (*Gerridae*) collected in Benin by Francoise Portaels *et al*. (22).

In addition to extensive field work on the occurrence of *M. ulcerans* in natural environments, the potential role of biting hemiptera as vectors of *M. ulcerans* has been extensively explored in a set of elegant laboratory studies conducted by Laurent Marsollier *et al*. (13, 15, 16). In these studies, dipteral larvae (Phormia terrae-novae) were injected with suspensions of *M. ulcerans* and subsequently fed to *Naucoris cimicoides*, collected from swamps in France (13). Marsollier and colleagues showed from these studies that *M. ulcerans* successfully colonized the insect over a period exceeding 90 days, causing no growth impairment or death of the insect. More interestingly, they showed through microscopy that whereas wild-type bacteria could be detected on the raptorial arms of naucorids, the majority of organisms were localized in the salivary glands of the insect. Finally, Marsollier *et al*. (15) also presented data supporting the role of the *M. ulcerans* cytotoxic macrolide toxin, mycolactone, in colonization of the insect.

Although these studies were of significance, it is difficult to determine their relevance to transmission of Buruli ulcer in Africa for a number of reasons. First, the species of Naucoridae used was from France, not Africa where Buruli ulcer is endemic. Second, the bacterial strain used for most of these studies is a member of the "ancestral" lineage of *M. ulcerans* rather than a member of the "classical" lineage associated with severe Buruli ulcer in Africa and Malaysia (12). To investigate the ability of *M. ulcerans* to colonize aquatic African hemiptera, we infected adult belostomatids collected in Ghana with a "classical" isolate of *M. ulcerans*. We also employed a mycolactone-negative mutant of *M. ulcerans* to determine the role of mycolactone in insect colonization. We showed that belostomatids can be persistently colonized by both mycolactone-producing and mycolactone-negative *M. ulcerans*; however, we obtained no evidence of replication within internal insect organs. We showed extensive colonization of the exoskeleton and showed that *M. ulcerans* is transmitted to prey via feeding. In our infection model, we demonstrated the significance of trophic-level transfer of *M. ulcerans* in the environment in which naturally infected mosquito larvae successfully

passed *M. ulcerans* up the food chain. These results provide a useful model for beginning to understand the ecology of*M. ulcerans* in West Africa.

### *3.2 Results*

*3.2.1. Mycobacterium ulcerans exhibits prolonged infection in African Belostomatids.*

We showed that throughout the 60-day study period, all of the belostomatids that had been infected with *M. ulcerans* 1615 GFP were shown to be positive for the bacteria as determined by either microscopy, PCR analysis, or both (Table 3.1). Based on microscopy and PCR, there was a 100% infection rate as determined by the presence of *M. ulcerans* in 12/12 insect guts after 24 h (Table 3.2 ). This amount declined significantly (P = 0) to 9% at 30 days p.i., with only one gut being AFB positive, and rose slightly to 30% at 60 days. This increase was not statistically significant (P = 0.8357). There was a low infection rate of the salivary glands assayed at all time points, with a mean of 12% insects shown to be positive for bacteria as determined by microscopy for *M. ulcerans*. By using PCR analysis, however, the average rate of infectivity over time was much higher (63%). The rates of infection in the head, raptorial arms, and thorax followed similar patterns. The largest number of *M. ulcerans*-positive insects for these three anatomical sections averaged 88% and occurred at 30 days p.i. This increase between 1 day p.i. and 30 days p.i. was statistically significant, with P values of 0.05, 0.0003, and 0.0004, respectively. Between 30 days and 60 days, even though there was a decrease in the number of insects that were positive for bacteria on the head, raptorial arms, and thorax, the difference was not significant.

There was some discrepancy between PCR and microscopy results for the percentage of insects positive for *M. ulcerans* (Tables 3.2, 3.3). There were significantly more insects shown to be positive as determined by PCR analysis in the head, raptorial arms, and salivary glands at 1 day p.i. (P = 0.02, 0.001, and 0, respectively). By using microscopy, at 30 days p.i. there were significantly more insects shown to be positive for bacteria on the head, raptorial arms, and thorax (P = 0.0006, 0.02, and 0.0006, respectively). Contrary to the decrease in the number of insects that were positive for *M. ulcerans* at 60 days p.i. in the gut, there was a significant increase determined by PCR

analysis (P = 0.002). All insect sections that were shown to be positive for bacteria by bright field microscopy were also positive for GFP bacilli, thus strengthening the observation. A total of three insects died prior to the day 30 and day 60 time points; however, the deaths were not attributed to any effect of *M. ulcerans* but rather to natural causes. A similar death rate occurred among the uninfected insects.

*3.2.2. M. ulcerans increases in external compartments and decreases in internal organs over 60 days.*

In order to quantitatively determine multiplication of *M. ulcerans* within the infected insects, dissected anatomical sections were homogenized, decontaminated by the modified Petroff's method, and cultured on M7H9 medium supplemented with OADC and 20µg/ml each of chloramphenicol and cycloheximide. These efforts, however, were mostly frustrated by the overgrowth of faster-growing bacteria and fungi present in many insects. The salivary glands produced little bacterial growth and no isolates of *M. ulcerans*. For this reason, a semi quantitative method for obtaining evidence for the growth of *M. ulcerans* was employed based on detection and enumeration of acid-fast and GFP positive bacilli present in dilutions of insect section homogenates. Results from these studies showed that the bacterial load on the exoskeleton (head, raptorial arms, and thorax) was greater than that in the internal organs (salivary glands and gut) at all time points (Fig. 3.1, 3.2, 3.3). Since the primary infection of the belostomatids was performed orally, it was expected that the insect guts would have a large amount of bacteria compared to other anatomical sites at early time points, and this phenomenon was indeed observed. However, bacterial density dropped significantly (P = 0.0003) in the gut by the 60-day time point. The mean bacterial density within the salivary gland remained below 10 detectable bacterial cells for all time points without any significant changes. In contrast, anatomical sections of the insect covered by a substantial exoskeleton showed a significant and steady increase in density of detectable bacteria over the 60-day study period with as many as 1,000 bacterial cells seen per viewing field at 1000× magnification (P values of 0.05, 0.01, and 0.019 for head, raptorial arms, and thorax, respectively).

*3.2.3. M. ulcerans is efficiently transmitted from infected belostomatids to blowfly larva through feeding*

Belostomatids feed by grabbing and immobilizing prey with their raptorial arms and sucking out prey contents with their stylet (28). In order to determine the ability of *M. ulcerans*-infected belostomatids to transmit *M. ulcerans* through grabbing and biting, blowfly larva exuviae, which were fed to infected belostomatids, were analyzed for the presence of *M. ulcerans* by using microscopy, PCR analysis, and culture (Fig. 3.4). Each insect prior to being sacrificed at 1 day, 30 days, and 60 days p.i. was given a naïve blowfly larva. Larva exuviae collected at each time point were pooled for analysis. *M. ulcerans* could be detected by using microscopy (Fig. 3.5) and PCR analysis (data not shown) in all exuviae. Even 60 days after infection with *M. ulcerans*, belostomatids were still able to transmit *M. ulcerans* to naïve blowfly larvae through feeding. The attempt to culture back from the blowfly larva exuviae was unsuccessful even after decontamination of samples due to overgrowth of native bacteria. In this experiment, 100% infectivity of maggot exuviae was found as determined by both PCR and microscopy, indicating a direct transmission of bacteria from the insects to their prey.

*3.2.4. Mycolactone is not required for the colonization of belostomatids.*

Insects that were infected with the mycolactone-negative strain MU1615::TN118g showed patterns of persistence within African belostomatids similar to those of MU1615g bacteria (Tables 3.1, 4 and 5). As stated earlier, each infected insect was dissected into five anatomical sections, homogenized, and assayed for the presence of the bacteria. There was a 100% infectivity of insect guts 1 day p.i. as determined by both microscopy and PCR. This efficiency decreased steadily over time to 25% insects at 60 days p.i. as determined by microscopy. However, PCR analysis showed there was a significant increase in the number of insect guts that were positive for bacteria ($P = 0.04$). Similarly, the salivary glands of the insects were the least infected, and there were no significant differences in the number of positive results as determined by either microscopy or PCR. There were comparable numbers of insects that were positive for bacteria on the head, raptorial arms, and thorax as determined by either microscopy or PCR, and there was no significant difference between the two methods, with the

exception of more positive results for bacteria on the head at 30 days and 60 days p.i., shown by microscopy. More importantly, there were very few significant differences between the number of insects positive for MU1615g and MU1615::TN118g (mycolactone negative), as determined by either microscopy or PCR (Table 6). Similarly, the external surfaces of the insects showed the highest bacterial density over time. At 1 day p.i.,there were significantly more insect raptorial arms positive for the mycolactone-negative strain than for the wild type (P = 0.0004). At 30 days p.i., there were significantly more insects with wild-type-infected raptorial arms and thoraces than there were mycolactone negative-infected insects (P = 0.003 and 0.0001, respectively). There were no significant differences between MU1615g and mycolactone-negative MU615::TN118g infected insects 60 days p.i. In the salivary glands and guts, the bacterial density decreased significantly over the 60-day period, and there was no significant difference between this observation and that of MU1615g infected insects.

### *3.3 Discussion*

The hypothesis that *M. ulcerans* is a vectored pathogen which is transmitted to humans via the bite of predaceous water bugs has received considerable attention (2, 13, 17, 24).The interpretation of the data presented in earlier studies has been complicated by the following three major issues: (i) the insect species used were not African species, (ii) the primary *M. ulcerans* used was not representative of the classical *M. ulcerans* from Africa, and (iii) none of the studies have provided provided comprehensive quantitative data on the location of the bacterium within the insect. In this study, we have used an African insect species that has been found to have positive PCR results for *M. ulcerans* in nature and a classical lineage strain of *M. ulcerans* to determine the following: (i) whether *M. ulcerans* persistently colonizes or grows within African predaceous water bugs, (ii) how the bacterium is partitioned within external and internal body parts, (iii) whether mycolactone plays a role in insect infections, and (iv) whether *M. ulcerans* can be transmitted by water bugs to prey within a food chain. Although, like Marsollier and colleagues, we show extensive colonization of the exoskeleton of belostomatids by *M. ulcerans* (Fig. 3.2, we do not have convincing evidence of bacterial growth and

replication in the internal compartments of belostomatids. We found very low numbers of *M. ulcerans* in the salivary glands of belostomatids at all times, in contrast to the work of Marsollier *et al.* We initially thought that the differential findings of our laboratory and those of Marsollier and colleagues might be due to the use of different lineages of *M. ulcerans*. However, when tested under comparative conditions, we found no major differences between the ancestral and classical strains in their abilities to associate with belostomatids (data not shown).

We also ruled out the possibility that the colonization of external compartments of belostomatids occurred as an artifact of the infection method. In previous studies, and in early work in our lab, *M. ulcerans* bacteria were injected into blowfly larvae. During this procedure, inoculum leaks out of the larva, producing a significant amount of surface contamination which could be transferred to the insect's raptorial arms when it grasps prey for feeding. We were able to avoid this problem by taking advantage of the fact that mosquito larvae readily ingest bacteria and in turn are consumed by predaceous water insects higher up the food chain. Nonetheless, in our work, as in the work of Marsollier and colleagues, considerable colonization of raptorial arms occurred (Fig. 3.2). Belostomatids use their arms for grooming their stylet as well as for grabbing prey (28). This behavior could also lead to colonization of the raptorial arms. In accordance with Marsollier and colleagues, we showed that infected insects could transmit *M. ulcerans* via feeding; however, this transfer of bacteria is most likely to have occurred through contact with the heavily colonized raptorial arms and other external parts rather than the salivary glands.

We do not find that the mycolactone toxin plays a significant role in the ability of *M. ulcerans* to persist within insects because both toxin-positive and isogenic toxin-negative strains persist equally. The evidence for the impaired ability of mycolactone-negative strains to colonize French naucorids rested on a 10-fold difference between wild-type and mycolactone-negative strains, which was minimal considering the length of the experiment (15). Despite the fact that we collected insects three times during field trips to Ghana, we never obtained sufficient numbers of naucorids to produce statistically sound data. We did, however, conduct limited studies with the small number of naucorids we were able to collect. Results from these studies were similar to those using

belostomatids in that we did not see any evidence of replication in the salivary gland, but the numbers of insects and time points were too few for statistical analysis.

The presence of the numerous and complex microbial flora found in belostomatids, especially in the gut, made it impossible to obtain any *M. ulcerans* cfu. Despite the fact that we detected few other bacteria in the salivary glands, we did not obtain a culture of *M. ulcerans*. The long-term instability of GFP under neutral pH suggests that the presence of fluorescent *M. ulcerans* in belostomatids is consistent with the presence of viable organisms. We did show long-term colonization of belostomatids by *M. ulcerans* using direct smear microscopy and PCR.

With respect to methodology, we did not always find a concordance between microscopy and PCR results. Similar results have been reported in other studies (1, 3, 8, 9, 10, 19). The direct smear method of detecting acid-fast bacilli has a sensitivity range of between 40 to 85% and a specificity of 67 to 100% (4, 8). The degree of sensitivity of PCR for the detection of pathogens within specimens has rendered it the method of choice for most studies (19). Where false positivity has been suspected with PCR, microscopy has been used to confirm the accuracy of positive PCR results in some cases (1, 9, 10). Generally, the power of detection of the bacterium of interest is increased by the use of both methods. Our findings support the hypothesis that predaceous aquatic insects may play an important role in maintaining *M. ulcerans* within food webs in the aquatic environment (2). In this respect, external contamination of insect skeletons could also play a role because we have observed tadpoles grazing on the surfaces of predaceous water bugs in microcosm environments (H. Williamson, unpublished data). We also cannot rule out the possibility that belostomatids may be involved in mechanical transmission of *M. ulcerans.*

Finally, this work suggests that *M. ulcerans* can live as a commensal on belostomatids (2, 33). It is surprising that neither mosquito larvae nor belostomatids suffer developmental or behavioral defects as a result of *M. ulcerans* infection. Likewise, *M. ulcerans* appears to survive on the exoskeleton of belostomatids and within the guts of mosquito larvae for long periods of time. This close association between bacterium and insect, which is neither detrimental nor beneficial, is the hallmark of commensalism in nature.

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### *3.5. Appendix*

**Table 3.1: Distribution of Acid-fast and PCR positive insects post infection with MU1615g**

| WT *M. ulcerans* 24hrs p.i. | Microscopy | | | | | PCR | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Head | Raptorial arm | Thorax | Salivary gland | Gut | Head | Raptorial arm | Thorax | Salivary gland | Gut |
| 1 | + | + | + | - | + | + | + | + | + | + |
| 2 | + | - | + | - | + | + | + | + | + | + |
| 3 | + | - | + | - | + | + | + | - | + | + |
| 4 | + | - | + | - | + | + | + | + | - | - |
| 5 | - | - | - | + | + | + | + | + | + | + |
| 6 | - | - | + | - | + | + | - | + | + | + |
| 7 | - | - | - | - | + | - | - | - | - | + |
| 8 | - | - | + | - | + | + | + | + | + | + |
| 9 | - | - | - | - | + | + | + | + | + | + |
| 10 | + | - | + | - | + | + | + | + | + | + |
| 11 | + | - | + | - | + | + | + | + | + | + |
| 12 | - | + | + | - | + | + | + | + | + | + |

| WT M. ulcerans 30 days p.i. | Microscopy | | | | | PCR | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Head | Raptorial arm | Thorax | Salivary gland | Gut | Head | Raptorial arm | Thorax | Salivary gland | Gut |
| 1 | + | - | + | - | - | + | + | + | + | + |
| 2 | + | + | + | - | - | + | - | + | + | + |
| 3 | + | - | - | - | - | + | + | + | - | + |
| 4 | + | + | + | + | - | + | - | - | + | - |
| 5 | + | + | + | - | - | - | - | - | - | - |
| 6 | + | + | + | - | - | - | + | + | - | - |
| 7 | + | + | + | - | - | - | - | - | - | - |
| 8 | + | + | + | - | - | - | - | - | - | + |
| 9 | + | + | + | + | + | - | - | - | - | - |
| 10 | - | + | + | - | - | - | - | - | - | - |
| 11 | + | + | + | - | - | - | - | - | - | - |

| *WT M. ulcerans6* 0 days p.i. | Microscopy | | | | | PCR | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Head | Raptorial arm | Thorax | Salivary gland | Gut | Head | Raptorial arm | Thorax | Salivary gland | Gut |
| 1 | + | - | - | - | - | - | - | + | - | + |
| 2 | - | - | + | - | - | - | + | + | - | + |
| 3 | - | - | + | - | + | + | + | + | - | + |
| 4 | + | + | - | - | - | - | + | + | - | + |
| 5 | + | + | + | + | - | + | + | - | + | - |
| 6 | - | + | + | - | - | - | + | + | + | + |
| 7 | - | + | + | - | - | - | + | + | + | + |
| 8 | - | + | + | - | - | - | + | + | + | - |
| 9 | - | - | - | - | - | + | - | + | + | + |
| 10 | - | - | - | - | - | - | - | + | + | + |

**Table 3.2: Number of infected belostomatids positive for MU1615g by microscopy and PCR (by anatomical site)**

| Days p.i. | No. of insects positive for *M. ulcerans* in the indicated compartment/total | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Head | | Raptorial arms | | Thorax | | Salivary glands | | Gut | |
| | Microscopy | PCR | Microscopy | PCR | Microscopy | PCR | Microscopy | PCR | Microscopy | PCR |
| 1 | 6/12 | 11/12[b] | 2/12 | 10/12[b] | 9/12 | 9/12 | 1/12 | 10/12[b] | 12/12 | 11/12 |
| 30 | 10/11[c] | 4/11 | 9/11[c] | 3/11 | 10/11[c] | 4/11 | 2/11 | 3/11 | 1/11 | 4/11 |
| 60 | 3/10 | 3/10 | 5/10 | 7/10 | 6/10 | 8/10 | 1/10 | 8/10[b] | 3/10 | 8/10[b] |

[a] At 1, 30, and 60 days p.i., 12, 11, and 10 insects, respectively, were sacrificed and assayed for the presence of the bacterium as determined by microscopy and PCR.
[b] More insects were determined to be positive for the bacterium by PCR. Shown is statistical significance at the 5% level ($P < 0.05$).
[c] More insects were determined to be positive for the bacterium by microscopy. Shown is statistical significance at the 5% level ($P < 0.05$).

**Table 3.3: Distribution of Acid-fast and PCR positive insects post infection with MU1615::Tn118g**

| *M.ulcerans* 1615::TN118 60 days | Microscopy | | | | | PCR | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Head | Raptorial arm | Thorax | Salivary gland | Gut | Head | Raptorial arm | Thorax | Salivary gland | Gut |
| 1 | + | + | + | - | - | - | + | - | + | + |
| 2 | + | + | + | - | + | - | + | + | + | + |
| 3 | + | + | - | - | - | - | - | - | - | + |
| 4 | - | - | - | - | - | - | + | + | + | + |
| 5 | + | + | - | - | - | - | + | + | - | + |
| 6 | + | + | + | - | - | + | + | + | - | - |
| 7 | + | + | + | + | - | - | + | + | + | + |
| 8 | + | + | + | - | - | - | + | + | - | + |
| 9 | + | - | + | - | + | + | - | + | - | + |
| 10 | + | + | + | - | - | + | + | + | - | + |
| 11 | + | + | + | - | + | + | + | + | - | + |
| 12 | + | + | - | - | - | + | + | + | - | - |

| *M.ulcerans* 1615::TN118 30 days | Microscopy | | | | | PCR | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Head | Raptorial arm | Thorax | Salivary gland | Gut | Head | Raptorial arm | Thorax | Salivary gland | Gut |
| 1 | + | - | + | - | + | - | + | - | + | + |
| 2 | + | + | - | - | - | - | + | + | + | - |
| 3 | + | + | + | - | - | - | - | - | - | + |
| 4 | + | + | + | - | - | - | - | - | - | - |
| 5 | - | - | - | - | - | - | + | + | - | - |
| 6 | - | - | - | - | + | - | + | - | + | + |
| 7 | + | - | + | - | - | - | + | + | + | - |
| 8 | - | - | - | - | - | - | - | - | - | - |
| 9 | - | - | - | - | + | - | - | - | + | + |
| 10 | + | + | + | - | + | - | - | - | + | - |
| 11 | - | - | - | - | - | - | - | - | - | - |
| 12 | + | - | - | + | - | - | - | - | - | - |

| *M.ulcerans* 1615::TN118 24hrs | Microscopy | | | | | PCR | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Head | Raptorial arm | Thorax | Salivary gland | Gut | Head | Raptorial arm | Thorax | Salivary gland | Gut |
| 1 | + | + | + | + | + | - | - | - | - | + |
| 2 | + | + | + | - | + | + | - | - | - | + |
| 3 | + | - | + | + | + | + | + | + | + | + |
| 4 | + | + | - | - | + | + | - | - | - | - |
| 5 | + | + | + | - | + | - | + | + | - | + |
| 6 | + | + | - | + | + | - | - | - | - | + |
| 7 | - | - | + | - | + | - | + | + | - | + |
| 8 | - | - | + | - | + | - | - | - | + | + |
| 9 | - | + | + | - | + | - | + | + | + | + |
| 10 | - | + | + | - | + | - | + | - | + | + |
| 11 | + | + | + | - | + | - | + | + | + | + |
| 12 | - | - | - | - | - | - | - | - | - | + |

**Table 3.4. Persistence of MU1615g in specific anatomic compartments as determined by microscopy and PCR**

| Days p.i. | Method | Section | *P* value[a] | *Z* value[b] | Days p.i. | Method | Section | *P* value[a] | *Z* value[b] |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Microscopy | Head | 0.6884 | 0.401 | 1 | PCR | Head | 0.0012 | −3.243 |
| 30 | Microscopy | Head | 0.0822 | −1.738 | 30 | PCR | Head | 0.0320 | −2.145 |
| 60 | Microscopy | Head | 0.0034 | 2.925 | 60 | PCR | Head | 0.5800 | 0.553 |
| 1 | Microscopy | Raptorial arms | 0.0150 | 2.432 | 1 | PCR | Raptorial arms | 0.0900 | −1.696 |
| 30 | Microscopy | Raptorial arms | 0.0219 | −2.292 | 30 | PCR | Raptorial arms | 1.0000 | 0.000 |
| 60 | Microscopy | Raptorial arms | 0.1025 | 1.633 | 60 | PCR | Raptorial arms | 0.4678 | 0.726 |
| 1 | Microscopy | Thorax | 1.0000 | 0.000 | 1 | PCR | Thorax | 0.0390 | −2.064 |
| 30 | Microscopy | Thorax | 0.0154 | −2.422 | 30 | PCR | Thorax | 0.6602 | −0.440 |
| 60 | Microscopy | Thorax | 0.7518 | 0.316 | 60 | PCR | Thorax | 0.8437 | 0.197 |
| 1 | Microscopy | Salivary glands | 0.2835 | 1.072 | 1 | PCR | Salivary glands | 0.0390 | −2.064 |
| 30 | Microscopy | Salivary glands | 0.4932 | −0.685 | 30 | PCR | Salivary glands | 0.6798 | 0.413 |
| 60 | Microscopy | Salivary glands | 0.8948 | −0.132 | 60 | PCR | Salivary glands | 0.2217 | −1.222 |
| 1 | Microscopy | Gut | 1.000 | 0.00 | 1 | PCR | Gut | 1.0000 | 0.000 |
| 30 | Microscopy | Gut | 0.1685 | 1.377 | 30 | PCR | Gut | 0.6798 | −0.413 |
| 60 | Microscopy | Gut | 0.3749 | 0.887 | 60 | PCR | Gut | 0.7854 | −0.272 |

[a] *P* values were calculated for the number of insects positive for bacteria as determined by either microscopy or PCR using the Mann-Whitney two-sample, rank-sum test.

[b] *Z* values correspond with the strains used. A positive value indicates greater significance with *M. ulcerans* 1615 GFP than *M. ulcerans* 1615::TN118 GFP. A negative value means greater significance with *M. ulcerans* 1615::TN118 GFP than *M. ulcerans* 1615 GFP.

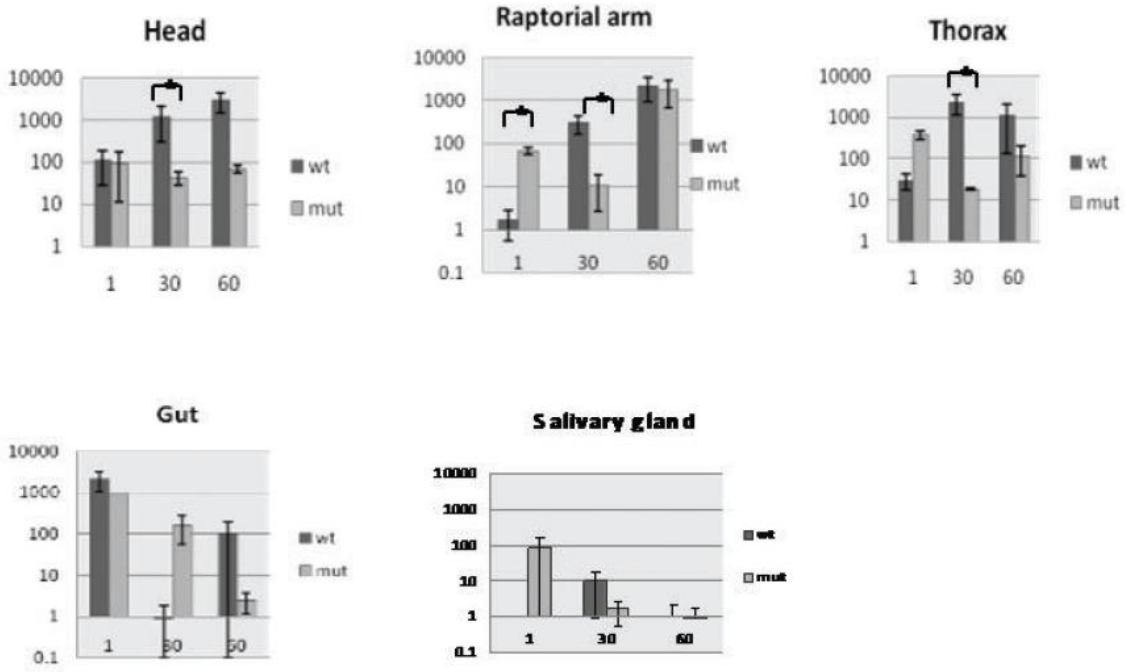


**Figure 3.1***:* **Average number of MU1615g and MU1615::TN118g bacterial cells per insect section.**

AFB abundance as determined by number of bacilli within 50 random viewing fields at 1000× computed from a Most Probable Number (MPN) direct smear preparation. Y-axis, number of bacterial cells; x- axis, days post infection. Head (A), raptorial arms (B), thorax (C), salivary glands (D) and gut (E).

*significant difference between MU1615g, dark gray bars and MU1615::TN118g, light gray bars.

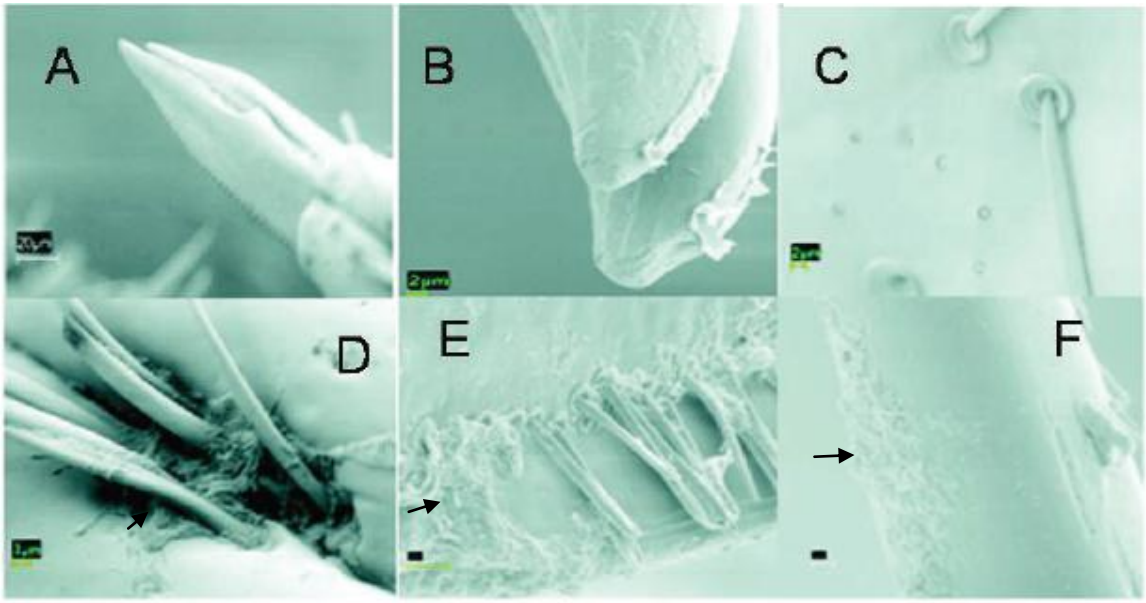


**Figure 3.2: Scanning elecron micrographs of uninfected (top panel) and *M. ulcerans* infected (bottom panel) belostomatids.**

(A and D) Stylet; (B) tip of raptorial arm; (C and F) setae of raptorial arm of uninfected insect; (E) rostrum of infected insect. Arrows indicate clusters adherent bacteria, 60 days p.i.

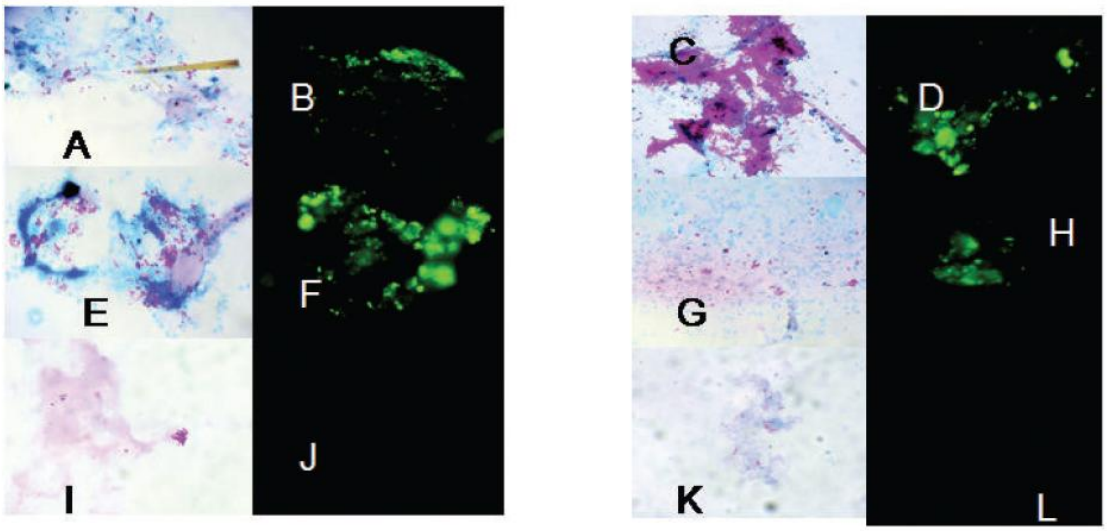


**Figure 3.3: Abundance of MU1615g in raptorial arms, gut and salivary glands.**

Location of acid-fast (bright-field microscopy) and GFP-labeled (epiflourescence microscopy) MU1615g in raptorial arms, gut and salivary glands of infected insects. Raptorial arms at 1d (A, B) and 60d (C, D) post infection. Gut at 1d (E, F) and 60d (G, H) post infection. Salivary glands at 1d (I, J) and 60d (K, L) post infection.

Arrows indicate clusters of acid fast and fluorescent bacilli respectively

Total magnification = 1000×

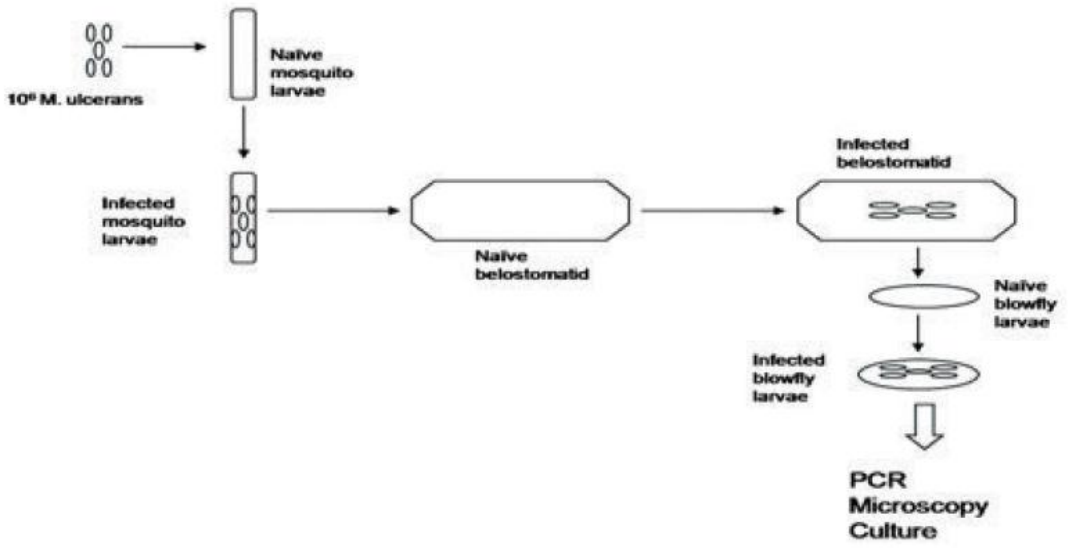


**Figure 3.4: Flow chart of experimenal design to analyze blow fly larvae exuviae for the presence of *M. ulcerans***

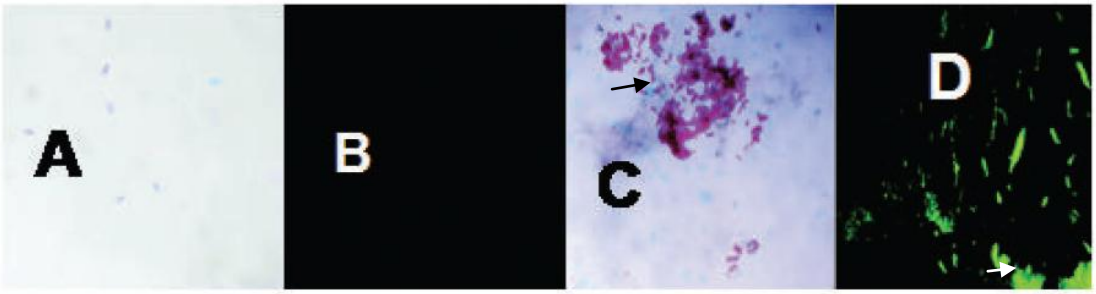


Figure 3.5: Presence of *M. ulcerans* in exuviae of maggots infected by belostomatids**.** Ziehl Neelsen stain (A and C) and observation under a GFP filter (B and D), of maggot exuviae collected from uninfected (A and B) and MU1615g infected (C and D) belostomatid post feeding. Total magnification = 1000X. Arrows indicate clusters of acid fast and fluorescent bacilli respectively.

# Chapter 4: Experimental infection of Medaka (*Oryzias latipes*) with *Mycobacterium ulcerans*: A model for transmission, pathogenesis and toxicity to fish.

### *4.1 Introduction*

Naucorids and Belostomatids have been implicated as insect vectors of Buruli ulcer. Both groups of insects are aggressive predaceous hemiptera, known to attack and immobilize prey that may even be larger than them (40). Both insects are known to consume small fish. Fish in Buruli ulcer endemic water bodies have been found positive for the *M. ulcerans* DNA. About 90% of this evidence has relied on the identification of *M. ulcerans* using primers designed for the insertion sequence IS2404 (10, 23). It has been shown, however, that IS2404 is not exclusive to *M. ulcerans* as previously thought (35, 46). Other organisms within aquatic water bodies including novel strains of *Mycobacteria* have also been found to be IS2404 positive. Recently, it has also been shown by variable nucleotide tandem repeat typing that the DNA isolated from most of the fish collected from endemic water bodies, identify novel mycobacterial species and not *M. ulcerans* (46).

Mycobacteriosis in fish has been well documented over the years (8, 22, 31) with the three most important pathogenic species being *M. abscessus, M. fortuitum* and *M. marinum*. More recently however, outbreaks of mycobacteriosis have been reported in various locations along the Mediterranean Sea and the Chesapeake Bay. These infections have been attributed to novel mycobacterial strains including *M. marinum DL, M. pseudoshottsii*, *M. chesapeake* and *M. shottsii*, which have all been isolated and characterized (20, 34, 35, 42). One striking revelation is that some of these novel strains possess a plasmid that encodes for variants of the virulent macrolide toxin, mycolactone, similar to the mycolactone produced by *M. ulcerans* (33). Although putative *M. ulcerans* DNA has been detected in fish, the ability of *M. ulcerans* to colonize fish has not been determined experimentally. Based on this evidence we have investigated the capability of *M. ulcerans* to produce an infection in Medaka (*Oryzias latipes).*

Medaka are small (2-3 cm long by 0.5-1cm wide) oviparous fresh water fish native to Asia and found primarily in Japan (38, 48). They are widely used as a laboratory animal in biological fields, especially useful for studying developmental biology (27, 47). Medaka are omnivorous and can be maintained on a variety of synthetic diets, water fleas, nauplia of brine shrimp, aquatic worms, dried unicellular green algae and

large protozoa. The maximal survival lifespan of Medaka in undisturbed environments is reported as 5 years (11), however under laboratory conditions, Medaka can live a maximum of 1 year (38).

Almost all aspects of Medaka biology have been repeatedly studied and published. Life-cycle, sexual behavior, spawning habits, embryological development, genetic inheritance, pathology, feeding habits and ecology of the fish have been well documented (11, 12,15). Subsequently, Medaka serves as a good model for the study of human disease, because they have an immune response and genes similar to humans (3). Medaka and human share 104 conserved systemic segments involving at least 3 orthologous gene pairs (27). In line with these research advances are a wide range of resources including extensive databases in toxicology, molecular genetics, and an existing transgenic line. Of particular importance is the See- through (ST) Medaka which are devoid of most major pigments, allowing organs to be observed in living individuals (45). This transgenic model has been used very successfully as a tractable experimental model for tuberculosis pathogenesis, using Medaka as the host for *M. marinum 1218* (3). The *M. marinum 1218* infection model can be manipulated to yield either acute or chronic infection in a dose dependant manner. The chronic infection model is similar pathogenically to *M. tuberculosis* infections in humans, resulting in slow but progressive granuloma formation in the liver and kidney, as well as inflammation of the spleen (3).

*M. ulcerans* causes a painless infection in humans and guinea pigs, characterized by cell death via apoptosis with no apparent immune response (14). In anole lizards, it produces three patterns of inflammatory response; a chronic granulomatous disease in which acid fast bacilli are predominantly intracellular, encapsulated granuloma, or a diffuse necrotizing granuloma in which most AFB are extracellular—similar to the characteristic lesion found in human infections (25). In mice, *M. ulcerans* infection is characterized by a persistent acute inflammatory response, necrosis, AFB resulting from lysed phagocytic cells and nerve damage (16, 28).

The only reported association of *M. ulcerans* with fish has been the detection of the bacterial DNA in fish collected from Buruli ulcer endemic areas. This is the first study to address the ability of *M. ulcerans* to produce an active infection in fish and

also provide information concerning the potential role of fish in the ecology of *M. ulcerans*.

### *4.2 Results*

#### *4.2.1. M. ulcerans establishes systemic infection in Medaka*

To determine the ability of *M. ulcerans* to establish an initial infection in Medaka, infected fish were sacrificed 1 wk p.i., dissected and observed by fluorescent microscopy for the green fluorescent marker on MU1615g and MU1615::Tn118g. We observed that the heart, kidney and liver of the infected fish fluoresced green compared to control (Fig 4.1 and data not shown) regardless of the dose of inocula administered. To further substantiate our findings, the dissected organs were homogenized and subjected to microscopy. Wet mounts of the homogenized organs viewed under an epifluorescent microscope revealed the presence of Gfp expressing bacteria (data not shown). Smears made from all dissected organs and stained with Ziehl – Neelsen also revealed the presence of acid-fast bacilli (Tables 5.1and 4.2). This observation was also comparable to the detection of bacteria DNA within the organs via polymerase chain reaction (Table 4.1 and 4.2). The fact that bacteria were present in all organs assayed suggests that *M. ulcerans* produces a systemic infection in fish, similar to *M. marinum* strains.

#### *4.2.2. M. ulcerans is both intracellular and extracellular in Medaka*

In human and guinea pig infection, *M. ulcerans* is largely extracellular in the lesions produced and does not form granulomas like other pathogenic mycobacterial infections. To determine the histopathology of *M. ulcerans* in Medaka, infected fish were sacrificed at 1, 8 and 23 wks p.i., fixed and stained with Ziehl-Neelsen and hematoxylin and eosin. *M. marinum* 1218g and MMDL infected medaka were used as positive controls. Negative control PBS-infected fish showed no presence of acid-fast bacilli or pronounced inflammatory response at all time points (Fig 4.2 A and B). MU1615 infected medaka were positive for very few intracellular acid-fast bacteria in the kidney, spleen and liver (Fig. 4.2 C) and data not shown) but showed little

inflammatory response (Fig 4.2 D). There were pockets of extracellular bacteria scattered in the guts of infected fish (Fig. 4.3 E and F). MU1615::Tn118g infected Medaka showed scattered pockets of intracellular and extracellular acid-fast bacteria diffuse inflammatory response in the kidneys and spleen (Fig 4.2 E and F, and data not shown). As was expected in both strains of *M. marinum* infected Medaka, acid-fast bacilli were both intracellular and extracellular (Fig 4.2 G, I and Fig. 4.3 C, E) with loosely associated granuloma formation in 1218g (Fig. 4.2 H and Fig. 4.3 D) to well organized granuloma in MMDL (Fig. 4.2 J and Fig. 4.3 F) (3, 44).

*4.2.3. M. ulcerans is avirulent in Medaka compared to M. marinum*

*M. marinum* is lethal to fish at concentrations above $10^5$ cfu and produces chronic granulomatous disease at concentrations below $10^3$ cfu (3, 18, 39). To determine whether *M. ulcerans* is pathogenic to Medaka, inocula was administered to the fish in a dose dependent manner ($10^2$ – $10^8$) and observed over time for the development of disease and subsequently death. Even at the highest dose of $10^8$, both MU1615g and MU1615::Tn118g infected fish exhibited no gross signs of disease and survived up to 8 wks p.i. when they were voluntarily sacrificed (Fig 4.4B). At low doses of $10^2$-$10^4$ *M. ulcerans* more than 70% infected fish survived up to 23 wks p.i, comparable to PBS control fish (Fig 4.6 and 4.7). In stark contrast to the above, 1218g infected fish all died before the end of the study and this occurred in a dose dependent manner. Fifty percent of Medaka infected with $10^4$ and $10^8$ cfu all died by 2 wks pi and 1wk p.i. respectively (Fig. 4.5A and 4.6). A similar observation was made for Medaka infected with *MMDL*, however the time to death was delayed in these fish as compared to *M. marinum* infected fish (Fig 4.5B and 4.6).

*4.2.4. Mycolactone is not required for M. ulcerans persistence in Medaka*

The pathogenicity of *M. ulcerans* has been attributed to the presence of the virulent toxin mycolactone produced by the bacteria. To determine the effect of mycolactone on the pathogenesis of *M. ulcerans* in Medaka, a mycolactone negative strain was used. Fish infected with either the wild type or the mycolactone negative *M. ulcerans* exhibited similar survival responses (Fig. 4.6, and data not shown). There was no significant difference in the numbers of fish that were positive for either bacteria at all

the time points assessed in this study (Tables 4.1 and 4.2). Even though Ziehl-Neelsen stain revealed more visible bacteria in MU1615::Tn118g infected Medaka compared to MU1615g Medaka in some cases, the amount of inflammatory response generated was comparable (Fig 4.2 and 4.3). Both strains had pockets of bacteria within the guts and few bacteria in the liver, heart and spleen

*4.2.5. M. ulcerans does not appear to replicate within Medaka*

*M. marinum* and *M. fortuitum* have been shown to replicate within both naturally and artificially infected fish (37, 41). These bacteria can be successfully isolated from infected fish due to the high numbers that result from colonization and replication. To determine if *M. ulcerans* replicates within Medaka, we tried to culture the bacteria from the organs of infected fish. However, our efforts were frustrated due to the overgrowth of faster growing bacteria native to Medaka. We therefore determined the increase in *M. ulcerans* DNA in infected Medaka over time using quantitative PCR and primers designed to amplify the enoyl reductase gene of the mycolactone producing plasmid. Our data suggests that there is no significant increase of *M. ulcerans* within Medaka over time (Fig 4.8 and 4.9). Rather, there was a slight but insignificant decrease in Medaka infected with $10^8$ and $10^4$ cfu MU1615g and $10^4$ cfu MU1615::Tn118g between 1 and 8 wks p.i. There was a significant decrease however in the Medaka infected with $10^8$ cfu of MU1615::Tn118g.

## *4.3. Discussion*

*M. marinum*, the progenitor of *M. ulcerans*, is a well characterized fish pathogen that successfully colonizes and replicates within infected fish (4, 9) and can be isolated by culture from water and fish. Occasionally, *M. marinum* may cause human disease when introduced via broken skin. The disease in man is characterized by granuloma formation localized to the site of infection and histopathology reveals primarily intracellular bacteria (8). On the other hand, *M. ulcerans* infection in man is localized with the formation of lesions due to necrosis of the subcutaneous fat tissue and bacteria are mostly extracellular (17, 19, 24). *M. marinum* has almost 98% sequence identity with most genes in *M. ulcerans*, but lacks the mycolactone producing plasmid

responsible for virulence. Also it appears that *M. ulcerans* is going through an evolutionary bottle neck by the loss of metabolic and stress response genes suggestive of the adaptation of the bacteria to a novel environmental niche (21).

Although the ecology and mode of transmission still remains unknown, the association of *M. ulcerans* with slow moving water in all geographic areas where it has been identified is the one major risk factor (1, 2, 5). *M. ulcerans*, however, cannot survive in water (personal communication, Pamela Small) and hence needs a host or reservoir within the environment where it can colonize and actively replicate. A wide scale study of various environmental organisms within water bodies in *M. ulcerans* endemic communities has revealed a high association of the bacterial DNA with biofilm, water filtrate, and pseudophytes (glass slides) (46). *M. ulcerans* DNA has also been identified in a wide range of aquatic invertebrates, of particular interest are Naucorids and Belostomatids (26, 29, 46), and fish (10, 23), all collected from *M. ulcerans* endemic water bodies. The diversity involved with the range of organisms positive for DNA suggests the possibility of transfer of *M. ulcerans* in a very complex food web within water bodies. As part of efforts to identify potential biological reservoirs of *M. ulcerans* in the environment, we have established models for further understanding the interactions between *M. ulcerans* and fish by the following: (i) artificially infecting Japanese Medaka with *M. ulcerans*, (ii) determining the pathogenicity of *M. ulcerans* in medaka, (iii) determining the role of mycolcatone in Medaka infection and (iv) determining whether *M. ulcerans* can actively colonize and replicate in Medaka. This is the first study to address the possibility of *M. ulcerans* to survive and replicate in fish

Our data suggests that *M. ulcerans* is capable of establishing an early infection in Medaka (Fig 4.1). We show by fluorescence microscopy that the infection is systemic by 1 wk p.i., spreading to the organs following intraperitonial injection of the inocula. We noticed that the Gfp signal was pronounced in MU1615::Tn118g infected fish compared to MU1615g and this is because the mycolactone quenches the fluorescence in the latter. Both wild type and mycolactone negative *M. ulcerans* can also be detected intracellularly and extracellularly within the kidney, gut, heart, spleen and liver of infected fish via acid-fast staining. These results are comparable to the early

systematic spread of *M. marinum* in artificially infected fish following intraperitoneal inoculation (3, 6, 13, 30, 32).

We also determined the pathology of *M. ulcerans* in medaka using increasing doses of bacteria for inoclua. Whereas an inoculum of $10^6$ cfu of *M. marinum* results in an LD50 by 1 wk p.i. (3), medaka injected with $10^8$ cfu of *M. ulcerans*, both wild type and mycolactone negative mutant, survived the entire 8 weeks of study (Fig 4. 6). Medaka infected with lower doses of *M. marinum* eventually develop a chronic infection characterized by well organized granuloma formation in the kidneys, livers and spleen, however there are no external signs of disease such as lesion formation. In *M. ulcerans* infection, there is no granuloma formation but there is the presence of scattered pockets of bacteria within the kidney, guts and peritoneum of infected fish. There is also a lack of pronounced inflammatory response and the fish appear healthy looking (Fig5.2). This observation may be different in other fish as hosts. For instance in *M. marinum* infection of zebra fish and gold fish, even though low doses result in chronic disease, infected fish develop lesions and appear bloated in comparison to control (3, 30, 41). In addition to these observations, mycolactone does not appear to confer additional virulence to *M. ulcerans* pathogenicity to Medaka. Our data shows no statistically significant difference between the genome forming units of both strains at 1, 8 and 23 wks p.i (Fig 4.8 and 4.9). Again, these results may be different in a different breed of fish.

A key finding from this study is that *M. ulcerans* does not appear to replicate in Medaka. Acid fast bacteria could be identified in fish sacrificed at 8 and 23 wks p.i., however, in comparison with *M. marinum* infected fish, there was no significant increase in the number of bacilli observed. Since we were unable to culture back form infected fish, we used PCR to determine increase in genome forming units of *M. ulcerans*. Our data shows an insignificant decrease in both wild type and mycolactone negative *M. ulcerans* (Fig 4.8 and 4.9). In order to avoid the bias conferred by the different growth rates of *M. ulcerans* and *M. marinum 1218, M. marinum DL* was used as a control. This fish pathogen produces mycolactone F, and is also classified as a slow grower. *M. marinum DL* fish infected with $10^8$ cfu all died by 10 days post infection, and low dose infected medaka, although not showing outward signs of

lesion formation and bloating, exhibited pronounced and well organized granuloma formation in the kidneys, liver and, spleen (Fig 4.2 and 4.3). Pockets of intracellular and extracellular acid fast bacilli were also identified within the granulomas. It is unclear whether the resulting pathology is due to mycolactone F or other immunogenic/virulent proteins in *M. marinum DL*. There have been conflicting reports on the virulence of *M.marinum DL* in fish compared to wild type *M. marinum* (20, 43, 44).

Our findings are novel and relevant to the elucidation of potential environmental reservoirs of *M. ulcerans*. To date, the association of *M. ulcerans* with fish has purely been based on the identification of DNA using PCR and primers designed to amplify the insertion sequence IS2404, previously thought to be unique to *M. ulcerans* (10, 23). Recent studies have revealed that this insertion sequence is present in other organisms, particularly novel species of slow growing mycobacteria that also have the mycolactone producing plasmid (35, 46). The latter discovery has also fueled the discussions as to the ability of *M. ulcerans* to colonize fish. This study is the first of its kind to describe the interactions that occur between *M. ulcerans* and fish. We cannot rule out the possibility of fish being reservoirs in the transmission chain because it appears that *M. ulcerans* persists in infected medaka, but we do not see any increase in bacterial load over time. The infection may be transient in Medaka but have a different characteristic is other laboratory fish models such as zebra fish and gold fish. It is possible that those host systems may elucidate a finer differentiation of acute and chronic infects on *M. ulcerans*, and possibly a role for mycolactone in pathogenicity.

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### *4.5. Appendix*

**Table 4.1: Infected fish ($10^4$) sections positive for acid fast bacilli and bacterial DNA (PCR) at 7 and 60dpi respectively**

| | | **MU1615g** | | **MU1615::Tn118g** | | **MMDL** | |
|---|---|---|---|---|---|---|---|
| | DAYS P.I. | 7 | 60 | 7 | 60 | 7 | 60 |
| **GUT** | Microscopy | 3/4 | 4/4 | 3/4 | 4/4 | 4/4 | 4/4 |
| | PCR | 1/4 | 4/4 | 2/4 | 2/4 | 4/4 | 4/4 |
| **HEART** | Microscopy | 0/4 | 1/4 | 1/4 | 2/4 | 1/4 | 4/4 |
| | PCR | 2/4 | 2/4 | 2/4 | 1/4 | 0/4 | 4/4 |
| **KIDNEY** | Microscopy | 3/4 | 2/4 | 2/4 | 3/4 | 1/4 | 4/4 |
| | PCR | 3/4 | 2/4 | 3/4 | 2/4 | 1/4 | 4/4 |
| **LIVER** | Microscopy | 1/4 | 3/4 | 1/4 | 4/4 | 3/4 | 4/4 |
| | PCR | 1/4 | 4/4 | 2/4 | 1/4 | 2/4 | 4/4 |
| **CARCASS** | Microscopy | 3/4 | 4/4 | 3/4 | 4/4 | 4/4 | 4/4 |
| | PCR | 4/4 | 4/4 | 3/4 | 2/4 | 4/4 | 4/4 |
| **SPLEEN** | Microscopy | 0/4 | 2/4 | 0/4 | 4/4 | 1/4 | 4/4 |
| | PCR | 1/4 | 2/4 | 1/4 | 4/4 | 0/4 | 4/4 |

**Table 4.2: Infected fish ($10^8$) sections positive for acid fast bacilli and bacterial DNA (PCR) at 7 and 60dpi respectively**

| | | MU1615g | | MU1615::Tn118g | | MMDL | |
|---|---|---|---|---|---|---|---|
| | DAYS P.I. | 7 | 60 | 7 | 60 | 7 | 60 |
| **GUT** | Microscopy | 3/4 | 3/4 | 4/4 | 4/4 | 4/4 | 4/4 |
| | PCR | 3/4 | 2/4 | 4/4 | 4/4 | 3/4 | 4/4 |
| **HEART** | Microscopy | 1/4 | 2/4 | 1/4 | 4/4 | 3/4 | 4/4 |
| | PCR | 3/4 | 3/4 | 1/4 | 4/4 | 2/4 | 4/4 |
| **KIDNEY** | Microscopy | 3/4 | 3/4 | 3/4 | 4/4 | 2/4 | 4/4 |
| | PCR | 3/4 | 3/4 | 3/4 | 4/4 | 2/4 | 4/4 |
| **LIVER** | Microscopy | 2/4 | 3/4 | 1/4 | 4/4 | 4/4 | 4/4 |
| | PCR | 3/4 | 2/4 | 3/4 | 4/4 | 4/4 | 4/4 |
| **CARCASS** | Microscopy | 3/4 | 3/4 | 4/4 | 4/4 | 4/4 | 4/4 |
| | PCR | 3/4 | 3/4 | 4/4 | 4/4 | 4/4 | 4/4 |
| **SPLEEN** | Microscopy | 3/4 | 3/4 | 3/4 | 4/4 | 2/4 | 4/4 |
| | PCR | 3/4 | 3/4 | 3/4 | 4/4 | 2/4 | 4/4 |

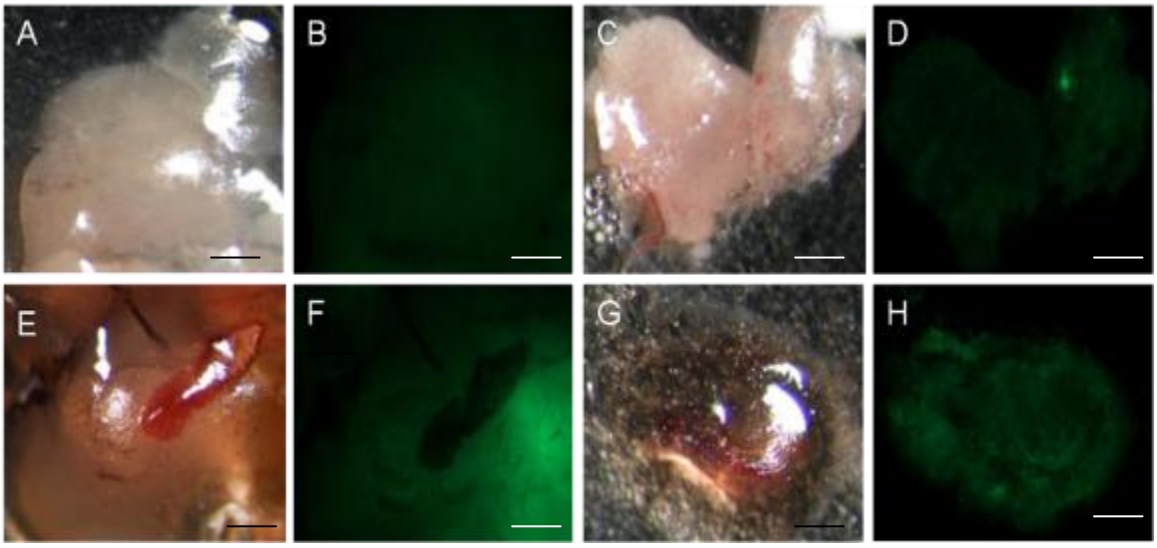


**Figure 4.1: Initial establishment of infection of Medaka by fluorescent microscopy.** Dissected organs from $10^8$ cfu MU1615g (top panel) and MU1615::Tn118g (bottom panel) infected medaka were examined for Gfp expressing bacteria 1 wk post-infection. A, C, E and G – bright field; B, D, F and H – fluorescent filter. A, B, E and F - liver; C, D, G and H – kidney.

Scale bars =10µm

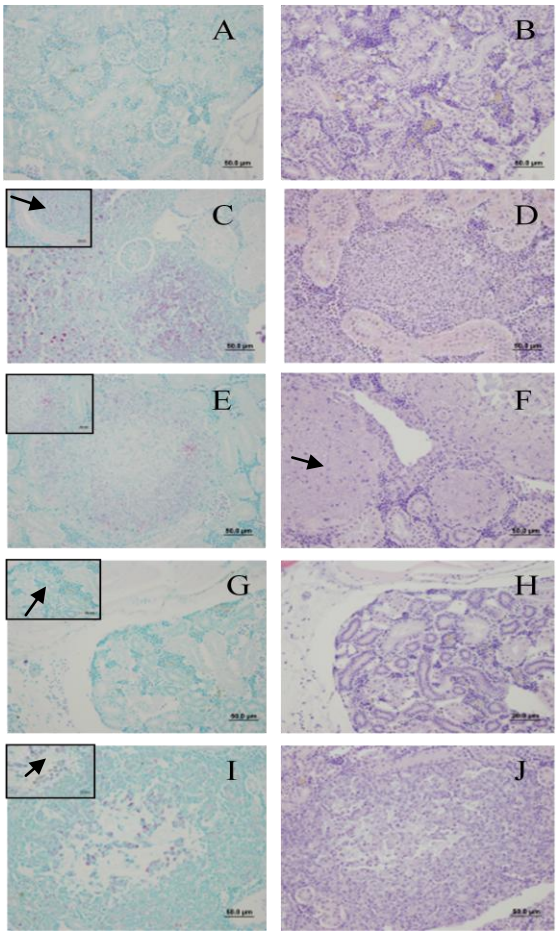


**Figure 4.2: Representative histopathology of Medaka infected with $10^4$ cfu of different strains of mycobacteria 8 wks post-infection.**

All sections were fixed and stained with Ziehl – Neelsen stain (left panels) and hematoxylin and eosin stain (right panels). (A and B) PBS negative control. (C and D) 1218g infected Medaka showing loosely associated granuloma (D) with intracellular and extracellular pockets of bacteria (C – arrow and inset). (E and F) MMDL infected Medaka showing well organized granuloma with necrotic centers (F – arrow) surrounded by bacteria. (G and H) MU1615g infected Medaka showing little inflammatory response (H) with few intracellular bacteria (G- arrow and inset). (I and J) MU1615::Tn118g infected Medaka showing diffuse inflammatory response (J) and scattered pockets of intracellular and extracellular bacteria (I – arrow and inset). Total magnification, ×200 for all panels and ×1000 for insets. Scale bars = 50 µm

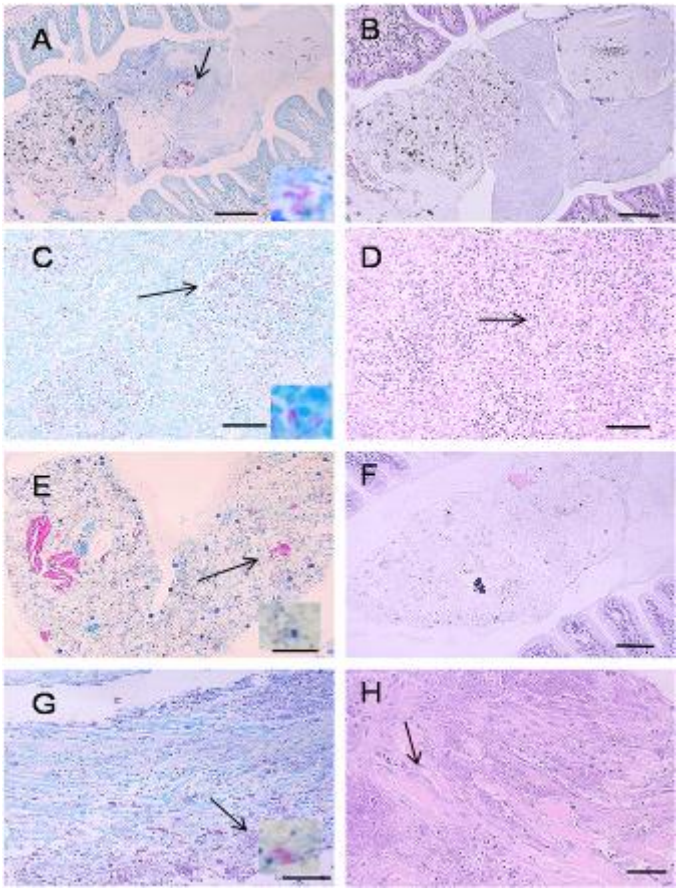


**Figure 4.3: Mycobacteria strains produce systemic intracellular and extracellular infection in Medaka.**

Histopathology of sections fixed and stained with Ziehl – Neelsen (left panels) and hematoxylin and eosin (right panels) for Medaka infected with $10^8$ cfu. (A and B) Gut of MU1615::Tn118g infected Medaka showing scattered extracellular bacteria (arrows). (C and D) Liver of 1218g infected Medaka showing well organized granuloma (D – arrow) surrounded by bacteria (C-arrow). (E and F) Gut of MU1615g infected Medaka showing scattered extracellular bacteria (arrows). (G and H) Spleen of MMDL infected Medaka showing diffuse granuloma (H-arrow) with mostly intracellular bacteria. Magnification, ×200 for all panels and ×1000 for insets. Scale bars = 50 µm

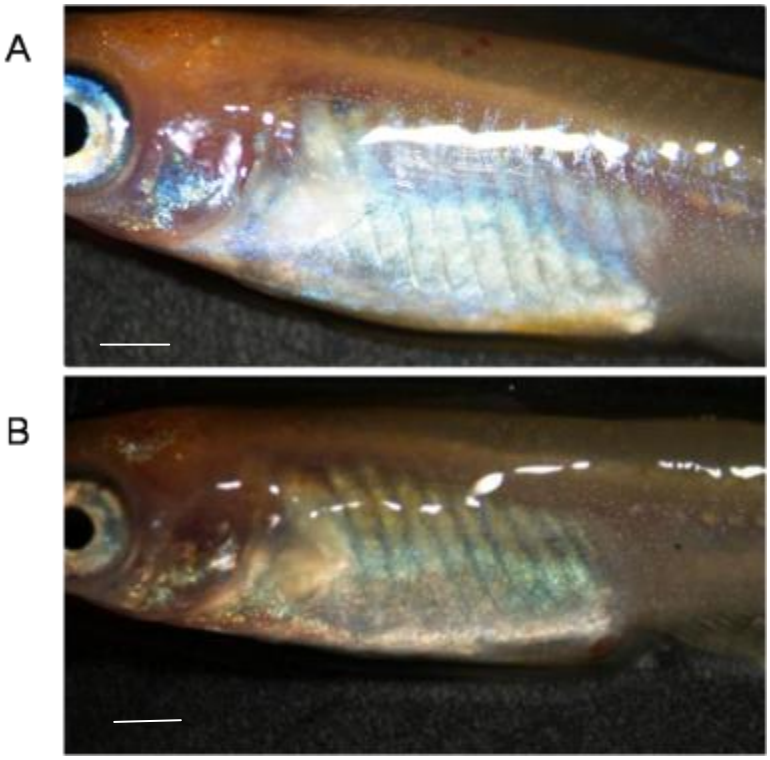


**Figure 4.4: Gross morphology of Medaka post infection.**

(A) Medaka injected with 30µl of PBS (negative control) at 8 wks post infection.

(B) Medaka injected with $10^8$ cfu MU1615g at 8 wks post-infection show no signs of disease or lesions. Scale bars = 100mm

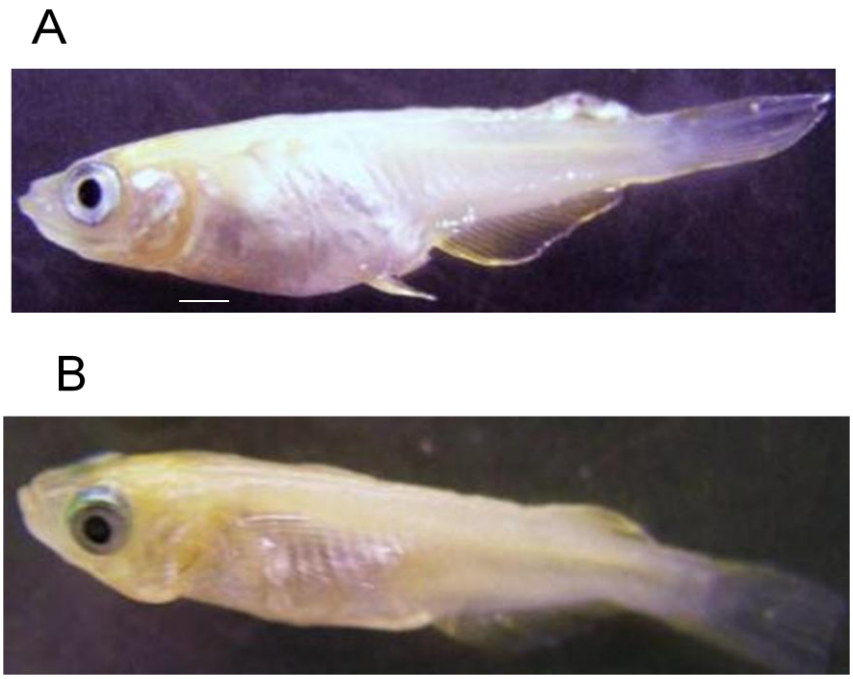


**Figure 4.5: Gross morphology of Medaka post infection**.

(A) Medaka injected with $10^8$ of 1218g at 4 wks post infection. (B) Medaka injected with $10^8$ cfu MMDL at 7 wks post-infection show signs of bloating. Scale bars = 100mm

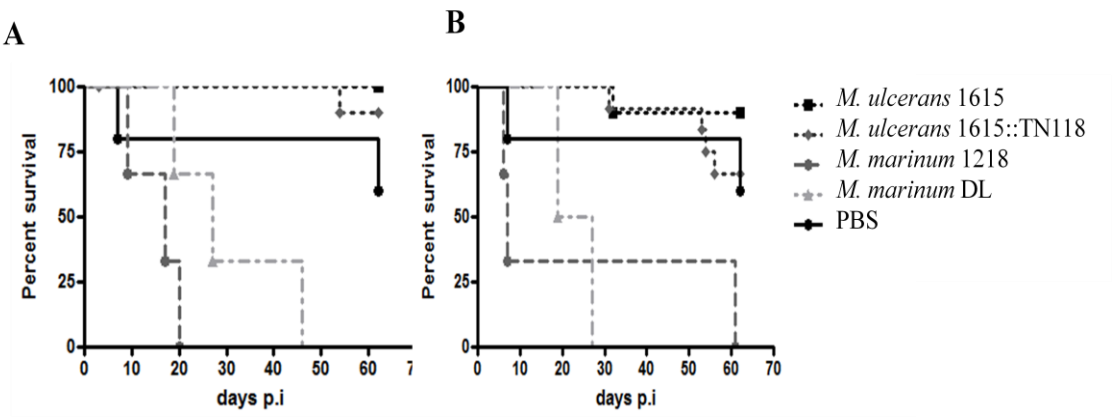


Figure 4.6: **Percent survival of Medaka infected with $10^4$ (A) and $10^8$ (B) cfu of different mycobacteria strains ( experimental design 2).**

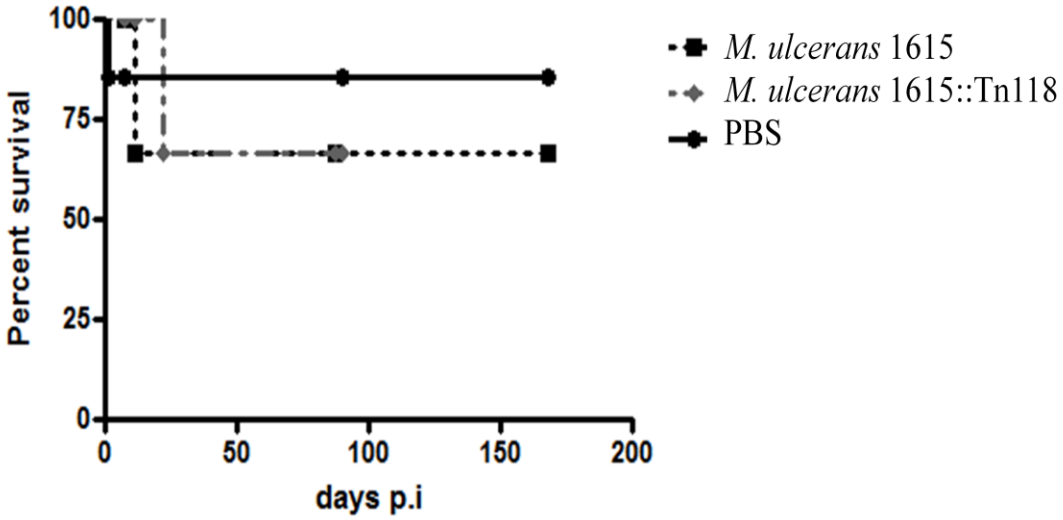


**Figure 5.7: Percent survival of Medaka infected with $10^4$ cfu of MU1615g and MU1615::Tn118 compared to control (experimental design 1)**

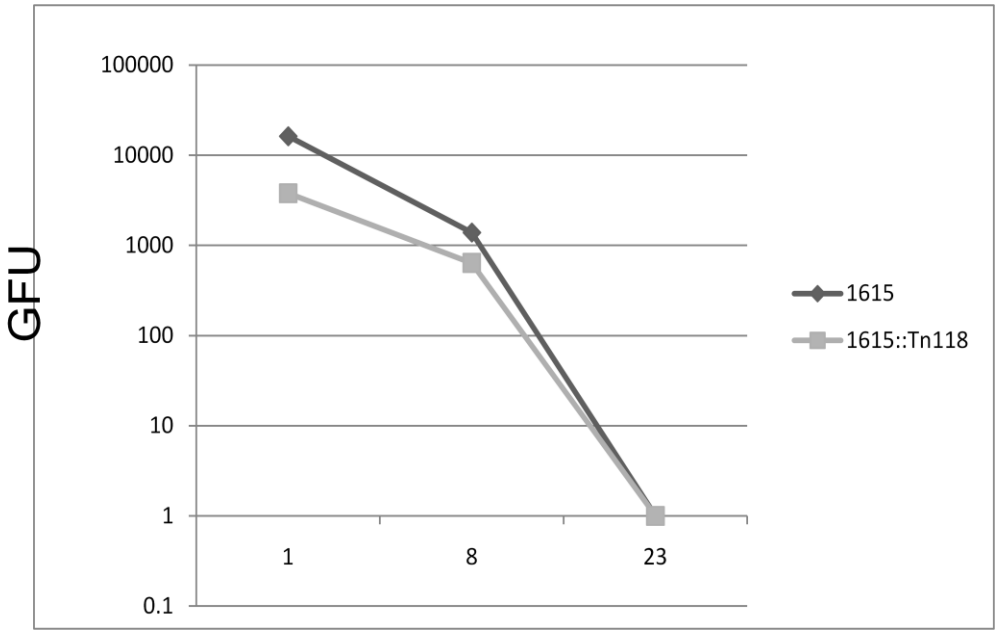


**Figure 4.8: *Mycobacterium ulcerans* persists but does not appear to replicate in Medaka (Experimental design 1).**

Mean log genome forming units (gfu) of MU1615g and MU1615::Tn118g infected ($10^4$ cfu) Medaka at 1, 8 and 23 wks p.i for ER-PCR positive Medaka as determined by qPCR using an internal probe for the ER gene. Data are means and standard deviations of the values obtained from infected medaka. P>0.05 for both strains (Mann Whitney test).

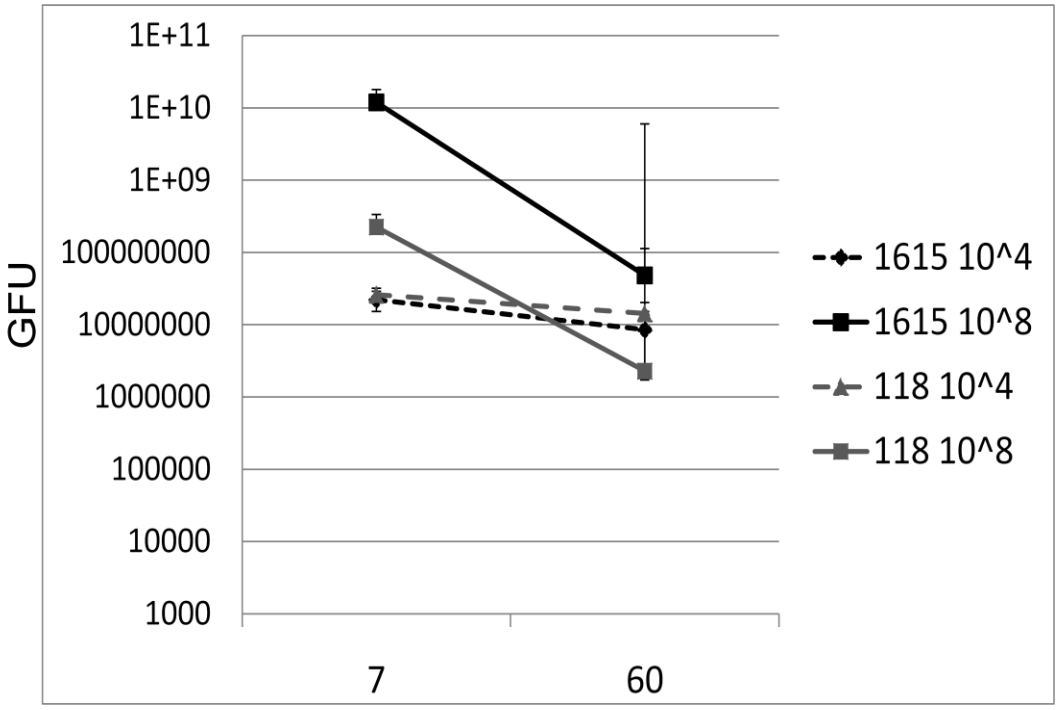


**Figure 4.9: *Mycobacterium ulcerans* persists but does not appear to replicate in Medaka (Experimental design 2).**

Mean log genome forming units (GFU) of MU1615g and MU1615::Tn118g infected Medaka at 1 and 8wks p.i for ER-PCR positive Medaka as determined by qPCR using an internal probe for the ER gene. Data are means and standard deviations of the values obtained from infected Medaka. P>0.05 for both strains (Mann Whitney test). R2 = 0.995 and 0.975 for MU1615g and MU1615::Tn118g respectively.

# Chapter 5: Fish monocytes as a model for understanding *M. ulcerans*-host pathogenesis

### *5.1 Introduction*

The ability of mycobacteria to colonize a eukaryotic host involves a long history of co-evolution and adaptation. In this process, the more rapid generation time of the bacteria compared to the host has enabled bacteria in many cases to adapt to their hosts without causing lethal infections. The hallmark symptom of mycobacterial infection in humans, fish and laboratory animals is the formation of granulomas (6). Granuloma formation is an immune mediated response used by the host to trap pathogens and other foreign substances. The primary cells involved in granuloma formation in mycobacterial infections are macrophages, but other cells such as lymphocytes, fibroblasts and bacteria are also important (1). The classical model of granuloma formation has been perceived as a host-protective event to prevent the spread of the infection (32, 39). However, recent data suggests that granulomas also play a role in expanding bacterial infection (7). Employing both *in vitro* and *in vitro* assays, researchers have shown that *M. leprae, M .tuberculosis, M. avium* and *M. marinum* can enter macrophages and subsequently replicate within phagosomes by interrupting phago-lysosome formation (4, 8, 13, 26 30).

*M. marinum* infection in fish has been widely used as a surrogate model for understanding the host-pathogen interactions between *M. tuberculosis* and its human host (3, 5, 7, 29, 39). It is pathogenic mycobacteria, closely related to *M. tuberculosis* with a much shorter generation time and has been used successfully in both *in vivo* and *in vitro* experiments. Since fish are natural hosts for *M. marinum* infection, their macrophages have been used to further tease out specific interactions that occur during acute and chronic infection (10, 15, 41). Macrophages are ubiquitously distributed in tissue and play a key role in the early immune response as well as in adaptive immunity. They are key mediators of the inflammatory response both in acute and chronic infection.

In humans, most *M. marinum* infections are cutaneous because *M. marinum* like *M. ulcerans* has a restricted low temperature growth requirement. *M. marinum* is very closely related to *M. ulcerans* and is thought to represent the ancestral line from which *M. ulcerans* evolved. There is 98% sequence identity of the 16SrRNA genes from *M. marinum* and *M. ulcerans*, and equally high homology between housekeeping and

structural genes from the two species (22, 23, 24, 31, 37, 38). With the ongoing search for the environmental reservoir of *M. ulcerans* and the phylogenetic relationship between *M. marinum* and *M. ulcerans*, the question has been asked whether fish can be a candidate involved in the transmission process or provide an ampilying reservoir in the environment. There have been reports of potential of *M. ulcerans* DNA in fish. Identification of *M. ulcera*ns DNA have been based on identification of an insertion sequence IS2404 in DNA extracted from fish collected in Buruli ulcer endemic areas (9). It has been shown recently that the IS2404 insertion sequence is not unique to *M. ulcerans* and is found in other organisms as well in other mycolactone producing mycobacteria which cause infection in fish and frogs. In this work we have shown experimentally that *M.ulcerans* does not replicate or cause disease in fish. *M. ulcerans* strains are believed to have evolved from *M. marinum* through reductive evolution (22, 23, 24, 32). Though through evolution, loss of genes makes *M. ulcerans* no longer able to infect fish, acquisition of mycolactone cannot compensate for this (37, 38). This raises two hypotheses why *M. ulcerans* does not colonize fish; (i) because mycolactone is not toxic for fish cells or (ii) because the genome reduction has resulted in loss of genes necessary for pathogenesis in fish. We have tested the effects of mycolactone on fish cells to address the first hypothesis.

Many models have been developed for studying the pathogenesis of *M. ulceranss* including guinea pigs, mice, bats, armadillos and anole lizards. The best disease model for understanding *M. ulcerans* pathogenesis is a guinea pig dermal model, in which lesions develop as a result of mycolactone secreted by largely extracellular bacteria (17, 18, 19, 20, 34). *M. ulcerans* causes a painless infection in humans and guinea pigs, characterized by cell death via apoptosis with no apparent immune response (17). In anole lizards, it produces three patterns of inflammatory response; a chronic granulomatous disease in which acid fast bacilli are predominantly intracellular, encapsulated granuloma, or a diffuse necrotizing granuloma in which most AFB are extracellular—similar to the characteristic lesion found in human infections (25). In mice, *M. ulcerans* infection is characterized by a persistent acute inflammatory response, necrosis, AFB resulting from lysed phagocytic cells and nerve damage (18, 27).

We have developed an *in vitro* model for understanding *M. ulcerans* pathogenesis as a result of mycolactone using gold fish (*Cyprinus carpio*) macrophages (CLC). This cell line has been well characterized (15) and is used as a model for mycobacterial host-pathogen interations (10). The cell line has an optimal growth temperature of 28°C, which is ideal for *M. ulcerans* growth. It has been shown that *M. marinum* can enter and replicate efficiently in CLC cells whiles *M. smegmatis* is killed intracellularly (10). We used acetone solubule lipids (ASLs) of 3 different congeners of mycolcatone; A/B produced by the African and Malaysian strains of *M. ulcerans*, E produced by *M. liflandii* which cause disease in frogs and F produced by *M. marinum DL* 240490 and *M. pseudoshotsii* which causes disease in fish. Acetone solubule lipids extracted from these mycobacteria are described as partially purified mycolactone and the sole activity of these lipids is due to mycolactone (16).This is the first study to address whether *M. ulcerans* and mycolactone are pathogenic to fish cells *in vitro*.

### *5.2 Results*

#### *5.2.1. Mycolactone is cytotoxic to CLC cells in vitro*

The cytotoxic effects of different congeners of mycolactone have been described for a variety of cultured cells including mouse fibroblasts and human neutrophils (2, 16, 17, 31, 34, 35). Mycolactone cytotoxicity is characterized by rapid necrosis at concentrations above 1 µg/ml within 4 h and delayed apoptosis at concentrations as low as 1ng/ml within 24 h. To determine the effect of mycolactone on CLC cells, acetone soluble lipids from MU1615, MMDL and XL5 was added in a dose dependent manner to a semi-confluent layer of CLC cells and observed microscopically for their distinct phenotypes. At concentrations above 10µg/ml, mycolactone A/B treated cells were visibly rounded and swollen by 24 h p.i. compared to cells treated with an ethanol control. This phenotype is significant of necrosis. At 10ng, cells treated with mycolactone A/B had mostly lost the confluent monlayer appeared apoptotic (Fig. 5.1). By 48 h post treatment, the cell monolayer had completely detached. Cytotoxic effects were also observed with

mycolactone F and E treated cells, however, the phenotype differed slightly from mycolactone A/B treated cells. At concentrations of 10µg and above, mycolactone E and F treated cells appeared mostly apoptotic with condensed nuclei with loss of cell structure (Fig. 5.1). These observations were also made for cells treated with mycolactone concentrations below 100ng, but were delayed. Also, at low concentrations of mycolactone E and F treated cells, some cells were able to regain their morphology after 48 h.

To determine the mechanism of mycolactone – mediated cell death by mycolactones A/B, E and F, we added acetone soluble lipids from MU1615, XL5 and MMDL to a semi-confluent layer of CLC cells in a dose dependent manner and tested for the ability to produce apoptosis and necrosis. Apoptosis is characterized by nucleosome enrichment and clearing of the cytoplasm, and was quantified using an ELISA kit at 24h post treatment with mycolactone. There was a dose dependent apoptotic effect on mycolactone A/B and F treated cells (Fig. 5.2). At concentrations of 100µg and 10µg, mycolactone A/B and F treated cells were at least 2-fold more apoptotic than mycolactone E treated cells. At the lowest concentration of 10ng there was limited apoptosis with no significant difference between the congener of mycolactone used. Mycolactone E treated cells did not appear to have marked differences in apoptosis regardless of the concentration of mycolactone used.

Necrosis of cells treated with mycolactone is characterized by cell rounding and swelling followed by membrane permeabilization and cell lysis. This phenotype was quantified by the release of LDH during cell lysis after treatment with mycolactone for 24 h. Mycolactone A/B treated cells exhibited the highest amount of apoptosis at 100µg and this amount significantly decreased with decreasing doses of mycolactone. Conversely, both mycolactone E and F treated cells releases significantly low amounts of LDH at 100µg. At all other concentrations, there was no significant difference between the amounts of apoptotic cells regardless of the mycolactone congener used. These results suggest that mycolactone A/B – mediated cell death is via both apoptosis and necrosis whereas cell death by mycolactones E and F is mostly mediated via apoptosis. It is also evident from these results that mycolactone A/B exhibits a more potent effect on CLC cells compared to mycolactone E and F.

### *5.3 Discussion*

The activity of mycolactone A/B on human macrophage and neutrophils, and mouse macrophage and fibroblasts has been investigated (2, 16, 30). The hallmark cytotoxic phenotypes include cell rounding and swelling with subsequent cell lysis at high concentrations and eccentric condensed nuclei with cleared cytoplasms at low concentrations. Further analysis shows that mycolactone causes cell death by both necrosis and apoptosis. These events explain to a large extent the localization of *M. ulcerans* at the site of infection, the non-systematic nature of the disease and the observation of extracellular bacilli in histopathological sections of fixed lesion tissue. In earlier studies we find that *M. ulcerans* does not cause disease in fish. Conversely, *M. marinum* DL causes disease in fish and makes mycolactone F whilst *M. liflandii* causes disease in frogs and makes mycolactone E. We are uncertain as to whether the lack of disease is due to the loss of genes in *M. ulcerans* or that mycolactone is not cytotoxic to fish cells. In order to understand the pathogenic potential of *M. ulcerans* to fish, we have investigated the toxicity of mycolactone for fish cells using CLC macrophages from goldfish. This model system has been used for understanding *M. marinum* pathogenesis as a surrogate model for *M. tuberculosis* pathogenesis (10). Studies of the immune defense mechanisms of fish have shown significant similarity with those of mammalian systems (11, 12). Fish have B and T – like lymphocytes, non specific cytotoxic cells (NK-like cells) and phagocytic cells including macrophages (11, 12). The latter have been shown to be involved in first line defenses just like in mammalian systems and are ubiquitously distributed throughout the fish (40).

Our data suggests that all three congeners of mycolatone are cytotoxic to CLC cells *in vitro*. The potency of mycolactone E and F appear to be reduced compared to that of mycolactone A/B. These results have been corroborated by a previous study which also showed a fold decrease in potency of mycolactone F on mouse fibrolasts (L929 cells) as compared to mycolactone A/B (31). Structurally, all three mycolactone make the same core but differ slightly in the side chain (28). Mycolactone A/B has the longest side chain of the three (15C) and has a hydroxyl group on carbon 12 that is absent in the other two congeners. Mycolatone F has the shortest side chain (13C), whilst mycolactone E has a

14 carbon side chain. The differences in the side chains have been associated with the level of potency of the mycolactones in their respective hosts. However, no study has been done to understand the relative degree of toxicity of each mycolactone within the same host.

We also have characterized the mechanism of CLC cell death due to mycolactone. We show that cell- mediated death due to mycolactone A/B is mostly by necrosis at high concentrations and apoptosis at low concentrations and is dose dependent. Again this phenomenon has been described for other cell types and our results are in concordance with previous observations (2, 16, 31). These results further support localization of the infection and the subsequent lack of dissemination because all the first line immune defense cells that take up the bacteria upon infection are killed by mycolactone. In contrast, we have observed that cell - mediated death due to mycolactones E and F is characterized largely by apoptosis. This might explain why *M. marinum* DL, the strain that makes mycolactone F, is able to cause disease in fish by the formation of granuloma. These observations support the fact that mycolactone A/B is the most potent congener of mycolactone.

In conclusion, we have investigated virulence of *M. ulcerans* for fish in two ways; (i) a fish model *in vivo* and (ii) a cell model *in vitro*. Studies conducted in our lab suggest that *M. ulcerans* may not be pathogenic in fish (this work). In contrast we have evidence that CLC macrophages from goldfish are susceptible to mycolactones. If mycolactone is made by *M. ulcerans in vivo* in fish, early cytotoxic events due to mycolactone A/B can alter the ability of *M. ulcerans* to replicate within fish macrophages and hence the inability of *M. ulcerans* to produce disease in fish. In contrast *M. marinum* DL, which makes mycolacttone F causes disease in fish. It is possible that the later has evolved in fish and has maintained it tropism for fish cells whilst *M. ulcerans* has evolved in other hosts and has subsequently lost its abitllity to colonize fish.

Other immune cells may also confer a supportive role in containing the infection caused by *M. ulcerans*. Generally, mycobacterial species are capable of being internalized by macrophages and epithelial cells, however, non-pathogenic strains are readily killed by the cell defense mechanisms (21, 34, 35). Pathogenic species on the other hand can overcome these defense mechanisms and subsequently replicate within

infected cells (26, 29). It is possible that the absence of disease in *M. ulcerans* infected fish could be due to the lesions in the chromosome of *M. ulcerans* created during its reductive evolution from *M. marinum*. Another possibility is that mycolactone is regulated *in vivo* and is not being made by *M. ulcerans* in fish, or if it is being made, the phagocytic cells are immediately killed by mycolactone and hence the bacteria cannot replicate and are subsequently killed. In human and guinea pig infections where mycolactone is being actively secreted, there are both apoptotic and necrotic cells. In histological sections of *M. ulcerans* infected Medaka, this observation is absent and there is very little influx of immune cells in areas where bacteria are found, suggestive of the absence of mycolactone.

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## *5.5 Appendix*

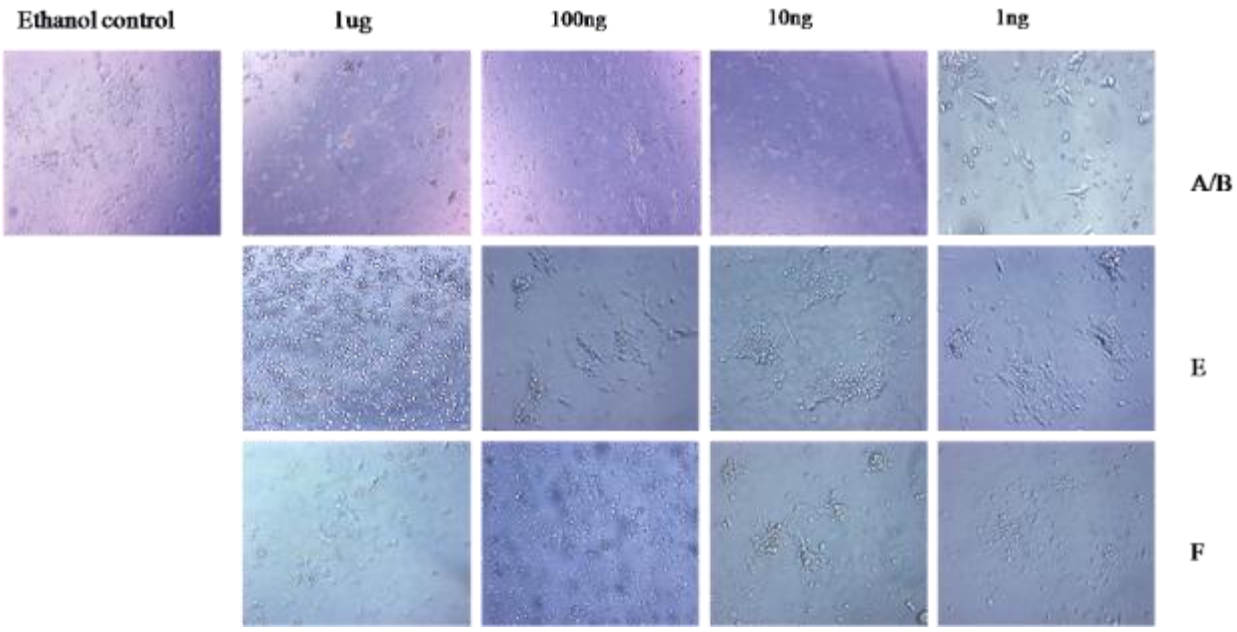


**Figure 5.1: Concentration dependent cytotoxicity of different congeners of mycolactone on CLC cells.**

Magnification ×200.

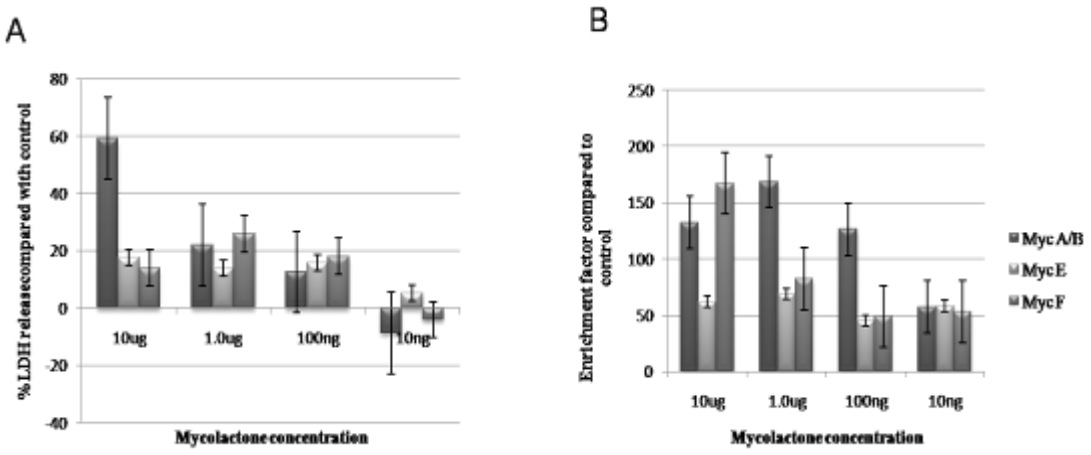


**Figure 5.2: Cytotoxicity of mycolactone measured by LDH release and nucleosome enrichment.**

(A) Culture supernatants were collected from wells containing CLC cells 4 h after treatment with mycolactone and the amount of LDH was measured using a Cytotox 96 assay kit (Promega). Data are means and standard deviations of the values obtained from triplicate samples; $P>0.05$ for all concentrations (Student's t test). (B) Apoptosis was assessed at 24 h with the cell death detection enzyme-linked immunosorbent assay kit (Roche) and expressed as fold enrichment of nucleosomes. Data are means and standard deviations of the values obtained from triplicate samples; $P>0.05$ for all concentrations (Student's t test).

# Chapter 6: Identification of Native Insect Bacteria Using culture and culture independent methods

### *6.1 Introduction*

Naucoridae and Belostomatidae are families of predaceous aquatic insects that belong to the order Hemiptera, also known as true bugs. They are found all over the world and there are few habitats without Hemiptera adapted to living there (9). Naucorids and Belostomatids thrive within a wide range of temperatures and climate conditions. The order is morphologically diverse. However all hemiptera have large compound eyes, four or five segmented antennae and have mouthparts that have been adapted for piercing or sucking. Their mandibles and maxillae are modified as needle-like stylets with two canals; one for delivering saliva and one for sucking fluid (11). The predatory suborders, to which Naucorids and Belostomatids belong, have well developed raptorial arms with which they grab and immobilize their prey prior to injection of paralyzing saliva.

Naucorids are most common in the tropics and there are about 150 described species (22). The most common genera include *Ambrysus*, *Aphelocherirus*, *Laccocoris*, *Pelocoris* and *Naucoris* of which the latter is dominant in Africa (23). There are approximately 200 described species of belostomatids and the most common genera are *Belostoma*, *Abedus*, *Lethocerus* and *Diplonychus*. The latter is dominant in Africa and prefers to live in the vegetative areas of stagnant water bodies. Previous work published by Portaels *et al* (18), found African naucorids and belostomatids among other aquatic non-predaceous insects to be positive for *Mycobacterium. ulcerans* DNA. To support this finding, Marsollier et *al*. carried out studies on naïve Naucorids collected in France, where he infected these insects in the laboratory with *M. ulcerans* and followed the infection over a period of 90 days (15). He showed that the bacteria successfully colonized the insects, causing no growth impairment throughout this period.

Insects are known to harbor a wide range of microbiota primarily in the gut and this property is useful in identifying various classes of insect- symbiont relationships (7, 8). The associations between these microbes and insects may either be parasitic, where the microbes benefit at the expense of the insect, mutualistic or symbiotic where both insect and bacteria benefit or none of the parties are harmed respectively. In the later cases, the microbes help in digestion and sequestering of nutrient for the insects that are on sub-optimal diets (2, 5, 6, 20). Just as the case is in humans, indigenous insect bacteria also play a role in withstanding the colonization of the gut by non-indigenous species

including pathogens (3, 7, 21). Insect bacteria are also known to play an important role in the generation or suppression of immune responses in the insects. In mosquitoes for instance, there is a link between the reduction in gut bacteria and increased production of *Plasmodium* oocysts (2). There are other multitrophic interactions that exist between insects and their environment as a result of their indigenous microbiota. The microbes within the gut of insects have the ability to adapt rapidly to changes in the insect diet and infection by non-indigenous bacteria. This is reflected in changes in enzyme profiles and alterations in population dynamics following feeding (3, 4).

Approximately 90% of insect-associated microbes have not been successfully cultured directly from their natural environments (1). This may be due to host specificity or the degree to which growth of these organisms is restricted. This problem can be overcome by the use of genomic approaches where organisms are identified at the DNA or RNA level (13, 14). These molecular methods are sufficiently accurate and are much less time consuming than attempts at culture. With this in mind, analysis of the normal flora of the naucorids and belostomatids was undertaken to provide important information on the potential interactions that might occur as a result of introduction of *M. ulcerans* to the insects. Information from these studies is also useful for developing techniques to suppress the growth of the insect normal flora in order to obtain *M. ulcerans* positive cultures. Finally identification of normal insect flora could provide a background for studying interactions between insect normal flora and *M. ulcerans*.

## *6.2 Results*

### *6.2.1. Isolation and characterization of bacterial Isolates*

For the isolation and identification of bacteria native to belostomatids and naucorids, serial dilutions of insect section homogenates were plated on LB and M7H9 media. LB media was used because it is rich in proteins. M7H9 mycobacterial media on the other hand is much less nutrient rich and was used to isolate potential native insect bacteria that might interfere with isolation of *M. ulcerans*. Bacterial colonies that formed in 24 – 48 h of incubation at 37°C were passaged on media up to three times in order to obtain pure cultures. The resulting isolates were subjected to antibiotic susceptibility tests

and growth on selective and differential media. Almost all of the bacteria found on and within the insects were gram negative (Table 6.1). No mycobacteria were isolated. The majority of isolates were Enterobacteriace cultured from the guts and thoraces of the insects. The distribution of the isolated bacteria species appeared to be random and there were many instances where bacteria with similar colony morphology were present in more than one tissue. The isolates that occurred in high frequency were *Salmonella sp*., *Klebsiella sp*., and *Proteus sp*. The only gram positive bacterium isolated was *Bacillus sp*. from the gut and raptoral arms of the insects.Approximately 90% of the isolates were resistant to ampicillin, penicillin, clindamycin and sulphonamides (Table 6.2). The most antibiotic resistant strain isolated was *Burkholderia sp*. This isolate was susceptible only to ciprofloxacin and chloramphenicol.

*6.2.2. Identification of bacterial strains using 16S rRNA sequencing*

In order to identify bacterial species that might be missed using culture dependent methods, the 16S ribosomal RNA was amplified directly from DNA isolated from the insect homogenates. Bacterial strains identified in Belostomatid homogenates are shown in Fig. 6.1 whilst those identified in Naucorid homogenates are shown in Fig. 6.2. Consistent with results from the culture dependent identification, about 80% of the sequenced bacteria had G+C contents between 40-50%, indicative of members of the family Enterobacteriaceae. In belostomatids, the most abundant bacterial strains were *Klebsiella pneumoniae* and *Bukholderia cepacia* (Fig 6.1). In the naucorids, there were no dominant bacterial strains however about 80% of the bacteria identified were enterobacteriaceae. There were 2 genera of gram positive bacteria; *Bacillus sp*. and *Geobacillus sp.* identified (Fig. 6.2). Common to both insects were *Klebsiella pneumoniae, Serratia marscenses* and *Burkholderia sp*.

We assessed the percentage abundance of each bacterial species identified by anatomical section of the insects. As was expected the guts of both Naucorids and Belostomatids had the highest bacterial diversity compared to the raptoral arms and salivary glands with at least 30% of insects being positive for more than 4 different types of bacteria (Fig 6.3). The most abundant strains isolated from the guts were *Proteus sp., Pseudomonas sp*. and *Comamonas sp*.The thoraces also had a significant amount of bacterial diversity with the

most abundant species identified being *Klebsiella pneumonia* and *Proteus vulgaris*. The salivary glands and raptoral arms had the least amount of bacterial diversity with only 10% of insect being positive for the identified bacteria in most cases.

*6.2.3. Phylogenetic diversity*

Phylogenetic diversity of bacteria identified by culture and by 16S rRNA sequencing was investigated. Bacteria were grouped according to phylotypes and the frequency of each occurring phylotype was scored against the total number of bacterial species identified (Fig. 4.4). The gamma proteobacteria phylotype occurred with the highest frequency among cultured isolates; 96%, and 90% for Naucorids and Belostomatids respectively. Among the bacterial strains identified by 16SrRNA sequencing, 90% and 50% respectively for Belostomatids and Naucorids were gamma proteobacteria. Delta proteobacteria were only identified among the sequenced isolates ad at low frequencies; 5% and 8% respectively for Belsotomatids and Naucorids. Aproximately 10% firmicutes were identified by both culture and sequencing. There was however no firmicute among the bacterial strains identified in Belsotomatids by sequencing. Beta proteobacteria were identified at a low frequency (~10%) by both sequencing and cutlture except among cultured bacterial isolates from Naucorids.

***6.3 Discussion***

In this study, the bacterial prevalence and diversity within belostomatids and naucorids was investigated using both culture dependent and culture independent methods. A broader diversity was noted among the culture independent method. This is not surprising as approximately 90% of bacteria have not yet been cultured (13, 17). Using classical microbiological tests, we were able to identify 45 different bacterial isolates to the genus level. Among these were largely members of the family Enterobacteriaceae in all the sections of the insects assayed, with the most frequent genera being *Salmonella, Klebsiella* and *Proteus*. All three genera have been repeatedly identified in similar studies (6, 10, 19, 20) by culture. Most of the the isolates were resistant to at least one antibiotic and this may be explained by the high rate of horizontal gene transfer of antibiotic resistance between bacteria found in the guts of insects (12). A significant level of resistance was noticed for amplicillin and penicillin. These antibiotics

are beta lactams that are effective mostly against gram positive bacteria and some gram negative bacteria. These results corroborate the observation that about 90% of the isolates were gram negative. The most resistant strain isolated was *Burkholderia cepacia*. *Burkholderia sp*. are ubiquitous environmental bacteria with important pathogenic species associated with cystic fibrosis (1). They are also well known for their resistance to a wide variety of antibiotics (16). We were unable to identify the bacteria to the species level, however, further biochemical testing can readily differentiate between pathogenic and non-pathogenic genera identified in this study for example *Salmonella sp.*

The bacterial strains identified via 16S rRNA sequencing also revealed a high frequency for members of the family Enterobacteriaceae. Similar to data obtained from using culture dependent methods, there was little difference between genera of bacteria identified on different anatomical sections of the insects. The most frequent bacteria identified in belostomatids were *Klebsiella pnuemoniae* and *Burkholderia cepacia* whilst there was no dominant genus among naucorids. As was expected, the guts of both insects had the most diversity of bacteria compared to other sections analyzed. Surprisingly, at least 3 salivary glands of the insects had more than one type of bacteria isolated by both culture dependent and culture independent methods. This organ is relatively sterile in most insects and *M. ulcerans* has been recovered via culture from artificially infected naucorids due to this reason (15).

Other genera of interest identified in this study were, *Acinetobacter*, *Serratia* and *Comamonas* which are both associated with human disease and also ubiquitous in the environment. All three above genera have been identified in insects (7, 6, 8, 13). We were unable to isolate many of gram positive bacteria by either culture dependent or culture independent methods. In other studies, gram positive bacteria, especially members of the family Enterococcus and Streptococcus, which are somewhat fastidious bacteria, have been frequently isolated from the guts of insects (6, 19). The media used in initial isolation of bacteria plays an important role in the bacteria eventually isolated. For instance blood agar is used mostly in to isolation of gram positive bacteria. In our study, the use of LB and M7H9 may have accounted for the high amount of gram negative bacteria isolated. There may have been a selective bias for interesting colonies during the initial screening of plates. For the culture independent method, the 16S rRNA primers

used were considered universal for bacteria, however, they may be biased to amplify only certain DNA sequences than others. This discrepancy may be also be explained by the fact that naucorids and belostomatids are aquatic insects and may have a different community of native bacteria.

We also described the phylotype diversity of bacteria present in Belostomatids and Naucorids. The gamma proteobacteria were the most abundant phylotype and this is true for other studies (14, 19, 21). The beta and delta proteobacteria were also represented in small amounts, however, it was surprising that no actinobacteria were identified in this study. *M. ulcerans* is a member of the actinobacteria phylum and their DNA has been identified in a small percentage of belostomatids and naucorids (18, 27). Evident from this study are the differences between bacteria identified by culture independent and culture dependent methods for the same sample. All four phylotypes identified in this study were represented in bacterial strains identified in Naucorids by 16SrRNA sequencing whereas only two phlyotypes were represented by culture dependent methods.

In conclusion, our data shows that there is significant native flora within belostomatids and naucorids. Of importance is the fact that almost 90% of the isolated bacteria grew well on M7H9 mycobacterial media and there will be possible interference with the isolation of *M. ulcerans* from infected belostomatids or naucorids. Also most of these bacteria have acquired significant resistance to most mainstream antibiotics. What remains unknown is whether these native bacteria confer any selective advantage or disadvantage to artificially introduced *M. ulcerans* to colonize and replicate within the insects.

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### *6.5. Appendix*

*Table 6.1: Identification of bacterial strains using culture dependent methods*

| Identification of section | Growth on LB | Growth on M7H9 | Gram stain | Growth on TSI | Growth on Citrate | Gelatin hydrolysis | SIM (motility/sulfur production/indole) | Urease | ID |
|---|---|---|---|---|---|---|---|---|---|
| Gut 1 | yes | yes | - | A/A/- | + | - | -/-/- | - | *Enterobacter* |
| Gut 2 | yes | no | - | n/a | + | + | n/a | + | *Burkholderia* |
| Gut 3 | yes | yes | - | A/K/+ | + | - | +/+/- | + | *Salmonella* |
| Gut 4 | yes | yes | - | A/K/+ | + | - | +/+/- | + | *Salmonella* |
| Gut 6 | yes | no | - | n/a | + | + | n/a | + | *Burkholderia* |
| Gut 7 | yes | yes | - | A/A/+ | - | + | +/-/+ | - | *E.coli* |
| Gut 8 | yes | yes | + | n/a | + | - | n/a | + | *Bacillus* |
| Gut 9 | yes | yes | - | A/A/+ | - | - | +/-/+ | - | *E.coli* |
| Gut 10 | yes | yes | - | A/A/+ | - | - | +/-/+ | - | *E.coli* |
| Gut 11 | yes | yes | + | n/a | - | - | n/a | + | *Bacillus* |
| Gut 12 | yes | yes | - | K/K/- | - | - | -/-/- | - | *Shigella* |
| FA 1 | yes | yes | - | A/A/- | + | + | -/+/- | + | *Serratia* |
| FA 2 | yes | yes | - | A/K/+ | + | - | +/+/- | + | *Salmonella* |
| FA 3 | yes | yes | - | A/A/+ | - | - | +/-/+ | - | *E.coli* |
| FA 5 | yes | yes | - | A/A/- | + | + | -/+/- | + | *Serratia* |
| FA 6 | yes | yes | - | K/K/- | - | - | -/-/- | - | *Shigella* |
| SG 2 | yes | yes | - | A/K/+ | + | - | +/+/- | + | *Salmonella* |
| SG 3 | yes | yes | - | A/K/+ | + | - | +/+/- | + | *Citrobacter* |
| SG 4 | yes | yes | - | A/K/+ | + | - | +/+/- | + | *Salmonella* |
| SG 5 | yes | yes | - | A/A/- | + | + | -/+/- | + | *Serratia* |
| SG 6 | yes | yes | - | A/K/+ | + | - | +/+/- | + | *Salmonella* |
| SG 7 | yes | yes | - | A/K/+ | + | - | +/+/- | + | *Salmonella* |
| SG 8 | yes | yes | - | A/K/+ | + | - | +/+/- | + | *Salmonella* |
| Tho 1 | yes | yes | - | A/A/- | + | + | -/+/- | + | *Serratia* |
| Tho 2 | yes | yes | - | A/K/+ | + | - | +/+/- | + | *Citrobacter* |
| Gut 5a | yes | yes | - | A/A/- | + | - | -/-/- | + | *Klebsiella* |
| Gut 5b | yes | yes | - | A/A/+ | - | - | +/-/+ | - | *E.coli* |
| Gut 6a | yes | yes | - | K/K/- | - | - | -/-/- | + | *Shigella* |
| Gut 6b | yes | yes | - | A/A/+ | - | - | +/+/+ | + | *Proteus* |
| FA 4a | yes | yes | - | A/A/- | + | - | -/-/- | + | *Klebsiella* |
| FA 4b | yes | yes | - | A/A/- | + | - | -/-/- | - | *Enterobacter* |
| FA 7a | yes | yes | + | n/a | + | - | n/a | + | *Bacillus* |
| FA 7b | yes | yes | - | A/A/- | + | + | -/+/- | + | *Serratia* |
| SG1a | yes | yes | - | A/K/+ | + | - | +/+/- | + | *Salmonella* |
| SG1b | yes | yes | - | A/A/- | + | - | -/-/- | + | *Klebsiella* |
| FA7aC | yes | yes | - | A/K/+ | + | - | +/+/- | + | *Salmonella* |
| FA7aY | yes | yes | - | A/A/+ | - | - | +/+/+ | + | *Proteus* |
| Gut5bp | yes | yes | - | A/A/- | + | - | -/-/- | + | *Klesbsiella* |

| Identification of section | Growth on LB | Growth on M7H9 | Gram stain | Growth on TSI | Growth on Citrate | Gelatin hydrolysis | SIM (motility/sulfur production/indole) | Urease | ID |
|---|---|---|---|---|---|---|---|---|---|
| Gut5bs | yes | yes | - | A/A/- | + | - | -/-/- | - | *Enterobacter* |
| SG1aY | yes | yes | - | A/K/+ | + | - | +/+/- | + | *Citrobacter* |
| SG1aC | yes | no | - | A/K/+ | + | - | +/+/- | + | *Salmonella* |
| SG4COL | yes | yes | - | A/A/+ | - | - | +/+/+ | + | *Proteus* |
| SG4SWA | yes | yes | - | A/K/+ | + | - | +/+/- | + | *Salmonella* |
| SG8Y | yes | yes | - | A/A/+ | - | - | +/+/+ | + | *Proteus* |
| SG8Ca | yes | yes | - | A/A/+ | - | - | +/+/+ | + | *Proteus* |
| SG8Cb | yes | no | - | A/A/- | + | - | -/-/- | + | *Klebsiella* |

**Table 6.2: Antibiotic testing of bacterial strains isolated using culture dependent methods**

| Identification of section | AM10a | CB100 | P10 | GM10 | S10 | E15 | TE30 | CIP | C30 | CC2 | SSS20 | AN30 | ID |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Gut 1 | 0 | 25 | 0 | 21 | 20 | 0 | 0 | 35 | 25 | 0 | 0 | 27 | *Enterobacter* |
| Gut 2 | 0 | 0 | 0 | 0 | 0 | 0 | 16 | 35 | 25 | 0 | 0 | 20 | *Burkholderia* |
| Gut 3 | 0 | 0 | 0 | 11 | 20 | 14 | 14 | 40 | 25 | 0 | 0 | 21 | *Salmonella* |
| Gut 4 | 0 | 0 | 0 | 13 | 19 | 12 | 0 | 32 | 25 | 0 | 0 | 21 | *Salmonella* |
| Gut 6 | 0 | 0 | 0 | 0 | 0 | 11 | 20 | 32 | 25 | 0 | 0 | 21 | *Burkholderia* |
| Gut 7 | 0 | 30 | 0 | 20 | 25 | 31 | 0 | 34 | 20 | 0 | 0 | 34 | *E.coli* |
| Gut 8 | 0 | 30 | 0 | 25 | 12 | 0 | 0 | 40 | 15 | 0 | 0 | 21 | *Bacillus* |
| Gut 9 | 0 | 27 | 0 | 31 | 24 | 32 | 0 | 40 | 21 | 0 | 0 | 32 | *E.coli* |
| Gut 10 | 0 | 30 | 0 | 26 | 25 | 31 | 21 | 44 | 22 | 0 | 0 | 30 | *E.coli* |
| Gut 11 | 0 | 30 | 0 | 24 | 15 | 0 | 0 | 43 | 16 | 0 | 0 | 25 | *Bacillus* |
| Gut 12 | 0 | 25 | 0 | 30 | 31 | 0 | 15 | 32 | 25 | 0 | 0 | 32 | *Shigella* |
| FA 1 | 0 | 0 | 0 | 33 | 30 | 0 | 12 | 45 | 25 | 0 | 0 | 21 | *Serratia* |
| FA 2 | 0 | 0 | 0 | 10 | 21 | 0 | 18 | 45 | 25 | 0 | 0 | 20 | *Salmonella* |
| FA 3 | 0 | 27 | 0 | 31 | 25 | 27 | 0 | 45 | 23 | 0 | 0 | 33 | *E.coli* |
| FA 5 | 0 | 23 | 0 | 24 | 17 | 0 | 0 | 42 | 24 | 0 | 0 | 21 | *Serratia* |
| FA 6 | 0 | 25 | 0 | 30 | 22 | 0 | 21 | 40 | 25 | 0 | 0 | 25 | *Shigella* |
| SG 2 | 0 | 0 | 0 | 25 | 15 | 12 | 12 | 40 | 25 | 0 | 0 | 30 | *Salmonella* |
| SG 3 | 0 | 30 | 0 | 23 | 23 | 0 | 0 | 44 | 26 | 0 | 0 | 31 | *Citrobacter* |
| SG 4 | 0 | 0 | 0 | 15 | 22 | 10 | 12 | 43 | 23 | 0 | 0 | 27 | *Salmonella* |
| SG 5 | 0 | 0 | 0 | 22 | 21 | 0 | 0 | 41 | 23 | 0 | 0 | 22 | *Serratia* |
| SG 6 | 0 | 0 | 0 | 11 | 18 | 11 | 21 | 40 | 25 | 0 | 0 | 25 | *Salmonella* |
| SG 7 | 0 | 0 | 0 | 12 | 18 | 14 | 10 | 32 | 25 | 0 | 0 | 23 | *Salmonella* |
| SG 8 | 0 | 0 | 0 | 11 | 20 | 12 | 6 | 35 | 25 | 0 | 0 | 21 | *Salmonella* |

| Identification of section | AM10a | CB100 | P10 | GM10 | S10 | E15 | TE30 | CIP | C30 | CC2 | SSS20 | AN30 | ID |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Tho 1 | 0 | 0 | 0 | 23 | 21 | 0 | 0 | 35 | 25 | 0 | 0 | 33 | *Serratia* |
| Tho 2 | 0 | 27 | 0 | 25 | 14 | 0 | 0 | 43 | 21 | 0 | 0 | 33 | *Citrobacter* |
| Gut 5a | 0 | 30 | 0 | 17 | 21 | 0 | 11 | 42 | 23 | 0 | 0 | 24 | *Klebsiella* |
| Gut 5b | 0 | 26 | 0 | 22 | 24 | 26 | 12 | 40 | 22 | 0 | 0 | 27 | *E.coli* |
| Gut 6a | 0 | 24 | 0 | 30 | 26 | 0 | 20 | 38 | 25 | 0 | 0 | 5 | *Shigella* |
| Gut 6b | 0 | 30 | 0 | 25 | 21 | 0 | 10 | 37 | 24 | 0 | 0 | 32 | *Proteus* |
| FA 4a | 0 | 24 | 0 | 24 | 21 | 0 | 12 | 41 | 23 | 0 | 0 | 21 | *Klebsiella* |
| FA 4b | 0 | 25 | 0 | 21 | 25 | 0 | 0 | 39 | 24 | 0 | 0 | 23 | *Enterobacter* |
| FA 7a | 0 | 23 | 0 | 23 | 22 | 0 | 0 | 40 | 25 | 0 | 0 | 30 | *Bacillus* |
| FA 7b | 0 | 0 | 0 | 31 | 21 | 0 | 0 | 41 | 25 | 0 | 0 | 2 | *Serratia* |
| SG1a | 0 | 0 | 0 | 12 | 19 | 12 | 21 | 44 | 21 | 0 | 0 | 25 | *Salmonella* |
| SG1b | 0 | 25 | 0 | 26 | 21 | 0 | 18 | 44 | 20 | 0 | 0 | 27 | *Klebsiella* |
| FA7aC | 0 | 0 | 0 | 10 | 25 | 12 | 12 | 45 | 25 | 0 | 0 | 24 | *Salmonella* |
| FA7aY | 0 | 24 | 0 | 23 | 22 | 0 | 11 | 45 | 24 | 0 | 0 | 31 | *Proteus* |
| Gut5bp | 0 | 25 | 0 | 33 | 21 | 0 | 12 | 45 | 23 | 0 | 0 | 26 | *Klesbsiella* |
| Gut5bs | 0 | 25 | 0 | 23 | 20 | 0 | 0 | 45 | 23 | 0 | 0 | 21 | *Enterobacter* |
| SG1aY | 0 | 30 | 0 | 24 | 22 | 0 | 0 | 42 | 25 | 0 | 0 | 24 | *Citrobacter* |
| SG1aC | 0 | 0 | 0 | 12 | 21 | 12 | 15 | 40 | 21 | 0 | 0 | 23 | *Salmonella* |
| SG4COL | 0 | 24 | 0 | 24 | 22 | 0 | 12 | 40 | 25 | 0 | 0 | 30 | *Proteus* |
| SG4SWA | 0 | 0 | 0 | 20 | 21 | 14 | 21 | 42 | 25 | 0 | 0 | 26 | *Salmonella* |
| SG8Y | 0 | 25 | 0 | 26 | 25 | 0 | 10 | 38 | 25 | 0 | 0 | 31 | *Proteus* |
| SG8Ca | 0 | 24 | 0 | 12 | 24 | 0 | 9 | 39 | 24 | 0 | 0 | 33 | *Proteus* |
| SG8Cb | 0 | 30 | 0 | 25 | 21 | 0 | 20 | 41 | 25 | 0 | 0 | 26 | *Klebsiella* |

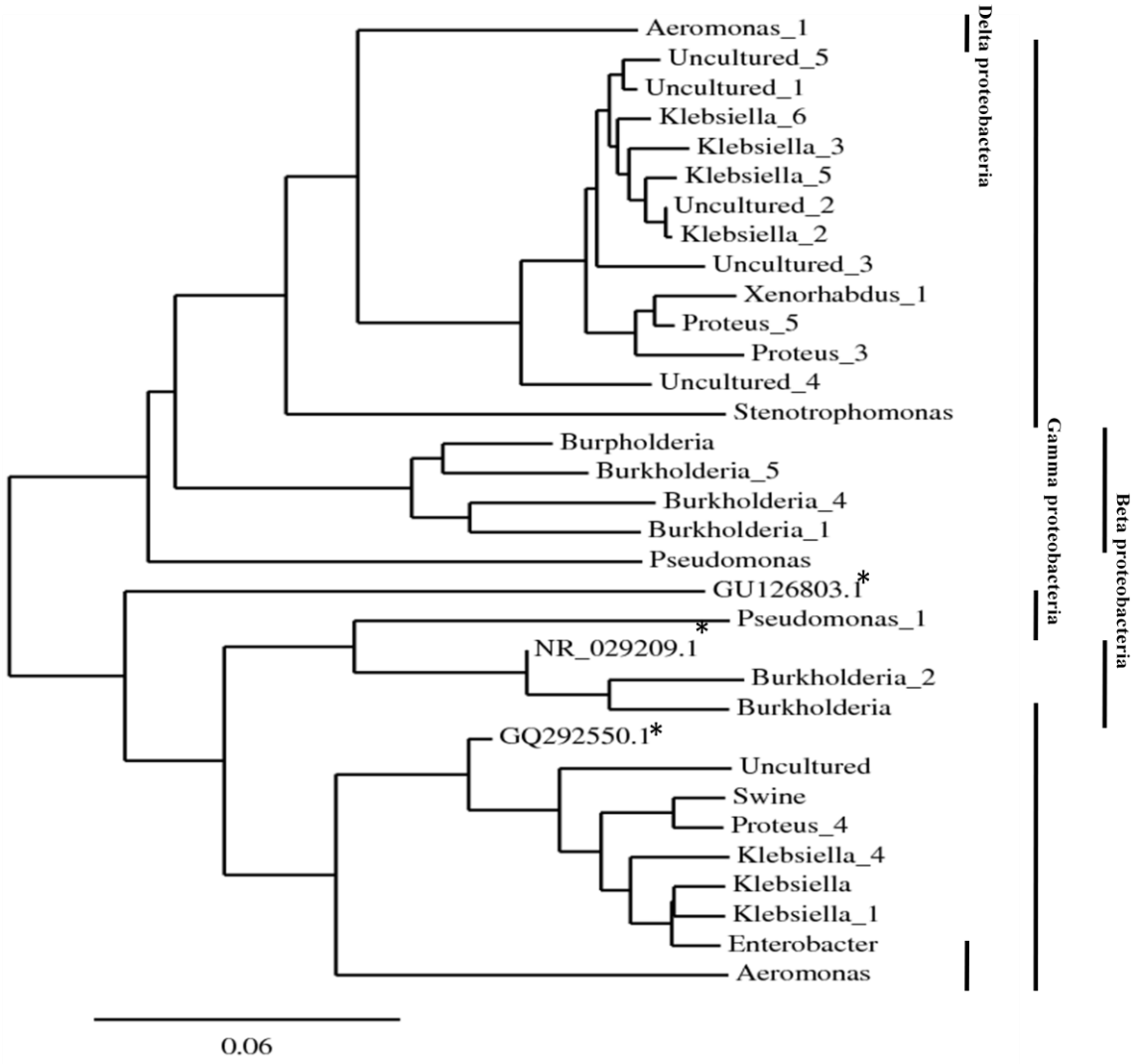


**Figure 6.1: Phylogenetic tree constructed for partial 16S rRNA gene of bacterial strains from Belostomatids**

* Reference strains; GU126803.1| *Klebsiella pneumonia*, NR_029209.1| *Burkholderia cepacia*, GQ292550.1| *Proteus vulgaris*

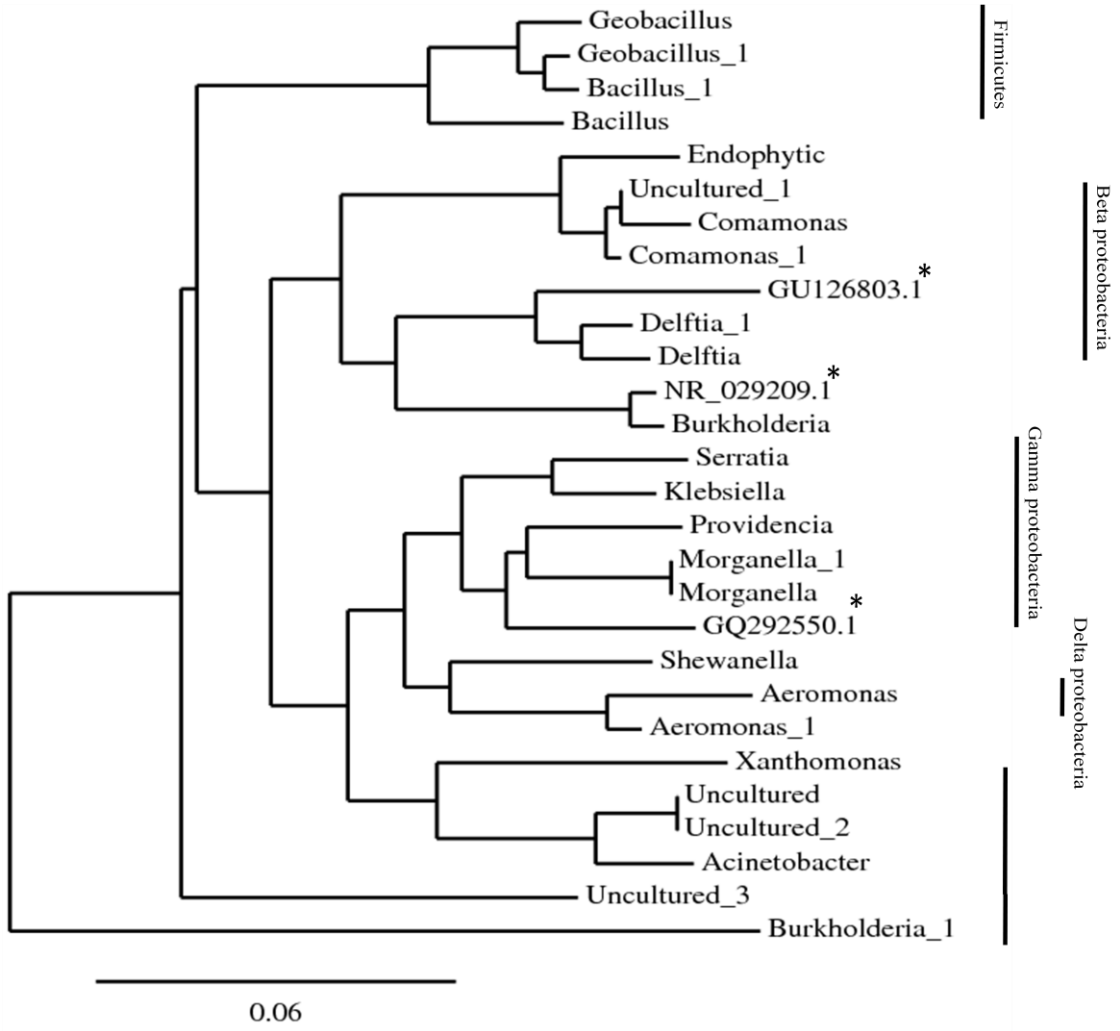


**Figure 6.2: Phylogenetic tree constructed for partial 16S rRNA gene of bacterial strains from Naucorids**

* Reference strains; GU126803.1| *Klebsiella pneumonia*, NR_029209.1| *Burkholderia cepacia*, GQ292550.1| *Proteus vulgaris*

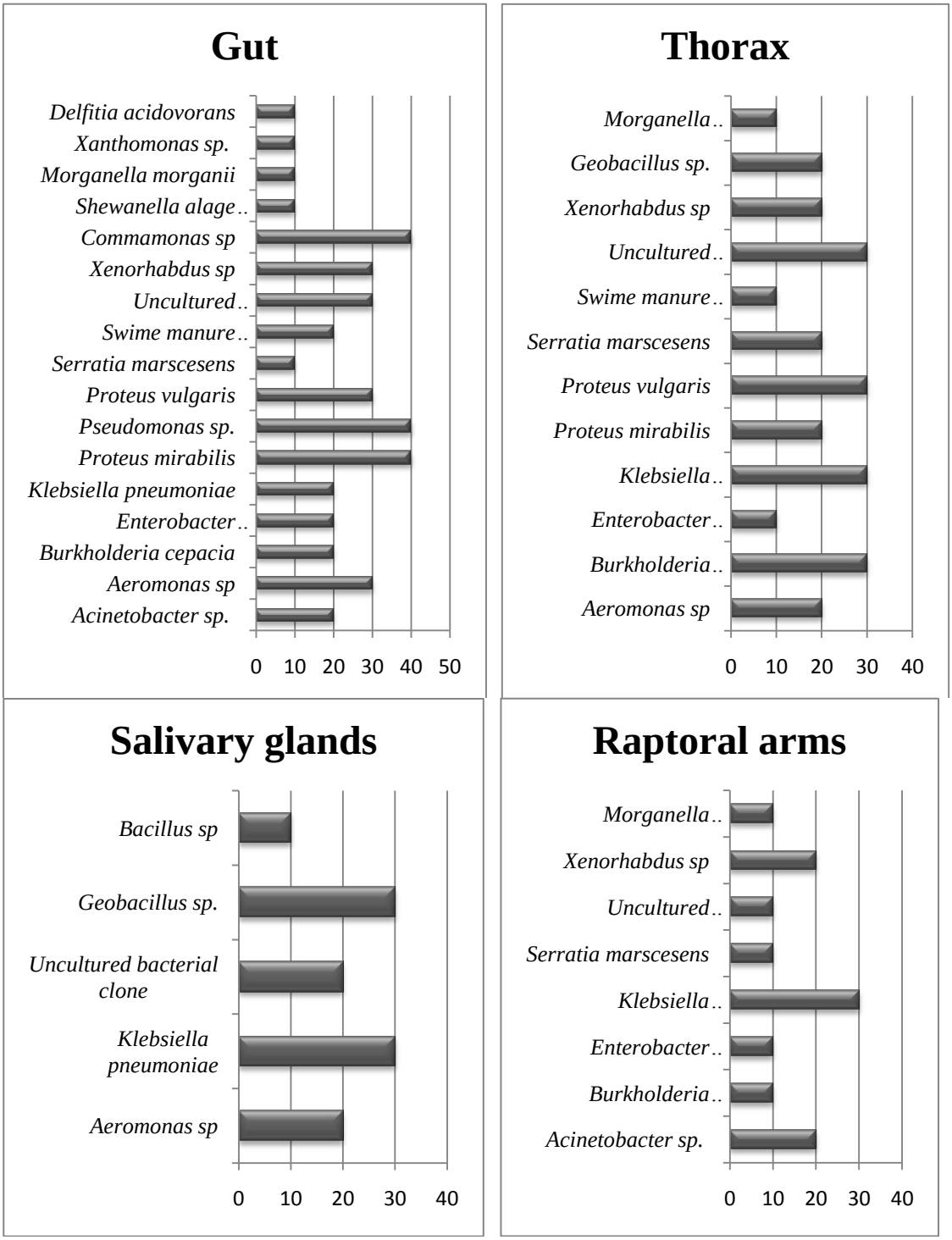


**Figure 6.3: Percentage of insects positive for each bacterial strains identified using 16S rRNA sequencing**

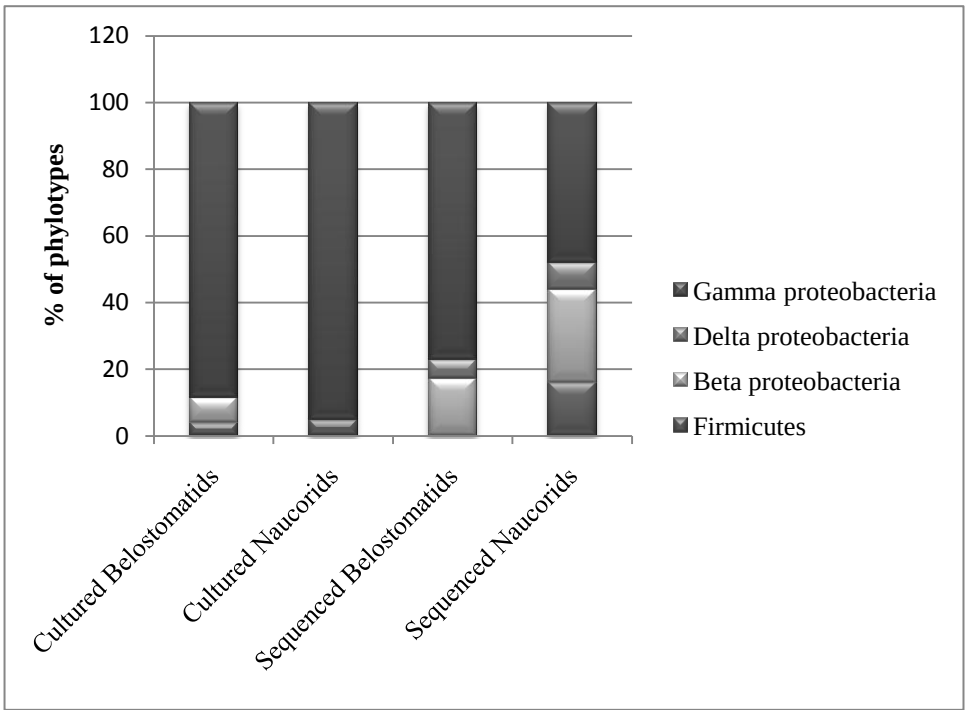


**Figure 6.4: Percentage abundance of phylotypes within culturable isolates and 16S rRNA gene library strains of bacteria in Belostomatids and Naucorids**

***Concluding remarks***

Buruli ulcer continues to remain a devastating disease in tropical rural parts of the world especially Africa. Both the disease and the causative organism, *Mycobacterium ulcerans* are of key interest to researchers because of the unusual patterns of the disease pathology and the biology of the bacterium repectively. Unlike other infections with environmental mycobacteria, the painless nature and slow progression of Buruli ulcer, makes it so devastating. Affected individuals seek health care only at the late and ulcerative stages of the disease. The stigma associated with the disease only goes to further retard its reporting. On the part of the causative organism, the fact that its DNA has been associated with many aquatic organisms, reveals a complex biology that makes it difficult to determine in which particular organism *M. ulcerans* is replicating. The hydrophobicity of the bacterium makes it concentrate at the air water interface in the environment and makes it readily form biofilms of the surfaces of many organisms.

Researchers have attributed the re-emergence of Buruli ulcer to the overlapping ecology of man and environmental mycobacteria. Man-made environmental disturbances, particularly deforestastion, urbanization and sandwining as well as natural flooding that occur due to rainfall patterns have been implicated as risk factors in the epidemiology of the disease. Noteworthy in the transmission of the disease is that person-to-person transfer of the bacterium is rare and that man becomes infected primarily through dierect contact with the environment. This signifies the true environmental nature of *M. ulcerans*, implying that it does not need the human host to proliferate in nature. The hypothesis that trauma to the skin is necessary for the introduction of *M. ulcerans* to the host hence bears significant validity. What remains to be addressed then is the identification of the environmental reservoir of the bacterium and the potential vectors that can cause injury to the skin and ultimately deliver an infective dose of *M. ulcerans* to unsuspecting human hosts.

Advances in research identifying various aquatic organisms that are PCR positive for *M. ulcerans* DNA suggests that the bacterium is being transferred within a complex food web. This implies two possibilities; (i) that the bacterium has a high diversity of metabolism, thus being able to proliferate in more than one organism, or (ii) that the

bacterium is contained within a secondary organism such as a protozoan, which is also associated with similar aquatic organisms as *M. ulcerans*. It is also important to note here the ongoing reductive evolution of *M. ulcerans* from its ancestor *M. marinum*. This has been characterized by significant genome contraction by the generation of pseudogenes and DNA rearrangement leading to the loss of coding sequences that may have allowed *M. ulcerans* to occupy similar niches as *M. marinum*. A good example is the truncation of the *crtB* locus in *M. ulcerans*. In *M. marinum*, this locus is responsible for the production of light induced carotenoids that protect the bacteria from direct sunlight (Stinear 2007). The absence of a full gene product in *M. ulcerans*, makes the bacterium sensitive to light indicating a change in its environmental niche. Other bacteria that have undergone significant genome contraction and niche specialization include *M. leprae*, *Yersinia pestis* and *Bordetella pertusis* (3).

The acquisition of the mycolactone producing plasmid is thought to have occurred by lateral gene transfer (5), however, the specific role for the toxin in the environment has not been elucidated. Is it possible that the toxin confers a selective advantage for niche domination in the environment? Is the toxin synthesized by the bacterium in the environment or only in the human host? Which phenomenon occurred fisrt; the genome downsizing or the acquisition of the plasmid? Why are the other mycolactone producing mycobacteria such as *M. liflandii* and *M. pseudoshottsii* associated with definite environmental hosts and what is the evolutionary history between these strains and *M. ulcerans*?

In the search for answers to the mode of transmission of *M. ulcerans* to man, it is imperative to experimentally test the findings in nature in a laboratory setting. The work presented in this study has sought to understand the role of insects and fish in transmission. We have shown that due to the waxy nature of *M. ulcerans*, it readily forms an extracellular matrix on the surface of Belostomatids, and hence can be directly introduced to unsuspecting humans that are accidentally bitten. We do not have conclusive evidence that the bacterium replicates within these insects but we cannot rule out the possibility of them being involved in the transfer of the bacterium in nature. The history with bacteria that are vectored primarily by insects involves a long evolutionary process of co-adaptation, and most of the insects are haematophagous. This is not the

case with Belostomatids or Naucorids; they are both predaceous. It is possible that aquatic insects may play other roles in the transmission process and future studies should specifically address the biology of the bacterium with respect to the biting insects.

We also show that fish may transiently contain *M. ulcerans* in the environment, however, unlike most environmental mycobacteria, *M. ulcerans* does not cause disease in fish (Japanese Medaka). Even though *M. marinum DL* and *M. pseudoshottsii* produce mycolactone and cause disease in fish, *M. ulcerans* specifically only produces disease in humans. Future studies should involve identifying whether *M. ulcerans* is pathogenic in other types of fish such as zebra fish and gold fish because they might exhibit different immune responses. We have also shown that mycolactone is cytotoxic to fish cells in vitro. Although the precise molecular mechanism of mycolactone on eukaryotic cells is unknown, it has been shown to accumulate in the cytosol eventually leading to cell cycle arrest and apoptosis (4). The effects of mycolactone are also ubiquitous, and not cell specific.

Despite the action of the toxin, the possibility of *M. ulcerans* living within protozoans is a hypothesis worth testing. Other environmental mycobacteria have been shown to replicate within *Acanthamoeba* (1). Recently, it was shown that mycobacteria are capable of foming spores (2). This may contribute to the persistence of *M. ulcerans* in the environment and also account for the dormancy and latency of the bacterium in nature. Much work is yet to be done to specifically understand the mode of transmission of *M. ulcerans*. This work is just a tip of the iceberg!

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***Vita***

Lydia Mosi received her Bachelor of Science degree with Honors from the University of Ghana Legon in 2003 with a concentration in Zoology and Biochemistry. She worked briefly at the Noguchi Memorial Institute for Medical Research, on identifying the distribution of the proposed insect vectors of Buruli ulcer transmission. In 2004, she began graduate studies in the Department of Microbiology at the University of Tennessee in Knoxville. She was awarded an award for outstanding graduate teaching assistant, Cokkianas outstanding graduate student award and the Association for Women in Science pre-doctoral award. She has also received several awards for travel including the David White travel award. She has also participated in several meetings involving microbiology and Buruli ulcer. She received her doctorate degree in Microbiology in December 2009. Following graduation, she plans to return to Ghana to work at the Noguchi Memorial Institute for Medical Research continuing her research into transmission of Buruli ulcer disease.