

Epidemiology of Buruli ulcer in the Mapé Basin of Cameroon

INAUGURALDISSERTATION

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch- Naturwissenschaftlichen Fakultät

der Universität Basel

von

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aus

Safnern (Bern)

Basel 2015

Originaldokument gespeichert auf dem Dokumentenserver der Universität Basel
edoc.unibas.ch

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät der Universität

Basel auf Antrag von

PD Dr. Maja Weisser und Prof. Dr. Gerd Pluschke

Basel, 15. Oktober 2013

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Dekan

*Dedicated to my wife
and my parents*

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Acknowledgments

First of all, I would like to thank Prof. Gerd Pluschke for the supervision of my PhD and his continued support of my work. Further I would like to thank Gerd for giving me the opportunity to establish a new Buruli ulcer field research site in Bankim and for allowing me to follow all interesting leads that developed from the work there.

I would also like to thank PD Dr. Maja Weisser from the University Hospital of Basel for being the co-referee of my PhD thesis and for taking time to read and understand my work.

At Swiss TPH I would like to thank Prof. Marcel Tanner for helping me to come back to Swiss TPH for my PhD and for always being available for discussions about future plans. Further at the institute, I thank all my colleagues in the Molecular Immunology Unit. Particularly I would like to thank Dr. Marie-Thérèse Ruf and Miriam Bolz for their support with several of my projects, for proofreading manuscripts and parts of this thesis and for the many discussions about science as well as other topics. Thank you also very much to Sarah Kerber for her dedication and invaluable assistance with all BSL-3 and other laboratory work as well as for always being flexible to adjust her schedule to take care of the most urgent tasks. With MiB/MaB/SaK/ThR we really had a great team and did some interesting science. Further, I would like to thank Arianna Andreoli for her help with field and laboratory activities including all the logistics of the field trips. I wish you a great continuation of the work in Bankim. Finally, thank you also to Dr. Nicole Scherr, Dr. Marco Tamborrini, Dr. Michael Käser, Dr. Araceli Lamelas Cabello, Dr. Katharina Röltgen, Dr. Xueli Guan, Jean-Pierre Dangy, Julia Hauser, Maja Jud, Raphael Bieri, Paola Favuzza, Andrea Vettiger, Emma Ispasanie and Angelika Silbereisen for scientific input and countless coffee break discussions. Also at Swiss TPH, I would like to thank Dr. Leticia Grize for her great statistical support on several projects and for her support with the writing of the manuscripts. Thank you also to Dr. Lukas Tanner for great scientific discussions as well as many coffee breaks and lunches both in Singapore and Basel. Further at Swiss TPH, I would like to thank all the administrative staff and in particular Christine Mensch for always being kind to answer questions and having a useful solution for any problem that arises.

Further in Switzerland, I would like to thank Prof. Pierre-Yves Bochud and Dr. Stephanie Bibert at the University Hospital of Lausanne for being interested in conducting a

Buruli ulcer host factor study. At the Labor Spiez, I would like to thank Dr. Nadia Schürch, Dr. Matthias Wittwer and Dr. Christian Beuret for their technical support with the processing of the environmental and host factor samples and for generously giving us access to their equipment.

At the Division for Tropical Medicine at the University of Heidelberg, I would like to thank Prof. Thomas Junghanss and Dr. Moritz Vogel for the great collaboration, their continued medical input on all our patients and for allowing me to be involved in a Buruli ulcer clinical trial in Cameroon. Thank you also to Moritz for agreeing to be the expert in my thesis committee, for the many discussions in Yaoundé, Ayos, Basel and on the phone about science, the logistics of various projects and many other topics as well as for appreciating my LaTeX and other programming.

In Ghana, I would like to thank Prof. Dorothy Yeboah-Manu for her support with the culturing of the difficult to culture *M. ulcerans* as well as her help with the host factor study.

In Cameroon I have to thank many people without whom my project would not have turned out as it has. First, I would like to thank Dr. Alphonse Um Book and his team in Yaoundé for their scientific, administrative and logistic support of all our activities in the country. Thank you also to Dr. Earnest Njih Tabah of the National Buruli ulcer Control Program of Cameroon for his support of my work. Thank you further to Alim Nouhou for helping with both BU and leprosy activities and for always having an uplifting comment ready. I would also like to thank Ferdinand Mou, Suzy Gaëlle Mayemo, Edgard Satougale and of course Jacques Christian Minyem of the Bankim Fairmed team for their great support of our activities, the close scientific and logistic collaborations, for the meals in Bankim as well as the great time and companionship together. Edgar, I would also like to thank for going beyond the duties of a driver and for helping with all our activities, taking us safely across every broken bridge and coming to pick us up after the break down of our motorcycles even in the middle of the night. Ferdinand, I would like to thank for supporting our local activities with everything at his disposal, for critically discussing our joint projects and for the runs in Bankim. Christian, I would like to thank infinitely for his great and very generous scientific as well as logistic and technical support of our activities in the area. Thank you also for continuing our activities in our absence with Swiss diligence, for representing us locally during these times and for, even if deep in the bush, always only being a quick email response, SMS or phone call away whenever some information was required. On a personal note, thank you for being a good friend and for the fun times together in Bankim, Yaoundé,

the rest of Cameroon as well as Switzerland. Thanks further for always being interested to go for Chinese food in Yaoundé and introducing me to “Tchop et Yamo” as well as the infamous and absolutely delicious restaurant with beefsteak and plantain in Yaoundé. Finally also in Cameroon I would like to thank all of the health care staff of the Bankim Health District and the surrounding areas. Specifically, I would like to thank Dr. Djeunga Noumen for supporting our activities in the Bankim Health District and Fidèle Gaetan Wantong for being an invaluable help to all of our activities. Fidèle, I would further like to thank for always being available to work with us when we were in Bankim, for taking me on the back of his motorcycle into the deepest bush to re-find patients and for always allowing us to easily connect with the local population.

Further, I would like to thank the Medicor Foundation of Liechtenstein for their valuable financial support of all my work.

On a personal level, I would like to thank my parents and my brother for always supporting me. I would also like to thank Mami and Papi for giving me all the opportunities of the world, supporting my studies in Calgary and Singapore and for always being there for me and my wife. I would also like to thank my friends in Switzerland, Canada, Singapore and around the world for their continued moral support and, where applicable, for sharing the experience of being a PhD student. Finally and foremost, I would like to express my deepest thanks to my wife, Peiling Yap. Peiling, I would like to thank for every day of her support during the last four and a half years. I would further like to thank Peiling for her scientific input to my work, for critically cross-reading not only my manuscripts but also this thesis and many important emails as well as for always being willing to discuss my results and bounce around ideas. Most importantly, I would like to thank Peiling for getting me through the tougher times of my PhD and for helping me to always keep my sanity and put things into the right perspective. With a lot of love for you, I look forward to many more years of our great scientific and private team work.

Summary

Buruli ulcer (BU) is a neglected tropical disease of the skin and subcutaneous tissue caused by *Mycobacterium ulcerans*. The disease has been reported from over 30 countries with most cases coming from West Africa. While it is commonly accepted that BU is not acquired by human-to-human transmission both the environmental reservoir of the pathogen and the mode of transmission to humans remain to be identified. Clinically BU presents with a wide range of forms which includes non-ulcerative lesions and ulcers with undermined edges. Much of the pathology of a *M. ulcerans* infection is believed to be caused by its unique ability to produce the macrolide toxin called mycolactone which causes tissue necrosis and local immunosuppression. BU can be diagnosed by microscopy, polymerase chain reaction (PCR), culturing and histology, however due to lack of access to laboratory facilities, cases are often diagnosed based on clinical symptoms only. Historically, BU was treated using wide scale excision, but since 2004 the WHO recommends the use of streptomycin and rifampicin daily for 8 weeks to treat the infection.

In the framework of this PhD thesis we have established a new BU field research site in the Mapé Basin of Cameroon and studied various aspects of BU epidemiology, differential diagnosis and transmission at this location. As a basis for our research, we conducted an exhaustive house-by-house survey for BU, leprosy and yaws in the Bankim Health District. Following the survey we closely monitored and studied all BU cases detected in the region. By supporting local laboratory diagnosis with real time-PCR (RT-PCR) and culturing, we were able to identify and describe a case of cutaneous tuberculosis which was initially diagnosed as BU. This patient highlighted the importance of further support to improve clinical differential diagnosis of BU in remote endemic areas. Eighty-eight of all the RT-PCR confirmed BU cases identified in the Mapé Basin in the course of our research were studied in detail. By mapping the patients' homes we were able to describe the distribution of BU in the area and to identify highly endemic communities. Based on the population age data collected in the survey we were also able to compute the age adjusted cumulative incidence rate of BU, revealing that children below the age of five were underrepresented among cases of BU. By analysing serological responses of patients and community members from BU endemic areas in the Mapé Basin and Ghana against an immunodominant antigen of *M. ulcerans*, we have observed a similar late onset of seroconversion, indicating that BU transmission intensifies when preschool children start

moving further away from home. To identify potential sites of *M. ulcerans* transmission, we screened sites of environmental contact at the homes and farms of laboratory confirmed BU patients for the presence of *M. ulcerans* DNA. In this analysis we identified three RT-PCR positive permanent village water sites and by studying one of these sites longitudinally in great detail we obtained evidence that *M. ulcerans* can persist in underwater detritus. This niche of *M. ulcerans* may represent an environmental reservoir and a source of infection of the pathogen. To further elucidate BU transmission pathways we have generated a set of clinical isolates of *M. ulcerans* from the Mapé Basin for a phylogeographic analysis of the distribution of the currently circulating haplotypes of *M. ulcerans* based on whole genome sequencing. By the routine culturing from clinical specimens and the evaluation of different transport media and decontamination methods we were further able to develop an optimized protocol for the primary isolation of *M. ulcerans* after long term storage of samples.

Our interdisciplinary research approach including biomedical and social science research elements, including future behavioural studies in young children, may eventually help to elucidate transmission and to improve control of BU.

Zusammenfassung

Buruli Ulkus (BU), ist eine vernachlässigte Tropenkrankheit der Haut und des Unterhautgewebes welche durch *Mycobacterium ulcerans* verursacht wird. Die Krankheit ist in über 30 Länder nachgewiesen worden und die meisten Fälle stammen aus West Afrika. Während es allgemein akzeptiert ist, dass BU nicht direkt von Mensch zu Mensch übertragen wird, sind sowohl das Reservoir von *M. ulcerans* in der Umwelt wie auch der Übertragungsweg des Bakteriums zum Menschen noch nicht bekannt. Klinisch präsentiert sich BU mit einem weiten Spektrum an Krankheitsbildern und kann sowohl zu geschlossenen Läsionen wie auch zu offenen Ulzera mit den typischen unterminierten Wundrändern führen. Die Pathologie von BU wird vor allem durch die einzigartige Fähigkeit von *M. ulcerans* bestimmt, ein Makroloidtoxin namens Mycolactone zu produzieren, welches zu Nekrose und Immunsuppression führt. BU kann durch Mikroskopie, Polymerase-Kettenreaktion (PCR), das Kultivieren der Bakterien oder histopathologische Untersuchungen diagnostiziert werden. Auf Grund fehlenden Zugangs zu diagnostischen Laboratorien werden jedoch BU Fälle oft nur klinisch diagnostiziert. In der Vergangenheit wurde BU durch chirurgisches Entfernen der Läsionen behandelt. Seit 2004 empfiehlt die Weltgesundheitsorganisation, dass BU mittels einer täglichen Kombinations-Chemotherapy bestehend aus Streptomycin und Rifampicin für acht Wochen behandelt werden soll.

Im Rahmen dieser Doktorarbeit, haben wir einen neuen BU Forschungs-Standort im Kamerunischen Mapé Becken etabliert und epidemiologische und diagnostische Aspekte der Krankheit sowie den Übertragungsweg untersucht. Als Ausgangsbasis für diese Arbeiten haben wir einen vollumfassenden Survey für BU, Lepra und Frambösie in dem Bankim Gesundheitsdistrikt durchgeführt. In der Folge haben wir alle neuentdeckten BU Fälle aus der Region systematisch erfasst. Durch die Unterstützung lokaler Labordiagnostik mittels quantitativer-PCR (RT-PCR) und das Kultivieren des Erregers konnten wir einen Fall von Haut-Tuberkulose identifizieren, welcher zunächst klinisch und mikroskopisch als BU diagnostiziert wurde. Dieser Fall zeigt wie notwendig es ist die lokalen Kapazitäten der klinischen Diagnose für BU zu verbessern. Insgesamt haben wir 88 RT-PCR positive BU Fälle, welche im Verlauf unserer Studie im Mapé Becken identifiziert wurden, im Detail untersucht. Wir haben die Häuser der Patienten

kartographiert und die Verteilung von BU in dem Gebiet beschreiben sowie hochendemische Regionen identifiziert. Basierend auf der Altersverteilung der Bevölkerung, welche wir in dem Survey erfasst haben, konnten wir die altersangeglichene kumulative Inzidenzrate von BU berechnen. Diese Analyse zeigte, dass unter fünf Jahre alte Kinder in der Altersverteilung der Patienten unterrepräsentiert sind. Unsere seroepidemiologischen Untersuchungen an Patienten und gesunden Personen aus BU endemischen Regionen Kameruns und Ghanas zeigten weiterhin, dass Kinder erst im Alter von etwa sechs Jahren vermehrt Immunantworten gegen *M. ulcerans* entwickeln. Dies deutet darauf hin, dass die Intensität der *M. ulcerans* Exposition zunimmt, wenn sich Kinder zunehmend weiter vom unmittelbaren Umfeld ihrer Elternhäuser entfernen. Um für die Ansteckung relevante Umweltreservoirs zu identifizieren, haben wir die Anbauflächen sowie die von BU Patienten im Dorf und in der Nähe der Anbauflächen aufgesuchten Wasserstellen auf das Vorhandensein von *M. ulcerans* DNA untersucht. Hierbei konnten wir drei RT-PCR positive permanente Dorfwasserstellen identifizieren. Eine detaillierte longitudinale Analyse von einer dieser Wasserstellen deutete darauf hin, dass *M. ulcerans* in Unterwasser-Detritus persistieren kann. Diese ökologische Nische könnte eines der Reservoirs von *M. ulcerans* darstellen. Um weitere Hinweise auf den Übertragungsweg von *M. ulcerans* zu erhalten, haben wir zusätzlich eine Stammsammlung von *M. ulcerans* Isolate aufgebaut. Die gewonnenen Isolate werden wir für phylogeographische und genomische Analysen zur räumlichen Verteilung der lokalen Haplotypen einsetzen. Durch das routinemässige Kultivieren von *M. ulcerans* aus klinischen Proben und das Evaluieren unterschiedlicher Transportmedien und Dekontaminationsmethoden konnten wir weiter ein optimiertes Protokoll für die Isolation von *M. ulcerans* aus klinischen Proben nach Langzeitlagerung entwickeln.

Unsere interdisziplinäre Forschung mit biomedizinischen wie auch sozialwissenschaftlichen Elementen, sowie zukünftige Verhaltensstudien an kleinen Kindern, werden schlussendlich hoffentlich dazu beitragen, den Übertragungsweg von BU zu identifizieren und die Kontrolle der Krankheit zu verbessern.

Chapter 1

Introduction

The World Health Organisation's (WHO) list of neglected tropical diseases (NTD) includes 13 conditions which are among the most frequent infections of the world's poorest inhabitants. Buruli ulcer (BU) is together with leprosy and trachoma, one of the three bacterial infections included in this set of diseases [1,2]. Starting around the turn of the last millennium, in parallel to large scale international efforts to achieve the Millennium Development Goals and reduce the worldwide burden of malaria, human immunodeficiency virus infection / acquired immunodeficiency syndrome (HIV/AIDS) and tuberculosis, measures to tackle NTDs have also been scaled up. For BU specifically, major efforts to tackle the world wide burden of this disease were initiated by the WHO's "Yamoussoukro Declaration on Buruli ulcer" in 1998 [3].

1.1 Epidemiology

The first report of BU was published in 1948 by MacCallum *et al.* describing the disease based on six patients observed in Australia. In this initial publication, the disease causing organism, later named *Mycobacterium ulcerans*, was also isolated [4]. Despite the first report from Australia, the international name of BU originates from later reports which identified the Buruli county of Uganda as a hot-spot for the disease in the late 1950's [5]. Around the same time of this first report of cases from Uganda, cases were also reported from various other African countries including areas that now belong to Democratic Republic of the Congo, Republic of the Congo, Gabon, Nigeria and Cameroon [6]. Cases were further reported from South East Asian countries such as Malaysia, Indonesia and Papua New Guinea as well as Central and South American countries such as Mexico and French Guiana [5,6]. Currently disease incidence is highest in West Africa, specifically in Côte d'Ivoire, Benin and Ghana [7]. In South America and South East Asia on the other hand, there are today only sporadic reports of new cases [8,9]. As shown in Figure 1.1, BU has been reported from over 30 countries on four continents and has an important public health impact [10,11]. The disease is considered the third most common mycobacterial disease after tuberculosis and leprosy [7] and in 2010 there were 4'888 cases notified worldwide [12]. Reports however suggest that this world wide case load may be a an underrepresentation of the true disease burden and BU may be more widely spread then what is currently known [7,12]. On the other hand

Portaels *et al.* also suggest that due to misdiagnosis (see below), some countries may also be over reporting cases [13].

In Cameroon, BU was first observed in 1969 in the Nyong area close to the towns of Ayos and Akonolinga [6]. Following this initial discovery of BU cases in southern Cameroon, other endemic areas, including the Bankim Health District (HD) in 2004 [14], have been discovered. Currently there are three major foci of BU in Cameroon; namely: Ayos/Akonolinga/Ngoantet, Bankim and Mbonge (A. Um Boock, personal communication).

As illustrated by the distribution of BU cases in Cameroon, the occurrence of cases in the endemic countries tends to be very focalized and incidence rates can vary greatly inside countries. Reports of cases must therefore be evaluated at local level [13]. In the endemic countries, BU cases occur in particular close to wetlands or areas with stagnant water, in rural regions or in locations where the environment has been disturbed by man-made alterations [12,15]. In some of these foci, the number of cases of BU, may even exceed the number of leprosy or tuberculosis cases [13].

BU disease occurs mostly in children between the age of 5 and 15 [7,10]. Contrary to some initial reports [5] which suggested that there are differences between the number of women and man suffering from BU, in most BU endemic regions no significant gender differences in BU incidence have been observed [13]. Most of the BU lesions occur on the extremities with roughly 80% of lesions on the lower extremities [10]. Analysis of the lesion distribution have further shown that in adults lesions most often occur on the extremities whereas in children, the trunk, head and neck are also affected [13].

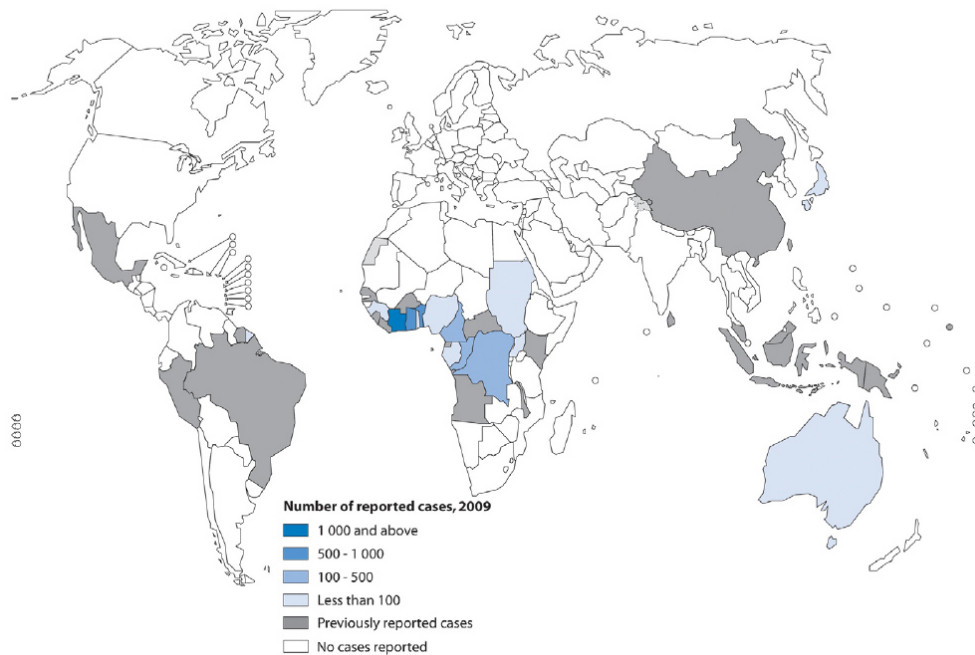


Figure 1.1: Global distribution of BU cases in 2009.
Figure adapted from Merritt *et al.* [10].

1.2 Causative agent

The environmental pathogen *M. ulcerans* is believed to have evolved from *Mycobacterium marinum* which lives in diverse aquatic and intracellular environments and occasionally causes granulomatous skin lesions in human [16]. In the course of its divergence from *M. marinum*, with which *M. ulcerans* shares 98.3% average genomic DNA sequence identity, *M. ulcerans* has undergone some major changes. Specifically the species emerged by acquiring the virulence plasmid pMUM by horizontal gene transfer [15]. A further bottleneck in the evolution of *M. ulcerans* was the uptake and expansion of the insertion sequences IS2404 and IS2606. The spread of multiple copies of these IS elements over the chromosome has led to extensive pseudogene formation, genome reduction and genome rearrangement in *M. ulcerans* [15]. The reductive evolution is thought to have reduced the ability of *M. ulcerans* to grow under low oxygen conditions compared to *M. marinum* and its resistance to direct sunlight. These changes are believed to indicate that *M. ulcerans* has adapted to a new niche in the environment which is different from the one occupied by its ancestor [10,15].

Within *M. ulcerans*, reductive evolution has led to the emergence of three lineages [17]. Lineage 1 *M. ulcerans* are fish and frog pathogens that do not usually cause disease in humans. Lineage 2 is the ecovar causing human disease in Japan and South America

and lineage 3 *M. ulcerans* strains cause human disease in Australia and Africa [17]. Further genetic analyses of the diversity of *M. ulcerans* strains which cause human disease, found that the pathogen is highly clonal and high resolution typing methods are required to differentiate strains circulating in the same endemic area [7,18]. Furthermore, the monomorphism of *M. ulcerans* limit our ability to monitor the spread of the pathogen, although it has recently been shown that by single-nucleotide polymorphism (SNP) typing, strains originating from an area smaller than 1000km² can be geographically clustered and separated into different haplotypes [18].

M. ulcerans, unlike all other mycobacteria, produces an immunosuppressive and necrotizing macrolide toxin called mycolactone. This toxin is responsible for most of the necrosis in the subcutaneous tissue in and around BU lesions [19]. Indeed, mycolactone alone has been shown to produce pathologies similar to those of *M. ulcerans* infections [19]. The toxin, which is secreted by *M. ulcerans* and diffuses into the infected tissue, has further been demonstrated to be essential for the virulence of *M. ulcerans* [10,12]. In addition to causing local tissue necrosis, mycolactone also has immunosuppressive properties and it is believed to kill infiltrating cells in human lesions [15,20]. *M. ulcerans* isolates from Africa produce the A/B form of the lipid toxin whereas Australian isolates produce the C form of the toxin and *M. ulcerans* from Asia produces the D form of mycolactone [7]. Strains of *M. ulcerans* from the various geographic regions that produce the different mycolactones differ in their virulence. In addition to the mycolactones produced by human pathogenic *M. ulcerans*, two additional chemical variants of the toxin, E and F, are produced by the fish and frog pathogens, which have previously also been referred to as *M. liflandii* and *M. pseudoshottsii* [15,20].

An additional noteworthy characteristic of *M. ulcerans* is its slow growth rate with an estimated generation time of 48 hours which greatly complicate the primary isolation of the pathogen (see below). Furthermore, *M. ulcerans* thrives in a cooler and fairly narrow temperature range from 28 to 34 °C, which likely explains why mainly cooler tissues like the skin are infected by the pathogen [10,15].

1.3 Reservoir and Transmission

Based on the distribution of BU cases among Rwandan refugees in Uganda in the 1960's and early 1970's researchers of the Uganda Buruli Group concluded that *M. ulcerans* was unlikely to be transmitted directly from person-to-person [5]. This opinion has mainly prevailed and it is now commonly accepted that infection occurs locally from an environmental source [21]. However to date the exact nature of the environmental reservoir and the mode of transmission of BU remain unknown and several hypothesis have been proposed [10].

As early as the 1960's and 1970's it has been noticed that clusters of cases occur in close proximity to rivers and swampy areas but researchers also found that direct contact with large water bodies was not required for BU transmission [22]. More recent case control studies to identify risk factors for BU in various endemic areas, have repeatedly confirmed the association of BU cases with stagnant or slowly flowing water [10,22]. Associated with increased risk in proximity to water bodies, it has also been suggested that the incidence of BU increases after man-made alterations of the environment such as dam construction, deforestation, intensified agricultural use as well as other changes that increase the risk of flooding or the amount of wetlands [10]. Failure to wear protective clothing and pour wound care have also been reconfirmed as risk factors for BU in several studies [10]. Individual studies have identified other risk factors which were however not confirmed in different settings [10].

Based on the age distribution of cases, it has been suggested that transmission of BU likely does not take place at the homes of the patients [10]. Initial studies on the transmission suggested that the pathogen is inoculated directly from the environment into small skin lesions [23]. Such small skin trauma could go unnoticed by the patients. Further, such a mode of transmission would necessitate an environmental reservoir of the pathogen from where the bacteria would be picked up. To identify this source of the infection, numerous studies have attempted to detect the pathogen in soil, on plants and in water. Although *M. ulcerans* can be cultured from human lesions, culturing from environmental sources is very difficult due to overgrowth with other microorganisms [10]. Our current understanding of the distribution of *M. ulcerans* in the environment is therefore based on the detection of *M. ulcerans* DNA in the environment. Although *M. ulcerans* DNA could be detected at numerous samples such as soil, biofilm, detritus, and

water filtrates [10,24], a clear environmental reservoir could not be identified. Furthermore, published data on the distribution of *M. ulcerans* in the environment is at times contradictory [25]. While in some studies none of the samples from the physical environment were positive for *M. ulcerans* [26], in other studies samples collected both from endemic and non-endemic areas were positive [24]. Because of the detection of *M. ulcerans* DNA in aquatic insects [26], the potential roles of insect vectors are also being investigated [26]. Specifically water bugs are suspected to play a role in the transmission of BU [26]. The first isolation of an environmental strain of *M. ulcerans* from an insect provided further evidence for their involvement [27] and various field and laboratory studies have attempted to elucidate the implication of water bugs in BU transmission [13]. However based on the fact that these insects do not regularly bite humans and the lack of direct causal evidence of their involvement, the role of water bugs in BU transmission remains questionable [10]. In Australia, studies have also implicated mosquitos in the transmission to BU [28]. In addition to experimental evidence from endemic areas, much has also been speculated on the possible nature of an environmental reservoir of *M. ulcerans* [29]. Based on characteristics of *M. ulcerans*, it has been suggested that an eutrophic water body with high amount of sedimentation may favour *M. ulcerans* growth because of the reduced rate of dissolved oxygen and UV penetration [10,29]. On the other hand animals could also act as the reservoir of *M. ulcerans* [10]. In Southern Australia such a scenario, where possums are believed to greatly contribute to the environmental source of the pathogen, has been described [30]. In Africa no such animal reservoir has been identified to date [31].

Ultimately various modes of transmission of BU, depending on the epidemiological setting, are also possible [10]. Targeted studies in highly endemic communities, particularly looking at the behaviour of the local populations, are urgently required to fully understand the mode of transmission of BU [10]. Once the mode of transmission is known, preventive measures can be developed and implemented in the communities affected [12].

1.4 Pathogenesis

BU is a necrotizing skin disease that is believed to usually start with a painless nodule [7]. The incubation period of the disease is estimated at 2-3 months [7]. From the initial nodular phase, the disease may progress by the massive destruction of the subcutaneous adipose tissue to a non-ulcerative oedema or plaque (Figure 1.2). As the disease progresses further the epidermis may collapse and characteristic ulcers with undermined edges develop (see Figure 1.2) [15]. As lesions advance they can lead to extensive tissue destruction with large numbers of extracellular *M. ulcerans* and minimal inflammatory response [15]. Further characteristics of *M. ulcerans* infected skin tissue are the presence of dead fat cells, so called “fat cell ghosts”, and epidermal hyperplasia [13]. In certain patients if left untreated, BU lesions progress in size to cause extensive skin tissue damage and may encompass entire limbs or large portions of the trunk [12]. Even after treatment such large ulcers result in extensive scars and may lead to permanent disabilities [12]. In the past reports have suggested that BU lesions are comparatively clean [15]. However recent reports now shown that secondary infections of BU lesions are not uncommon and necessitate additional treatment [34,35].

In an estimated 10% of cases BU is also manifest with multiple lesions and in rare cases *M. ulcerans* infection leads to osteomyelitis [7]. Further studies are required, but it is believed that particularly patients co-infected with HIV suffer from more severe presentations of the disease [22].

In the active phase of the disease, there is extensive necrosis at the site of the lesion, little inflammation and no infiltrating immune response. The lack of an immune response along the active rim of the lesions is likely due to the secreted mycolactone which prevents any immune cells from reaching the *M. ulcerans* in the lesion. In this phase, lesions may contain large amounts of usually extracellular bacteria. As the lesion heals under antibiotic treatment, granulomatous inflammation and organized lymphoid aggregates develop and bacteria and bacterial debris are typically found intracellularly [7]. During this “organizing phase” there is extensive infiltration of lymphocytes, macrophages and Langhans’ giant cells which eventually leads to fibrosis [13].



Figure 1.2: Clinical forms of BU.

Typical presentations of a nodule (A), plaque (B), oedema (C) and large ulcer (D) are shown. Images are adapted from the WHO [36].

1.5 Diagnosis

For BU laboratory diagnosis, there are currently four methods available. Microscopy can be used at the rural treatment centres to detect acid fast bacilli (AFB) after Ziehl-Neelsen staining. At reference laboratories, polymerase chain reaction (PCR) can be used to detect the *M. ulcerans* specific insertion sequence IS2404 or *M. ulcerans* can be cultured from clinical samples. Further at reference laboratories, tissue samples can be examined by histology to detect AFB. Based on its superiority in terms of sensitivity and specificity, PCR has become the gold standard of BU diagnosis [37]. However issues with false-positive results have recently been raised and strict quality control measures must be taken to ensure the accuracy of the obtained results (M. Eddyani *et al.*, PLoSOne, submitted). To increase accuracy of the diagnosis it is further important that samples are collected from all around the lesion [38,39] and it is suggested that each site is sampled at least twice [7].

In addition to supporting diagnosis, culturing of *M. ulcerans* is also used to assess treatment response and to monitor drug resistance [7,40]. Similarly, histology can be used to study the local immune response around the lesion and monitor the course of treatment [7,21,41]. Histopathology can further provide valuable insight into the state of the subcutaneous tissue of non-ulcerative lesions [41].

Because of difficulties with access to laboratory facilities, cases of BU in rural endemic areas are often diagnosed based only on clinical signs [15]. If performed by highly trained staff, the clinical diagnosis can achieve an accuracy of up to 95% [42]. However if performed by less specialized health care personnel, there are large discrepancies between clinical diagnosis and laboratory confirmation [7]. For the differential diagnosis, the nature of the edges of ulcerative lesions and the lack of fever as well as pain of small lesions, can be used as indications for BU [7]. Conditions such as tropical phagedenic ulcer, necrotising fasciitis, venous ulcer, diabetic ulcer, sickle-cell disease-related ulcers, yaws, cutaneous tuberculosis, leprosy, cutaneous leishmaniasis and malignant ulcer should be included in the differential diagnosis [2,43].

Because of these difficulties with the clinical diagnosis in routine settings, new point-of-care rapid diagnostic tests are urgently needed [2]. In particular a test to detect mycolactone in human lesions would be very specific and also allow for the monitoring of the viability of the infecting pathogen and thereby assessing treatment success [44].

1.6 Treatment

Historically BU was treated by surgical removal of the lesions. Because of the local dispersion of *M. ulcerans* into the healthy skin surrounding the lesion [45], such surgery needed to include a margin of healthy tissue to try to reduce the rate of re-lapse. Despite this measure, surgical treatment still resulted in relapse rates of around 20% [7]. The resulting satellite lesions then required multiple interventions and led to prolonged hospital stays [46].

As early as in the 1970's it was known that rifampicin can be used to treat small BU lesions [7]. However it was only in 2004, with experimental data in a mouse model and preliminary human data that the WHO started to suggest antibiotic therapy. Specifically current treatment guidelines recommend the use of 10mg/kg of oral

rifampicin and 15mg/kg of intramuscular streptomycin (S/R) daily for 8 weeks [7]. Although there were initial doubts [22], the use of S/R treatment appears to be well suited to treat BU and cases of recurrence are rare. Furthermore, toxic effects of S/R appear to be relatively rare [22]. Despite the efficacy of this antibiotic combination in killing the infecting pathogen, debridement and skin grafting is often required, particularly for larger lesion [22]. Because of the requirement of injections, efforts are on-going to establish a fully oral BU treatment with streptomycin replaced by clarithromycin [7]. With such a fully oral treatment regimen, blister packs with several days of treatment for the patient to take home, as for example used in the treatment of leprosy, could be implemented. However, wound care, which is increasingly being recognised as an important component in BU care, still requires major attention even with a fully oral treatment. Based on the temperature sensitivity of *M. ulcerans*, alternative treatment using heat is also being evaluated [47]. Furthermore, the development of a vaccine against BU is also on-going [12]. Early on in BU research it was shown that BCG has some potential to protect against osteomyelitis without an effect against cutaneous BU [13]. Further research is therefore required [12].

Even with adequate antibiotic treatment, large lesions may lead to disability and require physiotherapy to reduce the risk of permanent sequelae from joint contracture and poor scarring [2,7]. BU has been suggested to be able to self-heal in rare cases however often accompanied with deformities and functional limitations [22]. BU treatment may further lead to an immune system driven temporary worsening of the lesion or the development of secondary lesions. This phenomenon termed “paradoxical reaction”, can be resolved with simple wound care and changes to the treatment provided are unnecessary [21].

1.7 Control

In Cameroon, BU control is integrated with the control of leprosy, yaws and leishmaniasis into a national control program. This program collects epidemiological data about cases of BU from all around Cameroon and reports the statistics to the WHO. The program also advocates for the inclusion of BU into the training of future medical personnel (E. Njih Tabah, personal communication). However beyond these roles the National BU Control Program is limited by financial constraints and all remaining BU

control activities are conducted and in part financed (A. Um Boock, personal communication) by the African office of the Swiss NGO Fairmed. Philanthropic organizations play a key role in BU care in several African endemic countries [48]. In Cameroon, Fairmed coordinates and supports BU activities at all BU treatment centres except in Akonolinga, where the BU care facility is managed by Médecins sans Frontières (MSF). All Fairmed managed BU treatment centres are located at public health care facilities and are staffed by Ministry of Health employees. In addition to managing and distributing all drugs and other materials required for BU care, Fairmed trains, supervises and partially employs the staff working at the treatment facilities. In addition to treating cases, major health facilities in the endemic areas, which also act as BU reference facilities, supervise BU work in the surrounding health centres, where less severe cases of BU are treated as outpatients (A. Um Boock, personnel communication). For the Bankim endemic area, the Bankim district hospital coordinates BU activities in the Bankim health district and acts as a reference centre for cases coming from the surrounding districts.

1.8 Goal

For this PhD thesis we set out to establish a new BU field research site in the Mapé Basin of Cameroon and investigate epidemiological and microbiological aspects of the disease. We further aimed to contribute to a better understanding of the transmission of *M. ulcerans*.

1.9 Objectives

1. To describe the epidemiology of BU in the Mapé Basin of Cameroon, including the geographic origin and age distribution of patients as well as the localisation of their lesions on their bodies, in detail.
2. To study the onset of a serological response towards *M. ulcerans* in young children living in BU endemic areas in Cameroon and Ghana.
3. To study the distribution of *M. ulcerans* DNA at sites of regular environmental contact of laboratory confirmed BU patients.
4. To develop a protocol suitable for the primary isolation of *M. ulcerans* from clinical samples after long-term storage.
5. To raise awareness of issues with the differential diagnosis of BU in rural African treatment facilities.

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Chapter 2

Geographic Distribution, Age Pattern and Sites of Lesions in a Cohort of Buruli Ulcer Patients from the Mapé Basin of Cameroon

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Article published in:
PLoS Neglected Tropical Diseases

2.1 Abstract

Buruli ulcer (BU), a neglected tropical disease of the skin, caused by *Mycobacterium ulcerans*, occurs most frequently in children in West Africa. Risk factors for BU include proximity to slow flowing water, poor wound care and not wearing protective clothing. Man-made alterations of the environment have been suggested to lead to increased BU incidence. *M. ulcerans* DNA has been detected in the environment, water bugs and recently also in mosquitoes. Despite these findings, the mode of transmission of BU remains poorly understood and both transmission by insects or direct inoculation from contaminated environment have been suggested. Here we investigated the BU epidemiology in the Mapé basin of Cameroon where the damming of the Mapé River since 1988 is believed to have increased the incidence of BU. Through a house-by-house survey in spring 2010, which also examined the local population for leprosy and yaws, and continued surveillance thereafter, we identified, till June 2012, altogether 88 RT-PCR positive cases of BU. We found that, the age adjusted cumulative incidence of BU was highest in young teenagers and in individuals above the age of 50 and that very young children (<5) were underrepresented among cases. BU lesions clustered around the ankles and at the back of the elbows. This pattern neither matches any of the published mosquito biting site patterns, nor the published distribution of small skin injuries in children, where lesions on the knees are much more frequent. The option of multiple modes of transmission should thus be considered. Analyzing the geographic distribution of cases in the Mapé Dam area revealed a closer association with the Mbam River than with the artificial lake.

2.2 Author Summary

Buruli ulcer (BU) is an infectious disease caused by *Mycobacterium ulcerans* that is affecting mostly children in endemic areas of West Africa. Proximity to slow flowing water is a risk factor, but the exact mode of transmission of BU remains unclear. Man-made environmental changes, such as sand mining, damming of rivers and irrigation have been implicated with increases in disease incidence. Here we report findings from a survey for BU and continued case detection thereafter in the Bankim Health District of Cameroon. In this area, the local population believed that the damming of the Mapé River has led to the emergence of BU. In 28 months we identified 88 laboratory confirmed cases of BU. Studying these cases, we found that the age adjusted cumulative incidence of BU in the elderly is similar to that in children and that the distribution pattern of BU lesions neither matches mosquito biting patterns nor the distribution of small skin injuries. Multiple modes of transmission should therefore be considered. Our data further showed that the patients appear to have closer contact to the local Mbam River than to the artificial Mapé dam reservoir.

2.3 Introduction

Buruli ulcer (BU), a neglected tropical disease (NTD) of the skin, is caused by *Mycobacterium ulcerans* [1] and if untreated, can lead to disability. Worldwide, local BU incidence rates are highest in West Africa and Australia, where the classical lineage of *M. ulcerans* is found [2–4] and the disease occurs at different foci in the endemic countries. Both sexes can be affected by the disease and although individuals of all ages can get BU, most of the patients are less than 15 years old [5]. In Cameroon, BU was first described in 1969 in the Nyong river valley where during a cross-sectional survey in 2001, a total of 436 clinically diagnosed cases of active or inactive BU were found [6]. Since then, the Bankim Health District (HD) has been identified as an additional BU endemic area in Cameroon [7]. In this area, where our research has been carried out, the local population suspects that the creation of an artificial lake, by damming of the Mapé River in 1988, has led to an increase in BU incidence. Risk factors for BU include proximity to slow flowing water, poor wound care and not wearing protective clothing [8]. However, the exact mode of transmission has not yet been elucidated [9,10]. Clinically, BU presents with symptoms ranging from nodules, plaques and oedemas to ulcers [11]. The cytotoxic and immunosuppressive toxin, mycolactone, uniquely produced by *M. ulcerans*, is believed to account for most of the pathology of BU [12]. The severity of cases is classified into three categories, with ‘1’ being patients with small (≤ 5 cm diameter) lesions, ‘2’ patients with medium size lesions (5–15 cm) and ‘3’ being patients with large (> 15 cm) lesions, multiple lesions or lesions at critical sites [13]. Many BU cases identified in rural areas are still diagnosed based on clinical symptoms only, although the use of laboratory diagnosis is highly recommended by the World Health Organization (WHO). In 2004, the WHO introduced the use of the combination of streptomycin and rifampicin given daily for 8 weeks as treatment [14]. However, surgery and wound management remain critical aspects of BU care [15,16].

During our investigations of BU in the Bankim HD, we also examined the local population for two other NTDs of the skin, namely yaws and leprosy. Yaws is caused by *Treponema pallidum* (*T. pallidum*) subspecies *pertenuis*, and is transmitted through skin and mucous membrane contact [17,18]. After an initial single lesion, the disease progresses to secondary multiple lesions and in about 10% of cases it causes permanent disability [18]. Leprosy is caused by *Mycobacterium leprae*, which is believed to be transmitted by the respiratory route and can cause major disabilities through nerve

damage. Diagnosis of yaws and leprosy relies mainly on physical examinations [17,19] and treatment of both diseases is feasible with antibiotics [17,20].

The objectives of the present study were i) to conduct an exhaustive survey for BU, yaws and leprosy in the Bankim HD; ii) to continuously monitor the occurrence of BU in the Mapé Dam area; and iii) to examine the age distribution, geographic origin and distribution of lesions of the real-time polymerase chain reaction (RT-PCR) confirmed cases of BU to underpin future environmental and social science studies.

2.4 Materials and Methods

Ethical Statement

Approval for the survey and the subsequent continuous enrolment of cases was obtained from the Cameroon National Ethics Committee (N°041/CNE/DNM/09 and N°172/CNE/SE/2011) and the Ethics Committee of Basel (EKBB, reference no. 53/11). Participation in all aspects of the study was voluntary and all patients, independent of their study participation, were treated according to national treatment standards. All clinically confirmed cases who participated in the study provided written informed consent.

Study Area

The study was conducted in the Mapé Dam region of Cameroon (Figure 2.1) at two different geographical scales. The initial phase of the study was conducted in the Bankim HD which consists of seven Health Areas (HA): Atta; Songkolong, Somié, Nyamboya, Bandam, Bankim Urban and Bankim Rural. The health care infrastructure of the Bankim HD consists of one public district hospital, six primary and four private health centres (HC). All of these facilities employ two medical doctors and approximately 30 nurses. For the later part of the study, bordering regions in the 4 HD surrounding the Bankim HD (Nwa HD, Malantouen HD, Mayo Darle HD, Yoke HD) were also included in the study area. The main environmental features of the area are the Mapé Dam and the Mbam River.

Survey procedures

In early 2010 (March 22 to April 19), we conducted an exhaustive cross-sectional house-by-house survey for BU, leprosy and yaws in the 88 villages of the Bankim HD (Figure 2.1). Eleven teams of three trained field workers, namely one local nurse and two local community relays, were employed to interview all inhabitants. Field workers were trained for two days on the use of the questionnaire and the clinical signs of the three diseases investigated. At the household level, demographic information of all inhabitants was collected and posters with photographs of the clinical presentations of the diseases

were shown. Households with suspected cases were re-visited by staff with extensive experience in the diagnosis of BU, leprosy and yaws. From clinically confirmed BU cases, samples were collected for laboratory confirmation as follows. Two or three dry swabs were collected from ulcerative lesions or a fine needle aspirate (FNA) was drawn from non-ulcerative lesions [21]. To facilitate handling of FNA samples, they were transferred onto a swab.

Prospective BU surveillance

Following the survey, we continued to monitor the occurrence of all new cases of BU in the Bankim HD by community and HC based case referral and regular supervision until the end of June 2012. For this, a health worker, trained and experienced in the diagnosis of BU, regularly visited all HC in the Bankim HD and areas of the adjacent Malantouen HD. During these visits, suspected cases who independently came forward or who were referred to the HC by community or family members, were evaluated and if clinically confirmed, asked to come for treatment. Before treatment, swabs or an FNA were collected for laboratory confirmation as described above. In addition to demographic and clinical information, the houses where the patients lived for at least a year before disease onset were mapped using a GPS device. From the GPS device, coordinates were only recorded once the GPS receiver showed an accuracy of below 10m. Details of the location of the lesions on the patient's bodies were also collected and documented by photographs. Both clinically confirmed BU cases identified in the survey and during the continuous case detection were included in the cohort of patients investigated here.

Laboratory Confirmation of BU Cases

Samples were locally stored at 4°C before transport to the laboratory where definite BU diagnosis was obtained by insertion sequence (*IS*) 2404 RT-PCR. Analysis was done according to the protocol developed by Fyfe *et al.* [22,23]. In brief, swabs were transferred into glass bottles containing glass beads with 2-5mL of PBS, and the bottle vortexed for 1.5 minutes. From 1mL of the solution, DNA was extracted and RT-PCR performed. DNA was amplified in a StepOnePlus Real-Time PCR System (Applied Biosystems) and data analyzed using the Applied Biosystems StepOne Software (2.2.2).

Analysis of lesion distribution

Using published age specific relative body surface areas (RBSA) [24] and the number of patients in each of the age groups, the weighted average RBSAs of a model person (all ages), a model child (<15), and a model adult (≥ 15) were computed. If required to perform a Fisher's exact test, RBSA were converted to counts which add up to the observed number of lesions. The shape file used to analyze lesion localizations is found in Dataset S1.

Statistical analysis

Continuous variables were summarized as means and standard deviation or medians and interquartile ranges and categorical ones as counts and percentages. The Fisher's exact or Chi-squared tests were used to compare categorical characteristics between groups and Student t-tests or Mann-Whitney U-test in the case of continuous variables. Multiple comparisons were adjusted for using a Bonferroni correction. The software, SAS (SAS Institute, Cary, USA; release 9.3), RStudio (RStudio, Boston, USA, version 0.95.262) and R (The R Foundation for Statistical Computing; version 2.15.1) were used to perform the statistical analysis. Geographic data and the localisation of lesions were analyzed with ArcGIS ArcMap (Economic and Social Research Institute, Redlands, USA; version 10.0).

2.5 Results

Survey for BU, leprosy and yaws in the Bankim HD

In the course of the survey, a total of 48 962 individuals in 9 344 households (Figure 2.1) were interviewed. The population of one village (approximately 550 people) refused to participate in the study. Assessing demographics and living conditions in the Bankim HD (Table 2.1), we found that the local population is very young with an average age of 19.3 ± 17.0 (median = 14.0, interquartile range = 6.0 to 28.0), that 51.4% of the population are women and that overall, 61.2% of the population have attended school at some point in their lives. We further observed that Christianity is the most common (64.9%) religion and that, apart from the young members of society which are either students (32.2%) or children (23.5%), the most common professions in the district are farming (16.9%) and household work (17.4%). In terms of living conditions we found that there are on average 5.2 individuals living in each household and 26.8% of the households have a mosquito net. Further, our data showed that only 38.3% of the population have access to clean drinking water that comes at least from a fortified well and that the roofs and floors of the local houses are often very poorly constructed. Table 2.1 also shows that, the main local differences in the level of development in the HD exist between the six rural HA and the Bankim Urban HA (BA HA), which includes the town of Bankim (77.4% of the BA HA population) and nine small settlements around it. The higher level of development in the BA HA is reflected by the significantly higher percentage of people having gone to school at some point in their lives (p -value < 0.0001) or by the significantly better access to clean drinking water (p -value < 0.0001). Furthermore, in the BA HA the proportion of houses with better flooring (p -value < 0.0001) and walls (p -value = 0.0037) is also significantly higher compared to the other HA.

In the survey, we identified 32 cases of leprosy, 29 cases of yaws and 25 cases of BU based on clinical symptoms. With 32 cases of leprosy, the population-adjusted prevalence was at 6.5 cases per 10'000. The majority (70%) of the identified leprosy cases suffered from the multibacillary form of the disease and 22% of them were previously known but had abandoned their treatment and needed treatment re-initiation. Of the 29 yaws cases identified, 28% presented with the advanced symptom of hyperkeratosis and of the BU cases, 23% (6 cases) could be re-confirmed by RT-PCR.

Laboratory confirmation of BU

In the five months after the survey (April 2010 to August 2010), only two new RT-PCR reconfirmed BU cases were identified (Figure 2.2). Following this lag, between September 2010 and June 2012 (22 months) there was a steady flow of about 2.5 new RT-PCR confirmed BU cases per month from the Bankim HD. During this period, RT-PCR confirmed BU patients from the surrounding HDs (about 1.2 per month) also reported to BU treatment facilities in the Bankim HD. Overall, our study identified 157 clinically confirmed cases of BU of which 88 (56%) could be confirmed by RT-PCR. Of the non-confirmed patients, 48 (31%) tested negative in RT-PCR and of 21 patients (13%) no samples were collected. Gender ratio, age distribution, average disease duration prior to consultation, and proportion of category 3 cases were comparable between the RT-PCR positive and negative patients. Only age differed significantly (p -value = 0.034) between the RT-PCR confirmed and the non-confirmed cases with the average age of the confirmed cases being 21.2 and that of the non-confirmed ones being 29.3. To ensure the reliability of our conclusions we focused the remaining analysis only on the 88 RT-PCR confirmed BU cases. Age distribution (p -value = 0.4754) and professions (p -value = 0.5161) did not differ significantly between the population of the Bankim HD and the confirmed BU patients. The gender distribution among patients was moderately different (p -value = 0.061) from that of the overall population with a larger proportion of males among the confirmed BU cases.

Spatial distribution of BU cases in the Mapé basin

To better describe BU epidemiology in the Mapé basin we set out to identify the exact geographic origin of all 88 laboratory confirmed cases in our cohort. Based on information from the patients or their close relatives we were able to determine the HD of origin for 86 (98%) of the cases (Figure S2.1). For the remaining 2 cases we could only determine that they did not live in the Bankim HD for the year before the onset of symptoms, but we could not conclusively determine which HD they were from. Studying the distribution of cases by HD, we found that the proportion of category 1 cases among the patients originating from inside the Bankim HD (24/62) was significantly higher (p -value = 0.039) compared to the cases from the surrounding HDs (5/26; Figure S2.1). For the 62 cases that originated from within the Bankim HD we were also able to determine

their HA of origin. Using the population data as collected by the survey, we were then able to calculate the cumulative incidence rate of BU per HA in the Bankim HD during our study. As shown in Figure 2.3A, the cumulative incidence rate of BU in the Bankim HD is highest in the BR HA (5.08/1'000). The cumulative incidence rate in this HA is significantly higher compared to all other HA in the HD (p-value < 0.001). Interestingly the cumulative incidence rate is also significantly higher in the southern HA (BR, BA, BD, NY) compared to the northern HA (AT, SO, SG) of the Bankim HD (p-value < 0.001) (Figure 2.3A). Finally, for more detailed spatial analysis, the exact domiciles of 79 (89.8%) of the confirmed BU cases were mapped (Figure 2.3B). For 7 of the remaining cases (Bankim HD: 3 from the Bandam HA, 1 from the Somié HA; surrounding HD: 1 from the Malantouen HD, and 2 of unknown origin) we could not conclusively identify the exact house where they lived before the onset of BU. An additional two cases (1 each from the Nwa HD and Mayo Darle HD) are not considered in the analysis because they originated from outside of the Mapé basin. Based on the known exact origin of the cases that came from within the Bankim HD (n=58) and who were therefore identified by the same case finding strategy, a Kernel function was used to compute the density of BU in the Bankim HD (Figure 2.3B). This BU density map shows that most of the cases occur in the southern part of the Bankim HD, particularly along the Mbam River and in the area between the Mapé Dam reservoir and that river. The exact origins of 80.8% (21/26) of the BU cases from outside of the Bankim HD, indicate that the local BU focus expands outside of the Bankim HD, in particular westwards into the Malantouen HD (Figure 2.3B).

Age and gender distribution of cases

The median age of the 88 RT-PCR confirmed cases was 12.5 (interquartile range= 8.0 to 30.0). The age of patients ranged from 0.5 to 73, 52 out of 88 (59.1%) were children (age <15) and 11 (12.5%) were older than 50. The gender ratio of all cases was 1.44 male / female. In children this ratio was 1.89, in the 15 to 50 year olds it was 0.79 and in the above 50 year olds, it was 1.75. The age dependent variation in the gender ratio was not statistically significant (p-value = 0.20).

With the ages of the 62 (70.5%) cases of BU which originated from within the Bankim HD and the population age distribution as collected in the survey (Figure 2.4A

and Table 2.1), we computed the age adjusted cumulative incidence rate of BU in the Bankim HD for the period of the study. As shown in Figure 2.4B, we observed a low age adjusted cumulative incidence rate of BU in individuals aged below four years. The rate then peaked in children aged between four and < 14 years of age, with the 12 to <14 year olds particularly affected (34.4 cases per 10'000 inhabitants). Interestingly, the age adjusted cumulative incidence rate peaks again in the over 50 year olds (27.0 cases per 10'000 inhabitants; Figure 2.4B).

Localisation of BU lesions

In the laboratory confirmed BU patients studied here, 49/88 (55.7%) lesions occurred on the lower limbs, 27/88 (30.7%) on the upper limbs, 2/88 (2.3%) on the head and neck and 10/88 (11.4%) on the trunk. One of the trunk lesions occurred on the genitals. Two patients had multiple lesions and only the initial lesion was considered for analysis. The distribution of lesions differed significantly (p-value <0.001) from the relative body surface area (RBSA; Table S2.1). Interestingly, most of the lesions (52.3%) occurred in close proximity to joints with clusters around the ankles (19.2 %) and elbows (15.9 %; Figure 2.5A, 2.5B and Table S2.2). When analyzing the occurrence of lesions on the different body parts, we did not observe any statistically significant difference between lesions occurring on the right or left or front or back of the patients. However, when analyzing the occurrence of lesions on the joints, we did observe a statistically significant difference between the lesions on the front or back of the joints (p-value = 0.012), in particular there was a significant difference between the occurrence of lesion on the front or back of the elbow (p-value = 0.005). No such difference was observed when analyzing the joint lesions on the right or left of the patients' bodies. Analyzing the distribution of the lesions by body part, we found a moderately significant difference between males and females (p-value = 0.076) with the percentage of lesions on the trunk being significantly higher (p-value = 0.033) in males (Table S2.1).

The distribution of lesions by body parts in children (Figure 2.5C and Table S2.1) was significantly different (p-value = 0.009) from the RBSA of children. Interestingly, only children (n=2) had lesions on the head and neck. Overall, the lesions appear more dispersed in children (Figure 2.5C and 5D). While, the difference in the general lesion distribution by body parts between adults and children (Table S2.1) was not statistically

significant (p-value = 0.154), there was a significant difference (p-value = 0.011) between the distribution of lesions at joints in children and adults. In particular, most joint lesions in adults occurred at the ankle (36.1%), whereas most joint lesions in children occurred at the elbow (19.2%). Finally in adults, lesions occurred mainly on the front and back of the feet and the distribution also differed significantly (p-value = 0.004) from what is expected based on the RBSA of adults (Figure 2.5C).

2.6 Discussion

The 2011 Cameroon Demographic Health Survey (DHS) examined approximately 22'000 adults (>14 years old) and found that the population is very young with roughly 24% being 15-19 years old [25]. In the Bankim HD, we also observed a population that is strongly skewed towards young individuals and we found that living conditions and access to clean drinking water are very poor. Given the basic health infrastructure, these factors pose big challenges when addressing any health care related issues [26,27].

Although Cameroon has achieved nationwide leprosy elimination as defined by the WHO (< 1 case per 10'000 inhabitants) [28], our data showed that leprosy remains endemic in the Bankim HD. The substantial proportion of leprosy patients that had previously abandoned treatment further demonstrated that the oral treatment regimen requires better patient monitoring to achieve good compliance. In 2010 Cameroon reported 800 cases of yaws [29] and our survey confirmed that the Bankim HD is a yaws focus. Studies on the use of oral antibiotics have again raised hope for the eradication of yaws [30–32]. However, until eradication is possible, the focus of leprosy and yaws care should be early detection, complete cure and prevention of disabilities. To achieve this, front line medical staff needs to be trained on clinical diagnosis and efficient case management.

Characteristics of BU and the remote areas where it occurs have been suggested to necessitate active case searches for early case detection [33,34]. Indeed, house-by-house surveys have helped to elucidate BU epidemiology in Ghana and Ivory Coast [35,36]. In Cameroon a study around the Nyong River, identified 135 PCR confirmed cases of BU [6]. In the survey described here, the number of RT-PCR confirmed BU cases identified was smaller than expected. However, the lag of new cases during the first months after the survey indicated that the survey identified the cases present at that time. It cannot be excluded however, that a proportion of BU patients seeks to avoid contact with the formal health system. By continuous case detection, also accounting for the trust needed for cases to come forward [37], we identified 157 clinically diagnosed cases of BU (from April 2010 to June 2012). To increase validity of the findings [38,39], our analysis focused on the 88 (56.1%) RT-PCR confirmed cases among them. False negative RT-PCR results are unlikely since we analyzed multiple samples from each patient (data not shown). Although accurate BU clinical diagnosis is possible [40], misdiagnosis rates of up to 40%

have been reported emphasizing the pressing need for a point-of-care rapid diagnostic test [5,38,39,41–43].

Based on the number of BU cases in each of the HA in the Bankim HD and their respective populations, the BR HA was determined to have the highest cumulative incidence rate of BU in the Bankim HD. Furthermore, by detailed mapping of cases and through the use of a geographic information system (GIS), we identified hot-spots of BU transmission along the Mbam River. With only few cases living in the immediate proximity of only the Mapé Dam reservoir, our data does not support the suspected direct importance of this man-made lake. This does not exclude that environmental changes associated with the damming of the Mapé River may have had a more indirect effect on the spread of BU in the wider area. Whether the relatively large proportion of patients living in the town of Bankim (11/79 GPS mapped cases), contracted BU there, remains to be investigated. By also mapping cases from outside of the Bankim HD, we found that the local BU endemic area is larger than previously described [7]. Indeed it is possible that, because of the differences in case finding strategy inside and outside of the Bankim HD, our findings from outside the HD under represent the true degree of BU endemicity in the areas surrounding the Bankim HD. Further studies are therefore needed to investigate BU endemicity in the entire Mapé basin in more detail. Ongoing environmental and social science research at the identified hot-spots of disease is aiming to further elucidate the mode of transmission of BU.

BU affects individuals of all ages [15,34] but in the African endemic regions most patients are children [9,44]. However, when adjusting for the population age distribution, studies in Benin [45] and in Australia [46] showed that 75 to 79 year olds or the ≥ 74 olds, respectively, have the highest risk of contracting BU. Our data similarly showed that the age adjusted risk of BU is as high in the > 50 year olds as in children, a trend possibly associated with immunosenescence, the gradual deterioration of the immune system associated with natural age advancement [46,47]. It is interesting to note that cases among very young (< 5) children, which make up an even larger part of society than the 5 – 10 year olds, are relatively rare. This may indicate that compared to older children the very young children are less exposed to risk factors due to a smaller movement radius away from the house [10]. In the exposed individuals, host factors are likely to contribute to the degree of susceptibility [48]; seroepidemiological studies indicate that only a small proportion of exposed individuals develop clinical disease [49,50].

Detection of *M. ulcerans* DNA-positive mosquitoes in an Australian BU focus [46] as well as identification of the failure to wear protective clothing as a risk factor and of the use of mosquito repellent as a protective factor for BU [8], support the hypothesis that insects are involved in *M. ulcerans* transmission [10]. Most biting arthropods selectively feed at specific sites based on visual, physical or chemical cues such as distance of the ground, breath and skin temperature of the bait [51–55]. The resulting feeding patterns are often focused either on the feet and ankles or the head of the human subject [52]. Interestingly for vector transmitted parasitic diseases with local manifestations such as cutaneous leishmaniasis and filariasis, it has been found that the lesion distribution correlates with the biting sites of the responsible vectors [56,57]. BU lesions occur mostly on the lower limbs [15,45,58–60] and in adults, a focus on joints, specifically the elbows and ankles, has been reported [15,58]. Studies on the distribution of lesions also show that they are usually equally distributed between the left and right side of the body and compared to adults, children tend to have more lesions on the trunk [45,60]. Using GIS methodology we observed in this study that lesions cluster at specific locations on the limbs. We found that, particularly in adults, lesions occur mostly at locations where the skin is not commonly protected with clothing. As previously described, in females, which are more likely to cover their upper body with clothing, we found that there are less lesions on the trunk. In rural African villages children may often have their upper body exposed explaining the more dispersed distribution of their lesions.

Detection of *M. ulcerans* DNA in the environment [10] and identification of poor wound care and failure to wear protective clothing as risk factors for BU [8] have led investigators to speculate that transmission may alternatively occur by skin trauma and direct contact with *M. ulcerans* contaminated environment [10]. A study in Canadian children found that children 9 months and older have on average >3.5 recent skin injuries [61]. In 5 to 17 year olds injuries most often occur where the bones are close to the skin, i.e. at shins, knees, elbows and forearms. Injuries on the head were most common in children less than 5 years of age and lesions on genitals were rare in all ages [61]. While this study may have, due to differences in dress code and activities, limited relevance for Cameroonian children, it is remarkable that both BU lesions on the head in our cohort occurred in patients under the age of 5 [61]. While BU lesion distribution does not seem to correlate closely with the published distribution of insect bites, inoculation of skin injuries by a contaminated environmental source should lead, for example, to more lesions

on the knees. Based on these data, the option of multiple modes of transmission should be considered.

2.7 Acknowledgments

The authors would like to thank the population of the Mapé basin for their participation in the study. Further we would like to thank all the field workers, the entire Bankim FAIRMED team as well as Daniel ZeBekolo, Alim Nouhou, Jean Marie Nkenne and Joseph Kemmegne for their help with the field work. Also we would like to thank Peiling Yap for reviewing the manuscript and providing invaluable comments.

2.8 Tables

Table 2.1 Sociodemographic characteristics of the Bankim Health District population.

Characteristic	Health Areas							Total
	AT	SG	SO	NY	BD	BA	BR	
Individuals								
Inhabitants surveyed (n)	7 621	11 346	7 308	5 602	5 421	8 514	3 150	48 962
Age in years (mean ± SD)	19.9±17.0	19.8±17.2	20.2±18.2	18.2±16.3	17.4±16.5	19.3±16.1	19.1±16.5	19.3±17.0
Gender (% female)	49.8	50.9	52.9	53.1	51.7	51.1	51.1	51.4
Education* (%)	62.2	64.9	55.9	53.8	49.8	72.0	61.5	61.2
Religion (%)								
Christian	72.9	65.2	61.2	59.6	52.2	70.6	69.6	64.9
Muslim	26.7	34.6	38.8	40.4	47.8	28.7	30.2	34.8
Profession (%)								
Farming	25.6	13.3	17.0	12.5	13.7	12.7	32.9	16.9
Fishing	0.1	2.4	0.1	2.2	1.1	0.7	2.2	1.2
Household work	13.4	21.3	21.9	18.9	21.6	13.3	4.1	17.4
Student	32.9	33.6	26.8	30.6	24.0	40.5	33.2	32.2
Child	21.3	22.4	27.5	26.1	32.6	16.2	23.0	23.5
Households								
Number of households (n)	1788	2100	1332	1053	942	1532	597	9344
Individuals per household (mean ± SD)	4.3±2.9	5.4±2.9	5.5±3.3	5.3±3.0	5.8±3.7	5.6±3.4	5.3±2.9	5.3±3.2
Mosquito net** (%)	16.1	22.3	28.6	36.9	31.6	21.8	57.6	26.8
Clean drinking water*** (%)	27.0	34.5	47.9	17.2	25.0	76.7	23.2	38.3
Concrete or mood walls (%)	99.1	97.3	98.6	99.2	91.3	98.8	98.5	97.8
Tile or metal sheet roof (%)	40.6	40.8	32.0	39.4	31.4	78.1	42.8	44.7
Cemented or tailed floor (%)	8.0	10.9	7.8	9.7	6.8	41.6	11.6	14.3

*Attended school anytime during their life.

**Lived in household with a mosquito net.

***Had access to drinking water from a tap or a concrete fortified well.

SD: standard deviation.

AT = Atta.

SG = Songkolong.

SO = Somié.

NY = Nyamboya.

BD = Bandam.

BA = Bankim Urban.

BR = Bankim Rural.

doi:10.1371/journal.pntd.0002252.t001

Table S2.1: Lesion Distribution by Body Parts.

Lesion location*	All			Children (< 15 years old)			Adults		
	All (n=88)	Male (n=52)	Female (n=36)	All (n=52)	Male (n=34)	Female (n=18)	All (n=36)	Male (n=18)	Female (n=18)
Head or neck	2 (2.3)	2 (3.8)	0 (0)	2 (3.8)	2 (5.9)	0 (0)	0 (0)	0 (0)	0 (0)
Upper limbs	27 (30.7)	16 (30.8)	11 (30.6)	19 (36.5)	14 (41.2)	5 (27.8)	8 (22.2)	2 (11.1)	6 (33.3)
Trunk	10 (11.4)	9 (17.3)	1 (2.8)	7 (13.5)	7 (20.6)	0 (0)	3 (8.3)	2 (11.1)	1 (5.6)
Lower limbs	49 (55.7)	25 (48.1)	24 (66.7)	24 (46.2)	11 (32.4)	13 (72.2)	25 (69.4)	14 (77.8)	11 (61.1)

* number of patients with lesion at the given location and percentage in parenthesis

Table S2.2: Lesion on Joints.

Lesion Location *	All			Children (< 15 years old)			Adults		
	All (n=88)	Male (n=52)	Female (n=36)	All (n=52)	Male (n=34)	Female (n=18)	All (n=36)	Male (n=18)	Female (n=18)
Ankle	17 (19.3)	10 (19.2)	7 (19.4)	4 (7.7)	1 (2.9)	3 (16.7)	13 (36.1)	9 (50.0)	4 (22.2)
Elbow	14 (15.9)	9 (17.3)	5 (13.9)	10 (19.2)	7 (20.6)	3 (16.7)	4 (11.1)	2 (11.1)	2 (11.1)
Hip	3 (3.4)	3 (5.8)	0 (0)	2 (3.8)	2 (5.9)	0 (0)	1 (2.8)	1 (5.6)	0 (0)
Knee	3 (3.4)	3 (5.8)	0 (0)	3 (5.8)	3 (8.8)	0 (0)	0 (0)	0 (0)	0 (0)
Shoulder	2 (2.3)	1 (1.9)	1 (2.8)	1 (1.9)	1 (2.9)	0 (0)	1 (2.8)	0 (0)	1 (5.6)
Toe	1 (1.1)	0 (0)	1 (2.8)	0 (0)	0 (0)	0 (0)	1 (2.8)	0 (0)	1 (5.6)
Wrist	2 (2.3)	2 (3.8)	0 (0)	2 (3.8)	2 (5.9)	0 (0)	0 (0)	0 (0)	0 (0)
Not Joint	46 (52.3)	24 (46.2)	22 (61.1)	30 (57.7)	18 (52.9)	12 (66.7)	16 (44.4)	6 (33.3)	10 (55.6)

* number of patients with lesion at the given location and percentage in parenthesis

2.9 Figures

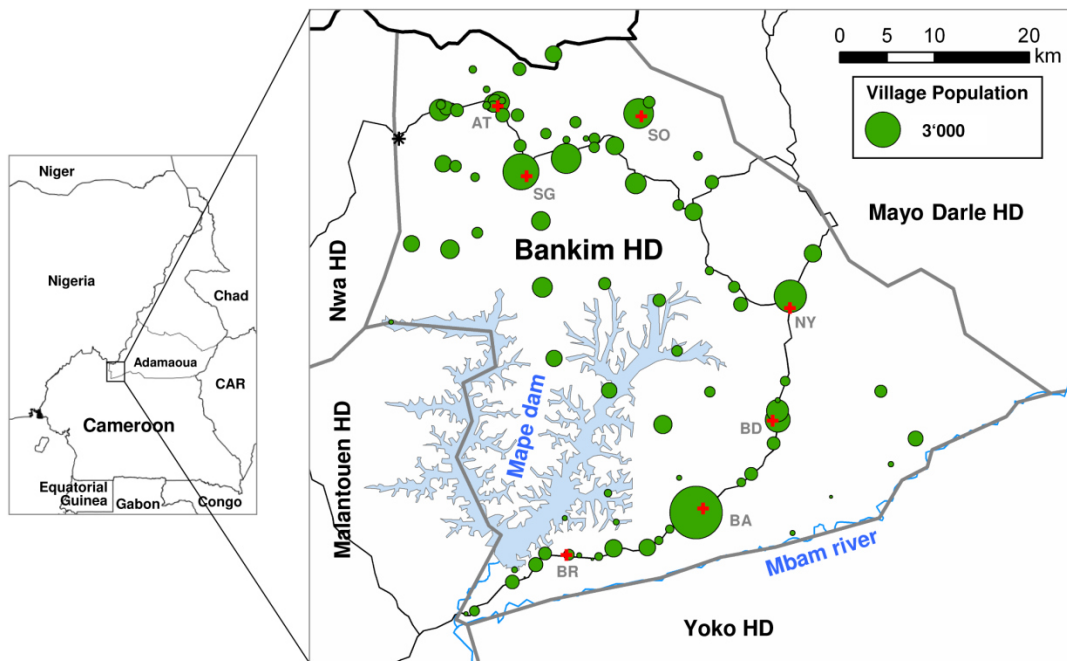


Figure 2.1: Environmental Features and Population of the Bankim HD.

The Bankim HD is located in the South-Western corner of the Adamaoua Region of Cameroon and encompasses most of the Mapé basin. The main environmental features of the area are the Mapé Dam reservoir and the Mbam River. The Bankim HD consists of 7 HA (red crosses depict the location of the main HC in each of the HA; BR: Bankim Rural; BA: Bankim Urban; BD: Bandam; NY: Nyamboya; SO: Somié; SG: Songkolong; AT: Atta) and is surrounded by four other HD. In early 2010 we conducted an exhaustive house-by-house survey and examined a total of 48'962 individuals in all HA of the district; population sizes of villages based on this survey are indicated by green circles. The village of Koumtchoum (black star; estimated population of 550) as a whole refused to participate in the survey and the village of Djaouro Tchi Arouna (470 inhabitants, located in the Somié HA between the HC of Somié and the town at the Nigerian border to the North-West of it) could not be mapped.

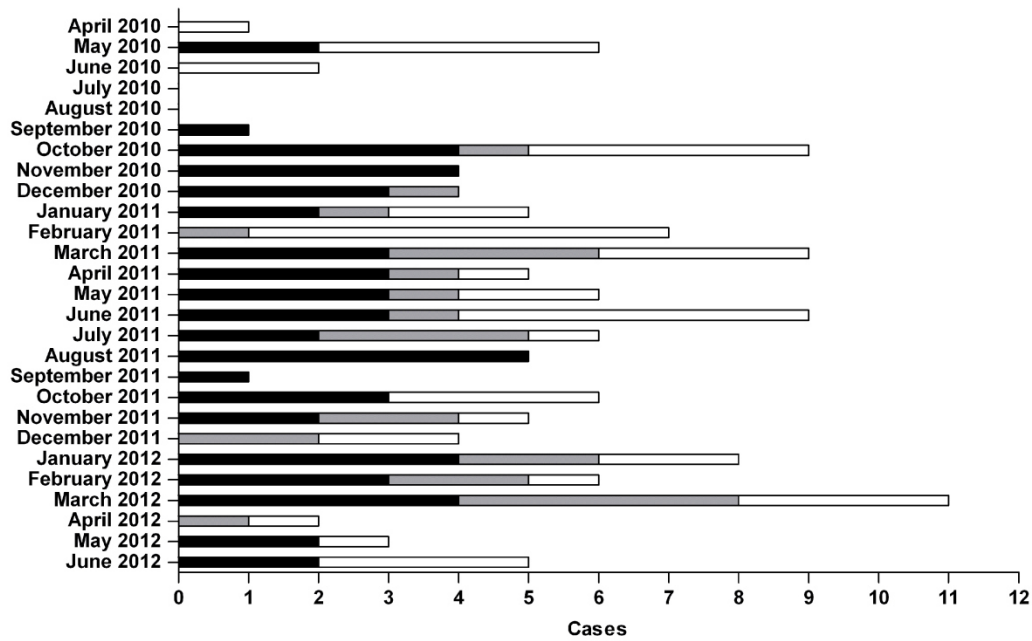


Figure 2.2: Identification of BU Cases in the Mapé Basin following the Survey.

In the 27 months following the exhaustive survey all clinically diagnosed BU cases in the Bankim area were included in a cohort study. Cases are separated into the RT-PCR confirmed cases which occurred inside (black) and outside (grey) of the Bankim HD. RT-PCR negative and non-laboratory examined cases occurring anywhere in the Mapé basin during the same time period are also shown (white).

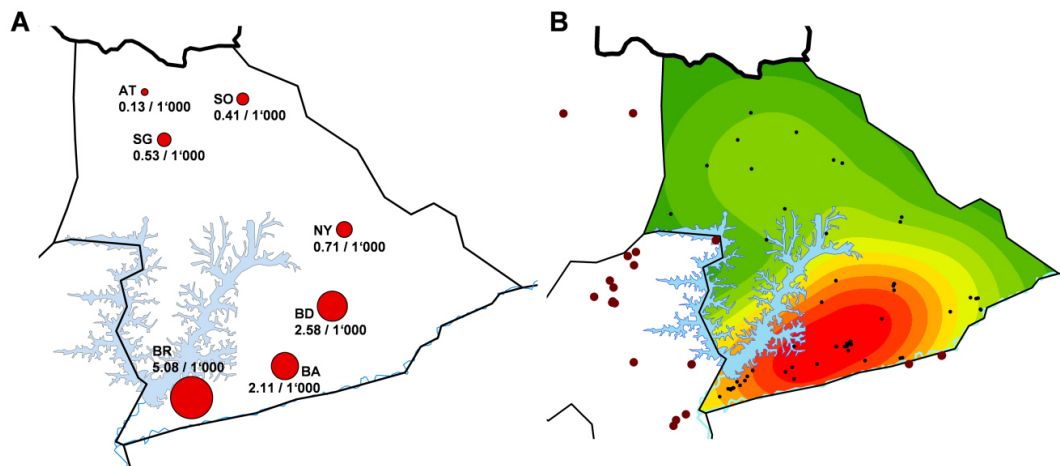


Figure 2.3: Geographic Distribution of BU in the Mapé Basin.

Based on the HA of origin of the 62 BU cases who originated within the Bankim HD and the population data collected in the survey, we computed the per HA cumulative incidence rate of BU in the Bankim HD (A). For detailed analysis, the places of residence of 58 (black points) of the 62 cases from the Bankim HD were mapped using a GPS device (B). For the remaining 4 RT-PCR confirmed cases which occurred inside the Bankim HD (3 from the Bandam HA, 1 from the Somié HA) we could not identify their home. Using a Kernel function the density of BU in the Bankim HD was computed based on the mapped cases (red: highest BU density). Panel B further shows the places of residence of 21 of the 26 RT-PCR confirmed cases of BU who originated from outside of the Bankim HD (brown points). For three of the remaining cases (1 from the Malantouen HD, and 2 of unknown origin) we could not identify their exact origin and two additional cases (1 each from the Nwa HD and Mayo Darle HD) are not shown because they originated from outside of the region shown on the map.

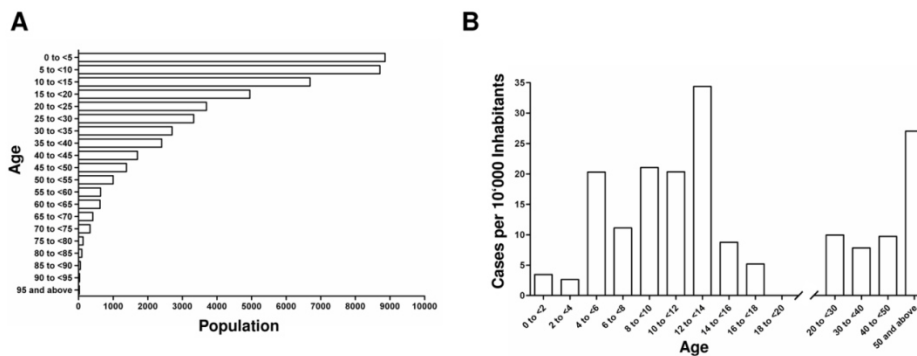


Figure 2.4: Population Age Distribution and Age Adjusted Cumulative BU Incidence Rate in the Bankim HD.

In the course of the exhaustive survey, data on the population age structure of the Bankim HD were collected (A). Using this age distribution and the ages of the RT-PCR confirmed BU cases which occurred inside of the Bankim HD ($n=62$), the age adjusted cumulative BU incidence rate (cases per 1'000 inhabitants) for the duration of the study could be computed (B).

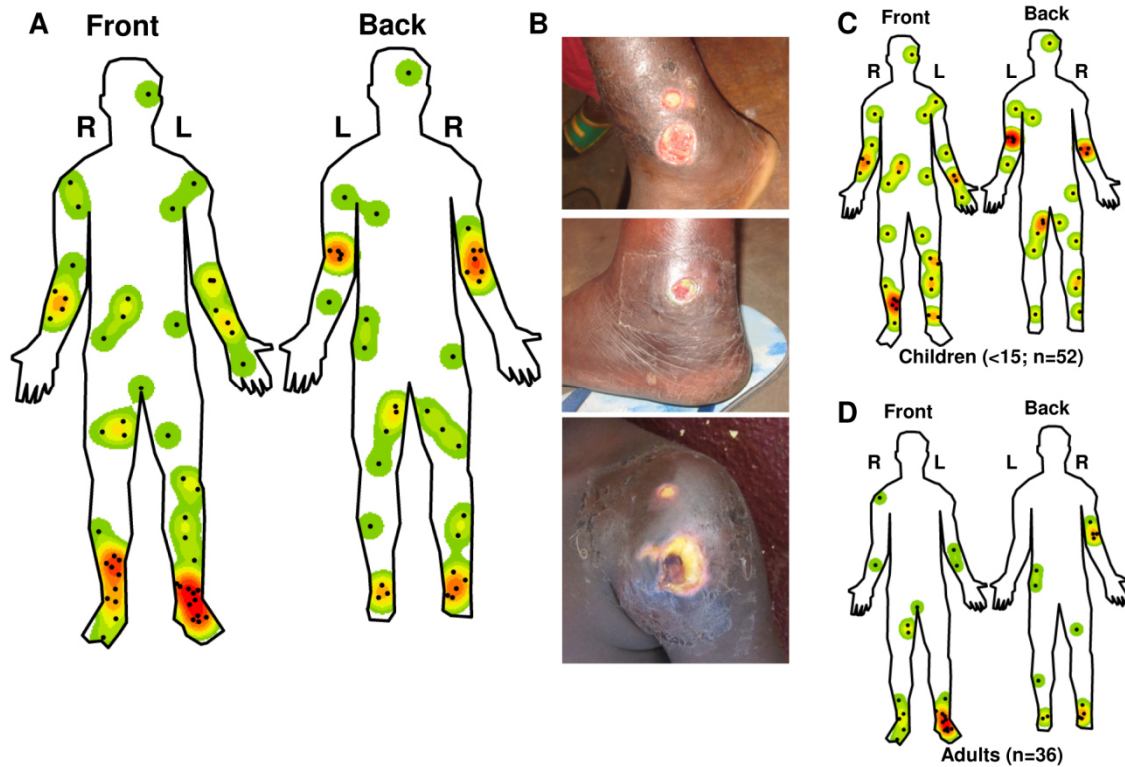


Figure 2.5: Lesion Localization.

The localization of the lesions of all the RT-PCR confirmed BU patients (88) were mapped in detail and Kernel function was used to create a heat map of the lesion distribution (A). The localization of the lesions on the front and back and left (L) and right (R) of the patient's bodies are shown. Studying the distribution of lesions, it was noted that they often occur at joints (B, example of two lesions on the ankle and one on the shoulder). Distribution of lesions in children (C, n=52) and adults (D, n=36) were also analyzed separately.

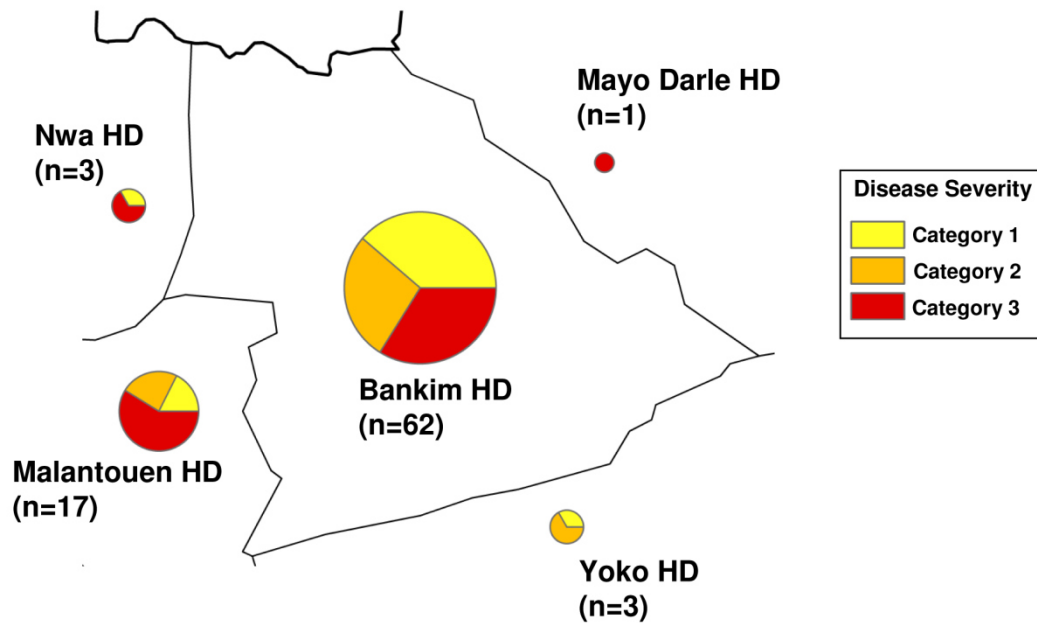


Figure S2.1: Disease Category by Health District.

Most of the RT-PCR confirmed cases that were identified in the Bankim area originated from within the Bankim HD (n=62). However, patients from all of the surrounding HD also came to Bankim for BU treatment (Malantouen: 17; Nwa: 3; Yoko: 3; Mayo Drale: 1). The number of cases that occurred in each of the HD are classified by disease severity (red: category 3, orange: category 2, yellow: category 1). Two RT-PCR confirmed cases (both category 3, both from outside of the Bankim HD) could not be displayed because the location where the patient first showed symptoms of BU could not be conclusively determined.

2.10 Supporting Information

Dataset S1: Shape File for the Analysis of Lesion Distribution.

The compressed folder contains the files of the body_FB shapefile which provides the outline of the front and the back of the human body which was used for the “mapping” and analysis of the lesion distribution.

Checklist S1: STROBE Checklist

2.11 References

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Chapter 3

Late onset of the serological response against the 18KDa small heat shock protein of *Mycobacterium ulcerans* in children suggests an association between age-related behavioural factors and exposure

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3.1 Abstract

A survey for Buruli ulcer (BU) in the Mapé Basin of Cameroon has revealed that the age adjusted cumulative incidence rate of BU in individuals below the age of four is very low, indicating that compared to other age groups, very young children are less exposed to *Mycobacterium ulcerans*. Here we determined serum IgG titers against the 18KDa small heat shock protein of *M. ulcerans* in more than 900 individuals living in BU endemic sites of the Densu River Valley in Ghana and the Mapé Basin in Cameroon. While none of the sera collected from children below the age of four had a detectable IgG titer against the shsp, the majority of sera contained high levels of antibodies against a domain of the *Plasmodium falciparum* merozoite surface protein 1. These data suggest that exposure to *M. ulcerans* increases at an age when children start moving further away from their homes and have more intense environmental contacts, including contact to water bodies at the periphery of their villages.

3.2 Author Summary

Although the causative agent of Buruli ulcer, a mycobacterium designated as *M. ulcerans*, had already been identified in 1948, transmission pathways and environmental reservoirs are still poorly understood. It is commonly assumed that the highly focal occurrence of *M. ulcerans* infections in endemic countries of West and Central Africa is associated with certain aquatic habitats. In addition, recent studies have indicated that currently unknown host or pathogen factors may contribute to the degree of susceptibility. While BU has long been generalized as a disease mainly affecting children <15 years, a comprehensive survey for BU in the Mapé Dam Region of Cameroon, taking account of the population age distribution, has revealed that the risk of BU is as high in the >50 year olds as in children and that cases among infants are relatively rare. In accordance with these findings, data of the present sero-epidemiological investigation suggest that children <4 years are less exposed to *M. ulcerans* possibly due to age-related behavioural factors such as a limited movement radius from their homes.

3.3 Introduction

The chronic skin infection Buruli ulcer (BU), cases of which have been reported from tropical and subtropical settings around the world, is caused by the emerging environmental pathogen *Mycobacterium ulcerans*. This species has diverged from the fish pathogen *Mycobacterium marinum* by evolving the ability to produce an immunosuppressive toxin, referred to as mycolactone, through the acquisition of a plasmid [1,2]. Over the course of the last few years other mycolactone-producing mycobacteria (MPM), have been identified in fish and frogs of widespread geographic origins and had been given diverse species names [3-6]. However, recent genome analyses indicate that all MPM are genetically closely related and can be divided into three principal lineages, which should all be considered ecovars of *M. ulcerans* [7].

Although DNA sequences of this agent could be detected in various aquatic environments of the affected countries, the disease is characterized by a highly focal occurrence [8-11]. Whether this circumstance can be attributed to host or pathogen factors is yet to be explored, as is the definite mode of *M. ulcerans* transmission [12]. Recent findings in south-eastern Australia, which is the only non-tropical area with local BU endemic sites, suggest that mammals may represent environmental reservoirs and that mosquitoes may function as vectors of *M. ulcerans* [13,14]. In West and Central African BU endemic areas, where most of the cases occur, it is controversially discussed whether infection takes place through trauma of the skin or insect bites, but definite evidence for either mode of transmission is lacking. A recent survey for BU in the Mapé Basin of Cameroon has revealed that the distribution pattern of lesions neither matches any of the published mosquito biting patterns, nor distribution patterns of small skin injuries found in the literature. Hence, the option of multiple transmission routes should be considered [15].

A further mystery contributing to the guesswork around *M. ulcerans* transmission is the close proximity of highly endemic and non-endemic sites as exemplified by adjacent communities located along the Densu River of Ghana. Interestingly, no significant difference in the exposure to *M. ulcerans* could be detected between residents of either BU endemic or BU non-endemic communities in this area when analysing their serum antibody titers against an immunodominant small heat shock protein (shsp) [11]. This preceding study, which has helped to identify a new low BU endemic site in the Volta Region of Ghana by screening blood sera of the local population for the presence of anti-shsp IgG titers, has shown that sero-

epidemiology provides an attractive approach to monitor exposure of populations to *M. ulcerans* [11].

One drawback of this initial investigation was that all study participants from both the Densu River Basin as well as the Volta Region were older than 5 years of age. Hence, it is still not known at which age immune responses against *M. ulcerans* antigens start to emerge. While it has long been generalized that in African BU endemic areas children below the age of 15 are worst affected by the disease [16], data from the above mentioned BU survey in the Mapé Basin of Cameroon showed that children <5 years old were underrepresented among cases, even though this age group and the severely affected 5-10 year olds were equally represented in the population under study [15].

In the present sero-epidemiological investigation anti-shsp IgG titers were determined in over 900 individuals from BU endemic sites in the Densu River Basin of Ghana as well as the Mapé Basin of Cameroon including more than 100 children <5 years of age. Results indicate that very young children are significantly less exposed to *M. ulcerans* than individuals of all other age groups, providing another cornerstone in the search for the mode of *M. ulcerans* transmission.

3.4 Materials and Methods

Ethics statement

Ethical clearance for the collection and testing of human blood samples from Ghana and Cameroon was obtained from the institutional review board of the Noguchi Memorial Institute for Medical Research (Federal-wide Assurance number FWA00001824) and the Cameroon National Ethics Committee (N°172/CNE/SE/201) as well as the Ethics Committee of Basel (EKBB, reference no. 53/11), respectively. Written informed consent was obtained from all individuals involved in the study. Parents or guardians provided written consent on behalf of all child participants.

Study areas

Study areas of the present serological investigation were located in two different BU endemic countries. In Cameroon serum samples were collected from all inhabitants of the BU endemic village of Mbandji 2 located in the Bankim Rural Health Area of the Bankim Health District.

Study participants and human blood sera collection

In Ghana, serum samples were collected from 101 laboratory confirmed BU patients (60 females and 41 males) as well as 404 age-, sex-, and home village-matched control individuals. Collected clinical data, which might have an impact on serological responses of the BU patients and/or the control individuals, included the clinical form of BU, disease category and number of lesions as well as the duration of lesions before treatment.

In Cameroon, blood sera were taken from 406 inhabitants of Mbandji2 with a nearly equal gender distribution. Re-sampling of 80 blood donors was carried out one year after the first blood collection. Demographic data as well as information on previous mycobacterial infections were recorded for all participants. While the majority of individuals had no history of mycobacterial infections, eight study participants reported to have had tuberculosis (2), leprosy (1) or BU (5).

The age distribution of study participants from Ghana and Cameroon is shown in Figure 3.1A and 3.1B, respectively.

ELISA

96-well Nunc-Immuno Maxisorp plates (Thermo Scientific) were coated with 0.25 μ g recombinant *M. ulcerans* 18 kDa shsp per well in 100 μ l phosphate-buffered saline (PBS) and incubated over night at 4°C. Plates were washed four times with washing buffer (dH₂O, 2.5% Tween20) before being incubated with blocking buffer 1 (5% skim milk in PBS) for 2 hours at room temperature (RT). After washing as described above, 50 μ l of 1:100 diluted human blood sera in blocking buffer 2 (1% skim milk in PBS) was added to each well and incubated for 2 hours at RT. Following a further washing step, 50 μ l of 1:8000 diluted goat anti-human IgG (γ -chain specific) coupled to horseradish Peroxidase (HRP, SouthernBiotech) in blocking buffer 2 was added to each well and incubated for 1.5 hours at RT. Plates were washed and 50 μ l TMB Microwell Peroxidase Substrate (KPL) was added per well. The reaction was stopped after 5 minutes using 0.16M sulfuric acid. The absorbance was measured at 450 nm in a Tecan Sunrise microplate reader.

Western Blot analysis

15 μ g of recombinant *M. ulcerans* 18kDa shsp or 5 μ g of a *Plasmodium falciparum* MSP-1 protein domain (amino acids 34-469 of PfK1) were separated on NuPAGE Novex 4-12% Bis-Tris ZOOM Gels with 1.0mm IPG well (Invitrogen) using NuPAGE MES SDS Running Buffer (Invitrogen) under reducing conditions. After electrophoresis the proteins were transferred onto nitrocellulose membranes using an iBlot Gel Transfer Device (Invitrogen). Membranes were blocked with 5% skim milk in PBS containing 0.1% Tween20 (PBS-T) and cut into thin strips. Membrane strips were then incubated with human blood sera at a 1:1000 dilution in PBS-T for 2 hours at RT. Strips were repeatedly washed with 0.3M PBS containing 1% Tween20 and after that incubated with 1:20000 diluted goat anti-human IgG (γ -chain specific) coupled to HRP (SouthernBiotech) for 1 hour at RT. After a second washing step, bands were visualized by chemiluminescence using ECL Western Blotting substrate (Pierce).

Data analysis

ELISA results were analyzed using GraphPad Prism software version 6.0 (GraphPad Software, San Diego California USA). Results are presented as box plots with a line for the median, edges for the 25th and 75th percentiles and traditional Tukey whiskers showing 1.5 times the interquartile distance. Dots on the graph represent individual points that lie outside that range. Differences between data sets were assessed by a Mann-Whitney and Dunn's multiple comparison test.

3.5 Results

***M. ulcerans* 18 kDa shsp specific serum IgG responses in BU patients and control individuals living in the BU endemic Densu River Valley of Ghana**

M. ulcerans 18 kDa shsp specific IgG titers were determined by ELISA in 101 BU patient sera as well as 404 sera from control individuals living in different communities of the BU endemic Densu River Valley in Ghana. Each serum sample was tested twice in two independent experiments (Figure S3.1, $R=0.039$ between duplicate experiments). No statistically significant difference ($p=0.3315$) could be detected by comparing ELISA OD values of the two cohorts (Figure 3.2A). While sero-responders were identified in all age groups >5 years, the vast majority of sera from both BU patients and control individuals had no detectable anti-18 kDa shsp titer. Interestingly, none of the sera from very young children (<5 years) exhibited a significant titer (Figure 3.2B). No relationship between ELISA OD values and clinical parameters of the BU patients (clinical form/category/duration/number of lesions) could be detected for both patients and controls, which can partly be explained by small sample sizes of the different categories (data not shown). Results for a subset of sera tested negative, moderately positive and highly positive by ELISA were reconfirmed by Western blot analysis, showing good agreement between ELISA OD values and Western Blot band intensities (Figure S3.2).

Age distribution of 18 kDa shsp specific serum IgG responses among individuals living in the Mapé Basin of Cameroon

We analysed 486 serum samples collected in Mbandji 2 located in the Mapé Basin of Cameroon, including 96 sera from children aged <5 years for the presence of IgG titers against the *M. ulcerans* 18 kDa shsp. In accordance with results from Ghana, individuals with high antibody titers were detected in all age groups >5 years, while none of the very young children showed any IgG titre above the background response (Figure 3.3). In order to reconfirm these data qualitatively and to be able to determine the age at which individuals start to mount immune responses against *M. ulcerans*, we tested all sera sampled from children below the age of seven by Western Blot analysis. While no specific bands representing IgG antibodies against the 18 kDa shsp, were detected for sera from children <4 years of age, sero-responders were found in all tested age groups >4 years. Since very week

IgG titers were recorded for some of the sera from four year olds, the approximate age of a possible seroconversion may be situated between three and five years (Figure 3.4). Accurate performance of the Western Blot analyses was confirmed by including a control serum (ELISA OD = 0.963) between each of the age groups. Analysis of the five most ELISA positive sera from older children aged between seven and twelve years showed strong Western Blot band intensities for some of the sera (Figure S3.3).

Analysis of serum IgG responses against a domain of the *Plasmodium falciparum* MSP 1 protein among infants living in the Mapé Basin of Cameroon

In order to assess the exposure of infant study participants living in the Mapé Basin to the mosquito transmitted parasite *P. falciparum*, IgG titers against an immunodominant domain of the malaria merozoite surface protein 1 (MSP 1) were determined by Western Blot analysis. In contrast to the striking lack of antibody titers against the 18 kDa shsp in children <4 years old, serum IgG responses against the MSP 1 domain were detected in all age groups tested. Strong band intensities were obtained for the majority of sera collected from children between one and seven years of age as well as for one of the infants <1 years old (Figure 3.5).

Serial analysis of anti-18 kDa shsp IgG titers among 80 residents of Mbandji 2

One year after the first serum collection in Mbandji2, 80 of the 406 study participants were re-sampled in order to analyse the stability of anti-18 kDa shsp serum IgG levels over time. While only minimal changes in antibody titers against the shsp were recorded overall, more individuals had a decreased serum IgG level after one year (Figure 3.6A). The most distinct changes, characterized by a high decrease of antibody titers between the two samplings, occurred in young adults, although no significant correlation between age and the change in OD could be observed (Figure 3.6B).

3.6 Discussion

A high degree of antigenic cross-reactivity among mycobacterial species complicates investigations on *M. ulcerans*-specific humoral immune responses. Nevertheless, the immunodominant 18 kDa shsp was shown to be a suitable serological marker for exposure to *M. ulcerans*. In one of our previous studies we observed the presence of antibody titers against this antigen in about 30% of healthy individuals living in BU endemic and non-endemic communities in the Densu River Basin of Ghana, indicating that infection with *M. ulcerans* may lead only in a proportion of exposed individuals to active disease [11]. Diverse outcome of infection with other mycobacteria such as *M. tuberculosis* and *M. leprae* has been associated with different host and pathogen factors. While only one study has investigated a possible association between BU and host genetics [17], various behavioural factors that may lead to increased susceptibility to the disease have been reported, with poor wound care, failure to wear protective clothing, and living or working near water bodies being the most common risk factors identified [18]. While the generalization persists that children <15 are most affected by the disease [16], a recent survey for BU in the Mapé Basin has revealed that the risk of BU is as high in individuals above the age of 50 as in young teenagers and that very young children are underrepresented among cases when adjusting for the population age distribution [15]. Data of our previous sero-epidemiological investigation focusing on the detection of serum IgG titers against the 18 kDa shsp suggested that individuals of all age groups >5 are equally exposed to *M. ulcerans* [11]. However, results of the present study, including more than 100 serum samples from children <5, show that none of the tested sera had an IgG titer against the shsp above the background level. Hence, children are likely to be considerably less exposed to the pathogen, which may be explained by certain behavioural factors connected with prevention from risk factors such as a smaller movement radius from the house.

Results of a case-control study carried out in a BU endemic region of south-eastern Australia indicated reduced odds of having BU for individuals who frequently used insect repellent and increased odds for those who were bitten by mosquitoes [19], which are associated with the transmission of *M. ulcerans* in this region. In African settings, the highly focal transmission of different *M. ulcerans* haplotypes in a BU endemic region [10,20,21] as well as the distribution pattern of BU lesions on the body, described for cases in the Mapé Basin of Cameroon [15], speak against an exclusive role of mosquito vectors in transmission. Further evidence for this assumption was supplied in this study by comparing antibody levels

against the *M. ulcerans* antigen to the titres against an immunodominant protein of the malaria parasite *P. falciparum* in very young children. In contrast to the absence of serum IgG against the *M. ulcerans* shsp, the majority of the children's sera contained high anti-MSP 1 domain IgG levels, indicating exposure to the mosquito transmitted malaria parasite already at a very young age. Hence, the option of different transmission routes in African and Australian settings should be considered. A sero-epidemiological investigation in south-eastern Australia as well as a BU endemic site in far north Queensland is planned to compare the exposure to *M. ulcerans* in different settings.

No significant difference could be observed by comparing antibody titers against the shsp between a BU patient- and control cohort. However, these results may be explained by the diverse group of BU patients recruited, including healed and active cases as well as cases with different clinical form, category and number of lesions. Due to the small sample size, no statistics could be applied to assess a relation between antibody levels and these different factors.

While the conducted sero-epidemiological investigations provided a good insight into the exposure to *M. ulcerans* in BU endemic areas at the population level, it reflects only a snapshot in time for the individual. Until today, mechanisms for the control of *M. ulcerans* infection are poorly understood. However, the fact that a considerable proportion of exposed, but healthy individuals develop specific humoral responses against *M. ulcerans* antigens together with the observations that BU can heal spontaneously [22] and that secondary *M. ulcerans* infection foci developing after successful treatment of the initial lesion heal without further treatment [23], suggests the existence of protective immunity. Although it is generally assumed that cell-mediated immunity plays an important role, antibodies could provide additional protection against the predominantly extracellular pathogen. More information on humoral immune responses against *M. ulcerans* could be obtained by serial analyses of serum samples from the same individual over time. Within the framework of our analyses, blood was re-collected from a limited number of participants one year after the first sampling. Results of this pilot study showed that antibody titers against the shsp were relatively stable in most of the individuals, while only a few distinct changes between the two samplings were observed. Interestingly, the absolute differences of the two serial ELISA OD values seemed to vary by age, with most pronounced changes in young adults - an age where the incidence of BU is believed to be the lowest [15]. Hence, we can only speculate that in this age group, enough pre-immunity has developed for the immune system to react, thereby preventing the development of the disease. However, due to the small cohort size of this pilot study, these

observations were not significant by tests of the homogeneity of variances. A future analysis of serially collected sera from individuals living in the Densu River Basin of Ghana over a two year period is expected to provide a better understanding of how the immune system functions to combat *M. ulcerans* infections. This may help to improve prevention and treatment strategies for BU.

3.7 Figures

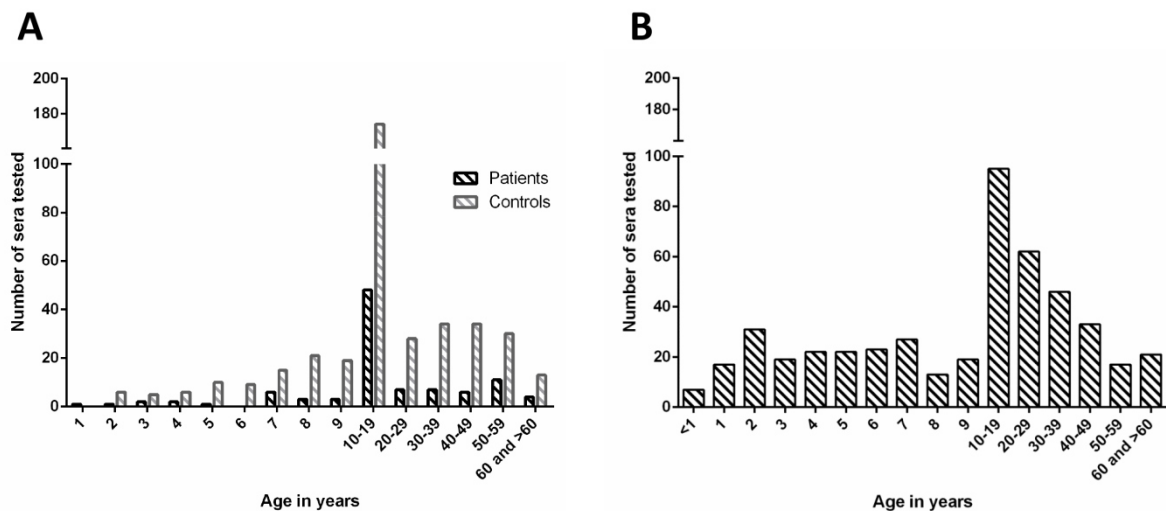


Figure 3.1. Age distribution of study participants.

A Blood sera were collected from 101 Ghanaian BU patients (black) and 404 control individuals (grey) of the different age groups shown. **B** In Cameroon, serum samples were collected from 406 individuals of which 80 were re-sampled after one year. The graph consists of all 486 data points.

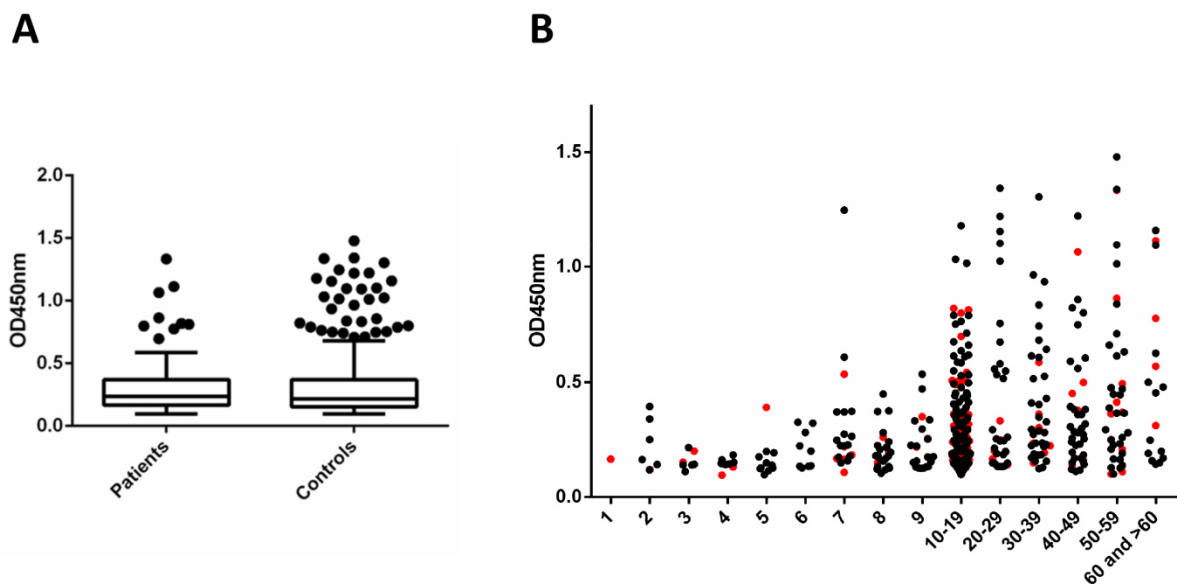


Figure 3.2. Anti-18kDa shp IgG titers in sera from Ghanaian BU patients and control individuals.

A Box plots showing OD values of sera (1:100 diluted) tested from Ghanaian BU patients (n=101) and control individuals (n=404) in an 18 kDa shp ELISA. No statistical difference between cohorts was observed. **B** The distribution of OD values for BU patients (red) and control individuals (black) is shown for different age groups. No IgG titres above the background level were observed for children below the age of 5 years.

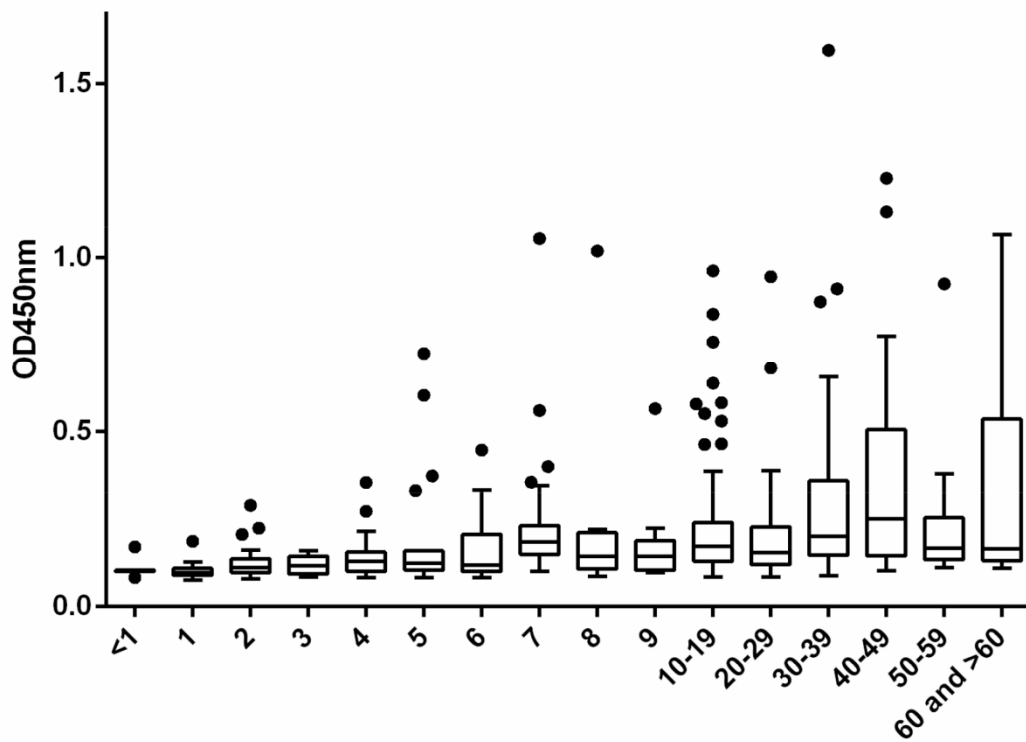


Figure 3.3. Anti-18kDa shsp IgG titers in sera from inhabitants of Mbandji 2.

OD values of serum samples (1:100 diluted) from inhabitants of Mbandji2 tested in an 18 kDa shsp ELISA are visualized for different age groups as box plots. No IgG titers above the background level were observed for children below the age of five.

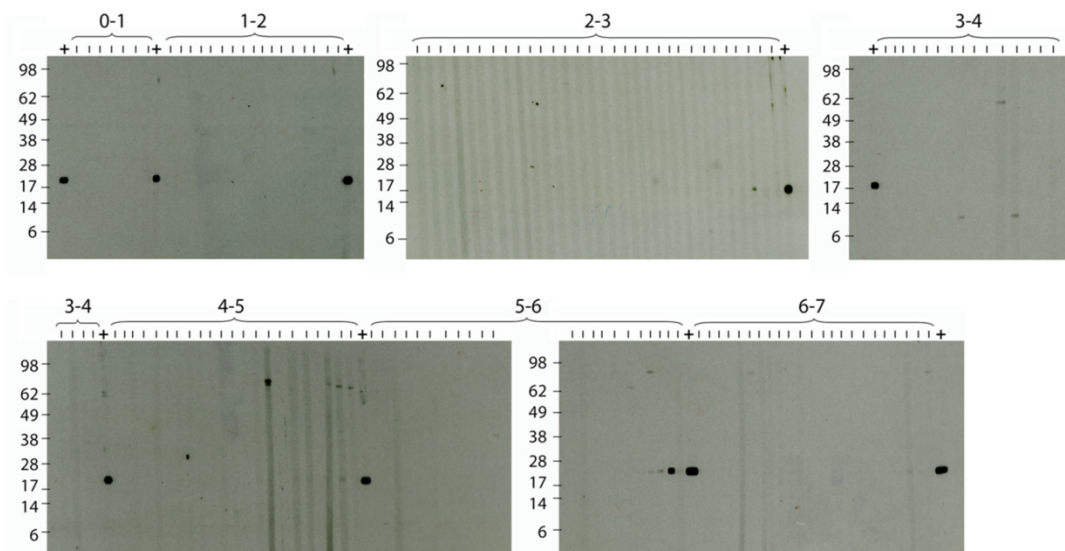


Figure 3.4. Western Blot analysis of specific anti- 18 kDa shsp IgG responses in children.

Sera collected from children living in Mbandji 2 were tested for the presence of specific anti-18 kDa shsp IgG titers by Western Blot analysis. No specific bands were detected for very young children below the age of four. An ELISA positive control serum (OD=0.963) was included between each of the age groups tested (+).

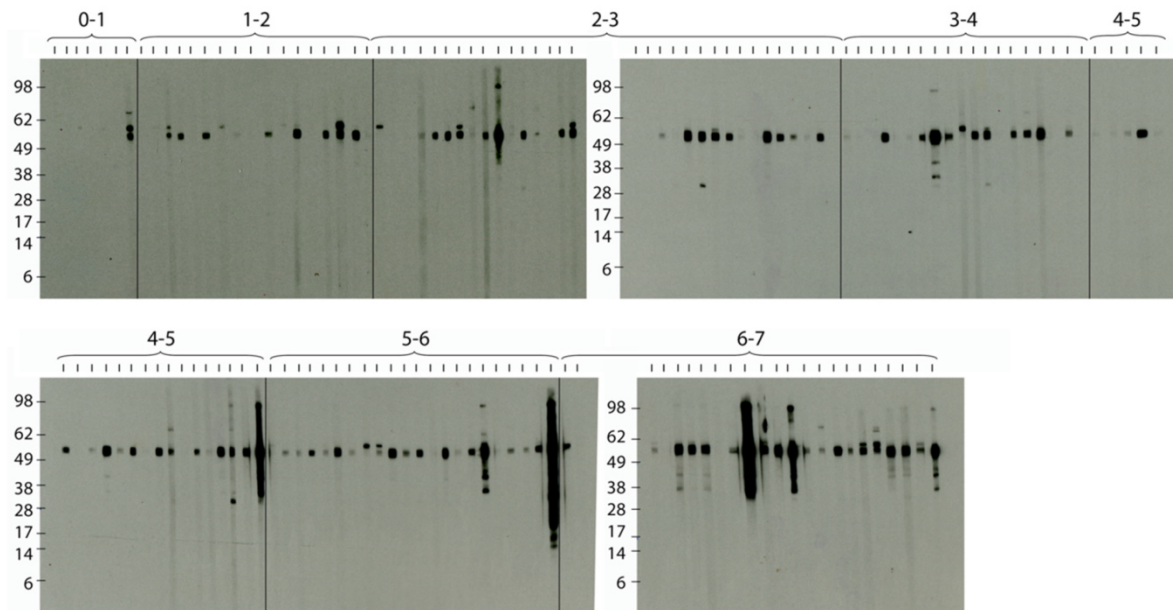


Figure 3.5. Western Blot analysis of specific anti-*P. falciparum* MSP 1 IgG responses in children. Serum IgG responses against a domain of the *P. falciparum* MSP 1 protein were analysed in sera from children living in Mbandji2 by Western Blot analysis. Specific bands were detected in the majority of individuals of all tested age groups >1 year.

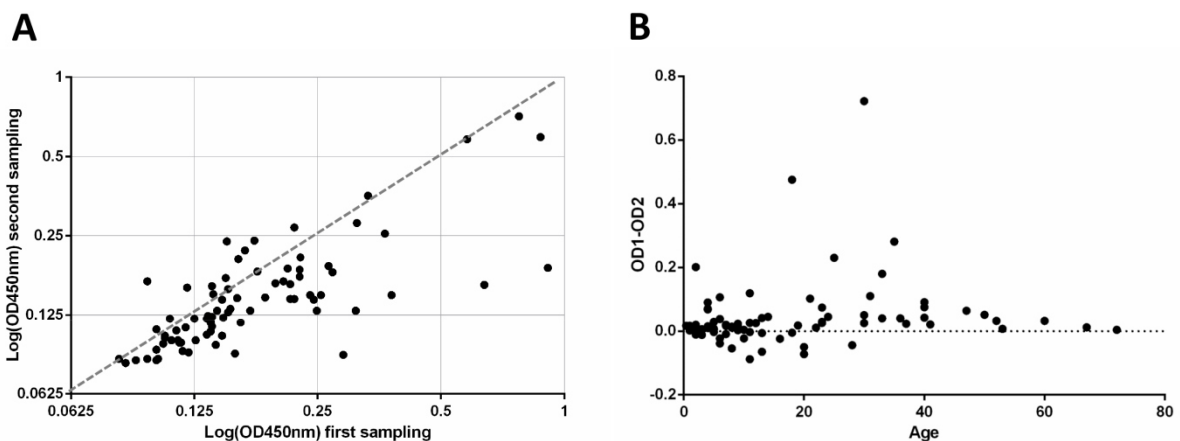


Figure 3.6. Anti-18kDa shsp IgG titers of serially collected serum samples.

A IgG titers against the 18 kDa shsp were determined in serial serum samples collected from 80 individuals. Minimal changes in antibody levels were observed and most individuals showed a decreased titer after one year. **B** Absolute differences in OD values between the two samplings are shown by age. A few distinct changes in antibody titers occurred in young adults although there was no significant correlation between Δ OD and age.

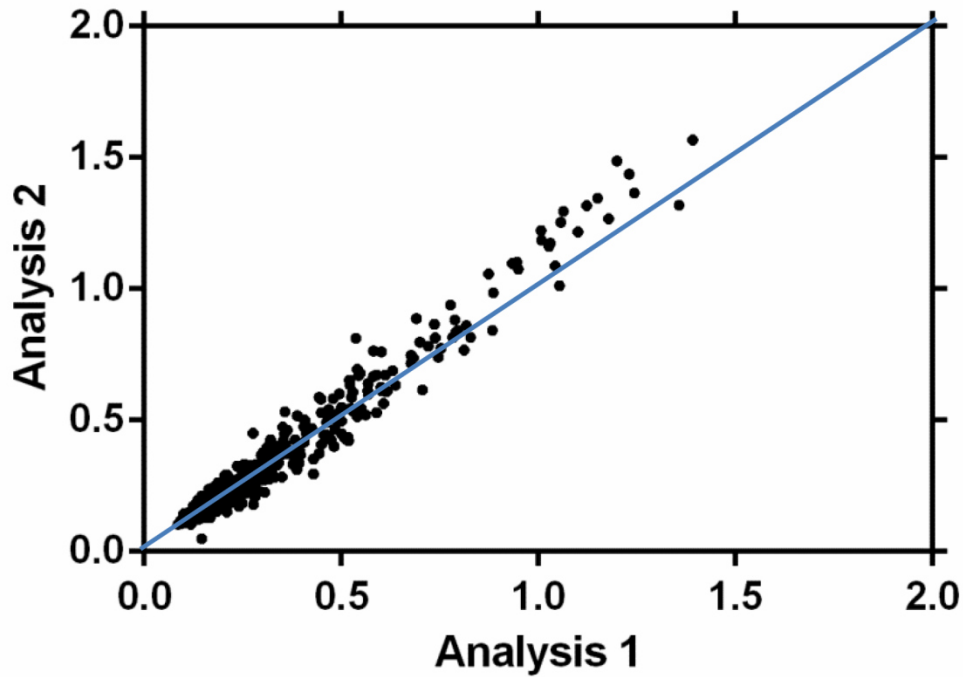


Figure S3.1. Duplicate ELISA testing of sera.

All serum samples collected from individuals living in the BU endemic Densu River Basin of Ghana were tested twice (analysis 1 and analysis 2) with a high correlation between duplicate experiments ($R=0.939$).

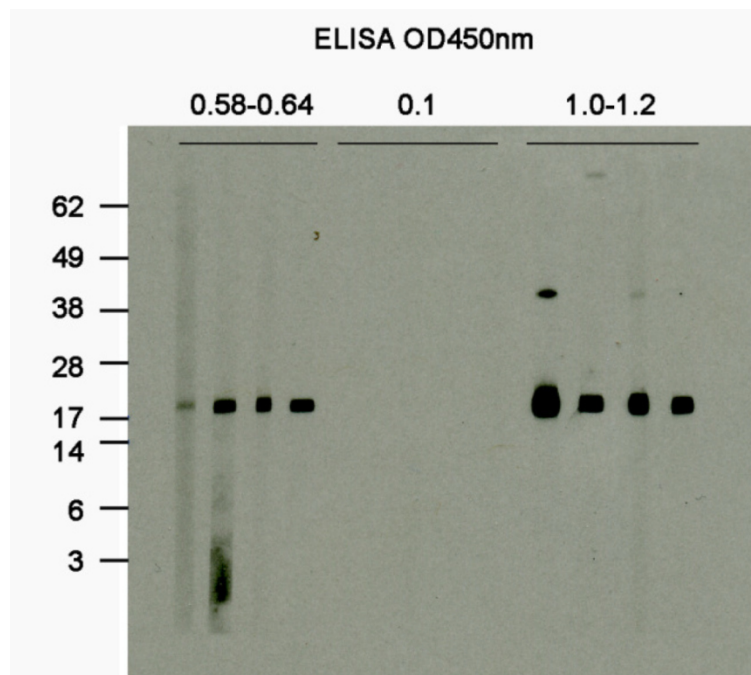


Figure S3.2. Reconfirmatory Western Blot of randomly chosen Ghanaian sera.

A subset of sera tested moderately positive (OD=0.58-0.64), negative (OD=0.1) and highly positive (OD=1.0-1.2) by ELISA were tested by Western blot analysis. Specific bands were detected for ELISA positive sera, while no signal was obtained for ELISA negative sera.

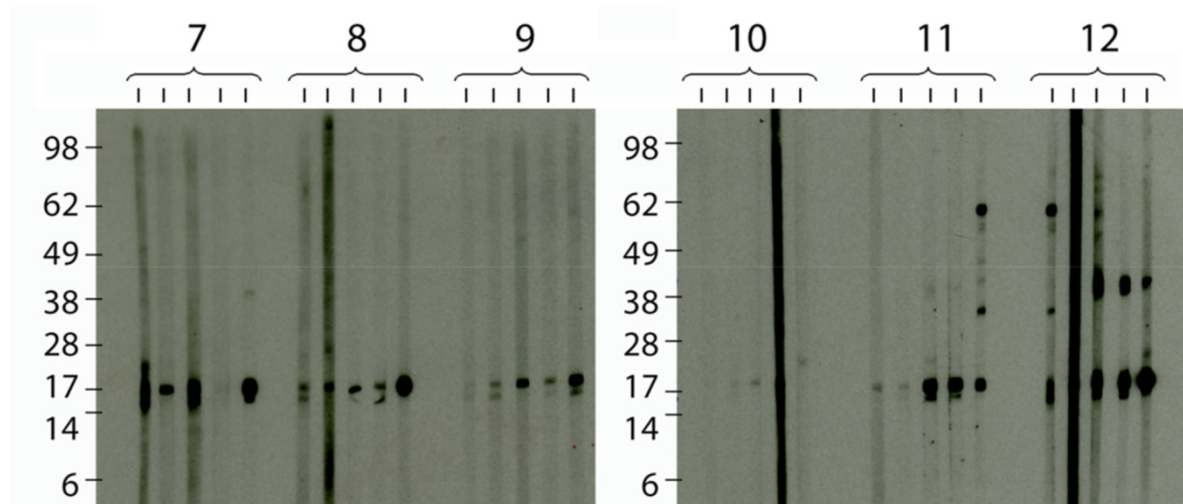


Figure S3.3. Western Blot showing specific anti-18kDa shsp IgG responses in older children.

The five most ELISA positive sera of each age group between seven and twelve were tested for the presence of specific anti-18 kDa shsp IgG responses by Western Blot analysis. Strong band intensities were observed for most of the sera.

3.8 References

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Chapter 4

***Mycobacterium ulcerans* persistence at village water site of Buruli ulcer patients**

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Manuscript submitted to:
PLoS Neglected Tropical Diseases

4.1 Abstract

Buruli ulcer (BU), a neglected tropical disease of the skin and subcutaneous tissue, is caused by *Mycobacterium ulcerans* and is the third most common mycobacterial disease after tuberculosis and leprosy. While there is a strong association of the occurrence of the disease with stagnant or slow flowing water bodies, the exact mode of transmission of BU is not clear. *M. ulcerans* has emerged from the environmental fish pathogen *M. marinum* by acquisition of a virulence plasmid encoding the enzymes required for the production of the cytotoxic macrolide toxin mycolactone, which is a key factor in the pathogenesis of BU. Comparative genomic studies have further shown extensive pseudogene formation and downsizing of the *M. ulcerans* genome, indicative for an adaptation to a more stable ecological niche. This has raised the question whether this pathogen is still present in water-associated environmental reservoirs. Here we show persistence of *M. ulcerans* specific DNA sequences over a period of more than two years at a water contact sites of BU patients in an endemic village of Cameroon. At defined positions in a shallow water hole used by the villagers for washing and bathing, hypertrophic detritus remained consistently positive for *M. ulcerans* DNA. The observed mean Ct difference of 1.45 between the RT-PCR for the insertion sequences IS2606 and IS2404 indicated that lineage 3 *M. ulcerans*, which cause human disease, persisted in this environment after successful treatment of all local patients. Underwater detritus may therefore represent a reservoir of *M. ulcerans* for direct infection of skin lesions or vector-associated transmission.

4.2 Introduction

Buruli ulcer (BU) is a neglected tropical disease of the skin and subcutaneous tissue caused by the environmental pathogen *Mycobacterium ulcerans*. The disease, which can affect all age groups and both sexes, has been reported in over 30 countries but is most frequent in West Africa. Typically, BU presents with ulcers with undermined edges but clinical manifestations also include nodules, oedema and plaques. Lesions can encompass entire limbs if patients report late for treatment [1]. The WHO recommends that all cases should be laboratory confirmed by microscopy, polymerase chain reaction (PCR), primary culturing or histology [2]. However, because of the limited access to laboratory facilities in BU endemic areas, cases are often diagnosed based only on clinical signs and there is a pressing need for a simple, sensitive and specific point-of-care diagnostic test [3]. Historically, BU was treated using wide scale excision of the lesions. Since 2004, the WHO recommends a combination therapy of daily streptomycin and rifampicin for 8 weeks as the standard treatment for BU [1].

The major risk factor for BU is proximity to stagnant or slow flowing water, but other factors such as poor wound care, and failure to wear protective clothing have also been identified in case-control studies [4]. It has further been reported, that man-made modifications of the environment may increase the incidence of BU [4]. Despite relentless efforts, both the reservoir and the exact mode of transmission of BU remain a mystery. Numerous investigations of the environment have attempted to identify the source of the pathogen with so far only limited success. Studies in Ghana and Benin, have examined environmental samples for the presence of the *M. ulcerans* insertion sequence (IS) 2404. Some of these studies have identified many IS2404 positive sites and found positive samples in both BU endemic and non-endemic areas [5]. On the other hand, a study from Ghana has reported that only very few samples were real time PCR (RT-PCR) positive [6]. These difficulties to conclusively identify the environmental reservoir of *M. ulcerans* and the fact that investigations on its genome have revealed that the pathogen has undergone substantial niche adaptation[7,8], have led investigators to look for animal reservoirs [4,7]. While to date no such reservoir has been detected in Africa, possums have been identified as an animal reservoir in the southern Australian BU endemic area [9]. The mode of transmission from an animal or environmental reservoir to human patients also remains to be elucidated. Both insect bites, from mosquitos or water bugs, and direct inoculation of bacteria into the skin

from an environmental reservoir after skin trauma have been suspected to be relevant for transmission [4] and several parallel modes of transmission may need to be considered [10].

The objective of the current study was to longitudinally monitor environmental contact sites of laboratory confirmed BU patients for the persistence of *M. ulcerans* DNA.

4.3 Materials and Methods

Ethical statement

Approval for this study was obtained from the Cameroon National Ethics Committee (N°041/CNE/DNM/09 and N°172/CNE/SE/2011) and the Ethics Committee of Basel (EKBB, reference no. 53/11). Participation was voluntary and all patients, independent of their study participation, were treated according to national treatment guidelines. All cases who participated in the study or their legal guardian provided written informed consent.

Study area, patient inclusion and patient confirmation

All RT-PCR confirmed cases identified in the Mapé Basin of Cameroon [10] between the beginning of December 2009 and the end of November 2011, were eligible for inclusion in this study. For definitive BU diagnosis, clinical samples were collected, DNA extracted and IS2404 RT-PCR performed as previously described [10–12]. Environmental sampling was performed between February 2011 and June 2013.

The main water bodies of the study area are the Mapé Dam and the Mbam River [10]. The region experiences two rainy seasons, a short one from mid-March to mid-May and a long one from mid-June to the end of September, with the rest of the year being dry.

Selection of environmental sampling locations and sampling procedures

Patients selected for in-depth investigation were interviewed to determine where they lived for the year before the onset of BU. Homes of as many non-participating RT-PCR confirmed cases as possible were also identified and mapped. If participating patients had a home both in their village and at their farm, an interview was used to determine where they spent more time. After achieving an accuracy of less than 10m, a GPS receiver was used to map the patient's home. Together with the patient, a close friend or relative, sites of regular environmental contact of the patient were then visited. The investigated and mapped sites included the patient's farm(s) and the location(s) where she/he obtained water while at home (VW: village water site) or at the farm(s) (FW: farm water site). Sites used to obtain water for drinking, cooking, bathing, cloth washing and dish washing were visited. At all sites, soil and

plant material was collected. At the water contact sites, a water sample was also collected. Samples collected at the farms were dry soil and plants growing on dry grounds. Plant and soil samples from the water contact sites, were collected from either in the water, at the water's edge or in the moist area around the water site.

Additionally, animal fecal samples were collected in the highly BU endemic village of Mbandji 2. Samples were collected around the homes of laboratory confirmed BU patients and included the feces of chicken, ducks, pigs, goats and sheep.

At two water contact sites located in Mbandji 2 (VW12 and VW13) we performed repeated and in-depth sampling over a period of more than two years (Supplementary Table 4.1). In addition to the samples collected at the initial time point ($t=0$) as described above, samples were collected from VW12 and VW13 at seven additional time points ($t=2.1, 4.8, 7.7, 10.5, 15.3, 20.3$ and 27.4 months). At the two initial time points, samples were collected from 3 sampling points at each VW site. At the remaining time points, samples were collected at 21-22 sampling points around VW12, with 3-5 sample replicates at each point. At the same time points, VW13 was sampled at 14-16 points with 1-3 sample replicates collected at each sampling point. Details of the sampling points and the number of replicates collected at each sampling point and at each time point are given in Figure 4.4, Supplementary Figure S4.1 and Supplementary Table S4.1. All samples, with the exception of points 7 and 13, which were plants on dry soil, were collected from inside the water or at the water's edge (Figure 4.4 and Supplementary Figure S4.1).

At the last follow-up time point ($t=27.4$ months), further soil samples from inside the water were collected around the log at site VW12. At each sampling point 3-5 replicates of the same type of sample were collected. At several sampling points on either side of the log, samples were repeatedly collected in the course of a few days (Figure 4.5). At sampling point 55, additional samples of various natures were collected.

All environmental samples were stored at 4°C until analysis.

Environmental DNA Extraction and RT-PCR

From the environmental samples, DNA was extracted and RT-PCR performed as previously described [11,12]. Briefly, approximately $200\mu\text{L}$ of each soil, plant and fecal

sample was transferred to a lysing tube and DNA extracted using the Fast DNA Spin Kit for Soil (MP Biomedicals, product number 116560-200) and a Precellys24 homogenizer (Bertin Technologies). For water samples, 1mL was transferred to a lysing tube, the tubes centrifuged for 10 min (14'000 rpm), the supernatant removed and the samples then processed like the other samples. All samples were at least once extracted by the above method. Some samples were also processed once by homogenizing them in lysing matrix E tubes in the presence of the MT buffer (MP Biomedicals) and Phosphate Buffered Saline (MP Biomedicals), pelleting debris (10 min at 14'000 rpm) and then extracting DNA from the supernatant with the QIASymphony (Qiagen) and the QIASymphony DSP Virus/Pathogen Midi Kit (Qiagen, product number 937055). For each extraction, a reagent control was included.

Extracted DNA (1 μ L of 100 μ L) was run twice in the IS2404 RT-PCR assays as previously described [11,12]. In the IS2404 RT-PCR, an internal positive control (IPC, Applied Biosystems) was included. Samples which were inhibited were diluted 1/5 and 1/10 and analyzed again. In each RT-PCR run both negative and positive controls were included. If a sample was positive in at least one of the IS2404 RT-PCR assays, DNA was extracted from a second aliquot of the same environmental sample. If again at least one of two parallel IS2404 RT-PCR assays was positive, the corresponding environmental sample was considered positive for IS2404. DNA extracted from these samples (1 μ L and 5 μ L) was then subjected to IS2606 and keto reductase (KR) RT-PCR as previously described [11,12]. If the extracts of a particular sample, were at least once positive for these two additional targets, the sample was considered positive for *M. ulcerans* DNA. All IS2404 positive samples that were not positive for both of the other targets were not considered to contain *M. ulcerans* DNA and were not included in the analysis. All RT-PCR assays were performed in a StepOne Plus Real-Time PCR System (Applied Biosystems) and analyzed using the StepOne Software (v2.2.2; Applied Biosystems). Samples extracted using the QIASymphony as well as samples analyzed on site in Cameroon, were RT-PCR tested for the presence of IS2404 by a Mastercycler Realplex 4 ep Gradient S (Eppendorf) and the data analyzed by Mastercycler ep Realplex (version 2.2).

Statistical Data Analysis

Statistical data analysis was performed using R (The R Foundation for Statistical Computing; version 2.15.1) and RStudio (RStudio, Boston, USA; version 0.95.262). Maps were drawn in ArcGIS ArcMap (Economic and Social Research Institute, Redlands, USA; version 10.0).

4.4 Results

Screening of environmental contact sites of BU patients for the presence of *M. ulcerans* DNA

From December 2009 to November 2011, 67 RT-PCR confirmed cases of BU were identified in the Mapé Basin of Cameroon. Of these patients, 46 were selected for in-depth environmental contact analysis based on their origin in the southern part of the Mapé Basin and their availability to participate in the study. The homes and farms as well as the VW and FW sites of the patients were mapped (Figures 4.1A and 4.1B). The median direct distance between the homes and farms was 1.5 km (interquartile range = 0.6 km to 5.3 km). While some patients lived permanently at their farm, others travelled more than 15 km to get from their home to their farm (Table 4.1). As shown in Figures 4.1A and 4.1B, many of the BU patients in the southern Mapé Basin moved south and east towards the Mbam River for their farming activities.

Environmental samples (171 soil, 153 plant and 109 water samples) were collected at the farms (n=49), FW (n=43) and VW (n=48) sites shown in Figure 4.1A. Of the soil and plant samples, 108/171 and 109/153 respectively, were collected in or around water. The remaining samples were collected from dry grounds. All environmental contact sites are numbered in Table 4.1; sites used by several patients are indicated by the same number. Additionally, pig, goat, sheep, chicken and duck fecal samples (n=24) were collected at 14 locations in the BU endemic village of Mbandji 2 (Figure 4.1B).

All environmental and fecal samples were tested by RT-PCR for the presence of the *M. ulcerans* specific IS2404 DNA sequence. Three VW sites (VW12, VW31 and VW54) and one duck fecal sample (F07) tested positive (Figures 4.1A and 4.1B). At locations VW31 (Figure 4.1C) and VW54 (Figure 4.1D), soil samples collected in the moist area around the water wells were positive. Both of these water sites were used by one BU patient each (Table 4.1). Water from VW31 was reported to be used for bathing and washing of clothing and water from VW54 was used for all purposes including drinking. At site VW12, used by three of the patients living in the village of Mbandji 2, both a soil and a plant sample collected at the water's edge, tested positive. Further details on the results of a longitudinal study at VW12 are provided below.

Persisting RT-PCR positivity of hypertrophic detritus after successful treatment of the identified local BU patients

As shown in Figures 4.1B and 4.2A, six BU patients were notified during the study period in Mbandji 2, which is situated between the Mapé Dam and the Mbam River (Figure 4.1E). The locations of the homes of these patients are shown in Figure 4.2B and characteristics of the patients, which are not related to each other, are listed in Table 4.2. Patients 06, 13, and 34, aged 9, 5 and 57, respectively, all used the RT-PCR positive VW12 site (Figure 4.2B and Table 4.1). Furthermore, the only positive faecal sample (F07; from a duck) was collected in close proximity of the home of patient 13 (Figure 4.2B). The other three patients from Mbandji 2 used primarily four other VW sites (Table 4.2, Figure 4.2B).

To better characterize IS2404 RT-PCR positivity in Mbandji 2, we performed detailed longitudinal analyses of VW12 and the close-by IS2404 negative site VW13 (Figure 4.1B and 2B). VW12 was a permanent small water body with a wooden log lying in it (Figure 4.3A). The water was shallow and flowed slowly from the left to the right. For most of the log, the left and right side of the water were not connected under the log; however at some points water could pass underneath the log. On the right side the vegetation was denser and a layer of detritus was accumulating. In contrast, the compacted ground on the left was not covered with detritus. Site VW12 was used by the local population – including patients 06, 13 and 34 (Table 4.1 and 4.2) – to wash clothing and for bathing. For these activities, locals stood in the water on the left side of the log. The father of patient 13 also reported that his daughter went to this site to play. In contrast, VW13 was used by the local population – including again patients 06, 13 and 34 – to obtain drinking water as well as water for cooking and bathing. In the front section of VW13, where there were planks of wood (Figure 4.3B), water emerged from several springs.

We collected and analysed environmental samples at eight time points over a period of 27.4 months at both VW12 (n=635) and VW13 (n=217) (Supplementary Table S4.1). Particularly at location VW12, substantial seasonal alterations of the environment over the study period were observed (Figure 4.3A and 4.3B). None of the 217 samples collected at VW13 tested positive in the IS2404 RT-PCR and only one of 108 samples taken from the sand pits to the north-west of VW12 tested positive (Figure 4.4B and 4.4C, Supplementary Figure S4.1 and Supplementary Table S4.1). In contrast, at 7/8 sampling time points, positive samples were obtained from at least one of the six positive sampling points identified at

VW12 (Fig. 4). In particular underwater soil samples collected at sampling point 37 were positive at 5/6 time points tested (Figures 4.4A and 4.4B). The average IS2404 RT-PCR Ct values of the positive samples varied between 34.0 and 38.4 (Figure 4.4B). As shown in Figure 4.4C, at the initial sampling time point, there was still one active case of BU (patient 34) using VW12 and there were still a total of three active BU cases in the entire village of Mbandji 2. However, from the third sampling time point on, no active BU case was using VW12 and from the fourth time point on, no active BU case was present in the entire village of Mbandji 2 (Figure 4.4C). Taken together IS2404 RT-PCR positivity at the VW12 site thus persisted for more than one year after successful treatment of all BU patients identified in the village of Mbandji 2 (Figure 4.4).

Having identified the deposit on the right hand side of the log lying in VW12 as an IS2404 hotspot (Figure 4.4C), we analysed the soil all around the log in more detail. While compacted and sandy ground was found on the left, the ground was covered with hypertrophic detritus on the right hand side of the log (Figure 4.5A). At the eighth sampling time point we collected three replicates of soil samples every 1.14m at a total of 14 sampling points all around the log (Figure 4.5B). Using on site RT-PCR, we identified sampling point 55 (Figure 4.5C) as being positive (data not shown) and then sampled this location as well as other points around the log repeatedly over the next 12 days (Figure 4.5C). All samples were analysed once back in the lab. While all 59 samples collected on the left hand side and at the back of the log were negative, 9/62 samples from the right side of the log were positive. Positive samples were identified at sampling point 55 and the neighbouring point 56 (Figure 4.5C) with an average IS2404 Ct value of 35.8 for all nine positive samples (Figure 4.5C). Additional sample types, including plants, roots or samples from the surface of the log, collected at sampling point 55 all tested negative (data not shown).

All the above mentioned IS2404 positive samples also tested positive in the IS2606 and the KR RT-PCR and the mean Ct difference (Δ Ct) between IS2606 and IS2404 (IS2606-IS2404) of the samples was 1.45 (95% confidence interval from 1.10 to 1.80). This Δ Ct indicated that the obtained PCR signal is not related to lineage 1 *M. ulcerans* bacteria, which are fish and frog pathogens and harbor only a few copies of IS2606, but that the PCR signal likely originates from lineage 3 bacteria, which are found in human lesions and contain 63-98 copies of IS2606 per genome [7].

4.5 Discussion

In African BU endemic areas, both the nature of the environmental reservoirs of *M. ulcerans* and the mode of transmission to humans have so far remained unclear. The physical environment, e.g. biofilms, and organisms such as amoeba, insects, fish and frogs have all been proposed as possible reservoirs for the pathogen [4]. Investigations in Southern Australia have identified mammals, specifically possums, as a local reservoir of *M. ulcerans* [9]. However, no such mammalian source of the pathogen has been detected in Africa thus far [13]. As for the transmission to humans, hypotheses include insect vectors and direct inoculation from the environment via small skin lesions. Parallel modes of transmission may, depending on the environmental and epidemiological setting, be relevant [4,10]. A recent review on BU transmission, found that more evidence is needed to conclude that insects are involved in *M. ulcerans* transmission [4]. Interestingly, *M. marinum*, the closest relative and ancestor of *M. ulcerans*, occasionally causes human infection by inoculation through small skin lesions which are often not remembered by the patient because of the long incubation period [14].

Although BU may occur at all ages, the relative risk for children below the age of five to develop the disease is lower than for older children [10,15]. This appears to apply across different endemic areas in Africa and may indicate that exposure to *M. ulcerans* is increasing, once children are taking up new activities away from their homes [10,16]. Such activities could include going to the farm to work or to water sources for household activities, to collect water or to play. With this in mind and since proximity to water bodies undoubtedly is a risk factor for BU [4], we set out to systematically test environmental, and in particular water, contact sites of laboratory confirmed BU patient. Specifically we collected plant, soil and water samples at the farms as well as village and farm water sites of patients and tested them for the presence of *M. ulcerans* DNA.

Due to the abundance of other and faster growing microorganisms in the environment, routine cultivation of *M. ulcerans* from environmental samples has mostly failed [17] and to date only a single *M. ulcerans* isolate from a water-strider, has been reported [18]. Attempts to culture from our samples were not successful also because of the overgrowth by other mycobacteria. By PCR using pan-mycobacterial and hsp65 primers [19,20] and DNA sequencing, we detected species such as *M. shimoidei*, *M. psychrotolerans* and *M. chubuense* in our preparations (data not shown). Because of these difficulties, RT-PCR for IS2404 is

commonly used to detect *M. ulcerans* in the environment. We applied most stringent quality control procedures with internal positive controls in each sample as well as negative and positive controls in each RT-PCR reaction. Further, we only considered an environmental sample positive if it was positive in two separate DNA extractions. With this approach we are confident that the positive samples truly contain *M. ulcerans* DNA. We can however not exclude, particularly given the heterogeneity of the environmental samples, that some positive samples may be missed. Although IS2404 is considered a specific marker for *M. ulcerans* [21], the existence of IS2404 positive *M. ulcerans* ecotypes (lineage 1) that are largely avirulent for humans complicates interpretation of RT-PCR data and requires that samples are also tested for the presence of IS2606 and that the difference between the IS2606 and the IS2404 Ct value is analyzed [7,11]. Because *M. ulcerans* ecotypes that cause human disease in Africa and Australia (lineage 3) harbor a higher number of IS2606 sequences than those of lineage 1, the ecovars can be separated based on the IS2606 to IS2404 Δ Ct [7]. All 41 IS2404 positive environmental samples collected in the course of this study also tested positive for IS2606 with a mean IS2606 to IS2404 Δ Ct of 1.45. This Δ Ct is well below the Δ Ct of 7 to 8 of the for humans non-pathogenic lineages 1 *M. ulcerans* strains [11]. Two of the IS2404 and IS2606 positive samples, both not included in the *M. ulcerans* DNA positive samples discussed in this paper, were negative for the RT-PCR of the lower copy number virulence plasmid associated KR sequence.

Our screening of environmental contact sites of laboratory confirmed BU patients revealed that they travel considerable distances to get to their farms and some of the patients further reported to spend several months there. Molecular typing studies of disease isolates may help to identify if the patients were infected close to their homes or farms [22].

By testing environmental samples, we identified two *M. ulcerans* DNA positive water wells (VW31 and VW54) in two different villages. In a third village we identified an *M. ulcerans* positive duck fecal sample (F07) and a positive open permanent water site (VW12). While this rate of environmental positivity is similar to what has been found in a study from Ghana [6], positivity was much higher in another study also conducted in Ghana [23]. It is interesting to note that all three positive sites were permanent as opposed to seasonal water sources. Obtaining water from such water sources has previously been shown to increase the risk for BU [24]. The positive duck fecal sample, merits further investigation to determine how waterfowl may contribute to the reservoir of *M. ulcerans*.

At VW31 and VW54 we did not investigate the local scenario any further and cannot exclude the possibility that these sites were contaminated with *M. ulcerans* DNA from the lesions of patients living close to the wells. However at VW12, located in Mbandji 2, we observed persistence of *M. ulcerans* DNA in underwater detritus for more than one year after successful treatment of the last BU patient. Continuous presence and case detection in the village allowed us to detect all local cases and it is therefore unlikely that the source of the environmental positivity was from bacteria recently spread from a human lesion. Interestingly, the more sandy ground on the left of the log at WV12 was never RT-PCR positive for *M. ulcerans* DNA and even on the right side of the log the distribution of *M. ulcerans* DNA was highly focalized (Figures 4.4 and 4.5), with samples taken from sites just a few meters apart giving different results. The persistent of RT-PCR positivity in the detritus is a strong indication that this micro-environment may represent a niche environment to which *M. ulcerans* has adapted in the course of evolution from the more generalist *M. marinum* [7,25].

The previously described age distribution of BU patients in the Mapé Basin [10] and the here described findings of *M. ulcerans* DNA at a village water sites, lead to the hypothesis that around the age of four, when children are beginning to be sent to fetch water, both exposure to *M. ulcerans* and the risk of contracting BU increases because of direct contact with the environmental source of the pathogen. Our data further suggest that, the detritus at the water site could represent a reservoir from where infection could take place through either direct contamination of skin lesions or through contamination or colonization of insect vectors.

4.6 Acknowledgments

We would like to thank all the patients, other members of the local community and personnel at the local health care facilities for their invaluable help and for participating in this study. We would also like to thank the staff of the FAIRMED offices in Yaoundé and in Bankim, in particular Ferdinand Mou and Edgar Satouglé for their support. Further we would like to thank Miriam Bolz, Patrick Bosshart, Jan Furrer and Daniel Gervasi for their help with the analysis of environmental samples. For their generous financial support we would like to thank the Medicor Foundation.

4.7 Tables

Table 4.1: Environmental contact sites of laboratory confirmed BU patients tested for *M. ulcerans* DNA.

Patient ID	Village Water (VW) ***	Farm (F) ***	Farm Water (FW) ***	Distance Home – F (km)
01	VW01, VW02, VW03	F not visited	FW01, FW02	NA
02	VW04, VW05	F01	NA *	0.63
03	VW06, VW07	F02	FW03	12.14
04	VW08, VW09	F03	FW04, FW05	14.31
05	VW10, VW11	F04	FW06	5.62
06	VW12 [#] , VW13	F05, F06	FW07	7.54; 6.19
07	VW14, VW15, VW16	F07, F08	NA *	0.34; 0.65
08	VW17	F09	FW08	0.69
09	VW18, VW19	F10	FW09, FW10	3.03
10	VW20, VW21	F11	FW11	2.27
11	VW22	F12	FW12	1.08
12	VW23, VW24	F13, F14, F15	FW13	5.37; 5.26; 1.40
13	VW12 [#] , VW13	F16	FW not visited	2.87
14	VW25	F17	FW14	1.57
15	VW26	F18	FW15, FW16	3.20
16	VW27, VW28	F19	FW17, FW18	11.96
17	VW29	F20	FW19, FW20	3.51
18	VW30	F21	FW21	0.55
19	VW31 [#]	F22	FW22	12.62
20	VW32	F23	NA *	0
21	VW not visited	F24	FW23	0
22	VW33	F25, F26	FW24	0.89; 1.20
23	VW34, VW35	F27	FW25	2.00
24	VW36, VW37	F not visited	FW not visited	NA

25	VW38	F28	FW not visited	5.66
26	VW39	F29	FW26, FW27	1.48
27	VW40, VW41	F30	FW28, FW29	7.32
28	VW42, VW43	F31	FW30, FW31, FW32, FW33	4.40
29	VW44, VW45, VW46	F32 **	FW34	3.60
30	VW47	F33	FW35	15.35
31	VW48	F34	NA *	0.30
32	VW49	F35	NA *	0.99
33	VW50	F36	FW36	1.83
34	VW12 [#] , VW13	F16	FW not visited	2.98
35	VW51	F37, F38	NA *	0.08; 0.21
36	VW52, VW53	F39	NA *	0.38
37	VW54 [#]	F40	NA *	0.24
38	VW55	F41	FW37	1.31
39	VW56, VW57	F42	NA *	0.59
40	VW58, VW59	F43, F44	FW38, FW39	1.13; 0.84
41	VW52, VW53	F45	NA *	0.79
42	VW60	F46	NA *	0
43	VW61	F47	NA *	0.02
44	VW not visited	F48	FW40, FW41, FW42	5.91
45	VW62	F49	FW43	13.52
46	VW48	F50	NA *	0.17

NA: not applicable

* Water carried to farm from home

** Location not tested by RT-PCR

*** VW, F and FW sites are individually numbered; sites which are shared between patients are identified by the same number

[#] Positive for *M. ulcerans* DNA

Table 4.2: Laboratory confirmed BU patients in Mbandji 2 during the course of the study.

Patient ID	Age	Gender	Clinical Form	Category	Disease Start Date*	Discovery Date	Treatment Start	VW used by the patient
06	9	M	nodule	1	unknown	13.04.2010	24.07.2010	VW12 and VW13
13	5	F	plaque	2	11.09.2010	06.11.2010	10.11.2010	VW12 and VW13
15	2	M	ulcer	3	26.10.2010	30.11.2010	03.12.2010	VW 26
33	11	M	ulcer	2	10.03.2011	05.05.2011	10.05.2011	VW 50
34	57	M	ulcer	1	01.03.2011	10.05.2011	12.05.2011	VW12 and VW13
40	42	M	ulcer	3	06.09.2009	07.08.2011	07.08.2011	VW 58 and 59

* Calculated based on information provided by the patient

Supplementary Table S4.1: Number of environmental samples collected at each sampling point of VW12 and VW13 at all sampling time points.

Sampling Point*	Sample Type	t = 0	t = 2.1 months	t = 4.8 months	t = 7.7 months	t = 10.5 months	t = 15.3 months	t = 20.3 months	t = 27.4 months
1	water	NA	NA	1	3	3	3	3	3
2	soil	NA	NA	1	3	3	3	3	3
3	plant	NA	NA	1	3	3	2	3	3
4	plant	NA	NA	1	3	3	3	3	3
5	water	1	NA	1	3	3	3	3	2
6	plant	1	NA	1	3	3	3	3	3
7	plant	NA	NA	1	3	3	3	3	3
8	soil	NA	NA	1	3	3	3	3	3
9	soil	NA	NA	NA	NA	3	3	3	3
10	plant	NA	NA	1	3	NA	NA	NA	NA
11	water	NA	NA	1	3	3	3	3	3
12	soil	NA	NA	1	3	3	3	3	3
13	plant	NA	NA	1	3	3	3	3	3
14	water	NA	NA	1	3	NA	NA	3	3
15	soil	1	NA	NA	3	NA	NA	NA	NA
16	plant	NA	NA	NA	NA	3	2	3	3
17	soil	NA	NA	5	3	3	3	3	3
18	water	NA	NA	4	3	NA	3	3	3
19	plant	NA	NA	5	3	3	3	3	3
20	plant	NA	NA	5	3	3	3	3	3
21	water	NA	NA	4	3	3	3	NA	2
22	soil	NA	NA	5	NA	3	3	3	3
23	water	1	1	NA	5	NA	NA	5	NA
24	soil	NA	NA	5	NA	5	5	NA	5
25	soil	2	1	4	5	5	5	5	5
26	water	NA	NA	NA	5	NA	NA	NA	NA
27	plant	2	3	5	NA	5	5	5	5
28	soil	NA	NA	5	NA	5	5	5	5
29	plant	NA	NA	NA	5	NA	NA	NA	NA
30	water	NA	NA	5	5	5	5	5	3
31	soil	NA	NA	5	5	5	5	5	5
32	plant	NA	NA	5	5	5	5	5	5
33	plant	NA	NA	5	5	5	5	4	5
34	water	NA	NA	4	5	5	5	5	5
35	plant	NA	NA	5	5	5	4	5	5
36	plant	NA	NA	5	4	5	5	5	NA
37	soil	NA	NA	5	5	5	5	5	5
38	water	NA	NA	5	5	5	5	5	5
39	plant	NA	NA	5	5	5	5	5	NA
40	plant	NA	NA	5	5	5	5	5	5
41	plant	NA	NA	5	5	5	5	5	5
42	plant	NA	NA	5	5	5	5	NA	5
43	plant	NA	NA	5	5	5	5	4	5

* Sampling points 1 to 16 are at VW13 and 17 to 43 are at VW12.

4.8 Figures

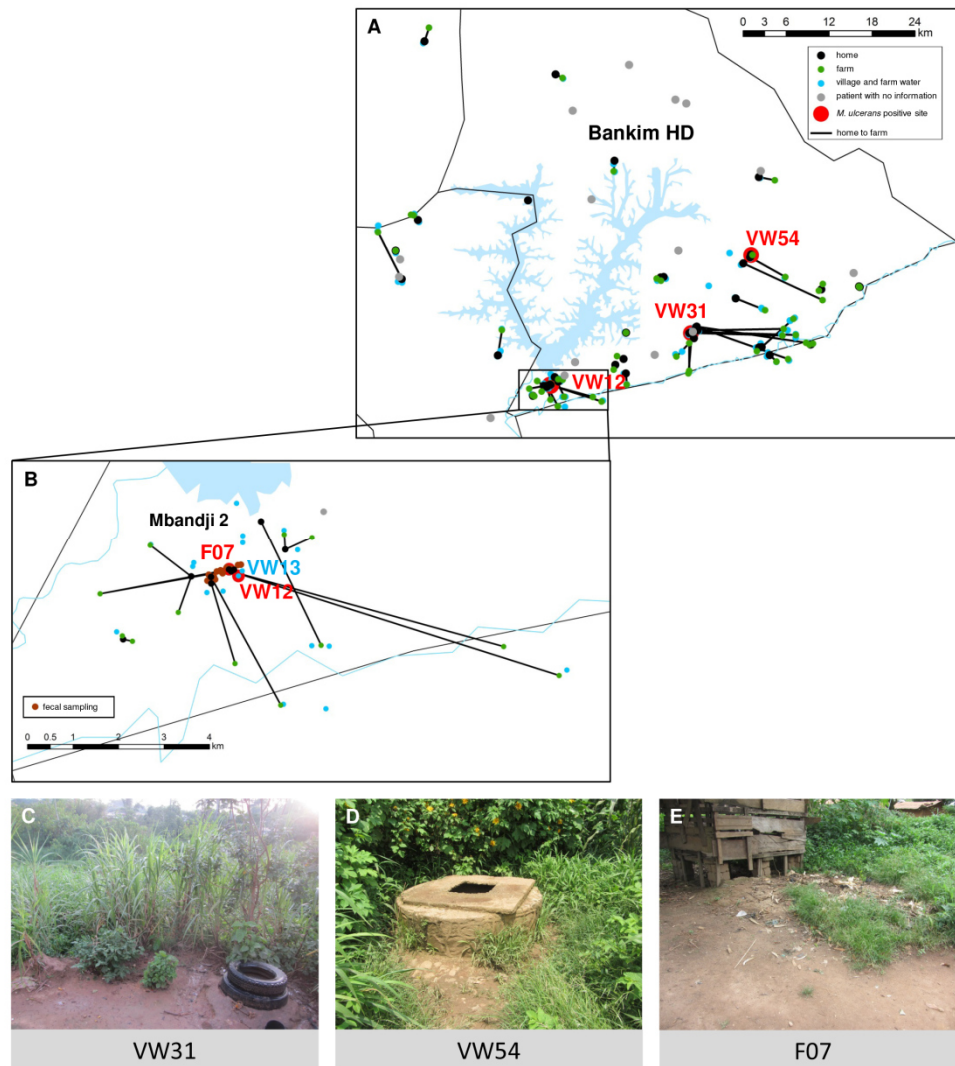


Figure 4.1: Environmental contact network of laboratory confirmed BU patients from the southern Mapé Basin.

Panel A and B (detailed view of the village of Mbandji 2) show the houses where the 46 laboratory confirmed BU patients in our study lived (black points), the farm(s) where they worked (green points) and the locations where they obtained their water (blue points) during the year before the onset of BU symptoms. The home of each patient is connected with their farm(s) as applicable. Homes of 17 of the 21 non-participating laboratory confirmed BU patients are shown in grey. At the farms and water contact sites, soil (n=171), plant (n=153) and water (n=109) samples were collected. Furthermore, in Mbandji 2 (B), animal faecal samples were collected around patients' houses (brown points). All samples were tested for the presence of *M. ulcerans* DNA and three village water sites were found to be positive (red points; VW12, VW31 and VW54). Further, at site F07 a positive duck faecal sample (red point) was collected. Photographs of locations VW31, VW54 and F07 are shown in C, D and E, respectively. Finally Panel B also shows a negative water contact site (VW13) which was studied in detail.

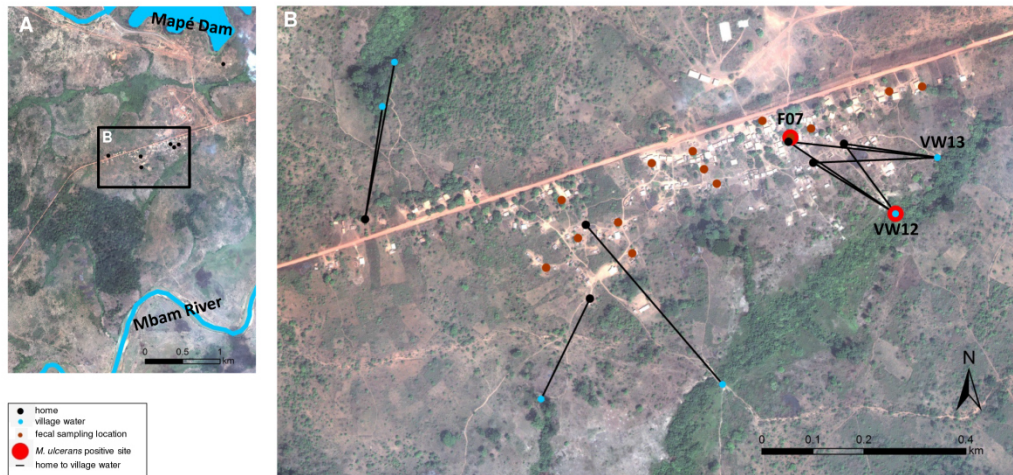


Figure 4.2: Water contact sites in Mbandji 2 which were investigated in detail.

Based on the high case number and the identification of two environmental sites which were positive for *M. ulcerans* DNA, water contact sites in Mbandji 2 were analysed in detail. The town is located between the Mapé Dam and the Mbam River (A). Panel B shows the locations of the homes of the 6 patients from Mbandji 2 in our study (black points) and each of the homes is connected with the village water site(s) used by the respective patient. Faecal sampling sites are also shown (brown points). Locations which tested positive for *M. ulcerans* DNA are highlighted in red (B). A positive (VW12) as well as a close by negative (VW13) village water sites were studied in more detail. Images are based on a 0.5m resolution WorldView-2 image take on March 12th 2011.



Figure 4.3: Alterations of the environment at locations VW12 and VW13 at the sampling time points.

Photographs of locations VW12 (A) and VW13 (B) are shown at selected environmental sampling time points.

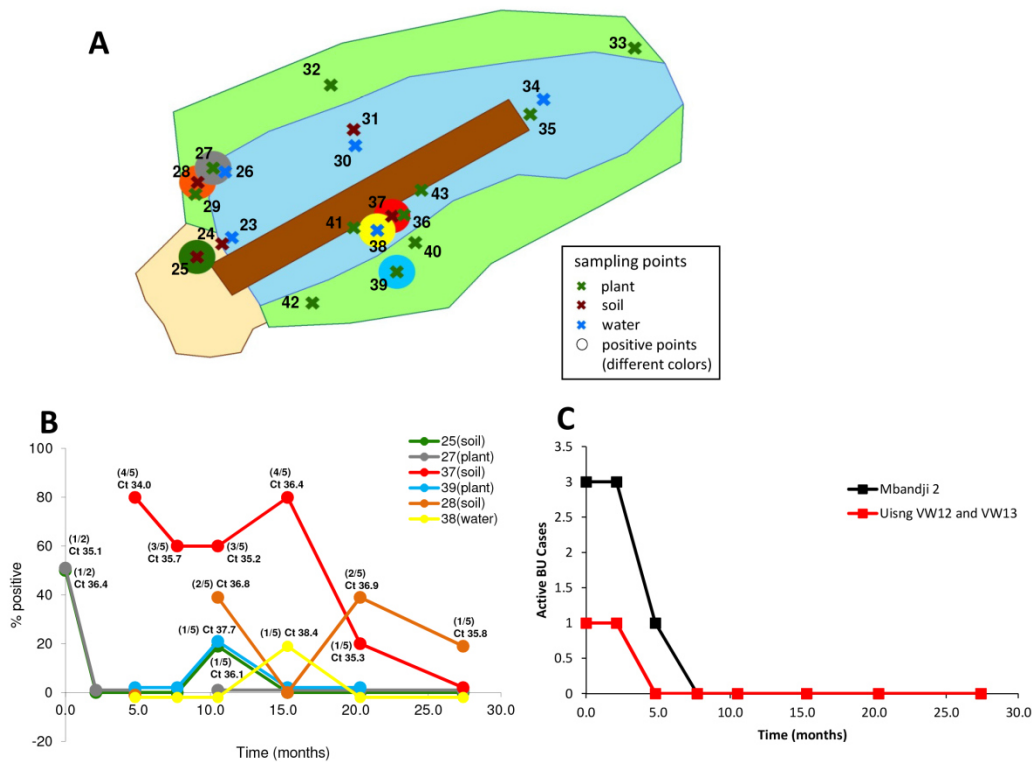


Figure 4.4: Persistence of *M. ulcerans* at a village water site of BU patients.

Panel A shows a diagram of the water hole at VW12A from where samples were collected at eight time points over a period of 27.4 months. Soil sampling points are shown as brown crosses, water sampling points as blue crosses and plant sampling points as green crosses. Supplementary Table S4.1 shows how many samples were collected at each sampling point and each time point. All samples were tested for the presence of *M. ulcerans* DNA by RT-PCR. At 7 sampling time points, *M. ulcerans* RT-PCR positive samples were identified at VW12 (B with positive sampling points identified by the larger coloured circles and C). Panel C (line colours correspond to the circle colours in panel B) further shows the rate of positivity of the collected sample replicates as well as the average Ct value for the IS2404 RT-PCR performed on the positive samples. Finally, panel C shows the number of active BU cases in the village of Mbandji 2 (black line) and the number of active BU cases using VW12 (red line) at the environmental sampling time points.

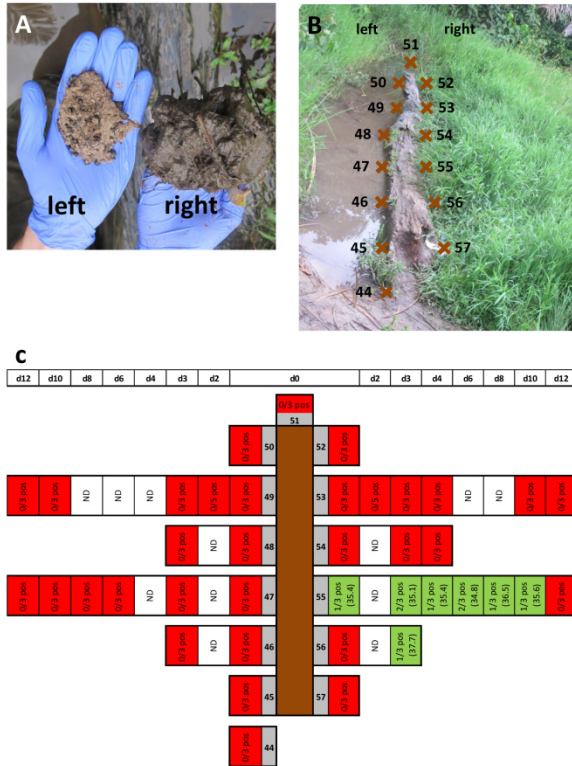
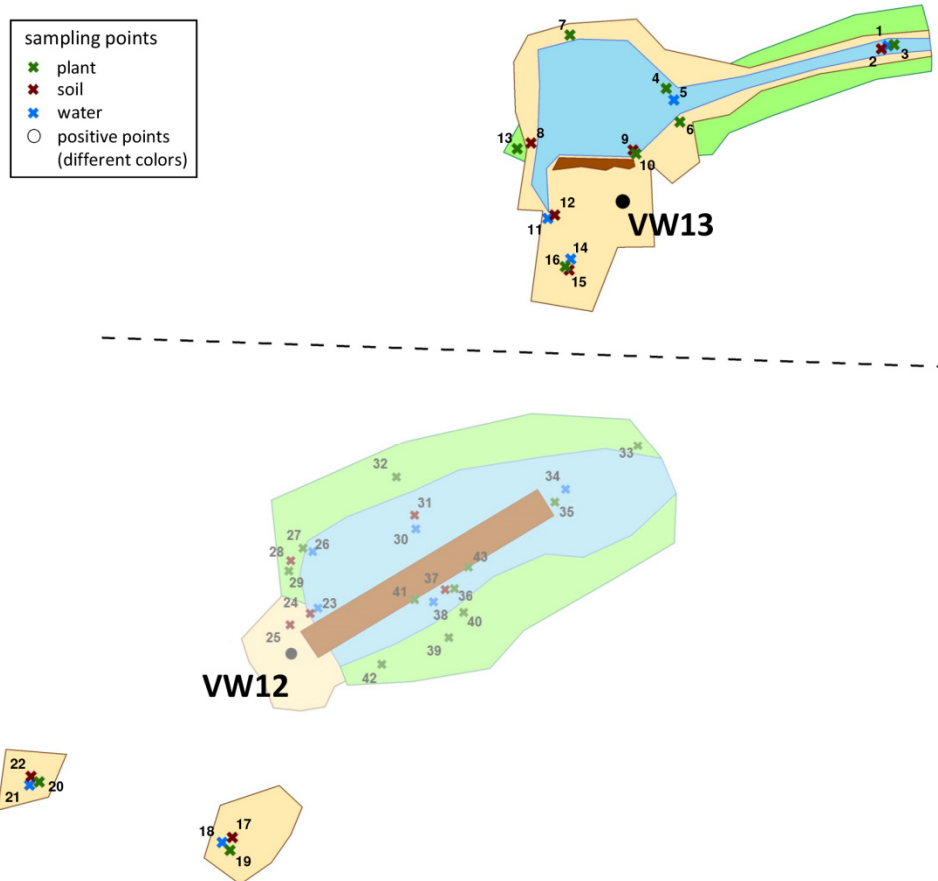


Figure 4.5: In-depth analysis of soil surrounding the log at VW12.

Panel A shows the nature of the soil on the right and the left hand side of the log. To better understand positivity of samples at location VW12, we performed sampling all along the log on either side (B; brown crosses indicate sampling points). Selected locations were re-sampled over the next 12 days as indicated in panel C. Panel C further shows the rate of positivity among the replicates collected at each sampling time point and the average Ct value of the IS2404 RT-PCR performed.



Supplementary Figure S4.1: Sampling points at VW13 and the sand pits at VW12.

Diagram of VW13 and the sand pits close to VW12 with the sampling sites; soil sampling points are shown as brown crosses, water sampling points as blue crosses and plant sampling points as green crosses. For details on the main water site of VW12 (transparent part) see Figure 4.4.

4.9 References

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Chapter 5

Primary Cultivation of *Mycobacterium ulcerans* from Cotton Swabs: Factors affecting contamination and specific growth after long term storage

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Manuscript ready to submit to:
Journal of Clinical Microbiology

5.1 Introduction

Buruli ulcer (BU), a neglected tropical disease of the skin, has been reported from over 30 countries worldwide with most cases occurring in West Africa. Clinically BU, which is caused by *Mycobacterium ulcerans* (MU), presents with both non-ulcerative lesions, such as nodules, plaques and oedema as well as ulcers and the major burden of disease falls on children between 5 and 15 years of age (1, 2). Despite years of research, both the reservoir and the mode of transmission of the environmental pathogen remain unknown (1). Currently available methods for laboratory diagnosis of BU are microscopy, polymerase chain reaction (PCR) for the MU specific insertion sequence 2404 (IS2404) or primary culturing. Based on the high sensitivity and specificity, the IS2404 PCR is considered the gold standard in BU diagnosis (2). Historically, BU was treated with surgery and tissue samples could easily be obtained for laboratory diagnosis. Since the introduction of streptomycin and rifampicin (SR) combination therapy in 2004 by the WHO, samples for laboratory testing are obtained by fine needle aspiration (FNA) from closed lesions and cotton swabs from ulcers (3).

Although primary culturing of MU can provide a definitive BU diagnosis, this method is strongly limited by the fact that colonies take 8-12 weeks to appear and even under optimal conditions it is not positive for all BU cases (4, 5). Culturing can therefore not be used for routine laboratory diagnosis of BU but as an auxiliary to other diagnostic methods and for studies on treatment efficiency, transmission and the development of drug resistances, primary isolation of MU remains crucial (6, 7). Because of the slow growth rate of the pathogen, primary culturing requires decontamination of clinical samples prior to culture inoculation to prevent the overgrowth with other microorganisms (5). Palomino and Portaels have shown that decontamination by the commonly used Protroff (NaOH) or reverse Petroff (HCl) methods or using oxalic acid (OA), all have a detrimental effect on the viability of cultured MU (5, 8, 9). The same study also found that PANTA, a mixture of the antibiotics -

Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azlocillin - did not have an inhibitory effect on the growth of MU and can therefore potentially be used to prevent the growth of other microorganisms (9). Once decontaminated, MU can be recovered by inoculation on Löwenstein-Jensen (LJ) media and incubation at temperatures between 30 and 33°C.

Two studies have previously shown that culturing from cotton swabs or FNA after short term transport in a semi-solid medium is feasible (10, 11). Eddyani *et al.* found that culture positivity using FNA was 17.6% which was not significantly different from the culture positivity from tissue samples (11). In the more recent study, Yeboah-Manu *et al.* evaluated the effect of decontamination by the Petroff method, OA or a combination of both on samples collected by cotton swabs. Processing samples within 24 hours, the two decontamination methods were found to be equivalent in terms of MU recovery but decontamination by OA showed a reduced rate of contamination and this was therefore declared as the preferred method (10).

For the current study we set out to determine the best procedure for the cultivation of MU from remote endemic areas after long term storage. Specifically, the objectives of the current study were to: (i) determine which transport medium, decontamination method or other factors decrease the chance of contamination of MU primary cultures and (ii) investigate which of the same factors can increase the chance of MU primary isolation. The method devised here is suitable for the establishment of an MU strain collection for research purposes following long term storage of samples coming from remote BU endemic areas.

5.2 Materials and Methods

Ethical statement and patient recruitment

Samples were collected from patients recruited in the Mapé Basin of Cameroon (12) between April 2010 and 2012 as well as from patients recruited for a BU clinical trial in southern Cameroon during the same period. Ethical clearance for the collection of samples was obtained from the Cameroon National Ethics Committee (N°041/CNE/DNM/09, N°006/CNE/SE/2010, and N°172/CNE/SE/2011) and the Ethics Committee of Basel (EKBB, reference no. 53/11). Participation was voluntary and all patients who participated in the study or their legal guardian provided written informed consent.

Sample collection, storage and transport

Prior to the start of treatment several FNA or cotton swabs were collected from each patient. To facilitate handling, FNA were transferred onto a cotton swab immediately after collection. Samples were stored dry, in 7H9 or in Amies (VWR International) transport medium. Sterile 7H9 (Difco Middlebrook, Becton Dickinson and Company) transport medium was home made to contain 0.5% Agar-Agar (Merck), 0.2% glycerol (Sigma), 2% PANTA (Becton Dickinson and Company) and 10% OADC Enrichment (Becton Dickinson and Company) (13). Locally the collected samples were stored at 4°C whenever possible. At 4-6 month intervals, samples were transported to the laboratory at ambient temperature. Once in the laboratory, swabs were stored again at 4°C.

PCR, decontamination and primary inoculation

For DNA extraction, swabs were transferred to glass bottles containing glass beads with 2-5mL of sterile PBS and the bottle vortexed for 1.5 minutes. From 1mL of the solution DNA was extracted as described by Lavender and Fyfe (14). To reduce the probability of false negative results, extracts were analyzed twice by IS2404 real-time PCR (RT-PCR) as previously described (14).

Decontamination of 1mL of wound exudate in PBS was performed with 1mL of 1M NaOH for 10 minutes (NaOH_10min) or 20 minutes (NaOH_20min) or with 1mL of 5% OA for 30 minutes (OA_30min) or 1 hour (OA_1h) at room temperature with occasional vortexing. Decontaminated extracts were neutralized with 20mL of sterile PBS, the decontaminated bacteria pelleted by centrifugation for 30 minutes at 3'000g, the supernatant decanted and the pellet re-suspended in a minimal volume of sterile PBS. Of the re-suspended pellet, 0.1mL was transferred to LJ (with glycerol; Becton Dickinson and Company) and/or LJ supplemented with 2% PANTA (LJ_PANTA).

Culture processing and *M. ulcerans* identification

All inoculated cultures were stored at 30°C until growth could be observed. Slants were regularly examined and discarded if contamination, i.e. overgrowth with other faster growing microorganisms, was detected. Inoculations where no growth was observed were kept for a minimum of 25 weeks before discarding. Suspected MU growth was confirmed by colony PCR using primers MU154 (5'-ggcagttacttactgcaca-3') and MU155 (5'-cggatcaagcgttcacga-3') and amplification for 32 cycles of 30 seconds at 94°C, 30 seconds at 60°C and 1 minute at 72°C. PCR products were resolved in a 1.5% agarose gel.

Statistical analysis

To identify factors that affect the rate of contamination and MU recovery in MU primary culturing, three statistical analyses, one with all inoculations and two with subsets, were performed. The first analysis identified differences between inoculations that did not contaminate versus those that did result in contamination (non-contamination vs. contamination). The data set used for this analysis included both RT-PCR positive and negative swabs and inoculations with any of the three possible outcomes; MU growth, contamination with another microorganism or no growth. Swabs with a negative RT-PCR result in this data set were assigned a Ct value of 76.80 (reciprocal of the midpoint between zero and the minimum of $1/Ct$). In the second analysis, a subset of inoculations was analyzed to identify factors that affect MU growth (MU growth vs. no growth). All inoculations with no realistic probability of resulting in MU growth, i.e. contaminated inoculations and inoculations from swabs that were RT-PCR negative were excluded for this analysis. Finally in the third analysis, we studied factors that affect the recovery of MU if some inoculations resulted in contamination (MU growth vs. contamination or no growth). This subset included all inoculations originating from positive swabs independent of their outcome, i.e. also including swabs for which growth may have been undetectable because of overgrowth from other microorganism. Workflows of the swabs included in the three data sets are shown in Figure 5.1 and the combinations of decontamination methods performed are listed in Supplementary Table S5.1.

For the identification of factors that contribute to the outcomes studied, generalized linear mixed models with patient identification and identification of each individual swab as random effects were used. Factors included in the one to one analysis were: transport medium, decontamination method, inoculated media, swab RT-PCR Ct value, per patient RT-PCR result, time from sampling to inoculation and the duration of the disease before

diagnosis. Factors that individually had a p-value of the association of less than 0.2 were included in the multivariate analyses. The software SAS (SAS Institute, Cary, USA; release 9.3), RStudio (RStudio, Boston, USA, version 0.95.262) and R (The R Foundation for Statistical Computing; version 2.15.1) were used to perform the analysis.

5.3 Results

Factors affecting the rate of contamination of MU primary cultures

Of the 443 swabs in the analysis for factors affecting the rate of contamination of MU primary cultures, 302 were RT-PCR positive with an average Ct value of 28.09 (Figure 5.1A). Of the 1125 inoculations from these swabs (Figure 5.1A), 7.82% resulted in MU growth, 52.53% yielded no growth and 39.46% resulted in contamination, i.e. overgrowth with other faster growing microorganisms. The median time to contamination was 5.0 (IQR = 4.0 to 11.0) days (Table 5.1). One-to-one analyses were used to identify factors that should be included in the multivariate analysis (Supplementary Table S5.2). When studying the combined effect of factor on the rate of contamination of MU cultures, we found that the transport medium had an overall significant (p-value: 0.006) effect on the probability of contamination. Specifically, dry swabs had a significantly increased probability of non-contamination with an odds ratio (OR) of 57.68; meaning swabs transported dry had 57.68 times less chance of contamination compared to swabs transported in Amies (Table 5.2). We found no significant difference in terms of the rate of contamination between swabs transported in 7H9 or Amiens. As also shown in Table 5.2 we found that there is no overall significant difference (p-value: 0.266) in the rate of contamination between the four decontamination methods evaluated here. The inoculated culture medium on the other hand significantly (p-value: <0.001) influences the rate of contamination, with cultures on LJ having a 3.79 (1/0.264) times higher probability of contamination than cultures inoculated onto LJ_PANTA. The multivariate analysis further showed that there is a significant (p-value: 0.007) interaction between transport medium and the inoculated media (Table 5.2). On the other hand, both an increase in the Ct value of the swab (p-value: 0.236) and the time from sampling to inoculation (p-value: 0.606) did not have a significant effect on the rate of the contamination of the cultures (Table 5.2). Overall this analysis to identify conditions that are

best to prevent contamination of MU primary culture, therefor suggest that swabs should be stored dry, any of the evaluated decontamination methods can be used, cultures should be inoculated into LJ_PANTA and neither the Ct value of the IS2404 RT-PCR nor the time from sampling to inoculation had any effect on the rate of contamination.

Factors affecting the rate of MU recovery in primary culturing

To identify factors that affect the recovery of MU, 440 inoculations from 220 swabs originating from 66 patients were analyzed. The average Ct value of the swabs was 27.86. Of all the inoculations included in the analysis, 20.00% (88/440) resulted in MU growth and the rest yielded no growth. The median time to growth was 67.0 (IQR = 55.0 to 105.2) days (Table 5.1). Because it was not possible to determine if the contaminated inoculations could have resulted in MU growth, those inoculations were not included in this analysis (Materials and Methods; Table 5.1). One-to-one analyses were again used to identify factors that should be included in the multivariate analysis (Supplementary Table S5.3). In the multivariate analysis, we found that the transport medium had an overall significant effect (p-value: <0.001) on the probability of MU growth (Table 5.3). Specifically, swabs transported dry had a significantly reduced chance of MU growth with only 0.023 times the chance of MU growth compared to samples transported in Amies (Table 5.3). Between swabs transported in Amies or 7H9 we did not detect any significant difference, although transport in Amies had a tendency to increase the chance of MU recovery (OR: 0.304; Table 5.3). Interestingly, in the multivariate analysis there was no significant difference in the chance of MU recovery between the decontamination methods evaluated (p-value: 0.519) and inoculation on either LJ or LJ_PANTA (p-value: 0.216). However, With an increase of the Ct value by one unit, the probability of MU growth was reduced by 12.1% (p-value: 0.044; Table 5.3) and with every

10 extra days of storage between sampling and inoculation, the probability of MU growth decreased significantly (p-value: 0.001) by 45.9%. Our analysis further detected a moderately significant (p-value: 0.074) interaction between the transport time and the transport medium, with Amies having the best chance of MU growth for an increase in transport time by 10 days. Overall, the analysis to evaluate factors that affect the recovery of MU therefore found that either storage in Amies or 7H9 are significantly better than keeping samples dry, the decontamination methods tested have an equivalent effect on the growth of MU and the MU recovery is not affected by inoculation of samples onto media containing PANTA. On the other hand, the analysis showed that both a one unit increase in the Ct value of the IS2404 RT-PCR and a 10 day increase in the storage time of the samples had a significant negative effect on the rate of MU recovery.

MU recovery versus no growth or contamination

For the identification of factors that affect MU recovery in a scenario where some cultures contaminate, 302 swabs from 72 patients with an average Ct value of 28.09 were analyzed. As shown in Table 5.1, 12.57% of the 700 inoculations in this analysis resulted in MU growth, 37.14% of the inoculations were contaminated and 50.29% did not result in any growth. The observed culture positivity corresponds to a per patient culture positivity of 43.06%. Analysis of these inoculations in a multivariate analysis with all factors that had a statistically significant effect on culture contamination or MU growth showed, that transport medium had a significant effect (p-value: 0.019) on the recovery of MU from primary cultures. Specifically, swabs transported in Amies showed the best recovery rate of MU, although not significantly better (95% CI of OR: 0.269 - 1.415) than 7H9. As also seen in the analysis of non-contamination (Table 5.2) and MU growth (Table 5.3), the decontamination

methods evaluated here, did not significantly vary in their effect on the chance of MU growth (p-value: 0.295; Table 5.4). On the other hand the use of the inoculated media had a significant (p-value: 0.003) impact on the chance of MU growth, with the use of LJ as opposed to LJ_PANTA reducing the probability of MU growth by 65.5%. As further shown in Table 5.4, a one unit increase in the Ct value away from the mean Ct value of the inoculated swabs (mean=28.09) decreased the chance of MU recovery by 10.8% (p-value: 0.011). Furthermore, consistent with the analysis of factors effecting *M. ulcerans* growth, the days from storage sampling to inoculation were again found to significantly (p-value: 0.006) affect the chance of MU recovery, with a decrease in the chance of MU recovery by 19.1% for every 10 extra days of storage compared to the mean per swab storage time of 80.2 days (Table 5.4, Figure 5.2). As shown in Figure 5.2, the predicted portability of *M. ulcerans* recovery decreases from 58% to 25% within 70 days of transport. Overall the analysis of the sample set consisting of only RT-PCR positive swabs but including all possible outcomes of the inoculations, showed that either of the moist transport medium increase the chance of MU recovery compared to samples kept dry, none of the decontamination methods is preferred and MU culturing is favored on LJ which are supplemented with PANTA. Further the analysis showed that both a one unite increase in the Ct value of the IS2404 RT-PCR and a 10 day increase in the storage time of the samples, negatively impacted the chance of MU growth.

5.4 Discussion

In the present study we evaluated the effect of various factors on the rate of MU recovery. For this we examined three sets of inoculations to identify factors that reduce the rate of contamination of the primary cultures, to determine which factors increase the rate of MU recovery in a scenario where none of the cultures contaminate and to evaluate the effect of factors on MU recovery in a real-life set of inoculations where there is some “undetectable growth” due to the contamination of cultures.

In a study on 1273 IS2404 PCR positive biopsies which were stored for up to 26 weeks in semi-solid transport medium, Eddyani *et al.* were able to achieve a culture positivity rate of 45.2% and thereby showed that the establishment of an MU strain collection from remote BU endemic areas from tissue samples is possible (13). Other studies using either tissue biopsies or cotton swabs and FNA and shorter transport time have also reported culture positivity rates around 50% with the rest of the cases, although clinically typical and PCR confirmed, remaining culture negative (5, 10, 11, 15, 16). With a per patient culture positivity rate of 43.1% in our study, we have therefore achieved, from cotton swabs and after long transport, MU recovery rates similar to what is reported in the literature. To increase MU recovery rates, Yeboah-Manu *et al.* have suggested that the collection of multiple samples per patient, which is feasible with the now routinely used and non-invasive sampling by cotton swabs and FNA, could increase the rate of MU recovery. Considering the third data set in our study (Figure 5.1C), we collected 4 (IQR = 2 to 6) swabs from most patients. This repeated sampling did indeed affected the rate of the recovery of MU in that there was a significant difference (p-value 0.003) between the number of samples collected from the culture positive (median: 5; IQR: 3 to 6 swabs) versus the culture negative (median: 2; IQR: 1 to 5 swabs) patients. To increase the chance of culture positivity, we have also ensured that lesion exudates are collected from all around ulcers. Even though BU lesions can be painless if there

is no secondary infection (17), and although it has been reported that the collection of FNA does not cause much discomfort for the patients (18), there is a limit to the number of samples that can be collected from a patient on one day. We recommend the collection of 5 samples per patient which should be tolerable and alternatively could be spread over 1-2 days.

In both our analyses for MU growth and for contamination of cultures, there was no significant difference between swabs transported in 7H9 or Amies medium. However, based on the reduced costs of Amies transport medium per swab compared to the 7H9 medium (approximately 0.7 USD per Amies swab and 2.8USD per 7H9 swab with PANTA and OADC), we recommend the use of Amies medium for the transport of swabs collected from BU patients.

In our analysis, the number of days for which samples were stored did not significantly affect the rate of contamination of the inoculated cultures but longer storage did significantly reduce the rate of MU recovery (Tables 5.3 and 5.4, Figure 5.2). This is potentially contrary to previous reports of culturing from tissue biopsies which found that that storage time did not affect the rate of MU recovery (13). Viability of MU may be supported if transported in a tissue biopsy, however given the similarity in the MU recovery rate of the above mentioned study and our results presented here, the reason for no-growth of MU from certain patients should be further investigated.

As can be expected, the CT value of the IS2404 RT-PCR had a significant effect on the rate of MU recovery (Tables 5.3 and 5.4). On the other hand neither the qualitative nor the quantitative RT-PCR result of the swab or the BU status of the patient had a significant effect on the rate of contamination of the primary cultures in the one-by-one or the multivariate analysis (Table 5.2 and Supplementary Table S5.2). In line with a recent report on secondary

infections of BU lesion (17), this finding does not support previous claims that BU lesion are less prone to contamination with secondary microorganism compared to other lesions.

In our analysis there was further no significant difference between the four decontamination methods evaluated, although in the univariate analysis for MU growth, NaOH for 10 minutes appeared to outperform the other decontamination options with borderline significance (p-value: 0.076, Supplementary Table 5.3). Because of the lack of difference between the decontamination methods and since 10min NaOH is the quickest decontamination method, we suggest this one be used. In a previous study on a small set of swabs that were processed within 24 hours, OA for 20 minutes was found to be most suitable based on its reduced rate of contamination (10). As shown by our analysis and also the lack of effect on MU recovery in the previous study (10), either decontamination method should allow for MU recovery. It must however also be noted that all decontamination methods have a strong negative effect on the viability of MU (9). As for the medium to inoculate, LJ_PANTA has a significantly (p-value: <0.001) reduced the rate of contamination (Table 5.2) and at the same time the mix of antibiotics does not significantly (p-value: 0.216) influence the probability of MU growth (Table 5.3). In fact, our analysis of MU growth vs. contamination or no growth including only RT-PCR positive swabs (Figure 5.1C), showed that the use of LJ_PANTA had a significant positive effect (p-value: 0.003) on the rate of recovery of *M. ulcerans*. This is similar to what has been found by Yeboah-Manu *et al.* where MU recovery was also significantly improved (p-value < 0.001) on LJ_PANTA compared to LJ alone (10).

By evaluating a small set of swabs, Yeboah-Manu *et al.* have previously been able to show that culturing from dry cotton swabs is feasible (10). In our study we confirmed have been able to confirm this finding. However the chance of *M. ulcerans* recovery is reduced by

68.9% compared to swabs transported in Amies (Table 5.4) and in our analysis dry swabs only achieved a per patient culture positivity rate of 13.1%.

In conclusion primary culturing of MU from cotton swabs after long term storage is possible. Based on our findings we suggest that samples should be transported in Amies medium, that they should be decontaminated in 0.5M NaOH for 10 minutes and that cultures should be inoculated onto LJ supplemented with 2% PANTA. As shown in the model presented in Figure 5.2, from inoculations of swabs with a RT-PCR Ct value of 27.8, that were transported in Amies medium, decontaminated using NaOH for 10 minutes and inoculated into LJ with PANTA, a probability of MU growth of 60% can be expected if samples were not stored at all. If the same samples are stored for 50 or 100 days the predicted probability decreases to 42% and 12% respectively. Furthermore, multiple samples (approximately 5) should be collected from each patient and only the PCR positive swabs should be inoculated for culturing.

5.5 Acknowledgments

We would like to thank all patients for participating in the study. Further, we would like to thank Fidèle G. Wantong, Dr. Djeunga Noumen, Dr. Moritz Vogel, Dr. Franklin Bayi and Prof. Thomas Junghanss for their support with the collection of the samples. Further we would like to thank the Medicor Foundation and the Dr. med. h.c. Erwin Braun Foundation for financially supporting this work.

5.6 Tables

Table 5.1: Outcomes of *M. ulcerans* primary culturing

Attribute	Non-Contamination vs. contamination #	MU growth vs. no growth ##	MU growth vs. contamination or no growth ###
<i>M. ulcerans</i> growth per inoculation (%)	88 (7.82)	88 (20.00)	88 (12.57)
<i>M. ulcerans</i> growth per patient (%)	31 (32.63)	31 (46.97)	31 (43.06)
Contamination (%)	446 (39.46)	-	260 (37.14)
No growth (%)	591 (52.53)	352 (80.00)	352 (50.29)
Days to primary outcome*	5.0 (4.0; 11.0)	67.0 (55.0; 105.2)	67.0 (55.0; 105.2)

Analysis of 1125 inoculations from 95 patients with contamination as the primary outcome.

Analysis of 440 inoculations from 66 patients with MU growth as the primary outcome.

Analysis of 700 inoculations from 72 patients with MU growth as the primary outcome.

* Median with IQR in parentheses.

Table 5.2: Association[§] between non-contamination and all factors of interest.

Factor	Value of factor	Odds ratio[§]	95% CI odds ratio	Overall effect (p-value)
Transport medium^{§§}	7H9	1.579	0.632 - 3.944	0.008
	dry	57.675	23.704 - 140.334	
	Amiens (ref. level)	1.000		
Decontamination medium	OA_1h	0.715	0.362 - 1.412	0.266
	OA_30min	0.540	0.260 - 1.122	
	NaOH_20min	0.879	0.383 - 2.016	
	NaOH_10min (ref. level)	1.000		
Inoculation media^{§§}	LJ	0.264	0.162 - 0.429	<0.001
	LJ_PANTA (ref. level)	1.000		
Swab RT-PCR Ct value	for an increase in 1 unit CT	0.992	0.978 - 1.006	0.236
Time from sampling to inoculation	for an increase in 10 day			0.606
Interaction of transport medium and days from sampling to inoculation	when 7H9 ^{§§§}	0.982	0.824 - 1.171	0.007
	when dry ^{§§§}	1.167	1.032 - 1.321	
	when A (ref. level)	0.799	0.643 - 0.993	

[§] Adjusted for random effects of the patient and swab identification.

^{§§} Interaction (p-value: <0.001) between transport medium and inoculation media was observed.

^{§§§} for an increase in 10 days from mean of days from sampling to inoculation.

Table 5.3: Association[§] between MU growth and all factors of interest with a relevant effect

Factor	Value of factor	Odds ratio[§]	95% CI odds ratio	Overall effect p-value
Transport medium	7H9	0.304	0.046 - 2.026	<0.001
	dry	0.023	0.004 - 0.143	
	Amiens (ref. level)	1.000		
Decontamination medium	OA_1h	0.414	0.102 - 1.683	0.519
	OA_30min	0.697	0.127 - 3.837	
	NaOH_20min	1.242	0.204 - 7.546	
	NaOH_10min (ref. level)	1.000		
Inoculation media	LJ	0.478	0.148 - 1.545	0.216
	LJ_PANTA (ref. level)	1.000		
Swab RT-PCR CT value	for an increase in 1 unit CT1	0.879	0.775 - 0.996	0.044
Time from sampling to inoculation	for an increase in 10 days			0.001
Interaction of transport medium and Days from sampling to inoculation	when 7H9 ^{§§}	0.531	0.320 - 0.881	0.074
	when dry ^{§§}	0.880	0.704 - 1.099	
	when A (ref. level)	0.541	0.347 - 0.844	

[§] Adjusted for random effects of the patient and swab identification.

^{§§} for an increase in 10 days from mean of days from sampling to inoculation.

Table 5.4: Model describing the association[§] between MU growth and all factors of interest with a relevant effect

Factor	Value of factor	Odds ratio[§]	95% CI odds ratio	Overall effect p-value
Transport medium	7H9	0.617	0.269 - 1.415	0.019
	dry	0.248	0.094 - 0.655	
	Amiens (ref. level)	1.000		
Decontamination medium	OA_1h	0.440	0.182 - 1.062	0.295
	OA_30min	0.529	0.203 - 1.375	
	NaOH_20min	0.739	0.236 - 2.313	
	NaOH_10min (ref. level)	1.000		
Inoculation media	LJ	0.345	0.169 - 0.703	0.003
	LJ_PANTA (ref. level)	1.000		
Swab RT-PCR CT value	for an increase in 1 unit CT1	0.892	0.818 - 0.974	0.011
Time from sampling to inoculation	for an increase in 10 days	0.809	0.695 - 0.941	0.006

[§] Adjusted for random effects of the patient and swab identification.

Supplementary Table S5.1: Parallel decontaminations performed on the samples included in the analyses

Combination of parallel decontamination methods	Non-contamination vs. contamination	MU growth vs. no growth	MU growth vs. contamination or no growth
NaOH 10 and OA 30min	19	3	13
NaOH 10 and OA 1h	87	44	86
NaOH 20 and OA 30min	61	25	33
OA 1h and OA 30min	70	23	32

Supplementary Table S5.2: Association[§] between Non-contamination and each of the factors of interest.

Factor	Value of factor	Odds ratio[§]	95% CI odds ratio	Overall effect (p-value)
Transport Medium	7H9	0.840	0.462 - 1.527	<0.001
	dry	24.314	13.268 - 44.555	
	Amiens (ref. level)	1.000		
Decontamination medium	OA_1h	0.853	0.465 - 1.567	0.021
	OA_30min	0.749	0.396 - 1.414	
	NaOH_20min	1.777	0.860 - 3.673	
	NaOH_10min (ref. level)	1.000		
Inoculation media	LJ	0.441	0.307 - 0.634	<0.001
	LJ_PANTA (ref. level)	1.000		
Swab RT-PCR Ct value	for an increase in 1 unit CT	0.990	0.978 - 1.002	0.119
Patient RT-PCR result	negative	0.799	0.379 - 1.682	0.554
	positive (ref. level)	1.000		
Time from sampling to inoculation	for an increase in 10 day	1.402	1.282 - 1.534	<0.001
Weeks before diagnosis*	for an increase by 1 week	0.992	0.976 - 1.007	0.285

[§] Adjusted for random effects of the patient and swab identification.

* Information as reported by the patient.

Supplementary Table S5.3: Association[§] between MU growth and each of the factors of interest.

Factor	Value of factor	Odds ratio[§]	95% CI odds ratio	Overall effect p-value
Transport medium	7H9	0.343	0.099 - 1.192	<0.001
	dry	0.004	0.001 - 0.015	
	Amiens (ref. level)	1.000		
Decontamination medium	OA_1h	0.373	0.135 - 1.032	0.076
	OA_30min	0.396	0.136 - 1.157	
	NaOH_20min	0.229	0.071 - 0.734	
	NaOH_10min (ref. level)	1.000		
Inoculation media	LJ	0.510	0.239 - 1.089	0.082
	LJ_PANTA (ref. level)	1.000		
Swab RT-PCR Ct value	for an increase in 1 unit CT	0.947	0.868 - 1.033	0.216
Time from sampling to inoculation	for an increase in 10 day	0.621	0.522 - 0.738	<0.001
Number of days of treatment before sampling	for an increase in 1 day	1.033	0.965 - 1.106	0.343
Weeks before diagnosis*	1 week	1.002	0.976 - 1.029	0.865

[§] Adjusted for random effects of the patient and swab identification.

* Information as reported by the patient.

5.7 Figures

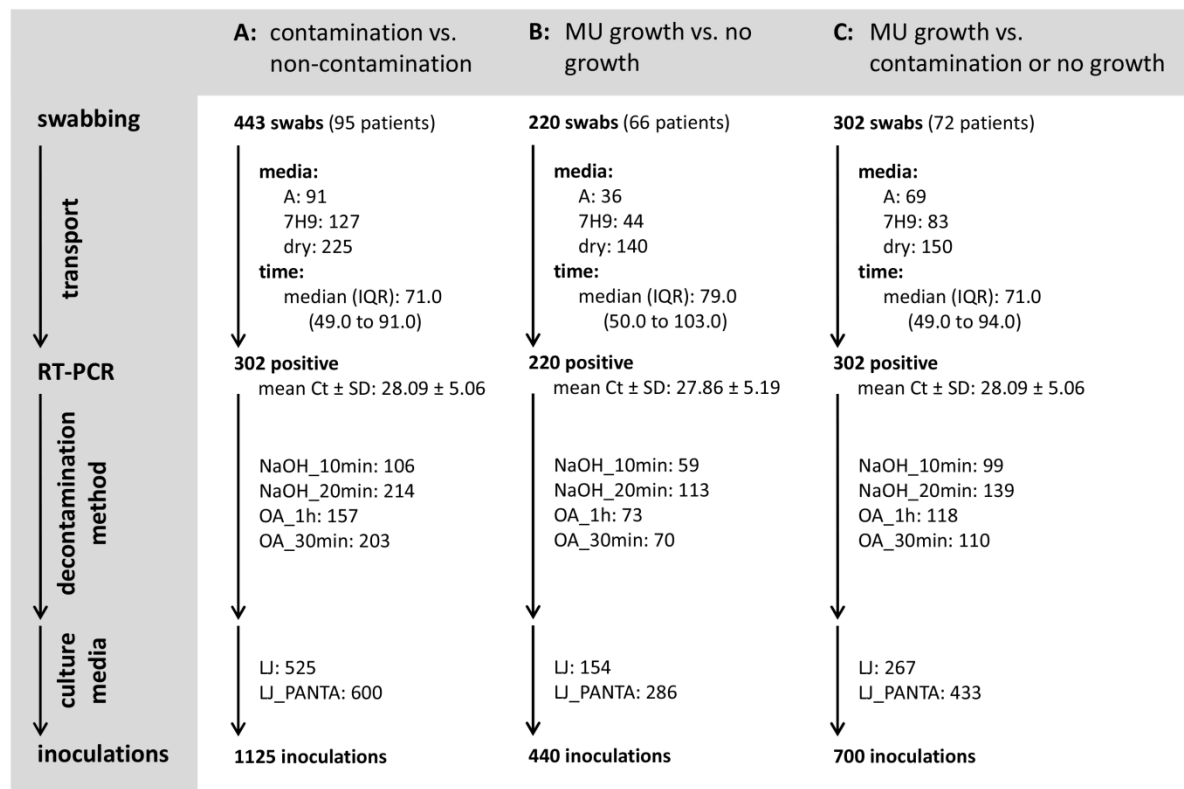


Figure 5.1: Workflow of swabs included in the statistical analysis.

The complete set of MU primary culturing inoculations was used to identify factors that affect the rate of contamination (non-contamination vs. contamination; A) of *M. ulcerans* primary cultures. A subset of the inoculations was used to identify factors that influence the growth of MU in the absence of any contaminated inoculations (MU growth vs. no growth; B) and a second subset was used to identify factors that affect MU growth if some of the inoculations resulted in contamination (MU growth vs. contamination or no growth; C). The number of swabs collected as well as the transport media and storage time are shown. Further, the number of RT-PCR positive swabs with their average Ct value are given and the numbers of decontaminations as well as inoculations performed are shown. Finally the number of total inoculations in each of the data sets is indicated.

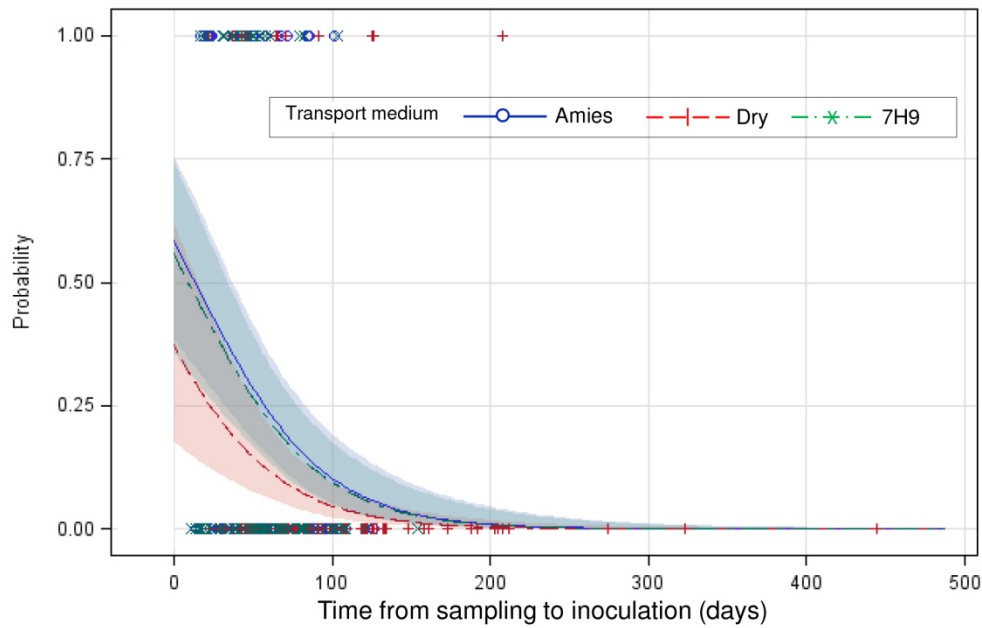


Figure 5.2: Predicted Probabilities for *M. ulcerans* Growth.

Based on the MU growth vs. no growth or contamination model we predicted the probability of MU growth as a function of transport time if samples are transported with the transport media indicated, decontaminated using NaOH for 10 minutes, inoculated onto LJ supplemented with PANTA and if the Ct value of the RT-PCR was 27.8. Mean predicted probability of MU growth and 95% confidence intervals are shown.

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Chapter 6

A Case of Cutaneous Tuberculosis in a Buruli Ulcer– Endemic Area

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This article has been published in:
PLoS Neglected Tropical Diseases

6.1 Presentation of Case

A 27-year-old male farmer presented himself to an integrated health centre in the Bankim health district of the Adamaoua region of Cameroon with two ulcerative lesions with undermined edges on the upper chest and neck as well as enlarged and indurated lymph nodes of the neck (see Figure 6.1). He had not sought any traditional treatment and indicated that the condition had been ongoing for one month. Given the known high prevalence of *Mycobacterium ulcerans* (*M. ulcerans*) disease (Buruli ulcer) in the Bankim health district [1], ulcer exudates were examined for acid-fast bacilli by Ziehl-Neelsen (ZN) staining at the local health centre and tested positive. Based on the positive ZN stain, a diagnosis of Buruli ulcer (BU) was made and the treatment recommended for BU by the World Health Organization (WHO) [2], daily rifampicin (600 mg *p.o.*) and streptomycin (1 g *i.m.*) for 56 days, was administered to the patient. Additional swabs of ulcer exudates were analyzed using the *M. ulcerans*-specific IS2404 quantitative polymerase chain reaction (qPCR) assay [3]. All four swabs obtained from the patient tested negative. Exudate swabs were also used for the initiation of a culture on Löwensein-Jensen medium after decontamination with 2.5% oxalic acid for 30 minutes at room temperature. After 8.5 weeks of incubation at 30°C, the optimal growth temperature of *M. ulcerans*, mycobacterial growth was observed. The cultured mycobacteria were ZN positive, but negative in the *M. ulcerans*-specific IS2404 qPCR. PCR amplification [4] and DNA sequencing of the rifampicin resistance determining region (RRDR) of the *rpoB* gene identified the strain as belonging to the *M. tuberculosis* complex; no rifampicin resistance conferring mutation in the RRDR was found. A qPCR identifying a single-nucleotide (A to C) change at position 2'154'724 further characterized the cultivated strain as belonging to Lineage 4 (Euro-American Linage) of *M. tuberculosis* [5]. Spoligotyping and analysis using the SITVITWEB database revealed that the strain belonged to the "T-family" [6] of *M. tuberculosis*, a spoligotype of Lineage 4 which encompasses all strains that are difficult to classify into other spoligotype families. While the strain therefore does not belong to the "Cameroon Family" of TB, the obtained spoligo type has previously been reported to occur in Cameroon [6].

Based on this laboratory diagnosis of an *M. tuberculosis* infection, the patient was re-examined 186 days after completion of the BU treatment. At this point, the ulcers had fully scarred (see Figure 6.1). However, the lymph nodes of the neck remained enlarged and indurated. A chest X-ray provided no evidence for pulmonary tuberculosis (TB), and the patient tested negative for human immunodeficiency virus (HIV) infection. Given the

laboratory results and the clinical presentation, the patient was retrospectively diagnosed as a case of cutaneous TB [7]. Given the insufficiency of the BU treatment to cure TB, the patient was started on the full regimen of the standard TB treatment recommended by the Cameroon National TB Control Program: two months of isoniazid, rifampicin, ethambutol, and pyrazinamide followed by four months of isoniazid and rifampicin.

6.2 Case Discussion

Ethical approval (clearance N° 041/CNE/DNM/09, 19/06/2009) to analyze patient specimens was obtained from the National Ethics Committee of Cameroon, registered under the N° IRB00001954. Written informed consent from the patient was obtained before specimens were used for reconfirmation of clinical diagnosis and detailed laboratory analysis.

BU disease presents with a variety of clinical manifestations including nonulcerative forms such as movable subcutaneous nodules, plaques, and oedema, which may eventually progress to ulcerative lesions with characteristic undermined edges. Without treatment, ulcers may enlarge considerably and involve entire limbs or large areas of the trunk [8]. It is believed that mycolactone, the macrolide toxin produced by *M. ulcerans*, largely contributes to the pathogenesis of BU disease [9]. The diversity in clinical presentation renders clinical diagnosis difficult. Of the four currently available methods for laboratory reconfirmation of BU [8], only one, ZN microscopy, is suitable as a point-of-care diagnostic test in the African endemic areas, which are usually remote and rural. However, its sensitivity is limited [10,11]. Cultivation of the slow-growing mycobacteria, histopathology, and PCR-based detection of *M. ulcerans* DNA can only be performed in central reference laboratories.

Given its wide range of clinical presentations, the differential diagnosis of cutaneous tuberculosis, which makes up 1%–2% of all TB cases worldwide [7,12], is also difficult. Both infectious and noninfectious diseases of the skin need to be considered when examining a potential case of cutaneous TB [13]. For a definite diagnosis, histological examination, PCR, or optimally isolation of *M. tuberculosis* is required. All of these diagnostic methods again require sophisticated reference laboratories [7,12]. Once diagnosed, patients can be treated with the regimen that is also used to treat pulmonary TB [7]. It is remarkable that a clinical manifestation very similar to that of BU disease was observed in the case presented here, although *M. tuberculosis* does not produce a potent macrolide toxin.

For patients from BU-endemic regions, ZN microscopy is usually accepted as reconfirmation of the clinical diagnosis and, to reduce costs, it has been suggested that only ZN negative swabs should be sent to a reference laboratory for analysis by PCR [11]. While the vast majority of ZN positive samples are also PCR positive, sensitivity of PCR is much higher than microscopy and many ZN negative samples still turn out PCR positive [11]. In BU-endemic areas as remote as the Bankim health district, clinical diagnosis by local medical staff is often considered sufficient for treatment decision. It has been shown that if clinical diagnosis is performed by highly trained and experienced staff, more than 90% of suspected cases can be reconfirmed by PCR [14]. However in regions where health care staff does not regularly encounter BU cases, a large proportion of suspected BU cases cannot be confirmed by laboratory tests [15]. Clinical overdiagnosis on one side, and true BU cases missed by relying only on ZN microscopy on the other side, can lead to over- or undertreatment of patients, respectively. It is therefore recommended that all BU cases should be laboratory confirmed [15]. If the decision is made to send only ZN negative swabs for confirmation to a reference laboratory, training of health care staff in BU differential diagnosis becomes even more important. Furthermore, such training should include the clinical presentation of other skin diseases, including other mycobacterial diseases. As demonstrated by the case presented here, lymphadenopathy—not a typical sign of BU—should lead to a more detailed clinical examination.

Overall, the case of a misdiagnosed patient with cutaneous TB in a BU-endemic area presented here further underscores the need for a simple, highly sensitive, and specific point-of-care diagnostic test for BU.

6.3 Learning Points

- Clinical diagnosis of Buruli ulcer should be supplemented with laboratory examinations.
- Microscopy, the only point-of-care laboratory test currently available, does not differentiate between Buruli ulcer and infections by other mycobacteria.
- Clinical presentation of other mycobacterial diseases should be included when training staff on the differential diagnosis of Buruli ulcer.
- There is a pressing need for a sensitive and specific point-of-care laboratory test for Buruli ulcer.

6.4 Acknowledgments

We would like to thank Fidèle Gaetan Wantong, Jacques Christian Minyem, and all the Bankim health care staff for their great support in the field. Thank you also to Marie-Thérèse Ruf and Katharina Röltgen for their valuable help in the laboratory.

6.5 Figure

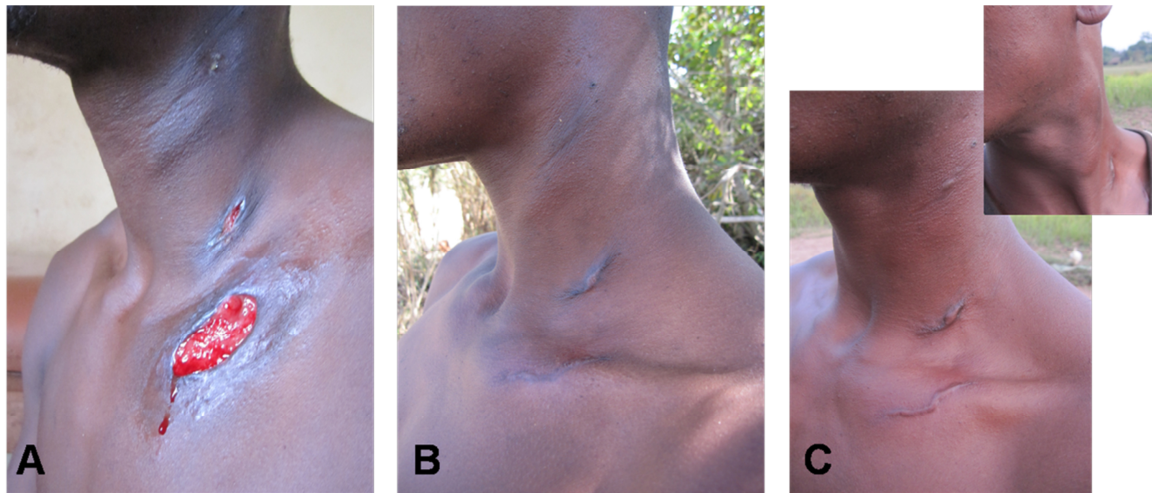


Figure 6.1. Clinical evolution of TB patient discovered in BU-endemic area. A 27-year-old male presented to an integrated health centre in a BU-endemic area in Cameroon with multiple lesions on the neck and upper chest. After acid-fast bacilli were observed in the wound exudates, the patient was diagnosed with BU and treated according to WHO guidelines. Figure 6.1A shows the patient on day 17 of BU treatment. The lesions healed following eight weeks of rifampicin/streptomycin combination therapy. Figure 6.1B and 6.1C show the patient 86 and 178 days after completion of BU treatment, respectively. Further laboratory analysis on the original wound exudates showed that the patient was suffering from TB as opposed to BU, and the appropriate long-term TB treatment was administered.

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Chapter 7

General Discussion and Conclusion

7.1 General remarks

Although BU was first formally described in 1948 in Australia [1], where it is called Daintree or Bairnsdale ulcer, the internationally used name of the disease originates from an area of the Mengo district of Uganda which was called Buruli (today the area is called Nakasongola). Twenty-eight of the 40 first BU cases from Uganda were discovered in this area in 1958 [2,3]. Much of what is known about the disease today, such as major risk factors, clinical and histopathological aspects of lesions and characteristics of the pathogen were initially discovered in the course of research conducted in the 1960's and 1970's [3,4]. Modern day BU research was then initiated by the launch of the WHO lead "Yamoussoukro Declaration on Buruli ulcer" in 1998 [5]. This new era in BU research is illustrated by a stark raise in the number of search results for "*Mycobacterium ulcerans*" in the MEDLINE database (<http://www.ncbi.nlm.nih.gov/pubmed/>) a few years afterwards (Figure 7.1). However despite continued research, BU remains mysterious with major aspects of both its pathology and transmission unknown. Specifically the unidentified reservoir, the unknown mode of transmission, the elusive natural history which leads to the different clinical forms and the only partially characterized mode of action of mycolactone all remain to be elucidated. In addition to these research questions, numerous aspects of BU care, such as early case detection, differential diagnosis, documentation of the patients, and treatment beyond antibiotics, remain to be optimized.

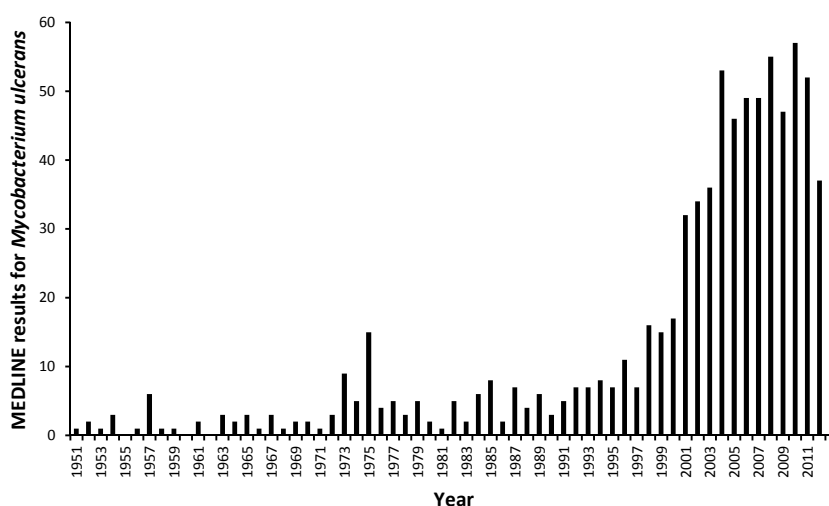


Figure 7.1: MEDLINE search results for "*Mycobacterium ulcerans*" between 1951 and 2012. Search was performed on 02. September 2013 and only publications published until the end of 2012 were included.

Within the scope of this PhD thesis we have established a new BU research site in the Mapé Basin, an emerging BU focus in a remote area of Cameroon [6]. With a survey in the entire Bankim Health District (HD) in 2010 and continuous surveillance thereafter, we studied the epidemiology of BU in the area in great detail (Chapter 2). The thereby gained understanding of the local scenario formed the basis for both completed and on-going biomedical (Chapters 3, 5 and 6 and Appendix 1) as well as environmental (Chapter 4) studies. On the next few pages, the relevance of our findings and observations for BU research and control will be discussed.

7.2 BU control and treatment in the Bankim HD: current status and possible improvements

A major concern in BU control is that patients should receive their treatment as early as possible to prevent lengthy hospital stays and debilitating deformities. Major efforts are therefore needed for early case detection [7]. Given the observation that many BU cases report very late, it is speculated that there are cases in remote communities that are not discovered by the health care system [8]. This is of course hard to prove or dismiss with evidence, although in some studies exhaustive surveys have identified a substantial number of new BU cases [9]. In the course of our work, we conducted an exhaustive house-by-house survey in early 2010 (Chapter 2). Despite including all communities in the Bankim HD, even the very remote ones that can only be reached by motorcycle, boat or foot and examining over 49'000 individuals, only 6 new cases of laboratory confirmed BU were discovered. In the future, surveys should be conducted only in selected communities chosen based on epidemiological data. Recently such a targeted two day survey on the western shore of the Mapé dam in the Malantouen HD, has identified 3 clinically confirmed BU cases that are currently receiving treatment and awaiting PCR confirmation (A. Um Boock, personal communication). Additional targeted surveys, for example along the Mbam River in the south eastern corner of the Bankim HD or in the area just to the south of the same river may also help to discover new cases with fairly little effort. Despite the small number of cases identified in our exhaustive survey, there is no evidence to suggest that BU cases were missed. As a matter of fact, not considering the identified cases of yaws (n=29) and leprosy (n=32), several other cases of – at times very severe – skin conditions were discovered in the survey. Furthermore, following the survey, there was a steady flow of new cases of BU discovered by passive case finding indicating that in the area around Bankim the community

appears to have well accepted the local BU control program and the personnel working for it. In addition to the passive case finding, two strategies are used to support continuous case detection. Firstly, health care providers engage with all actors in BU care, including traditional healers, and encouraging them to refer any case they encounter to a treatment facility. Secondly, the local population is sensitized using a health promotion video accompanied by the clinical evaluation of any skin lesions. These approaches to supplement case finding are likely to be more resource efficient compared to the very time, staff and money intensive exhaustive surveys. The impact of these strategies is currently being evaluated (F. Mou, personal communication). Sensitization in schools, local markets, churches or other places where communities – including those individuals that usually live in very remote areas – gather may further help to identify BU cases early.

In addition to improving case finding, tackling issues that lead to treatment seeking delays may also lead to earlier case detection. Both traditional and worldly concerns should be considered as causes of such delays. In addition to causing unnecessary procrastination of modern treatment, traditional treatments, such as topically applied herbal remedies or heat, may lead to severe burns of the skin and can substantially worsen the state of the lesions. Believing in a spiritual origin of the BU lesions may cause patients to seek care with a traditional healer. Financial constraints and convenience may also lead to the involvement of traditional healers. Given the remoteness of many of the communities in which BU occurs, the distance to the modern health care facility may be large and a traditional healer may, on the other hand, be working in the patient's village. However, some of the patients encountered at traditional healers in the course of this thesis, also travelled considerable distances to reach a healer with a reputation of treating BU. Potential costs of the modern treatment (see below), may further cause patients to consult traditional healers where services can be paid with natural goods or labour and where patients can get complementary housing and food. Traditional treatment has been shown to be involved in 50% of the treatment itineraries of a small cohort of BU patients in the Democratic Republic of Congo [10]. The same study did however also relativized the role of traditional healers by finding that it was more the staff at modern health care facilities – by wrong diagnosis – that caused treatment delays [10]. In the Bankim area traditional healers appear to play an important role in BU care and a system of collaboration, where they are given a few days to treat spiritual aspects of the disease and are then encouraged to refer suspected BU cases to the health care system, may represent a win-win-win situation. With this approach, the patients get their traditional believe concerns addressed, the traditional healer increases her/his credibility in the community by

collaboration with the local medical facility and the health care system receives the patient without much delay. Such an unconventional interaction between care providers is currently being evaluated in Bankim and showing some promising results (F. Mou and A. Um Boock, personnel communication). On the other hand, there may also be patients that don't want to come for modern treatment and BU control programs should focus on applying cost-effective measures to identify and treat those patients that are willing to accept antibiotic treatment. Even though antibiotic treatment and wound care is provided for free to BU patients, there are several factors that may lead to direct as well as opportunity costs that could cause patients to delay or later abandon treatment. These costs accrue because BU patients must come to treatment facilities daily for an injection during the antibiotic treatment and thereafter for wound care. Concerns include issues such as the inability of a caretaker of a child patient to leave behind other obligations and dependents and to relocate to the hospital or rent an apartment close to the treatment facility. The cost of daily transport by motorcycle taxi to the treatment centre could also deter patients. Being displaced for several weeks to receive treatment may further mean that meals must be bought which may exceed the financial capacities of patients. Appropriate counselling and measures to address these issues will hopefully allow the patients to come for treatment early and minimize the number of cases that abandon treatment. Specifically the provision of housing and food for patients that require such support may allow them to complete treatment. Attached to such support, it may also be possible to create a more extended care structure within which patients can provide psychosocial support to each other and the care for young patients can be shared if her / his caretaker has other obligations. Such a structure of extended support is currently also being evaluated in Bankim and results of its impact are pending (F. Mou and A. Um Boock, personal communication).

Some cases of BU that do not receive any modern medical treatment are speculated to be able to heal spontaneously or after only traditional treatment [11]. In the course of our work one patient that healed without modern treatment was identified. The 9 year old patient was first discovered in early 2011 (Figures 7.2A and 7.2B) at which time, he was RT-PCR and culture positive and the patient's parents accepted only traditional treatment. Seen again in late 2013, the ulcer on the elbow healed completely without any other complications and, to our knowledge, without receiving any modern BU treatment (Figure 7.2C). Such clean cases of healing without modern medical treatment are likely to be rare.

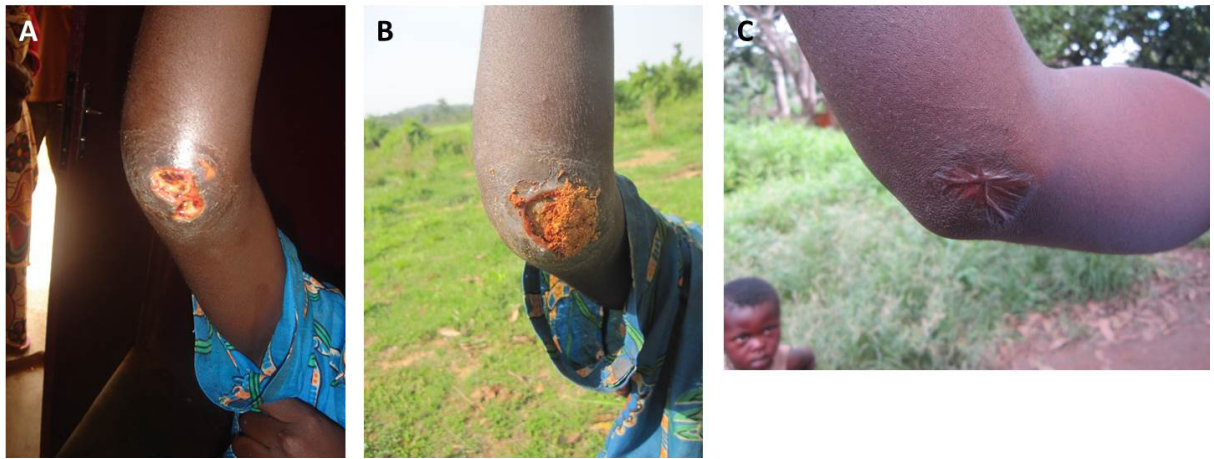


Figure 7.2: Case of spontaneous healing seen in Bankim HD.

RT-PCR and culture confirmed case of BU first discovered in March 2011 when the patient was seen twice (A and B) and only accepted traditional treatment (B). According to medical records the lesion scabbed completely by the end of July 2011. An image of the scar taken in late 2013 is shown in Panel C. According to our knowledge this patient did not receive any modern BU treatment.

Once a patient is registered at a treatment facility, it is of great importance for public health and research purposes that she / he is thoroughly documented. The relevance of this documentation may not be inherently apparent for the front line health care worker that is primarily concerned with the treatment of the individual patient. However only with detailed and reliable information about recent cases can the local BU scenario be truthfully described and useful conclusions drawn. With considerable efforts, we have established both an electronic and paper based data capturing system in the Mapé Basin and thereby greatly increase the quality of the available data. One aspect that required special attention in our setting was the village and health district of origin of the patients. Initially patients from outside of the Bankim HD were record as being from "outside Bankim" without any further details. For other patients, village names were not standardized, the village of the closest health centre as opposed to the village where the patient lived was recorded and insufficient effort was made to question the patient about where she/he lived before the start of treatment, i.e. not where they moved to for better access to treatment. Only after correcting these issues, were we able to identify small villages along the Mbam with several cases and BU endemic villages in the Malantouen HD. Given the crucial role of the collected information in research and public health, it is therefore of great importance that front line health care worker are trained to diligently document all cases.

In addition to the effective antibiotic treatment, wound care is essential for adequate clinical management of BU cases. The material and staff-time required for this care

contributes substantially to the costs of BU treatment. Although leaders of the African endemic countries have agreed in the “Yamoussoukro Declaration on Buruli ulcer” [5] that treatment of BU should be free, in particular wound management leads in many settings to considerable costs for the patients. Further, unlike for the amount of drugs, it is difficult for planners to predict how much wound care material will be required per case of BU. This makes the control program vulnerable to fraudulent claims and a stringent control system must be put into place. Measures to reduce cost such as sterilizing wound care material locally without being able to monitor the success of the process or by getting patients to wash their own bandages, a common practise at many treatment centres, may in the long run not result in any cost savings but in contrary lead to extra treatment cost due to delays in wound healing [12]. In addition to the necessary material, wound care also requires a suitable and sterile environment to perform wound cleaning and dressing. This dedicated area must provide enough space and be sufficiently equipped to handle the – at times – very large and contaminated wounds. There must also be a system of organizing the patients so that they can be efficiently, one after the other, taken care off. Once again medical and organizational training and supervision of front line health care workers and supervising staff is crucial to ensure appropriate care. Only recently has the WHO started to develop and implement standardized guidelines on how BU wounds – depending on factors such as their size or the presence of any secondary infection – should be cared for. Furthermore, procedures have to be implemented to safely dispose of the dirty wound care material. In the course of our environmental sampling, we tested two environmental samples from outside of the wound dressing room at the Bankim district hospital by IS2404 RT-PCR and one of the samples was positive (unpublished data). Such a contamination of the environment may increase the risk of infection and must be avoided by appropriately handling of waste material. Locally adopted solutions, for example by using bleach as a disinfectant, can minimize risks from used wound care material. Overall efforts required to provide basic wound care at rural health care facilities, such as the Bankim district hospital or any of the health centres in the area, are not trivial and should not be underestimated. Even seemingly simple aspects such as the logistics of transporting wound care material to the peripheral health centres must be well organized so that situations where staff in the periphery is forced to improvise can be avoided. With the prospect of an only oral treatment for BU by replacing streptomycin with clarithromycin [13], wound care will remain very important and standards must be maintained. Blister packs of antibiotics that are given to patients asking them to come back only once they are finished – as it is commonly done for leprosy patients – leads to the danger that wound care will be

neglected. Such negligence will unnecessarily prolong the wound healing process even further. Overall, current capacities in the Mapé Basin are only barely able to deal with the challenges of the large wounds and the wound care currently received by BU patients must be substantially improved. Excluding the option of creating a parallel health care system where the provision of wound care material for BU patients and the required expertise to treat the wounds are provided by a vertical structure, the necessary improvements to wound care would require investments in the health care system overall. BU control programs should not solely bear the cost of such changes since in addition to BU patients, all other patients with open wounds – caused by accidents, infections or chronic diseases – would profit from strengthened wound care expertise. However to get a rural African health care facility, such as the Bankim district hospital, to invest into wound care may be very difficult and local BU control measures may need to create the necessary hygienic conditions for appropriate wound care alone and preferably allow all patients with open wounds to profit from the established wound management system.

In addition to wound care, debridement of BU lesions is often required and large ulcers will not heal without surgery and skin grafting. This is illustrated in Figure 7.3 which shows a patient with a large open lesion discovered in Bankim in late 2009. Following antibiotic treatment for only 45 days, the patient returned to the village without any additional care. Her case was photo-documented in 2010, 2011 and for the last time in mid-2013 (Figure 7.3). Lesions of this size will require a skin graft which this particular patient is receiving now. It must be mentioned, that likely multiple factors, including the patient refusing any additional treatment after spending a long time at the health centre and the patient changing residence several times, contributed to the delay of this patient's skin graft. Offering wound debridement and surgery to patients in a timely fashion, possibly even during antibiotic treatment, is therefore crucial also for treatment adherence and to make the patients feel that their wound is healing. Conducting the necessary surgical interventions and skin grafting however requires even more sophisticated setups and additional equipment compared to simple wound care. Sufficiently trained staff and aseptic conditions are further required to provide the services and to keep the rate of graft rejection or other complications low. Creating such conditions in a health care facility like the Bankim district hospital, which suffers from regular power cuts and does not have access to running water, is complicated. Furthermore, surgeries will lead to additional and again difficult to plan costs. Detailed documentation of cases, which includes information such as how many days of wound care a patient received and if there was any surgery performed, may support planning for wound

care and surgical needs. Correlating the amounts of wound care material required - estimated based on the number of days of wound care received - and the need for surgery with factors such as disease category, should allow for quite detailed budgeting and overall planning.



Figure 7.3: Case illustrating that large ulcer will not heal without skin grafting.

Case was first discovered in late 2009, when the patient received antibiotic treatment. She was first photo documented in February 2010 (A) and after receiving antibiotic treatment left for the village. The patient was seen again in June 2011 (B) and in June 2013 (C) after which she is now receiving a skin graft.

An additional aspect of BU care that is often neglected, is the pain caused by the dressing and cleaning of the lesions and the lesions themselves. Reviews commonly state that BU lesions are painless [14], and this is likely true for nodules and possibly even for small plaques, oedemas and small ulcers. However, particularly lesions with secondary infections are painful [12]. Furthermore, large ulcers appear to be very painful based on the responses of the patients to wound care. Because pain medication is only rarely provided at the hospital, patients in Bankim have locally been reported to go to neighbourhood pharmacies or to traditional healers to get pain relieve medication. Even worse, patients may decide to abandon treatment if the pain becomes unbearable. All of this is highly undesirable for BU control and measures should be taken to provide the necessary pain management for the patients. To achieve this, staff at treatment facilities must be trained on the use of the appropriate analgesics and the drugs must be made available.

There are several additional issues and concerns that must be considered when treating BU patients. One such issue is the fact that especially large BU lesion are prone to secondary infections which can lead to a delay in wound healing [12] and may require additional antibiotic treatment [12,15]. As illustrated by a tetanus infection in a Bankim BU case in 2012, secondary infections of BU wounds may even be life threatening. In the past it has been speculated that BU lesions are less susceptible to secondary infections compared to other lesions [12]. However, in our studies on the cultivation of *M. ulcerans* from swab

samples, we did not find a statistically significant correlation between the amount of *M. ulcerans* DNA and the frequency of contamination with other microorganisms (Chapter 5).

Given the fact that lesions often occur on the joints (Chapter 2), prevention of disability (POD) is an additional important aspect of BU care. Especially after long treatment delays, contractures are frequently observed and can require lengthy physiotherapy to regain full functionality [16]. To be able to provide POD care, staff requires training and some basic equipment. Furthermore, POD is fairly time consuming and a larger treatment centre may require staff that is fully dedicated to this. A further insufficiently explored aspect of BU treatment is the care for patients with HIV and *M. ulcerans* co-infections, who are reported to often suffer from more severe forms of BU [17]. Although co-infections may be rare the rate of HIV infections in BU patients has been suggested to be above that of the general population (V. Yana Tijé, personal communication). However reference HIV rates used for such comparisons may not be fully reliable. In our cohort of 88 RT-PCR confirmed BU cases studied in great detail (Chapter 2), of the 41 tested patients five (12.2%) were co-infected (unpublished data). Guidelines for the management of BU/HIV co-infection are currently being prepared by the Global Buruli Ulcer Initiative of WHO. The success of BU care may further depend on the nutritional status of the patient, a factor that has been shown to influence wound healing [18]. Supplying appropriate meals for the patients would provide them with the essential nutrients that promote wound healing and as discussed above such support may also reduce treatment seeking delay and prevent some of the patients from terminating treatment prematurely. Finally, cases of BU should be followed-up for at least one year after the end of treatment. Such follow-ups will identify cases of relapse which may indicate the presence of drug resistance. New lesions discovered during follow-up may be caused by an immune reaction which would most likely resolve with adequate wound care or by re-infection that would need a new round of treatment [19]. Further, with BU lesions often encompassing joints, BU scars can be quite fragile and break open upon physical trauma. To prevent this, patients must be educated on the appropriate care for the scar, such as use of palm oil to keep the scar tissue soft. Furthermore, wound care must be provided if a patient comes back to the treatment facility with a re-opened scar. Follow-up visits are further important to detect any long-term side-effects of the drug treatment. Although it is reported that BU patients appear to be less susceptible to adverse effects of the drugs compared to other populations [13], this may also be an impression related to the lack of systematic and prolonged observation of the patients. Re-finding cases for follow-ups can however be complicated by the fact that some local communities are very mobile and contacting

individuals in very remote communities or having them adhere to scheduled appointments is difficult. Locally adopted approaches such as having the contact details of someone in the same village or a family member or friend that lives closer to the road may help to re-find patients or allow for messages to be passed to the patient telling them that they should come for a check-up. Having patients come to the facility with their transport reimbursed should be preferred over having health care workers, who require a *per diem* and have numerous other tasks, go out and find the patient with the possibility that the patient will not be at home.

7.3 Diagnosis of BU for research and public health purposes

The four currently available methods for laboratory diagnosis of BU are microscopy to detect acid fast bacteria (AFB) after Ziehl-Neelsen staining, PCR based detection of the *M. ulcerans* specific insertion sequence IS2404, culturing of *M. ulcerans* or identification of BU typical alterations of the skin by histology. A major difference between the first three and the last one of the methods is, that the first ones can be performed on samples obtained by minimally- or non-invasive collection of wound exudates by FNA or from below the undermined edges of the ulcer with a cotton swab. Histology on the other hand necessitates a tissue biopsy which can either be obtained specifically for this purpose or in the course of wound debridement or surgery. Microscopy, the only method that can be performed locally at health facilities in the periphery, is highly insensitive, with positivity rates between 40 and 78% being reported in the literature [20], and not 100% specific. A possible concern with a positive microscopy results is illustrated by the case of cutaneous TB presented in Chapter 6. Even though such cases may be rare, they have also been found in other BU endemic sites (D. Yeboah-Manu, personal communication). Culturing which can provide definitive BU diagnosis, is also far from 100% sensitive and more importantly takes a very long time. In our hands (Chapter 5) clinical isolates of *M. ulcerans* took between 6 and 25 weeks to grow on Löwenstein-Jensen medium. One cause for the low sensitivity of culturing is the overgrowth of the primary inoculations with other microorganisms, thereby depriving *M. ulcerans* of a possibility to grow (Chapter 5). Measures to prevent such contamination, e.g. by using dry swabs (Chapter 5) or decontaminating wound exudate [21], negatively impact the recovery of *M. ulcerans*. In our study aiming to optimize culturing, we have shown that transport with a simpler and cheaper transport medium called Amies, which is non-nutritious, may be better than the currently used medium (Chapter 5). As for the effect of the long term storage of samples, our data suggest that samples should be processed as soon as possible to increase the

chance of *M. ulcerans* recovery (Chapter 5). Despite the lack of diagnostic value, culturing does remain very important for studies that require clinical isolates. Such research includes for example investigations into the genetic diversity of *M. ulcerans* (Appendix 1). PCR, which has become the gold standard of BU diagnosis [22], is fast and highly specific if performed correctly in a quality controlled setting. Particularly RT-PCR, a method that was first applied to *M. ulcerans* detection by Rondini *et al.* in 2003 [23], achieves highly sensitive BU diagnosis. Although, the possibility of false negative PCR results cannot be fully excluded [24], it is likely quite low, at least if patients are sampled multiple times and specimens processed by experienced technicians. For correct sampling, samples collected by cotton swabs should be taken from all round the undermined edges to increase the chance of retrieving *M. ulcerans* DNA. Despite its advantages, PCR has limitations for use in very rural areas since it requires a sophisticated laboratory setup such as the “three room principal” to separate the individual steps of the process and is prone to contamination and false positive results. In many settings cases of BU are therefore currently only diagnosed retrospectively by PCR at a national reference laboratory. Furthermore, National BU Control Programs are struggling with the financial burden of having to pay for the analysis of the samples. In addition to the research value of laboratory diagnosis, it is also of great importance for public health purposes. All centres in Cameroon should therefore be given access to this type of diagnosis. In addition to fulfilling the requirements of the WHO (see below), staff at the centres could then also get feedback on the accuracy of their diagnosis. Finally histopathology has limited diagnostic value, since the focally distributed AFB are not necessarily detected in the part of the lesion being investigated. However like for culturing, histopathological studies are of great importance to improve our understanding of the pathology of BU and the immune response against *M. ulcerans*. Histopathology can for example aid in clinical decision making with respect to debridement by providing an insight into the extent of tissue damage or prolongation of antibiotic treatment by detecting the presence of clusters of intact AFBs after eight weeks of antibiotic treatment [25].

For confirmation of clinically diagnosed BU cases, the WHO highly recommends the use of PCR. Recommendations from the 2013 WHO BU meeting in Geneva demand that at least 70% of the BU cases reported by the endemic countries to the WHO should be PCR tested. Control programs are also asked to no longer report cases that tested PCR negative. WHO recommendations further suggest that countries should “strengthen laboratory capacities” to achieve the desired PCR test coverage rates. To achieve the proposed high rate of PCR confirmation, the financing of the test should further be discussed and the WHO

should provide support in the form of expertise to set-up and quality control the laboratories that are to conduct the tests.

Despite the recommendation of the WHO for more laboratory diagnosis cases of BU in the remote BU endemic areas of Cameroon are still diagnosed on clinical grounds only. Clinical diagnosis can achieve accuracies of about 95% if performed by highly experienced health care workers [26]. However routine clinical diagnosis as performed in the Mapé Basin, is much less accurate (Chapter 2). And even in other BU endemic areas, accuracies between 30-70% are reported in the literature (Chapter 2 and 6). In our main cohort of patients, 88 of 157 were laboratory confirmed reflecting a clinical diagnostic accuracy of 56%. According to WHO conditions such as tropical phagedenic ulcer, venous ulcer, diabetic ulcer, yaws, cutaneous tuberculosis, cutaneous leishmaniasis and malignant skin ulcer may be mistaken for BU lesions [27]. In Addition to these conditions, less experienced health care workers may also confuse common mycoses of the skin as BU lesions. A characteristic clinical feature of ulcerated BU lesions are the undermined edges, which are easily detectable by palpating the lesion or using a cotton swab. Cases of yaws, which often present with multiple lesions, and cases of venous or diabetic ulcers present with lesions that usually do not have undermined edges. As demonstrated in Chapter 6, lesions of patients suffering from cutaneous TB on the other hand do also present with undermined edges and it is more the location of the lesion that may be used to guide the differential diagnosis. For cutaneous leishmaniasis, local endemicity or a travel history of the patient will help to include or exclude this condition in the differential diagnosis. Systematic studies are required to further identify the conditions that are commonly mistaken for BU and to develop a list of key criteria that can be evaluated to reduce the risk of misdiagnosis. A study aiming to accurately diagnose all skin lesions observed in a BU endemic health district of Ghana has found that already basic clinical diagnosis can in many cases exclude BU and that simple wound dressing and re-examination of lesions after a few days may help to clarify the diagnosis of non-BU cases (T. Junghanss, personal communication). Such a wait-and-see approach is also applied in the differential diagnosis of leprosy [28]. However in rural communities, such as in the Bankim area, such a strategy may bear the considerable risk of the patient not returning for follow-up visits because they deem the treatment as being insufficient. If another bacterial infection is included in the differential diagnosis, combining wound care with a short course of antibiotic treatment may reduce the risk of losing the patient to follow-up. In addition to taking into account clinical features in the differential diagnosis, it is also important to consider the history of the lesion by asking the patient questions such as how and when it started and how

it has been treated by other health care professionals or traditional healers. Without any information from the patient, even trauma from motorcycle or machete accidents, cases of child abuse or snake bites may wrongfully be diagnosed as BU. It is however also true that caution must be taken when considering the information provided by the patients. Particularly with information obtained during a first questioning, when the patient just arrived at the treatment facility and local staff have not yet gained the trust of the patient, there is a considerable risk of getting wrong information. Patients may initially provide the answers they think the health care worker expects / wants to hear. Patients may further be unwilling to admit that they have consulted a traditional healer before coming to the formal health care facility or the patients may even be ashamed to admit how long they waited before presenting their lesion to a health care professional. In terms of their origin, patients may also be initially reluctant to tell the health care worker where they are from. It may further be truly difficult for patients to recall when the disease started and how it evolved over time. With this recall bias it must be considered that many of the patients live in villages where there are no daily newspapers and the day of the month may not be important to know. Patients may therefore not remember, if the lesion started 4, 6 or 8 weeks ago. On the other hand it is also very important how questions are asked and that sufficient time is given to the patient or a family member to answer. Especially if a possible answer is provided with the question, it is unlikely that the true opinion of the patient will be heard. Based on our experience, it can be helpful that patients are interviewed on several occasions and even by different people that during the patient's hospital stay have gained their trust. With this approach it is likely that information useful for the differential diagnosis can be obtained from the patient although in the end it is up to the health care professional to judge if the provided information is reasonable and trustworthy.

The high rate of false BU clinical diagnosis has implications for the patients, the public health system as well as for BU research. The most important implication for the patients is that they are denied the treatment they would actually require for an additional eight weeks. This implies a substantial opportunity cost, for example for a patient with ulcerative skin cancer who is deprived of the appropriate care she/he needs and instead receives a treatment that will not have any impact on the disease they are really suffering from. On the other hand, patients with a machete wound or yaws would only require good wound care or a single injection of benzathine penicillin respectively and their wound would be treated correctly whereas instead they receive eight weeks of unnecessary and potentially harmful antibiotics. For the health care system, false positive diagnosis of up to

50% means that substantial unnecessary costs occur. Finally for the researchers in the field, laboratory results may not be available at the time of conducting the work which implies that substantial efforts must be invested to study “patients” which then retrospectively have to be excluded from the analysis. To address this, clinical diagnosis should be improved and it may be helpful to observe patients in different endemic settings to better distinguish between noise and valuable information and allow for the discovery of parallels between the different settings (Chapter 3). Overall it is of great importance that all research is performed on PCR confirmed cases only so as to ensure the accuracy of the science being conducted.

To prevent all of these negative impacts of the falsely diagnosed BU cases, there is a great need to further train front line health care workers to better diagnose suspected cases of BU. Such training must be offered repeatedly to train new staff and re-refresh the knowledge of existing personnel. Further the training must be practical and should involve examination of current suspected cases or recently diagnosed ones. Once trained, the work of the diagnostic staff must be regularly supervised to ensure that there is an iterative learning process, in which front line medical staff can profit from the medical knowledge and diagnostic expertise of regional BU experts and laboratory results. Through the regular sharing of a short clinical description and images of the new patients, it can be made possible for experts at the central level to provide valuable input into the clinical diagnosis of BU in remote endemic areas. Using modern information technology and mobile internet connections, which today reach even extremely remote areas, such an exchange can be implemented. Given the necessary tools, efforts required from the front line health care worker as well as the central experts are minimal. We have set up such a system in the Mapé Basin and such a system for medical feedback is currently also being developed by the WHO (<https://who.telederm.org/>). As illustrated by our experience in Bankim, feedback to the periphery, supervision and guidelines on how the feedback is to be used, will be crucial for the success of the system. In addition to improving clinical diagnosis, better local lab capacities could also help to raise diagnostic accuracy although the applied test will have to be simple and sufficiently sensitive and specific to merit an effect on treatment decisions. Serological markers have shown to be suitable to detect exposure to *M. ulcerans* but are not suitable to detect active disease (Chapter 3). The development of a simple routine method to detect the *M. ulcerans* specific toxin mycolactone, is hampered by technical limitations, e.g. difficulties to produce sufficiently sensitive antibodies against the lipid structure. On the other hand diagnostic tests that detect *M. ulcerans* protein antigens in lesions by ELISA and eventually lateral flow assays look much more promising and may be available for field testing in the next few months (K.

Röltgen, personal communication). If high sensitivity and specificity can be achieved, such a rapid diagnostic test could greatly support differential diagnosis and be used to indicate to health care professionals if alternatives to the BU diagnosis need to be considered or if the clinical diagnosis of BU was correct.

Finally, in addition to improving BU diagnosis, the much more difficult to address issue will be to provide primary health care facilities with the necessary skills and capacity to care for patients suffering from the other diseases, i.e. cases that turn out not to be BU. Differential diagnoses, such as cancer, may require lengthy, complicated and expensive treatments that are not available in peripheral health care facilities. The welcome fact that BU treatment comes at no cost for the patient, may here even create some wrong incentives for health care providers causing them to offer this treatment to poor patients although in reality they would require an expensive treatment only available in a faraway referral hospital which is not assessable to them. Only broad range health system strengthening, with local capacity building and a functioning referral systems, would be able to address this concern.

7.4 Reservoir of *M. ulcerans* in the environment and site of BU transmission

Despite more than half a century of research, both the environmental reservoir and the mode of transmission of *M. ulcerans* remain to be identified. In fact, these two crucial elements of BU epidemiology are arguably the biggest mysteries of BU that remain to be resolved. Studies dating as far back as the early 1970's concluded that human-to-human spread of *M. ulcerans* was improbable and transmission from an environmental source much more likely [4,29,30]. A landmark study in the early 1970's by the Uganda Buruli Group further showed that BU transmission is very focalized and areas with high and low incidence can be just a few km apart [4]. To elucidate BU transmission it is therefore essential to have a very detailed understanding of the distribution of BU cases in an area to be able to identify foci of infection. Through detailed investigations in the Mapé Basin, we have been able to describe such areas of high disease incidence. Specifically, we have identified small communities along the Mbam River with several cases which may represent suitable locations to study BU transmission (Chapter 2). In our analysis the village of Bankim also appeared to be a focus of BU transmission. However many of the BU patients from there travelled considerable distances to their farms (Chapter 4) and could have gotten infected in these much more rural areas. In addition to realizing that BU transmission is very focalize, investigation

by the Uganda Buruli Group and other researchers showed that close proximity to slow flowing or stagnant water and contact with open water sources are risk factors for BU [4,29]. Investigations in various BU endemic regions have repeatedly re-confirmed this risk factor [31]. Poor wound care and failure to wear protective clothing have also been confirmed as risk factors for BU in several studies but other than that no conclusive information could be obtained from case-control studies [31]. Analysing the age range of people most affected by BU (see below), including sufficient numbers of patients and only including laboratory confirmed patients may help to identify new risk factors for BU in the future.

There is evidence that man-made alterations of the environment may increase the incidence of BU [32]. Based on the distribution of cases in the Mapé Basin, the reservoir associated with the Mapé Dam does not appear to play a major direct role in BU transmission in the region. We found that patients were more likely to be oriented towards the Mbam River as opposed to the Mapé Dam reservoir for their agricultural activities (Chapter 4). Further, there are only few cases of BU originating from the villages on the shore of the Mapé Dam in the central and northern part of the Bankim HD. On the other hand, there are patients living in the very small settlements located along the Mbam River (Chapter 2). For the risk of BU associated with water bodies, it must be noted that that proximity but not direct contact with the water appears to increase risk [29]. It is therefore possible that BU transmission takes place at water bodies much smaller than the Mbam River or the Mapé Dam close to the villages [29]. Overall, the collection of detailed geographic information of the origin of patients is a first step towards the tracing of BU transmission. Results help to decide where more targeted research should take place and which communities should be included in risk factor studies. With regards to risk factors of BU, it is also worth noting that given the fact that inhabitants of BU endemic village all live in very similar environments and perform similar work, it is likely that human genetic host factors also play a role in determining the susceptibility of an individual for BU. To date one host genetic risk factor study has investigated the role of a marker that has been associated with tuberculosis and leprosy. The study found that the SLC11A1 gene, which codes for the natural resistance-associated macrophage protein, plays a role in the susceptibility of an individual to develop BU [33]. A further such study to identify host genetic risk factors of BU in Ghana has been initiated and samples are currently being processed for analysis (G. Pluschke, personal communication).

In addition to identifying foci of transmission in the Mapé Basin, our detailed epidemiological investigation has also reconfirmed that most BU cases are between 5 and 15

years old (Chapter 2). Specifically 46.6% of the 88 patients studied were in this age range with 12.5% being younger and 40.9% being older. Based on the population age distribution which we determined in the exhaustive survey, we were able to calculate the age adjusted cumulative BU incidence rate for the cases that lived in the Bankim HD (Chapter 2). The distribution of this cumulative incidence rate revealed that the age adjusted cumulative incidence rate of BU was lowest among the zero to less than 4 year-olds with only 5.7% of the cases being in this age range. This is astonishing given that roughly 10'600 small children below the age of four, which represented 22.3% of the entire population, were living in the Bankim HD in early 2010 (Chapter 2). A similar underrepresentation of below 5 year-olds among BU cases was already reported in the early 1970's for cases from Uganda [4] and in a large scale study with 268 laboratory confirmed cases of BU from Ghana in 2009 [34]. However both of these studies do not go on to discuss this observation any further and even reviews commonly focus on the fact that BU affects mostly children between the age of 5 to 15 without any further considerations on why below 5 year-olds are less affected by the disease [35]. However, the population-adjusted age distribution of BU cases may in fact provide hints as to how and where transmission of the disease takes place. The age distribution appears to indicate that most below 5 year-olds do not engage in activities that expose an individual to the risk of contracting BU. Very young children are likely to stay closer to their homes and have reduced direct contact with the environment away from home, since they are carried to cover larger distances. Once children are more than four to five years old, they seem to be exposed much more intensely to *M. ulcerans* and start to contract the disease. Differences in the behaviour of below and above 4-5 year-olds may explain the difference in incidence of BU. A transition in behaviour around this age includes starting to walk around the villages and further away from home, going to the family farm to help with some basic activities and going to the village water sites to collect water for household use. Further studies are required to investigate to which extend the movement radius between children less than 4-5 years old is different from that of older ones and how this is increasing the risk of contracting BU.

Approaching the issue of exposure to BU from an immunological angle, Diaz *et al.* in our laboratory have previously been able to show that, although an antibody response specific for the *M. ulcerans* 18kDa small heat shock protein (shsp) cannot be used for diagnostic purposes, antibody titres against this antigen can be used to identify individuals or communities that have been exposed to the pathogen [36,37]. In the course of the work presented here, we have expanded, for the first time, the testing of sera to children below the

age of five and in addition to collecting sera from several hundred patients and controls from Ghana we also collected sera from multiple individuals living in the village of Mbandji 2, which is located in the Bankim Rural Health Area of the Bankim HD (Chapter 3). When sera were tested for anti-18kDa shsp antibody responses, similar to the age distribution of BU cases, children under the age of 6 were much less likely to be seropositive. This trend applied across both the Ghanaian and the Cameroonian endemic sites, indicating that there could be a similar behavioural transition between the very young and the older children in both BU endemic settings leading to seroconversion above a certain age (Chapter 3). Correlating the movement radius to the serological status of young children growing up in BU endemic areas may help to identify the activities that lead to increased exposure to *M. ulcerans*.

With the potential of BU transmission taking place away from the homes of the patients and at sites of environmental contact, we set out to study such sites in detail (Chapter 4). Specifically we visited and sampled the farms as well as the village and farm water sites of laboratory confirmed BU cases and attempted to detect *M. ulcerans* at these locations. Alongside the ability to culture *M. ulcerans* from the wounds of patients, attempts have been made to culture the pathogen from the environment to study its distribution in nature. These efforts remained unsuccessful until very recently when for the first time an environmental isolate of *M. ulcerans* could be cultured from an aquatic Hemiptera from Benin, by passaging through mice [38]. Unfortunately this approach is not suitable for routine use and because of these difficulties our current understanding of the distribution of *M. ulcerans* in the environment is based on the detection of *M. ulcerans* specific gene sequences by PCR. Although very sensitive, PCR has several caveats that need to be considered when interpreting the results. Firstly, the detection of *M. ulcerans* DNA in a sample does not necessarily mean that the corresponding sample contained viable bacteria. Even in the complex matrix of environmental samples, DNA is stable and may persist after the bacteria have died [39]. Secondly, there is a great risk of cross-contamination between samples, especially if many samples are processed, all steps of the processing are conducted in the same room or if conventional as opposed to real-time PCR is used. This risk has been highlighted in a study on the distribution *M. ulcerans* DNA in the environment which in contrast to earlier studies [40] only found few *M. ulcerans* positive sites [41]. The risk of contamination when analysing *M. ulcerans* samples was further raised by a quality control trial testing the accuracy of laboratories analysing environmental and clinical samples for the presence of IS2404 (M. Eddyani *et al.*, PLoSOne, submitted). An additional issue that has to be considered when interpreting environmental PCR results and that has not been sufficiently

addressed in the past, is the fact that there are microorganisms other than *M. ulcerans* that are IS2404 positive [42]. Only through the additional detection of the KR and IS2606 gene sequences and a specific difference between the multiplicity of IS2404 and IS2606 sequences can one conclude that the environment contains DNA originating from the ecovar of *M. ulcerans* that causes human disease [42,43]. Applying stringent conditions to ensure reliability of the RT-PCR results, we identified three *M. ulcerans* DNA positive village water sites used by laboratory confirmed BU patients in the Mapé Basin (Chapter 4). Further we found one positive duck faecal sample. From these findings we decided to study one of the positive village water sites as well as a close by negative site in great detail and over time. In doing so we identified detritus at the positive permanent village water site which may represent an environmental reservoir of *M. ulcerans* (Chapter 4). Interestingly an early BU transmission study on tsetse fly control workers showed that the use of small permanent water bodies to obtain water for household purposes increased the risk of getting BU [29]. Given the very focal distribution of *M. ulcerans* DNA in the environment that we have observed (Chapter 4), it is possible that we missed some positive sites and it may be valuable to examine further village water sites in villages that have recently reported cases as well as close by villages that have not reported any cases. If the role of village water sites is further confirmed, it would be interesting to examine the contact patterns of children of different ages with these sites. The positive waterfowl faecal sample raises the possibility of an animal reservoir of *M. ulcerans*, which unlike in Australia [44] has thus far not been identified in Africa [45], and also merits further investigation (Chapter 4).

By phylogeographic analysis of *M. ulcerans* isolates it may be possible to further narrow down the environment in which patient acquired their infection and to elucidate transmission networks of BU. Röltgen *et al.* have previously shown that even in a BU endemic area of less than 1000 km², strains of *M. ulcerans* can be differentiated based on single nucleotide polymorphism (SNP) typing [46]. From a total of 33 patients from the Mapé Basin we have isolated *M. ulcerans* and whole genome sequenced the isolates. Genomic data will allow for detailed phylogeographic strain-typing (Appendix 1). Of the same patients, we also collected detailed information on where they lived and worked for the year before the onset of BU symptoms (Appendix 1). Analysis of the strains is still on-going and we expect the phylogeographic analysis to further elucidate transmission by identifying groups of patients infected by the same haplotype. This may reveal whether patients are more likely infected around their homes or at their place of work (Appendix 1). Having typed the clinical isolates circulating in the Mapé Dam area, it would be of great interest to investigate if the *M.*

ulcerans in the environment, which we detected by RT-PCR (Chapter 4), have the same haplotype as the clinical isolates. As mentioned above, such investigations are however hampered by the fact that the routine culturing of *M. ulcerans* from the environment is an unsolved problem. Attempts to recover *M. ulcerans* from PCR positive environmental samples are on-going and with the right decontamination method and quick processing of the samples, this may be possible. As an alternative, next-generation DNA sequencing and metagenomic analysis could also be an option to type *M. ulcerans* strains in the environment [47,48]. However working with soil as a matrix for such analysis is not trivial and specific measures must be taken to ensure efficient analysis [49]. Comparative analysis of *M. ulcerans* positive and negative samples may further provide interesting insights on which types of bacteria or other organism share the specific ecological niche that *M. ulcerans* has adapted to.

In addition to the unknown environmental reservoir of *M. ulcerans*, the mode of transmission of the pathogen to humans also remains to be identified. Initial studies believed that small injuries likely play a major role in getting the pathogen into the skin of the patients [2,4,50,51]. However, since the hypothesis of contracting BU through skin trauma could not be fully established, speculations on the mode of transmission of BU continued. However, because of the long incubation period, estimated to be several months, patients may not remember having had small skin injuries at the site of their BU lesion. Interestingly, *M. marinum*, the closest relative and ancestor of *M. ulcerans*, occasionally causes human infection by inoculation through small skin lesions which are often not remembered by the patient [52]. In 1999, Portaels *et al.* were the first to suggest that insects could be involved in the transmission of BU. Specifically they tested plants and water bugs from BU endemic areas from Benin and Ghana and found all plants to be PCR negative but some water bugs were positive. Based on these findings they suggested that the water bugs could be involved in the transmission of BU by being passive reservoirs for the pathogen or by transferring *M. ulcerans* to human patients by bites or by contaminating trauma at skin sites with their faeces [53]. Several follow-up studies have argued for a direct implication of water bugs in BU transmission [54–56]. However their role has also repeatedly been questioned and a key role of water bugs in transmission of *M. ulcerans* seems rather unlikely [31,57]. More recently, mosquitos have been suggested to play a role in the transmission of *M. ulcerans* in Southern Australia [58]. However, certain insects may just accumulate *M. ulcerans* from the environment [53] and the detection of *M. ulcerans* DNA in insects may therefore simply be a proxy for contact with a close-by positive environmental site and not necessarily imply that the insects are involved in human inoculation with *M. ulcerans*. In addition to trying to

directly identify the vector of *M. ulcerans*, epidemiological data have also been used to narrow down the mode of transmission of BU. Similar to an epidemiological study in Kinyara in 1971 in Uganda [4], we attempted to contribute to the understanding of BU transmission by analysing the distribution of BU lesions on the body of the patients (Chapter 2). For this purpose we developed a novel tool for the analysis of lesion distribution by methods usually used for geographic mapping. Having both the option of direct inoculation into the skin and transmission by insect vectors in mind, we compared the observed lesion distribution with the distribution of small skin injuries in children and the biting sites of insects. Neither of the distributions clearly matched the BU lesion distribution we observed. Interestingly however there was a difference in the distribution of the lesions in children compared to adults with lesion in adults being more focused on the lower legs (Chapter 2). A similar trend was also observed by the Uganda Buruli Group in 1971 in male patients. In this study, which included 220 patients, however researchers also found a striking difference between male and female patients which they attribute to variations in the activities performed by the two genders [4]. In contrast, in our study of a smaller cohort of patients, we did not observe a significant difference between male and female patients (Chapter 2). Based on our findings, and as also suggested by other studies [31,53], we concluded that there may be multiple modes of transmission of BU. Further, in view of the difference in the onset of immune responses against *M. ulcerans* and *P. falciparum* that we observed in young children (Chapter 3), it appears that a potential insect vector for BU would be less commonly associated with the houses of patients compared to the malaria transmitting mosquitoes. The focal transmission pattern observed by Röltgen *et al.* [46] furthermore indicates that BU transmitting insects would need to be travelling only short distances. The role of water bugs has also been put into question based on the fact that these insects do not normally bite humans and bites are quite painful [31]. However, current evidence cannot exclude the role of insects in BU transmission and studies identify biting insects close to PCR positive environmental contact sites of BU patients may further help to explain the biggest mystery in BU research. Similar to the study conducted by Labbé and Caouette [59] on the distribution of small skin injuries in Canadian children, it would be interesting to monitor the distribution of skin injuries in individuals of all ages living in BU endemic areas. Given the differences in clothing worn and activities performed, the distribution of skin injuries in BU endemic areas is likely to be strikingly different from that of inhabitants of the developed world and the northern hemisphere.

7.5 Conclusion

Prevalence of BU, even in highly endemic communities, is far below the 30% or even over 50% reported for malaria and soil-transmitted helminths in communities similar to those affected by BU [60–62]. However the impact of a BU infection on an individual patients and their family is devastating. Even small BU lesions require lengthy treatment, especially large wounds often lead to permanent disabilities and all lesion types inevitably lead to some disruption in the productivity of the patient and possibly a caretaker. Although current antibiotic treatment can cure the *M. ulcerans* infection, wound management including the often required skin transplantations represents a major challenge and burden for the health system in rural Africa. To reduce the burden of BU on the individual patients and the affected communities, sensitization efforts are needed to promote early case reporting, capacities of treatment facilities such as their diagnostic capacity and the quality of care they provide, need to be strengthened. Further, research is required to elucidate the mode of transmission of BU so that populations living in endemic areas can be educated on how to prevent infection.

In this thesis, aspects of BU control and transmission were investigated in the Mapé Basin of Cameroon. Specifically, by investigating the age distribution of BU cases, we found that the number of cases in under five-year-olds is disproportionately low. In accordance with this we were further able to show that children below the age of six are less likely to have antibodies raised against *M. ulcerans* antigens compared to older children in the same communities. These findings indicate that transmission of BU takes place at locations away from home and beyond the small movement radius of very young children. Furthermore, close environmental contact may be required for transmission; something small children, that are being carried to get further away from their homes, are also experiencing less than older children. Indeed, we have identified three village water sites where *M. ulcerans* appeared to be present. Furthermore at one of these sites, which we studied in detail, we found that DNA of the pathogen persists over a period of more than two years. The RT-PCR positive detritus identified at this location may therefore represent an ecological niche and reservoir of *M. ulcerans*. On-going genetic analysis of the *M. ulcerans* strain circulating in the Mapé Basin will help to further elucidate transmission pathways and provide hints as to where the patients may have acquired the infection. In addition to studying these epidemiological and environmental aspects of BU, the work of this thesis highlighted the importance of correct differential diagnosis by reporting on a case of cutaneous tuberculosis that was initially misdiagnosed as BU. Further by large scale culturing effort we have been able to optimize

procedures for the primary isolation of *M. ulcerans* from human lesions and thereby facilitate the creation of strain collections that will enable further research on BU transmission and *M. ulcerans* pathogenicity.

Overall the research conducted in the course of this thesis, has increased our understanding of BU epidemiology and provides a solid basis for more targeted investigations into the transmission of *M. ulcerans* in the African context. Multicentre, comparative and interdisciplinary studies with large patient numbers stand the highest chance of resolving the remaining mysteries of BU.

7.8 References

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Appendix 1

Genetic Diversity of *Mycobacterium ulcerans* in the Mapé Basin of Cameroon

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A1.1 Project Summary and Plan

Buruli ulcer (BU) is a neglected tropical disease caused by *Mycobacterium ulcerans* that has been reported from over 30 countries with most cases originating from West Africa. The necrotising disease of the skin and subcutaneous tissue presents with a wide range of clinical forms including non-ulcerative and ulcerative lesions. Studies on the transmission of BU conclude that *M. ulcerans* is an environmental pathogen, human-to-human transmission is unlikely (1) and transmission may occur by an insect vector (2) or by direct inoculation of the pathogen into small skin wounds (3). However, despite years of investigations in numerous endemic areas, both the exact mode of transmission and the environmental reservoir of the pathogen remain to be identified and parallel modes of transmission may be possible (4, 5).

Various results of our studies on BU patients in the Mapé Basin of Cameroon provide further clues towards the identification of the environmental reservoir of *M. ulcerans*. Firstly, by comparing the age distribution of 88 RT-PCR confirmed cases with the age distribution of the local population, we found that children below the age of 4 are vastly underrepresented among BU cases (5). Similar trends in the age distribution of BU cases have also been observed in Ghana (6). Further, in a sero-epidemiological study of young children living in the Mapé Basin and a BU endemic area in Ghana we have found that children below the age of 6 are less likely to have an antibody response towards *M. ulcerans* compared to older aged children (K. Röltgen *et al.*, in preparation). Together these findings indicate that very young children are less exposed to risk factors of BU and that transmission likely takes place outside of the small motion radius of these very young children. Further transmission appears to require close contact with the environment, something very small children that are being carried to go further away from their homes would not experience. To investigate this hypothesis, we studied locations of regular environmental contact of laboratory confirmed BU patients and examined these locations for the presence of *M. ulcerans* DNA. We found that the soil around three permanent village water sites in the Bankim Health District (HD) were positive and at one longitudinally studied village water site, we identified underwater detritus as the potential reservoir of *M. ulcerans* (M. Bratschi *et al.*, in preparation). In the course of the same study, we also investigated where BU patients have their homes and farms and found that patients are fairly mobile, travel up to 15km to get from their home to their farm and at times spend several months living at their farms (M. Bratschi *et al.*, in preparation). With this wide motion radius of the patients there are several potential places where they could have contracted the infection.

The study described here, aims at further elucidating transmission of BU in the Mapé Basin by genomic analysis of the circulating *M. ulcerans* strains. Similar to a recent study conducted in Ghana, which showed that single nucleotide polymorphism (SNP) typing of *M. ulcerans* can be used for geographic clustering in an area of less than 1000km² (7), we plan to phylogeographically cluster strains and attempt to determine where the patients are most likely to have been infected. For the analysis, we isolated and whole genome sequenced a total of 86 *M. ulcerans* strains from 45 patients. In addition to strains from 33 patients from the Mapé Basin, this collection also included isolates from one patient from the Fouban HD which is located to the South East of the Mapé Basin and strains from 11 BU patients from two endemic areas in the South of Cameroon (Figure A1.1). For the cases from the Mapé Basin we also identified their exact place of residence and for 31 of these patients we mapped the location of the farms where they worked (Figure A1.2).

Whole genome sequencing of the strains has recently been finished and bioinformatic data analysis has been initiated. Methods that can be used to elucidate transmission networks of environmental pathogens will be used to performed detailed phylogeographic analysis of the strains isolated from the Mapé Basin. Further, comparisons between the strains from the Mapé Basin and those from Southern Cameroon will help to further our knowledge of *M. ulcerans* genetic diversity in the West African country. In addition to the strains that can be used for in-depth phylogeographic analysis, the sequenced isolates also include strains that were collected from two distant lesions from one patient and isolates obtained from a single patient over a period of more than a year. These strains allow us to investigate if patients with multiple BU lesions were infected multiple times or if a single infection can lead to multiple lesions. Further these strains may help to determine the *in vivo* mutation rate of *M. ulcerans* in the course of a human infection.

A1.2 Figures

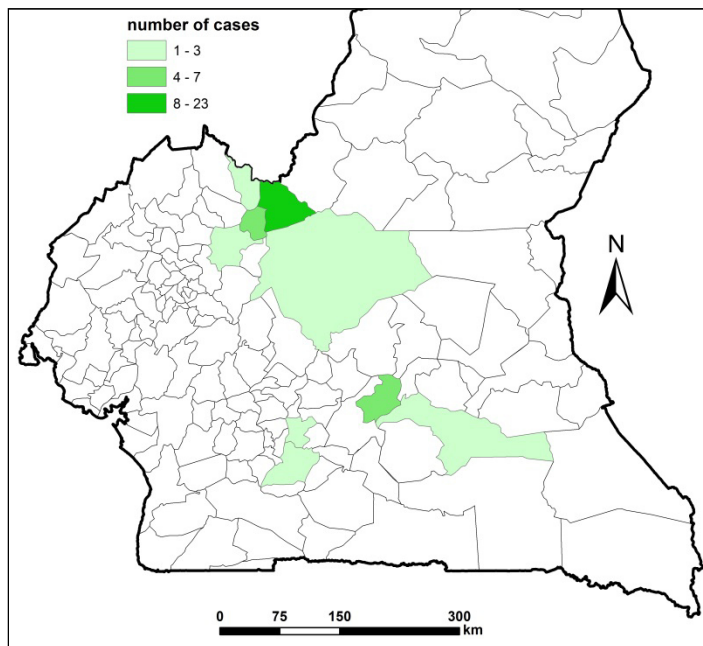


Figure A1.1: *M. ulcerans* strains per district.

To study *M. ulcerans* diversity in Cameroon, we collected 86 strains from 44 patients from various parts of the country. Districts of origin of the patients from whom strains were isolated are shown.

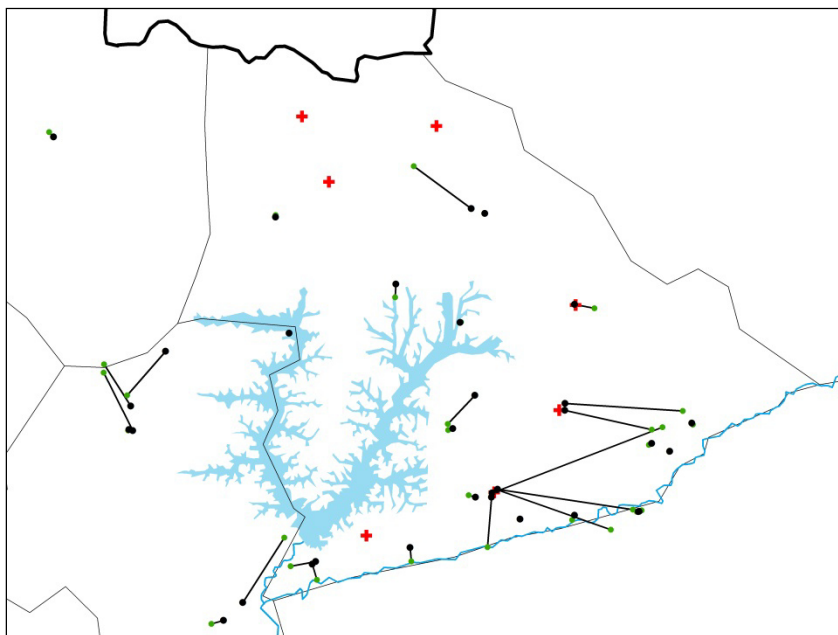


Figure A1.2: Geographic origin of patients from whom *M. ulcerans* strains were isolated.

Map shows the homes (black points) of the 33 patients from the Mapé Basin BU endemic area from which *M. ulcerans* strains were isolated for phylogeographic analysis. Locations of the farms (green points) of 31 of the patients were also mapped. Homes and farms of the same patient are connected. Locations of the 6 health centres and the Bankim district hospital are depicted with red crosses.

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Curriculum Vitae



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