Distribution of *Mycobacterium ulcerans* in Buruli Ulcer Endemic and Non-Endemic Aquatic Sites in Ghana

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Abstract

*Mycobacterium ulcerans*, the causative agent of Buruli ulcer, is an emerging environmental bacterium in Australia and West Africa. The primary risk factor associated with Buruli ulcer is proximity to slow moving water. Environmental constraints for disease are shown by the absence of infection in arid regions of infected countries. A particularly mysterious aspect of Buruli ulcer is the fact that endemic and non-endemic villages may be only a few kilometers apart within the same watershed. Recent studies suggest that aquatic invertebrate species may serve as reservoirs for *M. ulcerans*, although transmission pathways remain unknown. Systematic studies of the distribution of *M. ulcerans* in the environment using standard ecological methods have not been reported. Here we present results from the first study based on random sampling of endemic and non-endemic sites. In this study PCR-based methods, along with biofilm collections, have been used to map the presence of *M. ulcerans* within 26 aquatic sites in Ghana. Results suggest that *M. ulcerans* is present in both endemic and non-endemic sites and that variable number tandem repeat (VNTR) profiling can be used to follow chains of transmission from the environment to humans. Our results suggesting that the distribution of *M. ulcerans* is far broader than the distribution of human disease is characteristic of environmental pathogens. These findings imply that focal demography, along with patterns of human water contact, may play a major role in transmission of Buruli ulcer.

Introduction

*Mycobacterium ulcerans* is the cause of Buruli ulcer, a severe necrotizing skin infection (Figure 1). Although Buruli ulcer is globally distributed, it is an emerging infection primarily in Australia and West Africa [1]. The disease begins as a painless nodule or papule that, if left untreated, can lead to extensive ulceration that could cover 15% of the body [2]. Though the disease is not usually fatal, Buruli ulcer can lead to profound morbidity, especially within rural areas of West Africa where treatment options are limited. Though sex and age are not seemingly risk factors, women and children between the ages 5 and 15 are most often infected. Incidence of Buruli ulcer has increased over the last several years. For instance, in Ghana, the number of new cases reported has been 685 in 2003, 1021 in 2004, 1097 in 2005, and 1010 in 2006. True incidence data, however, is difficult to determine due to poor surveillance measures and case confirmation.

The major virulence determinant for *M. ulcerans* is a plasmid-encoded toxic macrolide, mycolactone [2,3]. Acquisition of the mycolactone plasmid is thought to have been a pivotal event in the evolution of *M. ulcerans* from an *M. marinum*-like ancestor [4]. Like *M. marinum*, *M. ulcerans* is an environmental pathogen. Although the exact mode of transmission for *M. ulcerans* remains unknown, person to person transmission is extremely rare and a large body of epidemiological data supports the hypothesis that infection results from exposure to aquatic environments [5,6,7]. Lack of direct person-to-person transmission is a characteristic *M. ulcerans* shares with other environmental pathogens such as *Francisella tularensis* and *Borrelia burgdorferi*. Environmental pathogens are maintained in the environment in the absence of humans. The distribution of such pathogens is far broader than the cases of human disease. For example the life cycle of *Borrelia burgdorferi*, the causative agent of Lyme disease involves several species of *Ixodes* ticks and a number of mammalian vectors. Human infections only occur through exposure to ticks; and humans are a dead end for infection. In some areas of the Western U.S., *Borrelia burgdorferi* is vectored by an *Ixodes* species which feeds primarily on lizards and rarely bites humans. In these areas, despite the abundance of *Borrelia burgdorferi* in the environment, human Lyme disease is extremely rare [8,9].

A major advance in deciphering the ecology of *M. ulcerans* resulted from the identification of an insertion sequence, IS2404, which is present in over two-hundred copies in *M. ulcerans* [10,11]. Early work showed that IS2404 was present in *M. ulcerans*, but
Author Summary

Buruli ulcer is an ulcerative skin disease caused by Mycobacterium ulcerans. Though usually not fatal, ulceration can cover up to 15% of the body, with treatment being costly and sometimes painful. Primary risk for Buruli ulcer in Africa is exposure to stagnant water, but the route of transmission is unknown. Detection of M. ulcerans in aquatic insects endemic sites suggests the presence of aquatic reservoirs. This article reports results from the first investigation into the ecology of M. ulcerans based on random sampling of both endemic and non-endemic aquatic sites. Development of a method for discriminating M. ulcerans from closely related mycobacterial pathogens made it possible to determine the distribution of M. ulcerans in aquatic environments. This article demonstrates the presence of M. ulcerans DNA in both endemic and non-endemic sites within aquatic insects, water filterate, and glass-slide biofilm communities. This article provides data suggesting that M. ulcerans is more broadly distributed than the human disease it causes. This study provides an initial step for future work on whether certain M. ulcerans strains are particularly successful human pathogens and suggests that research on specific human water contact factors may provide insight into the transmission of M. ulcerans.

absent in the closely related mycobacterial species M. marinum, and over 40 other mycobacterial species suggesting that the insertion sequence was specific for M. ulcerans [11]. In the past 15 years a large number of environmental samples collected from Buruli ulcer endemic regions in Australia and West Africa have been analyzed using IS2404 PCR. In Australia, IS2404 has been detected in water as well as from detritus collected from water bodies and, most recently, from trapped mosquitoes [12,13,14]. No acid-fast bacilli were reported and attempted cultures were negative. In 1999, Portaels et al. reported detection of IS2404 positive PCR from two groups of predaceous aquatic insects, Nauoridiae and Belostomatidae [15]. IS2404 PCR positive results have also been obtained from Nauoridiae and Belostomatidae collected in Ghana, Cote d’Ivoire and Benin [14,16,17]. In 2004, Marsollier et al. obtained IS2404 positive PCR results from 5/83 Nauoridiae collected in Cote d’Ivoire and, more importantly, successfully cultured an IS2404 positive mycobacteria from two of these. Although one of these isolates produced an ulcer upon injection into mice, both isolates were lost before they could be fully characterized. More recently, an exciting discovery was the culture and complete characterization of M. ulcerans from a Gerridiae, or water strider (in press). Gerridiae, like Nauoridiae and Belostomatidae are predaceous aquatic insects in the Order Hemiptera. Unlike Nauoridiae and Belostomatidae, Gerridiae are unable to bite humans. However, it is likely that Gerridiae along with other invertebrates share a food web with M. ulcerans. M. ulcerans has also been shown to form biofilms on aquatic plants [18]. A culture of an IS2404 positive mycobacteria was obtained from an IS2404 PCR positive plant (Family: Scrophulariaceae) collected from the Lobo River in Cote d’Ivoire and it has been suggested that snails may be transiently infected by feeding on this vegetation [18]. Contamination of this sample with M. szulgai prevented isolation of M. ulcerans. IS2404 positive samples include detritus, snails, and fish [15,17]. Taken together these results suggest that the ecology of M. ulcerans is complex and includes participation in a food web comprised of many different taxa and feeding groups.

Considerable speculation concerning the possibility of an insect vector for Buruli ulcer has followed from elegant studies in which laboratory infections of n auoridiae collected in France with M. ulcerans could be transmitted to mice from the bite of the infected insect [15]. However, these results have been interpreted with caution [19]. Most attempts to culture the organism from environmental sources have not been successful despite the fact that it is readily cultured from human tissues. None of the IS2404 PCR-positive insects identified in West Africa are blood feeders, making it unlikely that they could play a major role in transmission. Further, IS2404 has been found in several aquatic mycobacterial pathogens closely related to M. ulcerans such as M. liflandii [20], M. pseudohotviti [21], and a newly discovered clade of M. marinum [22] isolated from frogs and fish. Finally, environmental sampling has not been conducted in a systematic way and results from samples collected in non-endemic regions have not been conducted with the exception of one study in which an unspecified number of unidentified plants were collected from Cote d’Ivoire [18].

A problem inherent in the identification of pathogens in the environment is the difficulty of distinguishing the target species within a complex and largely unknown population of background microbial flora. Although it is impossible to have complete confidence that any PCR primer set targeting a specific gene sequence is 100% specific in this context, the use of multiple PCR targets is likely to increase specificity. The completion of the M. marinum and M. ulcerans genome sequencing projects has led to the identification of variable number tandem repeat (VNTR) sequences which have been very useful in detecting heterogeneity among M. ulcerans [23,24,25]. Results from these studies suggested that it might be possible to trace transmission pathways by matching VNTR profiles from environmental samples with those from M. ulcerans cultures obtained from patients in the same geographic area.

In this report we present data from a systematic collection of over 1400 environmental samples collected from both endemic and non-endemic regions of Ghana as part of a larger study aimed at defining the ecology of M. ulcerans. Using a tiered PCR based detection method we have mapped the distribution of M. ulcerans within 26 aquatic sites in Ghana. Samples analyzed include vertebrates, invertebrates, suspended solids from water filtrate, soil, and biofilms collected on glass slides. Preliminary evidence for M. ulcerans in environmental samples was obtained from PCR detection of the insertion sequence IS2404 [10] along with PCR detection of the enoyl reductase (ER) domain of the mycolactone toxin [26]. Variable number tandem repeat (VNTR) analysis of ER-PCR positive samples allowed the discrimination of M. ulcerans.

Figure 1. Buruli ulcer on the left limb following surgical debridement. Also shown is joint contracture of the left wrist from scarring caused by Buruli ulcer. doi:10.1371/journal.pntd.0000205.g001
from other mycolactone producing mycobacteria and also made it possible to match VNTR profiles from environmental samples with VNTR profiles obtained from patient isolates from the same region. Although \textit{M. ulcerans} has been detected in many Buruli ulcer endemic areas of West Africa using IS2404-PCR [17], this is the first study in which both endemic and non-endemic sites have been randomly and systematically sampled.

The major finding from this work is that \textit{M. ulcerans} and other mycolactone producing mycobacteria (MPM) are widely distributed in water bodies in endemic and non-endemic villages within the Ashanti and Greater Accra regions of Ghana. This is entirely consistent with \textit{M. ulcerans}'s position as an environmental pathogen. Although the human host may play a role in the dispersion of an environmental pathogen, the pathogen does not depend on the human host for dispersion. Thus the distribution of an environmental pathogen is always much broader than the distribution of disease. Further, these studies suggest that the presence of \textit{M. ulcerans} in the environment, while necessary, is not sufficient for Buruli ulcer.

\section*{Materials and Methods}

\subsection*{Bacterial strains}

Strains used in this study are listed in Table 1. \textit{M. ulcerans} strains were grown at 32°C for 4–6 weeks on M7H10 agar media. \textit{M. liflandii} was grown at 32°C in 5% CO\textsubscript{2} for 6 weeks on Bordet-gengou media. \textit{M. pseudoshotsii}, and \textit{M. marinum} DL strains were grown at 25°C for 6 weeks on Bordet-gengou media.

\begin{center}
\textbf{Table 1. Ghanaian isolates of \textit{M. ulcerans} and other mycolactone producing mycobacteria.}
\end{center}

| Species          | Strain | Source                      | Reference |
|------------------|--------|-----------------------------|-----------|
| \textit{M. ulcerans} | 1054   | Human Ghanaian isolate (Central Region) | This Work |
| \textit{M. ulcerans} | 1055   | Human Ghanaian isolate (Central Region) | This Work |
| \textit{M. ulcerans} | 1057   | Human Ghanaian isolate (Central Region) | This Work |
| \textit{M. ulcerans} | 1059   | Human Ghanaian isolate (Central Region) | This Work |
| \textit{M. ulcerans} | 1063   | Human Ghanaian isolate (Ashanti Region) | This Work |
| \textit{M. ulcerans} | Agy99   | Human Ghanaian isolate (Greater Accra Region) | [36] |
| \textit{M. marinum} | DL150991 | Sea bass Dicentrarchus labrax (Atlit-Mediterranean Sea, Israel) | [22] |
| \textit{M. marinum} | DL240490 | Sea bass Dicentrarchus labrax (Red Sea, Israel) | [22] |
| \textit{M. marinum} | DL045   | Sea bass Dicentrarchus labrax (Mediterranean Sea, Greece) | [22] |
| \textit{M. marinum} | DL300/04 | Sea bass Dicentrarchus labrax (Mediterranean Sea, Italy) | [26] |
| \textit{M. marinum} | DL180892 | Sea bass Dicentrarchus labrax (Ein Yahav, Israel) | [22] |
| \textit{M. marinum} | SA200695 | Sea bream Sparus aurata (Red Sea, Israel) | [22] |
| \textit{M. marinum} | CC240299 | Koi Cyprinus carpio (Ma’agan Michael, Israel) | [22] |
| \textit{M. marinum} | BB170200 | Silver perch Bidyanus bidyanus (Dor-Ma’agan Michael, Israel) | [22] |
| \textit{M. marinum} | CF030494 | Butterflyfish Chaetodon fasciatus (Red Sea, Israel) | [22] |
| \textit{M. marinum} | SR030597 | Rabbitfish Siganus rivulatus (Red Sea, Israel) | [22] |
| \textit{M. marinum} | Hybrid270995 | Red seabream Pagoius major (f) x Sparus aurata (m) (Red Sea, Israel) | [22] |
| \textit{M. Pseudoshotsii} | L15    | Sea bass Morone saxatilis | [21] |
| \textit{M. Pseudoshotsii} | L58    | Sea bass Morone saxatilis | [21] |
| \textit{M. liflandii} | X1128F | African clawed frog Xenopus tropicalis | [31] |
| \textit{M. liflandii} | X15    | African clawed frog Xenopus laevis | [31] |
| \textit{M. liflandii} | 1138   | African clawed frog Xenopus laevis, University of Massachusetts | This Work |
Lansing, Michigan for taxa identification. Additional belostomatids and naucorids were selectively collected by sweep sampling as above until an appropriate amount of each was obtained.

b. Collections of water filtrate. At each site, ten, 100–200 mL water samples were collected from mid-water column and passed through a 1.6 micron fiberglass filter (Whatman Inc). This filtrate was then passed through a 0.2 micron nitrocellulose filter (Whatman Inc). Three additional filtrate samples were collected from each site in which water was collected mid-water column and filtered directly through a 0.2 micron nitrocellulose filter. Filters were sealed in foil packets and kept in a cooler for transport in the field and to the University of Tennessee, Knoxville, Tennessee.

c. Soil sampling. Three samples of soil (approximately 5 grams) were taken from the floor of each water body. Two additional soil samples were taken from the riparian zone; one at the water edge and one 5 m from the water edge.

| Table 2. Presumptive identification of M. ulcerans in aquatic vertebrate and invertebrate samples in Ghana 2004–2006. |
|---|---|---|
| Order | Family | ER positive Samples¹ |
| Anura order | 4/31 |
| Anura Araneae | 8/27 |
| Coleoptera Noteridae | 1/58 |
| Coleoptera Hydrophilidae | 5/55 |
| Coleoptera Dytiscidae | 3/44 |
| Coleoptera Elidae | 2/5 |
| Coleoptera Scirtidae | 1/10 |
| Coleoptera Hydreaenidae | 2/14 |
| Diptera Chironomidae | 4/57 |
| Diptera Culicidae | 2/30 |
| Diptera Psychodidae | 1/1 |
| Ephemeroptera Protonoeidae | 4/24 |
| Ephemeroptera Caenidae | 3/28 |
| Ephemeroptera Baetidae | 2/44 |
| Gastropoda Physidae | 1/9 |
| Hemiptera Notonectidae | 3/37 |
| Hemiptera Belostomatidae | 5/41 |
| Hemiptera Nepidae | 4/21 |
| Hemiptera Naucoridae | 3/18 |
| Hirudinea | 3/25 |
| Lepidoptera Crambidae | 2/3 |
| Odonata Libellulidae | 4/26 |
| Oligochaeta | 2/24 |
| Osteichthyes | 1/24 |
| Ostracoda | 2/14 |
| Basommatophora Planorbidiae (Bulininae) | 2/29 |
| Bivalva Sphaeriidae | 1/1 |
| Bivalva Corbiculidae | 1/1 |
| Diptera Sciomyzidae | 1/3 |
| Diptera Syrphidae | 1/2 |

¹Large samples were tested individually. Smaller samples with numbers of individuals of three or above were pooled in sets of 3–15. Denominator represents total number of pooled or individual samples collected from the specific taxon.

d. Biofilm collection. Two sites were chosen for biofilm collection. Adigan was classified as a non-endemic site based upon incidence data from the Ghana Ministry of Health. Amasaman was classified as an endemic site for Buruli ulcer. Sixteen glass microscope slides were affixed to six PVC pipes using paper tape. The pipes with attached slides were submerged and anchored into the water body floor. Slides were collected at 3, 6 and 14 weeks and placed into individual 50 mL Falcon tubes (BD Biosciences). A sub-sample was taken from one side of each slide for DNA extraction and PCR (methods described below). The remaining side of the slide was stained for acid-fast bacteria using the Kinyoun’s staining according to the manufacturer’s instructions (Difco).

DNA extraction

DNA was extracted using a protocol adapted from Lamour and Finley [27]. Small invertebrates collected in Ghana were sampled in pools of groups of 3–15, whereas vertebrates and larger invertebrates were tested individually. Invertebrate samples were also collected from Tennessee. These samples were used as negative controls for PCR analysis. DNA was also extracted from M. ulcerans Agy99, M. marinum 1218, or water for use as positive and negative controls. Samples were vortexed in 400 μL lysis solution (100 mM Tris (pH 8.0), 50 mM EDTA, 500 mM NaCl, 1.33% SDS and 0.2 mg/mL RNase A) and one gram 1.0 mm glass beads (Sigma-Aldrich), then centrifuged. One hundred-fifty microliters of 5 M potassium acetate was added, and each sample was incubated at −20 °C overnight. Following centrifugation, supernatants were transferred to new tubes containing 0.66 M guanidine hydrochloride and 63.3% ethanol solution. The samples were then added to a MOBIO spin filter (MOBIO) in a 2 mL microcentrifuge tube (MOBIO). The flow-through was discarded and the filter was washed with 500 μL wash solution (10 mM Tris [pH 8], 1 mM EDTA, 50 mM NaCl, 67% ethanol), then further washed by the addition of 500 μL of 95% ethanol. The spin filter was dried by centrifugation, and then transferred to a new 2.0 mL microcentrifuge tube. Two-hundred microliters of elution solution (10 mM Tris [pH 8]) were added to the spin filters which were allowed to incubate at room temperature for 15 minutes. Following this, the DNA was eluted. The DNA was stored at −20 °C until further use.

DNA was subjected to amplification of IS2404, the enoyl reductase domain, and various variable number tandem repeat (VNTR) loci. Those yielding no amplification of the ER domain were diluted ten-fold twice for determination of inhibition of PCR.

Assay for sensitivity of IS2404 and ER

Dilutions were made of M. ulcerans Agy99 by first placing a loopful of cells into 1 mL of 1% SDS. Aggregates were broken by passing the suspension through a 25 gauge needle 10 times. One hundred microliters of the suspension was then transferred into a new tube containing 900 μL 1% SDS, and 10-fold dilutions were made. Ten microliters of each suspension was plated in triplicate onto M7H10 plates and allowed to incubate at 32 °C until further use.

Belostomatid samples, each with a wet weight of 160 mg, were placed in separate vials. These vials were spiked with dilutions of M. ulcerans DNA (prepared as above with the exception that M. ulcerans 1615 was used for this study) with predicted concentrations ranging from 10^5 CFU to 0.1 CFU. DNA was extracted as described.

Preparation of spiked samples for ER and VNTR analysis

In order to determine sensitivity of primer sets targeting ER and VNTR loci within environmental samples, belostomatid samples were spiked with serial dilutions of M. ulcerans DNA. Eight sacrificed belostomatid samples, each with a wet weight of 160 mg, were placed in separate vials. These vials were spiked with dilutions of M. ulcerans DNA prepared as described above. DNA was extracted as described.
Primers, PCR conditions, and sequencing

Primers used for this study are listed in Table 3. A 719 basepair fragment of the enoyl reductase (ER) domain, found on one polyketide synthase gene partially responsible for toxin production, was amplified for samples as well as for M. ulcerans Agy99 and 1615, M. marinum 1218, and water (as positive and negative controls) using a 50 μL reaction mixture containing 1 μL each of forward and reverse primer (1.0 μM), 10 μL 5× Go Taq reaction buffer (Promega), 1 μL 10 mM PCR nucleotide mix (Promega), 31.7 μL ddH2O, 1.6 units of Go Taq polymerase enzyme (Promega), and 5 μL DNA template. An average concentration of 10 ng/μL mycobacterial DNA was used for positive and negative controls. Cycling conditions began with an initial denaturation at 94°C for 5 minutes, 35 cycles of 94°C for 1 minute, 50°C for 45 seconds, 72°C for 1 minute, and a final extension of 72°C for 10 minutes. Primers and PCR conditions for amplification of VNTR MIRU 1 and 9, and loci 4, 5, 6, 8, 14, 15, 18, 19, 33, and ST1 as well as for IS2404 were as previously described [23,24,25,11].

The amplified DNA was subjected to gel electrophoresis using a 1.5–3.0% agarose gel and band sizes were compared using a 1 Kb DNA ladder (Invitrogen).

PCR products from all positive samples were cloned into the pCR2.1 Topo vector (Invitrogen) and sequenced using an ABI 3100 automated genetic analyzer (Applied Biosystems).

Results

Comparison of a mycolactone-based enoyl reductase (ER) PCR target to an IS2404 PCR target for detection of M. ulcerans

IS2404 PCR has been widely used for detection of M. ulcerans in the environment and patients because of the high copy number of the IS element (213) within the M. ulcerans genome [20] However, evidence from the M. ulcerans genome as well as results from restriction fragment length polymorphisms of IS2404 suggests considerable heterogeneity between copies, as well as the presence of incomplete copies which could lead to production of multiple products [29]. For this reason we developed a PCR method based on amplification of the ER domain of mlsA which encodes a polyketide synthase that produces the mycolactone core, and compared the sensitivity of ER PCR and IS2404 PCR using environmental samples, as well as M. ulcerans cultures.

In this study 319 invertebrate and vertebrate samples were analyzed using IS2404 PCR and the PCR products were sequenced. A PCR product of appropriate size was obtained from eight invertebrate samples. However, DNA sequencing showed that only four of the samples contained IS2404 DNA. Although adjustment of PCR parameters improved specificity somewhat, many non-specific products were still amplified. ER PCR of the initial eight IS2404 positive samples yielded four ER positive samples. DNA sequence results confirmed that all four ER positive samples contained ER sequence. Further analysis of DNA from 71 ER positive samples showed that ER DNA was the product in every case.

ER is present four times on the mycolactone plasmid. Although there is no evidence concerning plasmid copy number, most large plasmids are present in only 1 or 2 copies per cell making the copy number for the ER target 4–8 [3]. Because we initially assumed there was a clear correlation between copy number and PCR sensitivity, we were concerned that the lower copy number of the ER domain, with respect to IS2404, might influence the sensitivity of the method. Thus the relative sensitivity of ER and IS2404 PCR was evaluated using 10-fold dilutions of M. ulcerans culture. As few as 10–1 CFU of M. ulcerans could be detected using either method (Figure 2). These results suggested that ER PCR was adequately sensitive for detection of M. ulcerans in environmental samples where few copies of M. ulcerans might be present.

ER and VNTR primer sets are sensitive at low concentrations of M. ulcerans DNA

Sensitivity of the primer sets targeting VNTR loci and ER for environmental samples was also determined by spiking samples of belostomatids with serial dilutions of M. ulcerans DNA and performing ER and VNTR PCR. Results from this study show that ER and VNTR DNA could be detected at predicted concentrations as low as 0.1 CFU (Figure S2).

ER PCR based evidence of M. ulcerans in environmental samples from endemic and non-endemic villages in Ghana

During 2004–2006 1,068 invertebrate and vertebrate samples were collected from 14 endemic and 12 non-endemic sites with a focus on the Ashanti and Greater Accra regions of Ghana (Figure 3). Samples included material collected within 1 m².
Figure 2. Comparison of IS2404 and ER PCR for detection of *M. ulcerans*. Lanes represent serial dilutions of *M. ulcerans* 1615 from $10^7$ to $10^{-4}$ CFU detected using probes for IS2404 (A) or ER (B). Lanes are labeled: 1: 1KB ladder; 2: water blank for DNA extraction; 3: water blank for PCR; 4: *M. marinum* 1218; 5: *M. ulcerans* 1615 $10^7$ CFU; 6: *M. ulcerans* 1615 $10^6$ CFU; 7: *M. ulcerans* 1615 $10^5$ CFU; 8: *M. ulcerans* 1615 $10^4$ CFU; 9: *M. ulcerans* 1615 $10^3$ CFU; 10: *M. ulcerans* 1615 $10^2$ CFU; 11: *M. ulcerans* 1615 $10^1$ CFU; 12: *M. ulcerans* 1615 $10^0$ CFU; 13: *M. ulcerans* 1615 $10^{-1}$ CFU; 14: *M. ulcerans* 1615 $10^{-2}$ CFU; 15: *M. ulcerans* 1615 $10^{-3}$ CFU; 16: *M. ulcerans* 1615 $10^{-4}$ CFU; 17: *M. ulcerans* 1615 positive control for PCR.
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Figure 3. Sites sampled 2004–2006. Endemicity is based on human incidence of disease defined at the community level from data obtained from the Ghana Ministry of Health. **Location is approximate and endemicity is based on district level disease incidence data (GPS coordinates not available).**
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samples (N = 3) as well as those obtained by sweep sampling through vegetation. Identical sampling methods were used for all sites. Endemic sites yielded more samples than non-endemic sites. Of the 1,068 samples obtained, 572 (54%) were obtained from endemic sites whereas 496 (46%) were from non-endemic sampling sites. M. ulcerans DNA was detected in only 7% (78/1,068) of the total samples (Table 4) using ER PCR. From the 78 ER positive samples, 42 (54%) were from aquatic environments endemic for Buruli ulcer; whereas 36 (46%) samples were from non-endemic sites. The largest number of ER positive invertebrate samples was collected from Afuaman where 18 invertebrate pooled or individual samples were found to be positive. Six sites yielded only one ER positive pooled or individual sample. These included three endemic sites (Tontokrom, Bowkrom, and Amasaman) and three non-endemic sites (Bretsekrom, Dodowa, and Kreedons). Eight sites yielded zero ER positive invertebrate or vertebrate samples (five endemic and three non-endemic). The remaining eleven sites (six non-endemic and five endemic) had a range of 2 to 10 PCR positive pooled or individual invertebrate samples. All ER PCR positive results were confirmed by DNA sequencing.

ER positive DNA was detected in a broad spectrum of vertebrates and invertebrates representing 30 of the 89 taxa identified. Many taxa, such as Crambidae (moth) larvae and Araneae were found repeatedly positive at specific sites during the 2 year sampling period. Two pools of Crambidae larvae were found positive from Subin; one collected 2005 and the other collected 2006. Araneae have been found positive from sampling of Amasaman 2004, 2005, and 2006. Although some taxa, such as Belostomatidae and Naucoirdae have been found IS2404 positive by others [12,13,14] most ER PCR positive taxa reported in this study have not previously been identified as potential sources of M. ulcerans. M. ulcerans positive taxa represented a wide variety of functional invertebrate feeding groups and life stages (Table 2) [30]. Although most of the positive taxa represented predators, positive results were obtained from collector-gatherers such as those from the family Elmidae (beetle) and scrapers such as those from the family Baetidae (mayfly). A complete description of the demography and identification of positive taxa per site are presented in a separate paper (in preparation).

Previous reporting of M. ulcerans in Belostomatidae and Naucoirdae led us to selectively collect additional samples from these taxa. Seventy-one additional belostomatids and twenty additional naucoirds were obtained through selective collection. Of those, 3/71 (4%) belostomatids and 7/20 (35%) naucoirds were found to contain ER positive DNA.

Although these results suggest that M. ulcerans DNA is widely distributed in invertebrates, the majority of taxa identified (59/89) were repeatedly negative for M. ulcerans DNA (Table S1). In some cases where a taxon was represented by a single sample, such as with Calonoida (copepod), little can be said about the absence of M. ulcerans. In other cases such as with Coenagriniidae (damsel flies) and Pleidae (backswimmer), over 100 individuals were sampled. The absence of ER PCR positive results from these taxa is more meaningful.

Out of 260 samples of water filtrate tested (130 from non-endemic and 130 from endemic sampling sites), 97 (36%) were ER PCR positive. Sixty of the 97 ER positive filtrate samples (61%) were from areas non-endemic for Buruli ulcer, while 37 (38%) of the ER positive filtrate samples were from endemic areas. PCR was also conducted on 100 soil samples; 50 of which were from endemic sites and 50 from non-endemic sites. M. ulcerans DNA was detected in 3% (3/100) of the soil samples (Table 4). Each of these three samples was collected from the floor of the water body. Two of the three ER PCR positive soil samples were from an area endemic for Buruli ulcer (Nyame-Bekyere and Subin) while the third was from an area non-endemic for Buruli Ulcer (Abbeypanya).

**Table 4. Detection of M. ulcerans and MPM in environmental samples using ER and VNTR PCR.**

| Samples             | ER positive (%) | M. ulcerans VNTR profile (%) | MPM VNTR profile (%) |
|---------------------|----------------|-----------------------------|---------------------|
| Invertebrates/vertebrates | 78/1068 (7)   | 12/67 (18)                  | 3/67 (4)            |
| Water filtrate       | 97/260 (37)   | 8/82 (10)                   | 4/82 (5)            |
| Biofilm              | 37/47 (79)    | 17/37 (46)                  | 8/37 (22)           |
| Soil                 | 3/100 (3)     | 0/3 (0)                     | 0/3 (0)             |

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from mycolactone producing fish pathogens *M. marinum* and *M. pseudoshottsii* (Table 5).

Two separate VNTR profiles were identified among mycolactone producing *M. marinum* isolates and these were associated with different habitats (Table 5). Whereas fish from salt water had profile D, those from freshwater had profile E (Table 5). Profile D included one copy of MIRU1, four copies of locus 6, two copies of ST1, and two copies of locus 19 (1,4,2,2), and profile E had one copy of MIRU1, two copies of locus 6, one copy of ST1, and two copies of locus 19 (1,2,1,2). Despite the great geographical distance between the Red and Mediterranean Seas and the Chesapeake Bay, *MPM M. marinum* isolated from sea bass (*Siganus nivulatus*) and *M. pseudoshottsii* isolated from striped bass (*Morone saxatilis*) shared identical 1,4,2,2 VNTR profiles. VNTR analysis revealed a single VNTR profile for *M. liflandii* (1,2,2,1). These results showed that VNTR could be used to differentiate *MPM* found in environmental samples in Ghana.

*Table 5. VNTR profiles of *M. ulcerans* and other MPM isolates based upon numbers of repeats found at different loci (MIRU1, locus 6, ST1, and Locus 19 when applicable).*

| isolate   | VNTR Profile | MIRU1 | Locus 6 | ST1 | Locus 19 |
|-----------|--------------|-------|---------|-----|----------|
| *M. ulcerans* (Human Ghanaian isolates) |               |       |         |     |          |
| 1054      | B            | 3     | 1       | 1   |          |
| 1055      | B            | 3     | 1       | 1   |          |
| 1057      | B            | 3     | 1       | 1   |          |
| 1063      | B            | 3     | 1       | 1   |          |
| Agy99     | C            | 3     | 1       | 2   |          |
| 1059      | A            | 1     | 1       | 1   |          |
| *M. marinum* (Marine fish isolates) |               |       |         |     |          |
| DL150991  | D            | 1     | 4       | 2   | 2        |
| DL240490  | D            | 1     | 4       | 2   | 2        |
| DL045     | D            | 1     | 4       | 2   | 2        |
| DL180892  | D            | 1     | 4       | 2   | 2        |
| SA200695  | D            | 1     | 4       | 2   | 2        |
| SR030597  | D            | 1     | 4       | 2   | 2        |
| CF030494  | D            | 1     | 4       | 2   | 2        |
| 2790995   | D            | 1     | 4       | 2   | 2        |
| *M. pseudoshottsii* (freshwater fish) |               |       |         |     |          |
| L15       | D            | 1     | 4       | 2   | 2        |
| L58       | D            | 1     | 4       | 2   | 2        |
| *M. liflandii* (African, US imported frogs) |               |       |         |     |          |
| X128f     | F            | 1     | 2       | 2   | 1        |
| X15       | F            | 1     | 2       | 2   | 1        |
| 1138      | F            | 1     | 2       | 2   | 1        |

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*M. ulcerans* and other MPM are present in both Buruli ulcer endemic and non-endemic sites

To discriminate between *M. ulcerans* and other MPM, 78 ER-PCR positive samples collected from standardized sampling and 10 ER positive belostomatids and naucorids (3 belostomatids and 7 naucorids) that were selectively collected were tested for the presence and copy number of MIRU1, locus 6, ST1, and, if applicable, locus 19. Of these samples, VNTR profiles were obtained from 67 invertebrate/vertebrate samples (Table 6). The remaining 31 samples could not be VNTR typed presumably due to insufficient material. VNTR profiling showed that only 12 of these 67 samples (18%) had a VNTR profile which matched *M. ulcerans* (Table 6). Seven of these were collected from aquatic environments endemic for Buruli ulcer, and five of these were from non-endemic water bodies. *M. ulcerans* Profile A was identified in 9 different invertebrate species, whereas *M. ulcerans* profile C, found in the genome sequence strain *Agy99* was detected in specimens of a Nepidae (Order Hemiptera), a Belostomatidae and an unidentified spider. VNTR MPM profile D was found in three samples, including a tadpole (Anura) and two predacious aquatic insects (Coleoptera: Families Hydrophilidae and Dytiscidae, Table 4). *M. ulcerans* VNTR profile A and MPM profile D was obtained from different samples of Dytiscidae, Anura and Hydrophilidae. Both Anura and Hydrophilidae samples were collected from the same endemic site. The Dytiscidae samples were collected from two different endemic sites. *M. ulcerans* profiles A and C were identified in two separate Belostomatidae samples collected from separate sites, one endemic and one non-endemic. These results suggest that *M. ulcerans* and other MPM occupy the same water body.
VNTR analysis of 82 ER PCR positive water filtrates yielded 8 *M. ulcerans* positive samples. One of these was profile B whereas the other 7 typed as profile A. Four of these samples were from non-endemic areas, while the remaining four samples were from endemic areas. Four of the 82 ER PCR positive water filtrates yielded MPM profile E. Two of these were from endemic regions whereas two were from non-endemic sites. The identity of all *VNTN* products was confirmed by sequence analysis. Representative gels illustrating VNTR profiles from various sample types are given in Figure S1. These data suggest that human endemicity data do not reliably predict the presence of *M. ulcerans* in Ghana.

### Physical evidence consistent with the presence of mycobacteria can be obtained by collection of biofilm communities on glass slides

Ninety-six glass slides were submerged in water bodies associated with human use in the communities of Amasaman (endemic) and Adigon, (non-endemic). From these, 47 slides were collected at 21, 42 and 98 days. At 21 days, biofilm formation on slides collected from Adigon was sparse, but became progressively denser over the course of the experiment. In contrast, at Amasaman, the endemic site, biofilms were very dense by 21 days, but became less dense over the course of the study (Figure 4). Acid-fast bacilli were found on 45 of 47 slides (Figure 4). Microscopic analysis of the biofilm community showed the presence of diatoms and fungus as well as a mixed population of bacteria and considerable detritus. Acid-fast bacilli occurred in clusters or small groups, but were not associated with other flora present on the slide consistent with the ability of mycobacteria to adhere to glass [32].

### Adigon (non-endemic)

Of the 47 biofilm slides analyzed, 37 were ER PCR positive (Table 4). VNTR profiles of 17 (46%) of these matched *M. ulcerans*, while 8 matched VNTR profiles of other MPM. VNTR analysis of slides collected from Adigon at 21 days was not conducted because all samples were ER negative (Figure 5). Three of five ER positive slides (60%) collected at 42 days from Adigon had *M. ulcerans* VNTR profile A, whereas one of the slides had a VNTR profile matching other MPM (profile D). *M. ulcerans* VNTR profiles were not found at Adigon at 98 days although VNTR patterns matching MPM were found on two slides. One of these corresponded to *M. liflandii* (profile F) while the other matched that of MPM associated with fish (profile E).

### Amasaman (endemic)

Nine of the twelve (75%) ER positive slides taken from Amasaman at 21 days had a *M. ulcerans* VNTR profile matching profile A, whereas a VNTR profile matching that of *M. liflandii* (profile F) was found on two slides. Five of six (83%) ER positive slides taken at 42 days from Amasaman had *M. ulcerans* profiles. *M. ulcerans* was not detected on the slides taken from Amasaman at 98 days although three slides (60%) produced VNTR signatures matching fish-associated MPMs (D and E). These results show the evolution of biofilm communities through time. The absence of *M. ulcerans* at 98 days is particularly interesting and could be explained by spontaneous detachment of the biofilm, or by grazing by tadpoles or invertebrates.

### *M. ulcerans* DNA is not detected in ER negative environmental samples

The analysis of VNTR data from environmental samples is complicated by many factors not present when analysis is performed on a pure bacterial colony. DNA extracted from insects, frogs, fish or filters contains DNA from a complex population of organisms. If VNTR profiling is a valid tool for detection of *M. ulcerans* in environmental samples, ER negative samples should also be negative for *M. ulcerans* by VNTR PCR. If however, specific VNTR sequences are present in a number of different organisms, or in bacteria which do not produce mycolactone, VNTR analysis of ER negative sites could yield a *M. ulcerans* or MPM profile. For example, if 1 repeat of MIRU1, locus 6 and ST1 were present in each of three different bacteria within a single environmental sample, this sample would produce a VNTR profile consistent with *M. ulcerans*. If this were the case, it would be impossible to detect the organism by VNTR analysis.

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**Table 6. VNTR analysis of ER positive samples.**

| Order      | Family        | Description                 | Site Endemicity | VNTR profile | VNTR Profile Designation |
|------------|---------------|-----------------------------|----------------|--------------|-------------------------|
| Hemiptera  | Nepidae       | Predacious aquatic insect   | N              | MU 3,1,2     | C                       |
| Araneae    |               |                             | N              | MU 3,1,2     | C                       |
| Hemiptera  | Belostomatidae| Predacious aquatic insect   | N              | MU 3,1,2     | C                       |
| Hemiptera  | Belostomatidae| Predacious aquatic insect   | E              | MU 1,1,1     | A                       |
| Anura      |               | Tadpole                     | E              | MU 1,1,1     | A                       |
| Lepidoptera| Crambidae     | Moth larvae                 | E              | MU 1,1,1     | A                       |
| Odonata    | Libellulidae  | Predacious aquatic insect   | N              | MU 1,1,1     | A                       |
| Coleoptera | Dytiscidae    | Predacious aquatic insect   | N              | MU 1,1,1     | A                       |
| Diptera    | Culicidae     | Mosquito pupae              | E              | MU 1,1,1     | A                       |
| Hemiptera  | Notonectidae  | Predacious aquatic insect   | E              | MU 1,1,1     | A                       |
| Odonata    | Protoneniaidae| Damselfly larvae            | E              | MU 1,1,1     | A                       |
| Coleoptera | Hydrophilidae | Predacious aquatic insect   | E              | MU 1,1,1     | A                       |
| Anura      |               | Tadpole                     | E              | MPM1,4,2,2   | D                       |
| Coleoptera | Hydrophilidae | Predacious aquatic insect   | E              | MPM1,4,2,2   | D                       |
| Coleoptera | Dytiscidae    | Predacious aquatic insect   | E              | MPM1,4,2,2   | D                       |

Profiles A–C: *M. ulcerans* (MU); Profile D: MPM associated with fish N: non-endemic, E: endemic.

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VNTR analysis of environmental samples would have little value in the identification of *M. ulcerans*. To address this possibility, VNTR analysis was performed on two sets of ER negative samples. The first set consisted of ER negative DNA from 35 samples representing a broad spectrum of samples collected at many different sites. The second set of samples was a complete sample set of 34 samples from a single ER negative site. Invertebrate, vertebrate, water filtrate, and soil samples were represented in each set. Though some of these samples produced bands for an individual locus, none of these samples produced a *M. ulcerans* VNTR profile.

*Mycobacterium ulcerans* and MPM are widely distributed within endemic and non-endemic sites in the Ashanti and Greater Accra regions

Twenty-six sites were sampled from 2004–2006 (Table 7). Fourteen were endemic and twelve were non-endemic. These sites represented water bodies from south-central regions in Ghana with a focus on the Greater Accra and the Ashanti regions. All samples from seven sites were ER negative suggesting the absence of any MPM including *M. ulcerans*. Six sites had samples with DNA insufficient for VNTR analysis. VNTR profiling was performed on the remaining thirteen sites, seven endemic and six non-endemic sites. *M. ulcerans* profile A was found in 6 of the endemic sites. Three endemic sites had only one VNTR profile: Ampa Abena and Nyame-Bekyere had *M. ulcerans* profile A, and Subin had MPM profile E. Two or more VNTR profiles were found within the same water body at four of the endemic sites. Bonsaaso was found to contain *M. ulcerans* VNTR profiles A, B and C. Along with *M. ulcerans* profile A, Bowkrom and Afasman also had MPM profiles E and D, respectively. Amasaman was found to contain two *M. ulcerans* VNTR profiles (A and B), one of the MPM *M. marinum* VNTR profiles (profile D), and the profile corresponding to *M. lindsayi* (profile F).

Samples from six non-endemic sites produced VNTR profiles. However, there was less diversity of VNTR profiles from the non-endemic sites than endemic sites. Four of these sites were represented by one *M. ulcerans* profile (either profile A or C), and one of the sites, Afienya, had only a MPM *M. marinum* VNTR profile (profile E). Adigon was the only non-endemic site which yielded multiple VNTR profiles. VNTR profiles of *M. ulcerans* (profile A), MPM *M. marinum* (profiles D and E), and *M. lindsayi* (profile F) were all obtained from biofilm samples collected in Adigon.

VNTR profiles representing *M. ulcerans* and other MPMs were obtained from sites from both the Greater Accra and the Ashanti regions (Figure 3). *M. ulcerans* and MPM VNTR profiles were found within the same site more frequently in the Greater Accra region than in the Ashanti region. *M. ulcerans* VNTR profiles A, B and C (1,1,1, 3,1,1 and 3,1,2 respectively) were found in both the Greater Accra and the Ashanti regions. MPM *M. marinum* profile D was found only in the Greater Accra region, whereas profile E was found in both regions. Profile F (M. lindsayi) was found in two sites of the Greater Accra region.

**Discussion**

In this paper we present results from a large scale study of *M. ulcerans* in the environment. Although a number of studies have reported the presence of *M. ulcerans* in environmental samples from endemic regions [13,16,17,33], this is the first study where standardized ecological methods were used to reduce sampling bias, and the first to include longitudinal data from both Buruli...
ulcer endemic and non-endemic sites. One of the mysteries of Buruli ulcer is the close proximity of endemic and non-endemic villages. For example, whereas the disease is rarely reported from the Ga East district of the Greater Accra region in Ghana, it is endemic in the Ga West district despite the fact that endemic and non-endemic villages may be separated by only a few kilometers (Figure 3). Since the climate, rainfall, plant populations and ethnic groups in Ga East and Ga West are similar it has been difficult to understand the differential occurrence of Buruli ulcer within these regions.

The most important finding from this study is that, whereas Buruli ulcer occurs within discrete geographic village foci within endemic regions,\textit{M. ulcerans} is widely distributed in water bodies in both endemic and non-endemic villages in the Greater Accra and Ashanti regions. This is consistent with its position as an environmental pathogen. We have also been able to repeatedly detect the presence of \textit{M. ulcerans} within some sites over a 27 month framework suggesting the long term survival and presence of \textit{M. ulcerans} in specific locations. These results clearly show that the focal occurrence of Buruli ulcer within the Greater Accra and Ashanti regions cannot be explained by the presence or absence of \textit{M. ulcerans} in the environment. Thus other factors such as demography and human behavior may be important in the epidemiology of Buruli ulcer in these regions.

In contrast, there are large geographic areas in West Africa such as the Volta region of Ghana, or drier Northern parts of Ghana, Benin and Togo, where Buruli ulcer has never been reported. It has been assumed that the absence of Buruli ulcer from these regions is based on environmental constraints which restrict the growth of \textit{M. ulcerans} or potential reservoir species. Results from an on-going project in the Volta region confirm this hypothesis in that we have failed to reveal a single \textit{M. ulcerans} positive sample out of hundreds of invertebrate, water filtrate or macrophyte samples from 20 sites sampled. The absence of Buruli ulcer in Volta is explained by the absence of \textit{M. ulcerans} (work in progress).

In a clinical setting the use of a single PCR target for detection of a pathogen is widely accepted. However, the use of a single PCR target for identification of bacteria in an environmental sample is rarely adequate. In Ghana, analysis of many IS\textit{2404} positive samples revealed the presence of mycolactone producing mycobacterial species (MPMs) other than \textit{M. ulcerans} as had been predicted [29]. In contrast, in Australia IS\textit{2404} PCR appears to be specific for \textit{M. ulcerans} because of the absence of other MPMs [33]. Here we provide the first evidence for the presence of MPMs in West Africa and show that MPMs and \textit{M. ulcerans} share aquatic environments. The pathogenic potential of MPM for humans is unknown, although the lower growth temperature of some of these species makes them unlikely human pathogens [26]. The fact that...
the strain complexity of MPMs and *M. ulcerans* is greater in endemic areas and greatest within the Greater Accra region is an intriguing finding which needs further investigation.

The use of geographic-specific VNTR profiles in following chains of transmission is extremely important since the heterogeneity of *M. ulcerans* isolates appears to differ within different West African countries [23,24,25]. For example, data based primarily on patient isolates from Benin led to the conclusion that there was a single West African *M. ulcerans* clone. However, several biovars have been identified in Ghana [23]. Our results agree with those of Hilty et al [23] in showing the presence of at least 3 different VNTR profiles in Ghana. Thus it is important when discriminating between *M. ulcerans* and other MPM that a geographically representative set of patient isolates is used.

Our initial concerns regarding the effect of low target copy number on the sensitivity of PCR methods reflected our naivete regarding PCR theory. We had not considered that the efficiency of the PCR reaction depends on many factors including the efficiency of primer binding, the length of the product and local DNA conformation or that because the reaction is exponential, the first few targets bound may rapidly become the major products. Experimental results confirm this theory since others have found no difference between the use of IS2404 PCR and that of 16sRNA PCR for detection of *M. ulcerans* in human samples despite the enormous difference in copy number [34] and results from VNTR analysis of clinical isolates show gel bands with an intensity never reported for IS2404 PCR [23]. It is possible that the fact that VNTR sequences are non-coding segments of DNA may make them more accessible to primer binding.

Our studies confirm the presence of *M. ulcerans* in predacious aquatic insects including Belostomatidae and Naucoridae families reported by Portaels et al [15] and extend these findings by showing that VNTR profiles from these insects match those of human isolates of *M. ulcerans*. Belostomatids were common in many sites sampled throughout the year. However, even where large numbers of Belostomatidae were collected the rate of *M. ulcerans* infection was very low. In Ghana, despite repeated season sampling the numbers of naucorids found were very low (paper in preparation). Evidence for the role of naucorids as potential *M. ulcerans* vectors comes from studies in Cote d’Ivoire [16]. Insect population studies are needed to confirm whether naucorids are more abundant in Cote d’Ivoire than in Ghana.

Our results show that *M. ulcerans* is widely distributed within invertebrate communities in aquatic environments. However, none of the *M. ulcerans*-positive, predacious invertebrates are hematophagous; thus the frequency with which humans are bitten would be expected to be quite low [35]. Although potential trophic relationships exist between several taxa studied (belostomatids, for example, feed on many other invertebrates and vertebrates and also cannibalize each other), it will take considerably more work to elucidate chains of transmission within the environment. Results presented here are based on determining the presence or absence of *M. ulcerans* in an environmental sample. Further studies need to be conducted using quantitative PCR methods to determine which species are most heavily infected and thus are more likely to serve as vectors.

Although it has been reported that snails and fish may harbor *M. ulcerans* [17] our results suggest the possibility that many of the IS2404 positive mycobacteria detected are MPM other than *M. ulcerans*. In our studies *M. ulcerans* was never detected in fish or snails, although other MPM were identified in later studies. The most consistently *M. ulcerans*-positive samples detected were filtered water and biofilms on glass slides. This suggests that exposure of open lesions to infected water cannot be ruled out as a potential source of infection.

A general problem regarding detection of *M. ulcerans* in environmental samples is that evidence has come almost solely from detection of *M. ulcerans* DNA and under-estimation of *M. ulcerans* could result due to the presence of PCR inhibitors. Our results suggest that current methods are effective in eliminating PCR inhibitors since dilution of samples did not result in the detection of many additional ER positive samples and none of those detected through dilution could be confirmed by sequencing. Despite the broad spectrum of samples we did not find evidence for inhibitors in any particular taxa or sample type tested. Nonetheless, the possibility exists that the number of positive *M. ulcerans* positive samples may be underestimated.

The use of slide biofilms for trapping mycobacteria in the environment has proven particularly useful since it provides preliminary physical evidence for the presence of mycobacteria (AFB staining) along with molecular evidence, and facilitates

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### Table 7. ER and VNTR results from all samples obtained from endemic and non-endemic sites sampled.

| ENDEMIC | Site/Community Name | District | ER pos | VNTR Profile |
|---------|---------------------|----------|--------|--------------|
| Pakro   | Akwapim S           | +        | NA     |
| Bowkrom | Akwapim S           | +        | A,E    |
| Amasaman| Ga                  | +        | A,B,D,F|
| Afuman  | Ga                  | +        | A,D    |
| Nyame Bekyere | Amanse W | +        | A       |
| Tontokrom | Amanse W       | +        | A       |
| Bonsaaso River | Amanse W | -        | ND      |
| Bonsaaso Pond | Amanse W       | +        | A,B,C  |
| Ampa Abena | Denkyira          | +        | A       |
| Subin   | Denkyira            | +        | E       |
| DobloGonno | Ga               | -        | ND      |
| Ablekuma| Ga                  | -        | ND      |
| Watreso | Amanse W            | -        | ND      |
| Akotoshie| Ga                | -        | ND      |

| NONENDEMIC | Site/Community Name | District | ER Pos | VNTR Profile |
|------------|---------------------|----------|--------|--------------|
| Dodowa     | Dangme W            | +        | NA     |
| Ayikumen   | Dangme W            | -        | ND     |
| Adumanya   | Dangme W            | -        | ND     |
| Asebi      | Dangme W            | +        | A      |
| Keedmos*   | Ga                  | +        | NA     |
| Odumse     | Dangme W            | +        | A      |
| Abbeypanya | Dangme W            | +        | C      |
| Afienya    | Dangme W            | +        | E      |
| Weja       | Ga                  | +        | NA     |
| Adwuman    | Denkyira            | +        | A      |
| Bretiekrom | Amanse W            | +        | NA     |
| Adiglon    | Ga                  | +        | A,D,E,F|

ND: VNTR not performed. NA: Insufficient DNA for VNTR profiling. Profiles A–C: *M. ulcerans*; Profiles D–F: other mycolactone-producing mycobacteria.

*Endemicity based upon district rather than community level incidence of human disease.*

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longitudinal studies. The numbers of slides used and placement of PVC pipes are crucial because of the inevitable loss of slides through changes in water level, or disturbance by animals or humans. There was a decrease of DNA samples from slides giving through changes in water level, or disturbance by animals or PVC pipes are crucial because of the inevitable loss of slides...

References
1. Van der Werf TS, Stienstra Y, Johnson RC, Phillips R, Adjei O, et al. (2005) Mycobacterium ulcerans disease. Bull World Health Organ 10: 785–91.
2. George KM, Chatterjee D, Gunawardana G, Welty D, Hayman J, et al. (1999) Assessing water-related risk factors for Buruli ulcer disease. Bull World Health Organ 10: 785–91.
3. Stinear TP, Pryor MJ, Porter JL, Cole ST (2005) Functional analysis and virulence of Mycobacterium ulcerans. Science 309: 683–97.
4. Yip MJ, Porter JL, Fyle JA, Lavender CJ, Portaels F, et al. (2007) Evolution of Mycobacterium ulcerans and other mycolactone-producing mycobacteria from a common Mycobacterium marinum progenitor. J Bacteriol 189: 2021–9.
5. Marston BJ, Diao MO, Horsburgh CR Jr, Diomande I, Nanas OK, Coleman S (2004) Emergence of Buruli ulcer disease in the Daloa region of Côte d'Ivoire. Am J Trop Med Hyg 71: 387–92.
6. Raghunathan PL, Whitney EA, Asamoa S, Stienstra Y, Taylor Jr TH, et al. (1999) Identification and characterization of IS2404 and IS2606: two distinct repeated sequences for detection of Mycobacterium ulcerans by PCR. J Clin Microbiol 37: 1058–23.
7. Ross BC, Johnson PD, Oppedissano F, Marino L, Sievers A, et al. (1997) Detection of Mycobacterium ulcerans in environmental samples during an outbreak of ulcerative disease. Appl Environ Microbiol 63: 4135–8.
8. Stinear T, Davies JK, Jenkin GA, Hayman JA, Oppedissano F, et al. (2000) Identification of Mycobacterium ulcerans in the environment from regions in Southeast Australia in which it is endemic with sequence capture-PCR. Appl Environ Microbiol 66: 3206–13.
9. Portaels F, Shenier P, Guimaraes-Peres, Fonteyne PA, Meyers WM (1999) Insects in the transmission of Mycobacterium ulcerans infection. Lancet 353: 986.
10. Marsson I, Robert R, Aubry J, Saint Andre JP, Konakou H, et al. (2002) Aquatic insects as a vector for Mycobacterium ulcerans. J Med Microbiol Rev 19: 708–727.
11. Stinear T, Ross BC, Davies JK, Marsson I, Robins-Browne RM, et al. (1999) Mycobacterium ulcerans disease. Bull World Health Organ 10: 785–91.
12. Johnson PDR, Azuolas J, Lavender C, WishartElwyn, Stinear TP, et al. (2007) Emergence of Buruli ulcer disease in the Daloa region of Côte d'Ivoire. Am J Trop Med Hyg 71: 387–92.
13. Swanson SJ, Neitzel D, Reed KD, Belongia EA (2006) Cofections Acquired from Insects. Clin Microbiol Rev 19: 708–727.
14. Fikrig E, Narasimhan S (2006) Bacteria traveling incognito? Microbes Infect 8: 1390–9.
15. Ross BC, Marsson I, Oppedissano F, Edwards R, Robins-Browne RM, et al. (1997) Development of a PCR assay for rapid diagnosis of Mycobacterium ulcerans infection. J Clin Microbiol 35: 1089–1106.
16. Stinear T, Davies JK, Marsson I, Robins-Browne RM, et al. (1999) Mycobacterium ulcerans disease. Bull World Health Organ 10: 785–91.
19. Silva MT, Portaels F, Pedrosa J (2007) Aquatic Insects and Mycobacterium ulcerans: An association relevant to Buruli ulcer control? PLoS Med 4: e63.

20. Trott KA, Stacy BA, Lilland BD, Diggs HE, Harland RM, et al. (2004) Characterization of a Mycobacterium ulcerans-like infection in a colony of African tropical clawed frogs (Xenopus tropicalis). Comp Med 54: 309–17.

21. Rhodes MW, Kator H, McNab A, Deshayes G, Reyart JM, et al. (2005) Mycobacterium pseudohodshottii sp. nov., a slow growing chromogenic species isolated from Chesapeake Bay striped bass (Morone saxatilis). Int J Syst Evol Microbiol 55: 1139–47.

22. Ucko M, Colomn A (2005) Mycobacterium marinum infections in fish and humans in Israel. J Clin Microbiol 43: 892–5.

23. Hilty M, Yehoah-Manu D, Boakye D, Mensah-Quainoo E, Rondini S, et al. (2006) Genetic diversity in Mycobacterium ulcerans isolates from Ghana revealed by a newly identified locus containing a variable number of tandem repeats. J Bacteriol 188: 1462–5.

24. Stragier P, Ablordey A, Meyers WM, Portaels F (2005) Genotyping Mycobacterium ulcerans and Mycobacterium marinum by using mycobacterial interspersed repetitive units. J Bacteriol 187: 1639–47.

25. Ablordey A, Swings J, Huban C, Chemlal K, Locht C, et al. (2005) Multilocus variable-number tandem repeat typing of Mycobacterium ulcerans. J Clin Microbiol 43: 1546–51.

26. Ranger BS, Mahrous EA, Mosi L, Adhaumili S, Lee RE, et al. (2006) Globally distributed mycobacterial fish pathogens produce a novel plasmid-encoded toxic macrolide, mycolactone F. Infect Immun 74: 6037–43.

27. Lamour K, Finley L (2006) A strategy for recovering high quality genomic DNA from a large number of Phytophthora isolates. Mycologia 98: 514–17.

28. Stinear TP, Seemann T, Poido S, Frigui W, Reyset G, et al. (2007) Reduction evolution and niche adaptation inferred from the genome of Mycobacterium ulcerans, the causative agent of Buruli ulcer. Genome Res 17: 192–200.

29. Chemlal K, De Ridder K, Fonteyne PA, Meyers WM, Swings J, et al. (2001) The use of IS2404 restriction fragment length polymorphisms suggests the diversity of Mycobacterium ulcerans from different geographical areas. Am J Trop Med Hyg 64: 270–273.

30. Merritt RW, Cummins KW (2006) Trophic relationships of macroinvertebrates. In: Hauer FR, Lamperti GA, eds (2006) Methods in Stream Ecology (2nd ed.). UK: Elsevier. pp 583–609.

31. Mve-Obiang A, Lee RE, Umstot ES, Trott KA, Grammer TC, et al. (2005) A newly discovered mycobacterial pathogen isolated from laboratory colonies of Xenopus species with lethal infections produces a novel form of mycolactone, the Mycobacterium ulcerans macrolide toxin. Infect Immun 73: 3307–12.

32. Steed KA, Falkinham JO (2006) Effect of growth in biofilms on chlorine susceptibility of Mycobacterium avium and Mycobacterium intracellulare. Appl Environ Microbiol 72: 4007–4011.

33. Fyfe JA, Lavender CJ, Johnson PD, Globan M, Sievers A, et al. (2007) Development and application of two multiplex real-time PCR assays for the detection of Mycobacterium ulcerans in clinical and environmental samples. Appl Environ Microbiol 73: 4733–40.

34. Guimaraes-Peres A, Portaels F, De Rijk P, Fissette K, Pattyn SR, et al. (1999) Comparison of two PCRs for Detection of Mycobacterium ulcerans. J Clin Microbiol 37: 206–8.

35. Merritt RW, Benbow ME, Small PLC (2005) Unraveling an emerging disease associated with disturbed aquatic environments: the case of Buruli ulcer. Front Ecol Environ 3: 323–331.

36. Stinear TP, Mve-Obiang A, Small PL, Frigui W, Pryor MJ, et al. (2004) Giant plasmid-encoded polyketide synthases produce the macrolide toxin of Mycobacterium ulcerans. Proc Natl Acad Sci USA 101: 1345–9.