FISH AND AMPHIBIANS AS POTENTIAL RESERVOIRS OF *MYCOBACTERIUM ULCERANS*, THE CAUSATIVE AGENT OF BURULI ULCER DISEASE

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ABSTRACT

FISH AND AMPHIBIANS AS POTENTIAL RESERVOIRS OF *MYCOBACTERIUM ULCERANS*, THE CAUSATIVE AGENT OF BURULI ULCER DISEASE

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Buruli ulcer is a skin disease associated with exposure to certain tropical water bodies. Much remains unknown about the reservoir and transmission of this disease. Previous studies have suggested that fish may concentrate *M. ulcerans* in their gills and intestines and then serve as passive reservoirs of the bacteria. The current study was designed to expand on earlier studies by using a PCR-based assay targeting the enoyl reductase (ER) domain of the plasmid responsible for mycolactone production to screen multiple species of fish and amphibians from multiple water bodies where *M. ulcerans* has been previously detected. This was done to test the hypothesis that fish and amphibians serve as natural reservoirs of *M. ulcerans*, with some taxa or feeding guilds being more likely to harbor the pathogen than others. ANOVA and non-metric multidimensional scaling showed no fish or amphibian species or feeding guild served as a reliable indicator of the presence of ER-positive mycobacteria in a water body. However, specimens from certain water bodies were observed to have higher ER-positivity rates than others. *M. ulcerans* in an adult frog was found and confirmed with VNTR analysis. This is the first reported finding of the bacterium in a wild adult amphibian and suggests that amphibians may warrant further study.

DEDICATION

Non nobis Domine, sed Tuo Nomine da gloriam.

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Introduction

Mycobacterium ulcerans MacCallum is the causative agent of Buruli ulcer disease (MacCallum et al. 1948). In humans, Buruli ulcer disease manifests first as a small nodule and can progress to extensive skin ulcerations. This disease tends to occur in tropical and subtropical areas and is especially prevalent in the West-African countries of Benin, Ivory Coast, and Ghana (W.H.O. 2008). On a large scale, Buruli ulcer disease has been correlated with agricultural land use (Wagner et al. 2008). Local outbreaks have been linked to exposure to certain water bodies but the reservoir(s) and method of transmission of *M. ulcerans* have not been determined (Aiga et al. 2004; Jacobsen and Padgett 2010; Merritt et al. 2010; Raghunathan et al. 2005; Sopoh et al. 2010).

M. ulcerans is believed to have originated from the closely related *Mycobacterium marinum* Aronson, a common environmental bacterium (Stinear et al. 2000). Though *M. ulcerans* is also traditionally considered to be an environmental pathogen, recent analyses show that the genome of *M. ulcerans* is contracting, consistent with an evolutionary shift from a free-living ancestor to use of the more stable environment provided by a specific host (Stinear et al. 2007). In addition, *M. ulcerans* has acquired a virulence plasmid, pMUM001, which allows *M. ulcerans* to produce mycolactone. Mycolactone is a toxin that may play an important role in allowing *M. ulcerans* to use specific hosts. For example, salivary glands of certain aquatic insects can be colonized by wild-type *M. ulcerans*, but not by mutant mycolactone-negative *M. ulcerans* (Marsollier et al. 2005). PMUM001 occurs only in *M. ulcerans* and a few closely related mycolactone-producing mycobacteria recently discovered in fish (Rhodes et al. 2005; Ranger et al. 2006) and frogs (Trott et al. 2004). Though currently given separate species names,

it has been argued these other mycolactone-producing mycobacteria are simply different strains of *M. ulcerans* (Pidot et al. 2010).

Possible hosts of *M. ulcerans* include predaceous aquatic insects (Marsollier et al. 2002), fish (Kotlowski 2004), snails (Marsollier et al. 2004), terrestrial mammals (Fyfe et al. 2010) and mosquitoes (Johnson 2009). In aquatic environments *M. ulcerans* may be concentrated by small filtering organisms, such as filter feeding insects, which are then preyed upon by larger organisms, causing further concentration of the bacteria (Portaels et al. 1999; Merritt et al. 2005). Marsollier et al. (2005) suggested that *M. ulcerans* may be transferred to humans through the bite of aquatic Hemiptera. However, this is unlikely to be the primary method of transmission (Benbow et al. 2008), nor have these insects been shown to be a vector of any pathogen associated with animals in nature (Merritt et al. 2010). More recent experimentation by Mosi et al. (2008) showed colonization by *M. ulcerans* of the exoskeleton of biting water bugs (Hemiptera: Belostomatidae) that had fed on *M. ulcerans* infected mosquito larvae. This indicates that insects may still play a role as a reservoir in the transmission cycle.

Previous studies have suggested that fish may concentrate *M. ulcerans* in their gills and intestines and then serve as passive reservoirs of the bacteria (Portaels et al. 2001; Eddyani et al. 2004; Kotlowski 2004). In these studies, the gills and intestines of several fish were found to be positive for IS*2404*, an insertion sequence that occurs 209 times on the *M. ulcerans* chromosome and four times on pMUM001 (Stinear et al. 2007). This insertion sequence was previously thought to be specific to *M. ulcerans*, but is now known to occur in other mycobacteria (Yip et al. 2007). The use of the gills and intestine in the above studies raises the question of whether fish were actually colonized by *M. ulcerans*, or whether it was simply being trapped by their gills and passing through their digestive tracts. Feeding trials with frogs have shown that mycobacteria

can survive passage through the digestive tract and be passed out through the feces (Mok et al. 1987). Testing of internal organs other than the digestive tract is needed to show whether wild fish are actually becoming infected with *M. ulcerans*.

In addition, it is possible that *M. ulcerans* may be localized in organs other than the intestine and gills. Experimental exposure of *Danio rerio* Hamilton to *M. marinum* and *Mycobacterium peregrinum* Bojalil showed that these mycobacteria are primarily acquired through the intestine, and then disseminate to other internal organs. Though mycobacteriosis in fish can potentially involve any of the organs, it most commonly involves the kidney and liver (Belas et al.1995). With fish artificially infected with *M. marinum* and *M. peregrinum*, mycobacteria were subsequently recovered from the intestines, livers, and spleens of infected fish, but never from the gills (Harriff et al. 2007). It is possible that testing only the gills and intestines of fish may have resulted in an underestimation of rates of infection.

The discovery of mycolactone-producing mycobacteria in frogs has also raised the question of whether or not *M. ulcerans* might also occur in frogs. Many tadpoles feed by filtering or scraping small algal particles, and could conceivably acquire and concentrate *M. ulcerans* during this process, as suggested by Merritt et al. (2005). Though historically considered to be an extracellular mycobacterium, *M. ulcerans* is capable of growing inside amphibian cells at 28ºC (Drancourt et al. 2002).

It is unclear if the fish tested in earlier experiments were actually positive for *M. ulcerans* or if the positive IS*2404* tests resulted from the presence of other mycobacteria. More specific PCR methods have recently been developed. Enoyl reductase (ER) PCR targets the ER domain of the pMUM001 plasmid (Williamson et al. 2008), which only occurs in mycolactone producing mycobacteria. This target occurs in only four places on the plasmid, making ER-PCR

less sensitive but more specific than IS*2404* PCR (Durnez et al. 2009). Variable number tandem repeat (VNTR) DNA typing is also being used to distinguish between *M. ulcerans* and other mycolactone-producing mycobacteria. VNTR loci occur in varying numbers, with some occurring only once in the genome making this the least sensitive, though highly specific, PCR detection method (Lavender et al. 2008). Additionally, positive assay results are dependent on currently known VNTR profiles, and it is likely that additional profiles exist.

The current study was designed to expand on earlier studies by screening multiple species of fish and amphibians from multiple water bodies where *M. ulcerans* has been previously detected. This was done to test the hypothesis that fish and amphibians serve as natural reservoirs of *M. ulcerans,* with some taxa or feeding guilds being more likely to harbor the pathogen than others. As such, it was predicted that certain fish and amphibian taxa could be used as indicator species for the presence of environmental conditions favorable to *M. ulcerans*. In addition, because fish and amphibians are mobile (increasing the chance of contact with the bacterium), and provide a stable environment for the bacterium, PCR testing of fish and amphibians would allow for more consistent detection of *M. ulcerans* in a water body than other environmental sampling methods such as water filters. To this end, a survey of fish and amphibian taxa was conducted in *M. ulcerans* positive and *M. ulcerans* negative sites. Specimens were collected from *M. ulcerans* endemic areas for subsequent PCR screening for mycolactone-producing mycobacteria, and positivity rates were compared for internal organs versus external swabs, between different taxa, and between feeding guilds.

Methods

Study Location

This study was carried out as part of a larger study on the ecology of *M. ulcerans* in West Africa. In July 2008, samples were collected in Ghana, along a gradient from the Volta Region, where no *M. ulcerans* or mycolactone-producing mycobacteria have been detected, to the Greater Accra area, where *M. ulcerans* and other mycolactone-producing mycobacteria have been detected at multiple locations (Williamson et al. 2008). A second sampling season occurred August 2009, in the Ga West and Tema districts of Accra, Ghana, where *M. ulcerans* was previously detected (Williamson et al. 2008).

Collection and Preservation of Specimens

Three sampling techniques were employed at each site to maximize the variety of taxa captured. Three collapsible live bait traps (Promar model TR-501, Gardena, CA) were deployed at each site and baited with commercial catfish dough bait (Berkley, Spirit Lake, IA), and the traps were set as far apart as possible around the site. Traps were submerged for approximately one hour at each site. In addition, a seine (Cabela's Inc., Sidney, NE) was used when the sites were wadeable and without abundant aquatic vegetation. A D-net (Bioquip Products, Rancho Dominguez, CA) was used to sample marginal areas and within aquatic plant beds. Approximately one hour of combined seining and D-net sampling was performed at each site. When adult frogs were observed at a site, they were captured with D-nets. Up to 30 individuals of each fish and amphibian taxon present at the site were captured, and excess individuals were released. The one exception to these methods was Lake Weija (2009 Site 9), where live fish were purchased from local fishermen. Fish and tadpoles were euthanized with CO_2 and adult

frogs were euthanized with benzocaine hydrochloride (IACUC AUF # 06/08-090-00). Buckets used to hold captured fish and amphibians were cleaned and treated with RNase away™ (Molecular Bioproducts, Inc., San Diego, CA) between sites.

In addition to collecting fish and amphibians, 500 ml of water was also collected and filtered at each site during season two, to test for the presence of *M. ulcerans*. This water was pre-filtered through a 1.6 μm fiberglass filter (Whatman Inc., Kent, UK) to remove large particles and then filtered through a 0.2 μm nitrocellulose filter (Whatman Inc., Kent, UK). If the filter became clogged multiple filters were used. The filter apparatus was cleaned with RNase Away™ between each site. The filters were saturated with 95% ethanol for preservation and placed in foil wrappers for transport to Michigan State University, where they were stored at -20ºC until processing.

In 2008, all tadpoles were preserved in 10% formalin and all fish were preserved in 95% ethanol, and all organisms from a single site were grouped together. For 2009, half of all specimens (fish and tadpoles) were preserved in 10% formalin and half were preserved in 95% ethanol. Specimens in ethanol were either preserved individually or pooled into groups of 3 depending on size.

Fish and amphibians were identified to the lowest taxonomic level possible. Fish were identified to genus and in most cases species level using Dankwa et al. (1999). Amphibians were identified to the lowest possible taxonomic level using Channing and Howell (1999) and Schiotz (1999). In most cases this was the generic level. Many tadpoles cannot be identified to the species level, and the tadpoles of numerous species are not known. In addition, tadpole morphology changes during development, and tadpole keys are based on fully grown tadpoles (Channing and Howell 1999). Specimens were dissected and the intestines and kidneys removed

and stored in 95% ethanol. Instruments were cleaned between specimens using 95% ethanol followed by application of RNase Away™.

DNA Extraction

Year 2009 samples preserved in 95% ethanol were screened for *M. ulcerans* DNA. All DNA extraction and PCR procedures were performed in a hood, under sterile conditions. Negative controls were used throughout the process to ensure sterility and assess possible contamination. *M. ulcerans* cells preserved in 95% ethanol were used as a positive control to ensure that procedures were successful. For the dissected intestine and kidney samples, ethanol was poured off of each sample and reserved in a sterile container. The wet mass of the remaining sample was recorded and the sample was then homogenized with the previously reserved ethanol in a glass tissue grinder. For small samples (less than or equal to 20 mg wet mass), the entire sample was used for DNA extraction. For large samples, homogenate containing approximately 20 mg of tissue was used for DNA extraction. In order to remove the ethanol from the samples before DNA extraction, samples were centrifuged and the ethanol was removed. The remaining pellet was rinsed with TE, centrifuged, and the supernatant was removed.

Extraction was first attempted using the one tube method designed for extraction of *M. ulcerans* in aquatic insects, mollusks, and fish (Kotlowski et al. 2004). However, satisfactory detection was not achieved with this method. DNA was then extracted using a method optimized for environmental mycobacteria (Käser et al. 2009), with the exception of the final step, in that DNA was re-suspended in 25 µl TE instead of 100 µl water. Extractions were performed in groups of 18 samples, and each group included a negative and a positive control. Extracted DNA

from a subset of 27 intestine samples and 23 kidney samples was spiked with *M. ulcerans* DNA prior to PCR analysis to determine the level of PCR inhibition. Due to the presence of inhibitors, it was necessary to further purify the extracted intestinal DNA according to manufacturer's instructions using PowerClean® DNA Clean-Up Kits (MO BIO Laboratories, Inc., Carlsbad, CA), and eluting with 50 µl of the provided elution solution. Negative and positive controls were also cleaned using this procedure. Because the clean-up of the intestine samples resulted in more dilute DNA than that of the kidney samples, 8 µl of intestine sample DNA versus 4 µl kidney sample DNA was used per 25 µl PCR mixture.

PCR and Gel Electrophoresis

PCR was performed targeting the enoyl reductase domain of the plasmid responsible for mycolactone production. The 25 µl PCR cocktails contained 2 µl of each primer (10 µM) (F: 5'-GAGATCGGTCCCGACGTCTAC-3', R: 5'-GGCTTGACTCATGTCACGTAAG-3'), 12.5 µl FailSafe™ 2x PCR buffer (EPICENTRE Biotechnologies, Madison, WI), 0.5 µl Go Taq polymerase enzyme (Promega, Madison, WI), and either 4 µl of template DNA and 4 µl PCR water (for kidney samples), or 8 µl template DNA (for intestine samples). PCRs were run using primers and cycling conditions described in Williamson et al. (2008). Samples were loaded along with positive and negative controls onto 0.8% TBE agarose gels stained with Ethidium Bromide and fragment sizes were compared to a 1 Kb DNA ladder (Invitrogen, Carlsbad, CA). ER-positive samples were then sent to the University of Tennessee for VNTR analysis as previously described (Williamson et al. 2008).

Serial dilutions were made from a stock sample of *M. ulcerans* cells preserved in 95% ethanol. The stock solution contained approximately 10^7 CFUs/ml. Aggregates of bacteria were

broken apart by passing the bacterial suspension through a 25 gauge needle 10 times (Williamson et al. 2008) and eight 1:10 serial dilutions were made. To determine whether sensitivity was changed by the addition of fish tissue, the internal organs of a commercially purchased goldfish were dissected and processed using the previously mentioned techniques. 180 µl of 0.1 mg/µl (wet mass) tissue homogenate was spiked with 20 µl of each of the previously made dilutions before being subjected to the Käser (2009) extraction procedure and ER PCR.

Data Analyses

For data collected in 2008, a Jaccard dissimilarity matrix was constructed separately for both fish and amphibian communities. Separate non-metric multidimensional scaling (NMS) analyses were performed on fish and amphibian communities using species presence or absence data to determine if these communities were related to ER positivity. A scree plot was used to determine the optimal number of dimensions to characterize the data. Once the optimal number of dimensions was determined, 1000 initial starts were used for subsequent analysis. Additionally, an ANOSIM was performed using 10,000 permutations to determine if there were differences between ER positive and ER negative communities and an indicator species analysis was performed to determine if specific taxa could be used to predict ER positivity. The above statistical tests were performed using R version 2.13.1 (http://R-project.org, R Foundation for statistical Computing, Vienna, Austria), with the ecodist (Goslee and Urban 2007), labdsv (Roberts 2010), ellipse (Murdoch and Chow 1996), BiodiversityR (Kindt and Coe 2005), and vegan (Oksanen et al. 2011) packages. A site was considered to be ER positive if any ER positive filters had ever been recovered from the site (Heather Williamson, unpublished data). For 2009, a separate one way ANOVA was performed on arcsine square root transformed

percent positive values for fish and amphibians, with site and species as factors. For fish an additional ANOVA was performed using feeding guild as a factor. One location (Djorse) consisted of two different water types, a lotic stream next to a lentic pond. Due to the differing habitats, the location was classified as two different sites (Sites 1 and 2) in the analyses. Site 6 (Mensah Bar) was not included in season 2 analyses due to consistently ambiguous ER results for that site. Results were considered to be significant at $\alpha=0.05$. For significant ANOVAs, Tukey Kramer post-hoc multiple comparison procedures were performed. JMP® Statistical Discovery Software, version 7.0 (www.jmpin.com, SAS Institute, Inc., Cary, NC) was used for ANOVAs.

Results

A total of 624 fish, representing 13 genera and at least 17 species, and a total of 350 amphibians, representing 10 genera, were collected (Tables 1 and 2). A scree plot showed a three dimensional solution in the NMS analysis. NMS analyses showed that ER positivity was not associated with fish or amphibian community structure (Figures 1 & 2). ANOSIM verified these results (fish: p-value = 0.089191; amphibian: p-value = 0.38766). Indicator species analysis showed a significant association of fish species *Barbus sublineatus* with ER positivity (p-value = 0.038), however it had a low indicator value of 0.4333.

Of the 271 intestine samples that were subjected to DNA extraction, the resulting DNA from 141 samples (52%) was visibly discolored and showed PCR inhibition. A sub-sample of 27 intestinal DNA extractions that were not visibly discolored was spiked with *M. ulcerans* DNA to determine the level of inhibition. Of these, 63% showed signs of inhibition. Of the 23 kidney samples randomly selected and spiked with *M. ulcerans* DNA, none showed evidence of inhibition. Spin columns were used in an attempt to remove inhibitors from the discolored kidney samples. While this did result in removal of most of the brown coloration from the samples, the PCRs remained completely inhibited. The MO BIO cleanup kit removed both the brown coloration and the PCR inhibition.

With the one-tube procedure, the lowest detection limit using ER PCR was approximately 1,600 CFUs. With the Käser extraction procedure, the lowest detection limit was 0.32 CFUs. Addition of goldfish tissue homogenate did not decrease the detection limit, but additional non-specific binding was observed.

Negative controls consistently showed lack of contamination and positive controls consistently showed that the extraction and PCR were successful. Site 6 (Mensah Bar) was excluded from statistical analyses due to ambiguous PCR results for that site. PCRs of specimens from this site consistently produced a fragment which was slightly larger than the expected ER fragment, but was so close in size that it was not possible to conclusively determine whether the specimens were ER positive. Overall ER positivity rates were 54% (128 / 238). For fish, ER positivity was 39% (25 / 65), tadpoles were 59% (96 / 162), and adult frogs were 64% (7 / 11). Positivity rates for all specimens from a single site ranged from a low of 6% at site 1 to 74% at site 8 (Figure 3). ANOVA results showed ER positivity rates for fish were significantly different among locations (p-value = 0.0054), with a Tukey test indicating that specimens from sites 2, 3, and 4 were most highly positive. ER positivity rates were not significantly different between fish species or among feeding groups. Amphibian positivity rates were not significantly related to location, genus identification or life stage (adult versus tadpole). When fish and amphibian data were combined, positivity was not significantly related to site or to group (fish versus tadpole versus adult frog).

Of the 128 positive specimens, 118 were identified as positive based on DNA from the intestine. Five specimens had positive results from both the intestine and the kidney, and 5 had positive results only from the kidney.

Twenty-seven external surface swabs were analyzed, and 4 of these were ER positive. Of the 27 total swabs, 14 came from fish or amphibians in which either the kidney or intestine was ER positive. Of these 14, only one external swab was also positive. The other 3 ER positive external swabs came from fish and amphibian specimens in which the kidney and intestine were ER negative.

One 0.2 μm nitrocellulose filter was tested from 7 sites sampled in season 2. Only the filter from site 6 (Mensah Bar) tested ER positive, despite ER-positive specimens being collected from every site.

One hundred seventy ER positive samples were sent to the University of Tennessee for VNTR analysis. Three of these were successfully VNTR typed. Two were determined to be *Mycobacterium liflandii*, a mycolactone-producing mycobacteria pathogenic to frogs. These were recovered from the intestine of a *Hyperolius* tadpole (Site 10), and the intestine of a predatory cichlid, *Hemichromis bimaculatus* (Site 2). One specimen, an adult frog from the genus *Leptopelis*, (site 3) was confirmed to be positive for *M. ulcerans*. This is the first known finding of *M. ulcerans* in an adult amphibian.

Discussion

Site ER positivity was not statistically associated with specific fish or amphibian communities and showed no association between species identification and ER positivity. However, this sampling scheme was qualitative in nature, representing a single point in time. To fully characterize fish and amphibian communities, more intensive sampling methodologies would be required. According to these data, the presence or absence of certain fish and amphibian species appears unlikely to be useful as an indicator for the presence of *M. ulcerans* in a water body.

Species and functional groups did not influence positivity rates in fish, but with fish data, certain sites were significantly linked to positivity. This may suggest that the environmental distribution of *M. ulcerans* is a part of the ecology of highly focal waterbodies, but it may also be an artifact related to small sample sizes in those locations. Fish appear likely to come into contact with the bacterium regardless of feeding guild. Additionally, while it is not apparent that fish act as replicative reservoirs for *M. ulcerans*, they do accumulate bacteria at levels detectable by standard PCR, and offer a potential tool for environmental screening. Functional group analyses were not performed for amphibians because little is known about the specific feeding methods of tadpoles, and because all adult frogs are predators. However, neither genus nor site was significantly linked to positive ER status among amphibians.

The overall fish ER positivity rate (39%) was higher in this study than IS*2404* positivity rates reported in previous studies (10% in Eddyani et al. 2009 and 20% in Kotlowski et al. 2004). This was unexpected considering the much higher sensitivity of IS*2404* PCR. Possible reasons for the difference include annual and seasonal variation in positivity rates, increased sample sizes

in the current study, and differing extraction methods. For example, the one-tube extraction method used by Kotlowski was attempted in the current study, but the Käser extraction method was chosen because it resulted in detection limits that were over 5,000 times more sensitive. Site was shown to be an important factor in the current study, so number and location of sites could also explain the difference. Non-specific binding was observed, particularly in the intestine samples, so it was possible that this could have produced some false positives in samples from this study. Contamination is always a possibility, but every effort was taken to avoid contamination, and negative controls consistently showed a lack of contamination. In addition, samples were processed in batches that usually included both kidney and intestine samples. If contamination had occurred it would have impacted the results of both, not just intestines. The fact that almost all of the kidney samples were negative provides evidence that contamination was not a factor. The possibility of PCR inhibition in previous studies must be considered, especially given the high amount of inhibition initially observed in the present study.

For amphibians, it is important to consider the slow rate of development of *M. ulcerans* and the transience of the aquatic tadpole stage. It has been shown that *M. ulcerans* can replicate within amphibian cells (Drancourt et al. 2002). Timing of tadpole development is highly variable, depending on species, temperature, food availability, and density. Some *Hyperolius* species go through metamorphosis after 5-6 weeks, while some *Kassina* species can take up to 10 months (Channing and Howell 2006). *M. ulcerans* or other mycolactone-producing mycobacteria acquired by tadpoles with very short aquatic stages would potentially have very little time to replicate while the amphibian remained in the tadpole stage, and might not be detectable until after the frog has gone through metamorphosis. For this reason, adult frogs may be better targets than tadpoles for future studies. The single specimen in which *M. ulcerans* was

verified by VNTR typing was an adult frog. This is the first report of *M. ulcerans* in a terrestrial amphibian, and may represent a potential aquatic-terrestrial linkage. While *M. ulcerans* has traditionally been associated with aquatic habitats, it has recently been found in terrestrial mammals in Australia (Fyfe et al. 2010).

The majority of positive ER results came from intestine samples for both fish and frogs. External swabs had an overall ER positivity rate of only 15%, suggesting that while bacteria may sometimes adhere to the external surface of an organism, ER positive intestine results were actually due to bacteria in the intestine, and not due to external contamination of samples. The very low rate of ER positive kidney samples suggests that bacteria were acquired during feeding and were passing through the intestine without causing a systematic infection. If the bacteria were acquired through injection, such as during attack by predators, it would likely be detected in the kidney as well (Mosi 2009).

Water filters had lower overall positivity rates than fish and amphibians. Unfortunately, only a single filter could be processed from each site where water filtrate was collected due to time constraints, though multiple sites were tested. One of 7 filters (14%) tested ER positive. That filter was from site 6 (Mensah Bar), a site that produced ambiguous ER results and was therefore removed from the fish and amphibian analyses. The 14% positivity rate for filters was consistent with the 15% positivity rate for external swabs. Both of these would likely represent bacteria suspended in the water column. Water filters have been used as a way to detect *M. ulcerans* in water bodies (Williamson et al. 2008, Vandelannoote et al. 2010). However, this method could potentially be missing *M. ulcerans*-positive locations simply due to the small quantity of water filtered. If *M. ulcerans* was unevenly distributed in the water body, water samples could prove to be inconsistent. Use of fish or amphibians may be a more effective way

to determine whether *M. ulcerans* is present in a water body due to the animals' mobility and subsequently greater chance of bacterial contact. Additionally, a greater detection rate in fish guts versus water samples in this study suggests some retention within fish intestines, but further research is needed to determine the residence time of *M. ulcerans* within fish guts. Preliminary study by Mosi (2009) found that *M. ulcerans* could be detected in the guts of 9% of artificially infected belostomatids 30 days post infection and in 30% of guts 60 days post infection, though these findings are difficult to interpret due to small sample sizes.

Despite finding relatively high ER positivity rates in fish and amphibians, very few samples were successfully VNTR typed. There are two potential explanations for this. First, there were likely very low proportions of *M. ulcerans* or other mycolactone-producing mycobacterial DNA in the samples, and many of the samples yielded low overall DNA quantities. DNA was further depleted by early extraction issues and repeated PCRs. Many of the ER bands were very faint, consistent with small quantities of DNA. VNTR amplification is less sensitive than ER PCR, so it is likely that DNA could be detected by ER amplification but not by VNTR amplification. Lavender et al. (2008) reported low sensitivity for VNTR of environmental samples, with over 100 genomes μL^{-1} DNA required for identification. Fyfe et al. (2010) reported that at least 10^5 organisms/gram must be present for successful VNTR analysis. Low quantities of DNA in fish are consistent with the conclusion of Mosi (2009) that *M. ulcerans* does not replicate in fish. For fish, it appears that *M. ulcerans* and other mycolactone-producing mycobacteria are likely encountered during feeding, but then pass through the digestive tract. This could be further investigated by testing fecal material separately from intestinal tissue.

A second possibility for low success of VNTR typing was false ER positive results. As discussed above, it was unlikely that contamination was an issue, given the low rate of ER positivity among kidney samples that were processed along with the intestine samples. However, it was possible that non-specific binding may have caused false positives. Non-specific bands were observed in many intestine samples, and some were close in size to the expected ER band size. This could be dealt with in future studies by re-designing primers, varying PCR protocols and reagent concentrations, trying different polymerases, or by using different PCR targets. Verification through sequencing would also be an important tool for determination of positive PCR identity

The relatively high levels of PCR inhibition experienced during the course of this study reinforce the need to consider inhibition when dealing with environmental samples. Inhibition was likely caused by some combination of polysaccharides, polyphenols, and/or humic acids. Polysaccharides are especially problematic in plant materials. They co-precipitate with DNA during ethanol precipitation, thereby remaining in the extracted DNA (Sharma et al. 2002). Polyphenols and humic acids are the most common inhibitors of environmental samples, especially samples including soil particles, and lakes with colored water. They can cause brown color in extracted DNA, and covalently bind to DNA, which blocks the action of DNA polymerase (Wilson 1997).

Methods to remove these inhibitors include precipitation of polysaccharides with high concentrations of NaCl and Sarcosyl, or with SDS and potassium acetate. Polyphenols and humic acids can be removed with Hexadecyltrimethylammonium bromide (CTAB) based extractions, Sephadex or other biogel columns, cesium chloride density centrifugation, gel electrophoresis, or Polyvinylpolypyrrolidone (PVP) (Dong et al. 2006). Commercial purification

kits such as the MO BIO kit used in this study are also used. However, because the methods vary in effectiveness, there is a need for a standardized extraction method for use in *M. ulcerans* studies in order to be able to meaningfully compare results across studies.

Numerous extraction methods have been used to test for *M. ulcerans* (Boom et al., 1990, Durnez et al. 2009, Fyfe et al. 2007, Käser 2009, Ross et al. 1997, Williamson et al. 2008). The Käser (2009) method was chosen for this study specifically because it was a recently developed, optimized combination of well-known methods, was developed with testing of environmental samples in consideration, was relatively inexpensive, and was validated with insect samples. However, despite working well on control fish tissue, the method did not remove environmental inhibitors from actual field samples. Other studies have also found problems with inhibition. Vandelannote et al. (2010) found inhibition in 50/148 environmental samples based on Ct values when using the Modified Boom extraction method (Boom et al. 1990, Durnez et al. 2009). While using a commercial kit extraction, Fyfe et al. (2007) detected inhibition in some soil samples extracts with the use of Ct values. The samples were re-analyzed after 10-fold dilution. While dilution is feasible in the presence of high amounts of target DNA, environmental samples may contain very small amounts of *M. ulcerans* DNA, which may not be detectable after dilution. It should also be noted that dilution fails to remove inhibitors that are bound to the DNA, and is an unsatisfactory treatment of nucleic acids contaminated by numerous environmentally-derived inhibitory compounds.

Conclusion

In this study *M. ulcerans* in an adult frog was found and confirmed with VNTR analysis. This is the first reported finding of the bacterium in a wild adult amphibian and suggests that amphibians may warrant further study. Amphibians cannot be ruled out as reservoirs due to the transience of their aquatic stages and higher ER-positivity rates as compared to fish. No fish or amphibian species or feeding guild served as an indicator of the presence of ER-positive mycobacteria in a water body. However, certain waterbodies were observed to have higher ER-positivity rates than others. While not useful as indicator species, fish and amphibians may still be useful for the detection of ER-positive mycobacteria. Future studies on the environmental distribution of *M. ulcerans* and other mycolactone-producing mycobacteria would benefit from the development of a standardized extraction method carefully tested to ensure removal of inhibitors.

APPENDIX

Table 1: Taxa collected in 2008 survey of areas considered likely to be ER positive (P) or ER negative (N) based on water filter data.

Location	Fish	Count	Amphibians	Count
Site 1 (P) Otinibi	*Barbus sublineatus*	19	*Hyperolius* sp.	1
	Cyprinodontidae	1	undetermined	3
	Sarotherodon sp.	1		
Site 2 (P) Danfa	*Barbus sublineatus*	6	none	
	Hemichromis bimaculatus	8		
	Sarotherodon sp.	6		
Site 3 (P) Teiman	*Hemichromis bimaculatus*	3	none	
	Parachanna obscura	1		
	Sarotherodon sp.	21		
Site 4 (P) Afiaman	*Brienomyrus brachyistius*	1	none	
	Hemichromis bimaculatus	1		
	Hemichromis fasciatus	1		
	Sarotherodon sp.	2		
Site 5 (P) Kotoku	*Sarotherodon* sp.	5	*Afrixalus* sp.	11
			Hyperolius sp.	6
Site 6 (P) Nsakina	*Protopterus annectens*	1	*Afrixalus* sp.	13
			Hyperolius sp.	1
Site 7 (N) Pampamwie	*Barbus parablabes*	6	*Leptopelis* sp.	6
	Barbus sublinatus	25		
Site 8 (N) Ata Kofi	*Barbus parablabes*	9	*Afrixalus* sp.	5
	Barbus sublineatus	26	*Amnirana* sp.	6
			Hyperolius sp.	9
Site 9 (N) Titiaka	*Barbus trispilos*	1	*Amnirana* sp.	3
	Larval fish	1	*Bufo* sp.	2
	Sarotherodon sp.	1	*Leptopelis* sp.	4
Site 10 (P) Wawaso	*Barbus macrops*	1	none	
	Barbus sublineatus	9		

Table 1 (cont'd)

Site 11 (N) Asato	Cyprinidae	3	*Amnirana* sp.	31
Site 12 (P) Okaniase	*Barbus sublineatus*	9	none	
Site 13 (N) Adaklu	*Barbus macrops* *Barbus trispilos* *Sarotherodon* sp.	25 11 1	none	
Site 14 (N) Adaklu	*Barbus trispilos* *Fundulosoma thierryi*	19 7	*Afrixalus* sp. *Hyperolius* sp.	1 10
Site 15 (N) Adaklu	*Aphyosemion walkeri* *Barbus macrops* *Barbus parablabes* *Sarotherdon* sp.	10 7 27 4	*Afrixalus* sp. *Hyperolius* sp.	5 1
Site 16 (P) Agodeke	*Aphyosemion petersii* *Barbus parablabes* *Barbus trispilos* *Propterus annectens*	15 4 26 1	none	
Site 17 (P) Abutia Kloe	none		*Afrixalus* sp. *Hemisus* sp. *Hyperolius* sp. *Kassina* sp.	6 1 6 1
Site 18 (P) Wayanu	*Barbus parablabes* *Barbus sublineatus* Larval fish *Syndontus* sp.	2 11 1 2	*Bufo* sp. *Hyperolius* sp.	1 2
Site 19 (N) Laweh Kope	*Barbus macrops* *Brienomyrus brachyistius* *Hemichromis fasciatus* *Sarotherodon* sp.	30 2 1 7	none	
Site 20 (N) Asutuare Junction	*Barbus macrops* *Hemichromis bimaculatus* *Sarotherodon* sp.	28 4 10	*Hyperolius* sp.	4

Table 1 (cont'd)

Site 21 (N) Asebi	*Barbus macrops* *Hemichromis fasciatus* *Sarotherodon* sp.	41 1 11	none	
Site 22 (N) Dedenya	Cyprinodontidae *Hemichromis bimaculatus* *Sarotherodon* sp.	2 1 5	*Hyperolius* sp.	22
Site 23 (N) Mensah	*Fundulosoma thierryi*	9	None	
Site 24 (N) Saduase	none		*Afrixalus* sp. *Chiromantis* sp. *Hyperolius* sp.	1 1 15
Site 25 (N) Oyibi	*Sarotherodon* sp.	31	none	
	Total Fish:	**524**	**Total Amphibians:**	**178**

Table 2: Specimens collected in 2009 to be screened for *M. ulcerans*. All samples were collected from districts considered to be endemic for Buruli Ulcer Disease.

Location	**Fish**	**Count**	**Amphibians**	**Count**
Site 1 (Djorse stream)	*Barbus sublineatus* *Hemichromis bimaculatus* *Epiplatys dageti*	1 4 2	*Bufo* sp.	10
Site 2 (Djorse pond)	*Hemichromis bimaculatus*	1	*Afrixalus* sp. Ranidae	12 4
Site 3 (Otuaplem)	*Hemichromis bimaculatus* *Clarias anguillaris*	3 1	Ranidae *Leptopelis* sp. *Afrixalus* sp. *Hemisus* sp. *Xenopus* sp.	2 1 4 10 12
Site 4 (Sarpeiman)	*Hemichromis bimaculatus* Unidentified larval fish	3 1	*Bufo* sp. *Afrixalus* sp. *Hyperolius* sp.	13 1 8
Site 5 (Seduase)	none		*Afrixalus* sp.	9
Site 6 (Mensah bar)	*Barbus macrops* *Sarotherodon* sp.	15 19	none	
Site 7 (Achiaman)	*Sarotherodon* sp. *Clarias anguillaris* *Hemichromis bimaculatus*	23 1 2	*Hyperolius* sp. Ranidae	17 1
Site 8 (Kwashikuma Marsh)	*Hemichromis bimaculatus*	5	*Afrixalus* sp. *Hyperolius* sp. Unidentified tadpole Ranidae	6 38 1 3
Site 9 (Lake Weija)	*Sarotherodon* sp.	15	none	
Site 10 (Kwashikuma Pond)	*Sarotherodon* sp.	4	*Afrixalus* sp. *Hyperolius* sp.	10 10
	Total fish:	**100**	**Total Amphibians:**	**172**

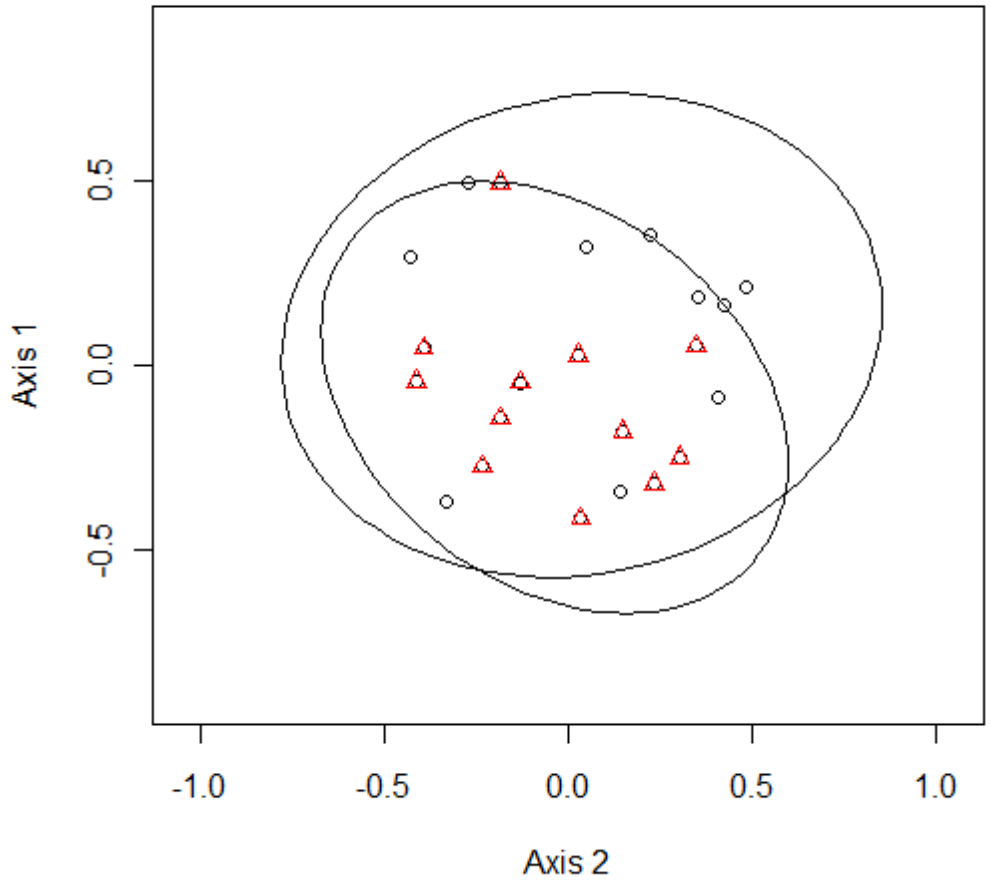

Figure 1: NMS ordination plot with three dimensions represented on two axes of fish communities using species presence or absence data from 2008 in relation to ER positivity. Triangles represent ER positive communities and circles represent ER negative communities. Ellipses represent 95% confidence intervals. Stress: 0.1568934. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this thesis.

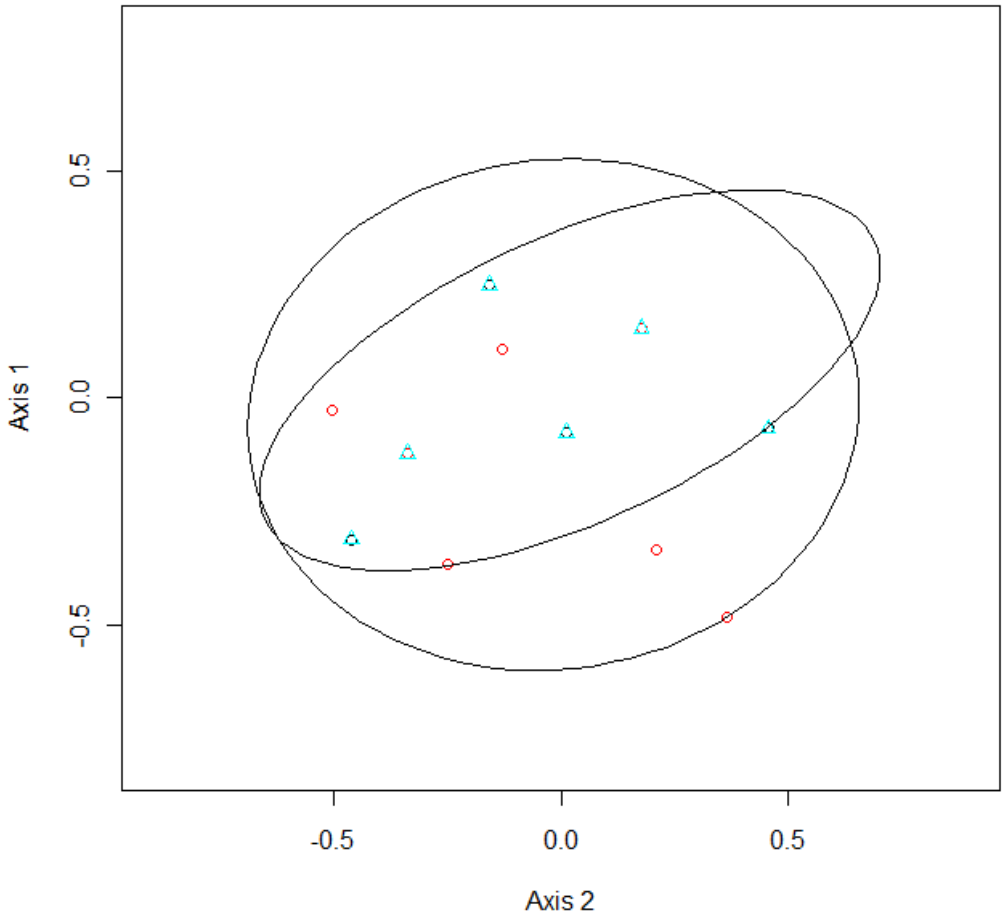

Figure 2: NMS ordination plot with three dimensions represented on two axes of amphibian communities using species presence or absence data from 2008 in relation to ER positivity. Triangles represent ER positive communities and circles represent ER negative communities. Ellipses represent 95% confidence intervals. Stress: 0.06834609.

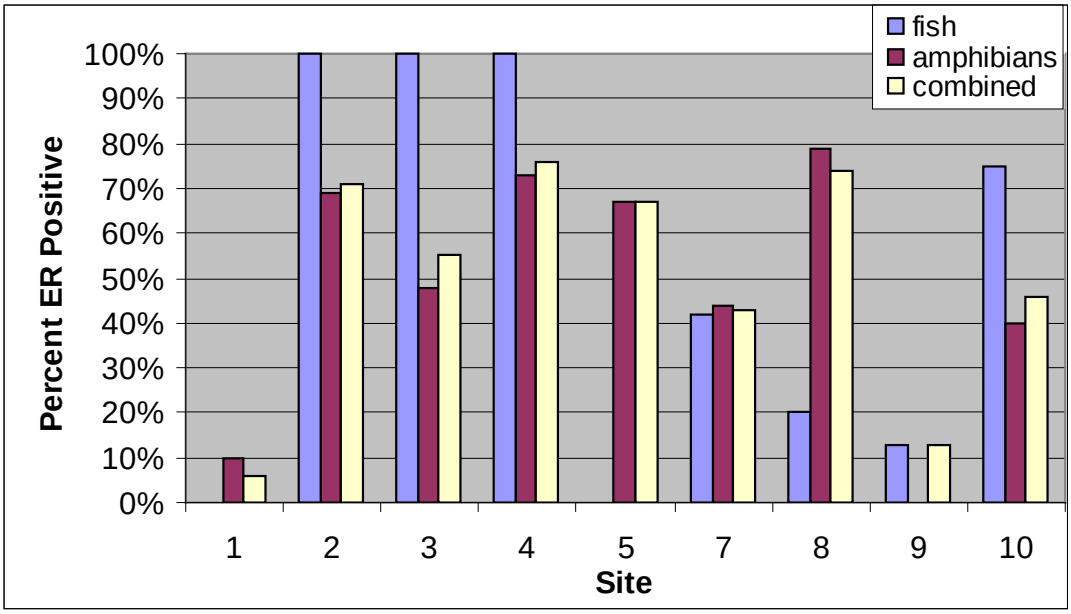

Figure 3: ER positivity rates of fish and amphibians and combined data for specimens collected in 2009.

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