

Epidemiology of Buruli ulcer in the Offin river valley of Ghana

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*Dedicated to anyone who
has ever had Buruli ulcer*

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Summary

Buruli ulcer (BU) is a debilitating skin disease caused by *Mycobacterium ulcerans*. In the last decades, the disease has been reported from 34 countries with endemic foci typically occurring in rural areas where access to medical health facilities is a challenge. Since the exact mode of transmission of the pathogen is still not fully elucidated, early case detection and treatment of patients are key factors to control the disease.

The first ever national active BU case search conducted in Ghana in 1999 identified the Offin river valley as one of the most BU endemic regions in Ghana. Based on recent anecdotal accounts indicating unstable transmission of *M. ulcerans* along the Offin river, we conducted as part of this PhD study, an exhaustive case search and household survey of 13 selected communities along the river. We observed an overall decline in the prevalence of BU. Subsequently, we installed an active surveillance system enabling the continued monitoring of the emergence of cases at the household level. By this system we were able to demonstrate that integration of control of several neglected tropical diseases such as leprosy and yaws was feasible and should be pursued to maximize the limited resources available for the control of these neglected tropical diseases.

Until now, reservoirs and/or vectors of *M. ulcerans* are yet to be identified particularly for endemic settings in Africa. In the course of this PhD thesis, we conducted sero-epidemiological studies, assessing the exposure of populations sampled from Ghana and Cameroon to the pathogen by measuring humoral responses against the *M. ulcerans*-specific 18kDa small heat shock protein. We observed that exposure to *M. ulcerans* begins at approximately four years of age, coinciding with the age when children move out of their households and have more intense contact with the environment. Furthermore, by comparing the age when first humoral immune responses to *M. ulcerans* and to other pathogens with different modes of transmission occur, we observed an earlier onset of serological response to antigens of the mosquito transmitted malaria parasite *P. falciparum* and of soil transmitted *Strongyloides* helminths. In contrast, exposure to antigens of water transmitted *Schistosoma* worms shared a similar pattern of late onset of immune response with what we observed for *M. ulcerans*. Our data indicate that transmission of

M. ulcerans occurs by contact with environmental sources of the pathogen outside of the small movement range of very young children.

In recent years, the prevailing assumption was that the reservoir of *M. ulcerans* is somewhat fixed in space due to the highly focal occurrence of BU outbreaks. In this regard, a strong link between genotype and geographical origin of clinical *M. ulcerans* isolates has been reported. We compared whole genome sequences of a limited collection of clinical *M. ulcerans* strains, isolated from individuals living in the Offin river valley and identified two co-existing clonal complexes not separated in time and geographical location along the Offin river. To this observation we infer the presence of a reservoir of infection that is more mobile than previously assumed.

Since prevention is complicated by elusive transmission pathways combined with the lack of a vaccine, the control of BU relies on adequate treatment of patients. To date, rifampicin is the only effective drug against BU. However, rifampicin hepatitis is a commonly reported side effect of rifampicin which is known to be aggravated in tuberculosis patients with pre-existing infection with the hepatitis B virus (HBV). We assessed the burden of HBV in the BU endemic Offin river basin by analyzing serum samples of the general population for the presence of the hepatitis B surface antigen (HBsAg). The overall serum HBsAg prevalence was high with 8% of the population being chronic carriers. If stratified by age, we observed a low serum HBsAg rate of 1.8% among children below 12 years of age compared to 11.1% for participants older than 12 years. By phylogenetic analysis based on the pre-S/S region of HBV, we could classify all isolates obtained from the Offin river basin as genotype E and serotype *ayw4*. In addition we identified two main HBV/E clusters. While one cluster was composed of only strains from the Offin river basin, the second cluster was in addition to Offin isolates also made up of strains from other parts of Ghana and West African countries like Niger, Nigeria and Benin. We conclude that transmission of HBV along the Offin river is mainly horizontal and recommend strict adherence to vaccination protocols and periodic screening of populations within the river basin.

Altogether, the multi-disciplinary approach of this PhD thesis to investigating various aspects of *M. ulcerans* transmission and epidemiology has resulted in key finding which add to the existing knowledge of the pathogen in Ghana and globally.

Zusammenfassung

Buruli-Ulkus (BU) ist eine durch *Mycobacterium ulcerans* hervorgerufene, verheerende Hauterkrankung. Das Auftreten dieser Krankheit wurde in den letzten Jahrzehnten aus insgesamt 34 Ländern gemeldet, wobei die Infektionsbrennpunkte typischerweise in ländlichen Regionen liegen, in denen der Zugang zu medizinischen Gesundheitseinrichtungen eine Herausforderung darstellt. Da die Übertragungswege des Erregers bis heute nicht vollständig geklärt sind, ist die Erkennung und Behandlung von Patienten in möglichst frühen Krankheitsstadien die primäre Strategie, um die Krankheit zu bekämpfen.

Die erste in Ghana durchgeführte landesweite Suche nach BU Patienten im Jahre 1999 hat das Offin-Flussgebiet als eines der Gebiete in Ghana identifiziert, in dem BU am häufigsten auftritt. Aufgrund von jüngsten Einzelberichten, die andeuten, dass das Auftreten von Infektionen durch *M. ulcerans* entlang des Flusslaufs schwankend ist, haben wir im Rahmen dieser Doktorarbeit, eine umfassende aktive Suche nach BU Patienten und eine Haushaltserhebung in 13 ausgewählten Gemeinden entlang des Flusses durchgeführt. Dabei konnten wir einen allgemeinen Rückgang der BU Prävalenz verzeichnen. Nachfolgend haben wir ein aktives Überwachungssystem etabliert, das uns ermöglicht, das Auftreten von Fällen auf Haushaltsebene kontinuierlich zu verfolgen. Mit Hilfe dieses Systems konnten wir zeigen, dass eine kombinierte Bekämpfung von mehreren vernachlässigten Tropenkrankheiten wie zum Beispiel Lepra und Yaws möglich ist und weiterverfolgt werden sollte, um die begrenzten Mittel, die für die Bekämpfung dieser vernachlässigten Krankheiten zur Verfügung stehen bestmöglich einzusetzen.

Bis heute konnten Infektionsquellen und/oder mögliche Überträger des Erregers insbesondere in BU endemischen Gebieten Afrikas nicht vollständig identifiziert werden. Im Verlauf dieser Arbeit haben wir sero-epidemiologische Studien in Ghana und Kamerun durchgeführt, indem wir Antikörper-Antworten gegen ein *M. ulcerans*-spezifisches Antigen gemessen haben, um Rückschlüsse auf die Exposition von Bevölkerungsgruppen gegenüber dem Erreger ziehen zu können. Dabei haben wir festgestellt, dass Kinder ab einem Alter von etwa vier Jahren, die langsam beginnen sich freier zu bewegen und vermehrt Berührungspunkte mit der Umwelt haben, erstmals mit *M. ulcerans* in Kontakt kommen. Beim Vergleich des Alters in dem erste

Immunantworten gegen *M. ulcerans* und gegen andere Krankheitserreger mit unterschiedlichen Übertragungswegen einsetzen, haben wir festgestellt, dass serologische Reaktionen auf Antigene des von Mücken übertragenen Malaria-Erregers *Plasmodium falciparum* und der durch Kontakt mit verseuchter Erde übertragenen *Strongyloides* Würmer, früher einsetzt. Im Gegensatz dazu, zeigte sich dass die Immunantwort gegen Antigene der durch verseuchtes Wasser übertragenen *Schistosoma* Würmer - wie für *M. ulcerans* beobachtet - erst später einsetzt. Unsere Daten weisen darauf hin, dass die Übertragung von *M. ulcerans* durch Kontakt mit Umweltquellen des Erregers ausserhalb des kleinen Bewegungsbereichs von sehr jungen Kindern stattfindet.

In den vergangenen Jahren wurde allgemein angenommen, dass die Infektionsquelle von *M. ulcerans* geografisch relativ fixiert ist, da Ausbrüche der Krankheit typischerweise extrem fokal auftreten. In diesem Zusammenhang, wurde auch berichtet, dass eine enge Verbindung zwischen Genotyp und geografischer Herkunft der klinischen *M. ulcerans* Isolate besteht. Wir haben die Genomsequenzen einer begrenzten Anzahl von klinischen *M. ulcerans* Stämmen, die von Bewohnern des Offin-Flussgebiets isoliert wurden verglichen und zwei gleichzeitig vorkommende klonale Komplexe, die weder zeitlich noch räumlich voneinander getrennt waren, detektiert. Wir schliessen daraus, dass die Infektionsquelle von *M. ulcerans* mobiler zu sein scheint, als bisher angenommen.

Da die Herkunft und Übertragungswege von *M. ulcerans* unklar sind und bis heute kein Impfstoff verfügbar ist, wird BU weniger durch Prävention, als vielmehr durch die adäquate Behandlung von Patienten bekämpft. Bis heute ist Rifampicin das einzig wirksame Medikament gegen BU. Rifampicin-Hepatitis ist jedoch eine häufig auftretende Nebenwirkung, die dafür bekannt ist, dass sie in Tuberkulose Patienten, die mit dem Hepatitis B Virus (HBV) infiziert sind, sich noch schwerwiegender auswirkt. Wir haben die Krankheitslast durch HBV im BU-endemischen Offin-Flussgebiet erfasst, indem wir getestet haben, ob Blutseren der allgemeinen Bevölkerung das Hepatitis B Oberflächenantigen (HBsAg) enthalten. Die Gesamtprävalenz des HBsAg in den Seren war mit 8% hoch. Eine Unterteilung in Altersgruppen hat gezeigt, dass Kinder unter 12 Jahren mit einer 1.8% HBsAg-Rate vergleichsweise weniger betroffen waren als Studienteilnehmer über 12 Jahren, die eine HBsAg-Rate von 11.1% aufwiesen. Mit Hilfe von phylogenetischen Analysen, die auf der Sequenzanalyse der sogenannten pre-S/S Region des HBV basierten, konnten wir alle Isolate aus dem Offin Flussgebiet als Genotyp E und Serotyp *ayw4* klassifizieren. Zudem konnten wir zwei primäre HBV/E Komplexe identifizieren. Während

ein Komplex ausschliesslich Stämme aus dem Offin Flussgebiet beinhaltete, setzte sich der zweite Komplex nicht nur aus Offin Stämmen, sondern auch aus Isolaten aus anderen Teilen Ghanas und West Afrikas, wie zum Beispiel Niger, Nigeria und Benin zusammen. Unsere Schlussfolgerung ist, dass die Übertragung des HBV entlang des Flusslaufes primär auf horizontalem Weg erfolgt und wir empfehlen daher eine strikte Einhaltung der Impfeempfehlungen und eine regelmässige Untersuchung der Bewohner des Flussgebiets.

Zusammengefasst, hat der multidisziplinäre Ansatz dieser Doktorarbeit, der die Untersuchung verschiedener Aspekte der Übertragung und Epidemiologie von *M. ulcerans* zum Ziel hatte, wichtige Erkenntnisse geliefert, die massgeblich das schon vorhandenen Wissen über den Erreger in Ghana und auch weltweit erweitern.

Chapter 1

Introduction

1.1 History and Epidemiology

In an account of his expedition to identify the source of the Nile river, James Grant described a swelling that developed on his right leg in 1861. He reported his lesion as discharging profusely, evolving into an ulcer which healed slowly but left him with residual scars and contractures. These descriptions are considered to be consistent with the edematous form of BU. Later in 1897, Sir Albert Cook described cases of what was initially thought to be tubercular ulcerations in Kampala Hospital in Uganda. The same hospital in 1910 also recorded a case with an ulcerated septic leg and a swelling which was also consistent with the edematous form of BU. In 1940, a two year old boy with ulcers on his leg was admitted to a hospital in Bairnsdale, Australia. Examination of a biopsy sampled from his lesion revealed histological features that were not befitting classical tuberculosis. This led to a further probe and based on these features being consistently observed in five other patients, the first definitive clinical description of the disease, which was later locally referred to as Bairnsdale ulcer [1], was published in 1948 [2]. Around the same period, hundreds of similar ulcers were reported from Africa with the majority coming from the then Belgian Congo (now Democratic republic of Congo) and Uganda. However it was not until the late 1950s and 1960s that the cases were published. Most cases by then were reported from the Buruli county of the then Mengo district in Uganda which led investigators to refer to the disease as Buruli ulcer [3]. The first probable case identified in Ghana was in the year 1971 [4]. By the 1980's several endemic foci were reported in many countries with West Africa recording the majority of cases [5–8]. By 1998, BU had become a global concern and Ghana as an example had recorded nearly 1,200 cases in four of its ten regions after a 5 year surveillance. In the same year the World Health Organization (WHO) launched the Global Buruli Ulcer Initiative (GBUI) to promote awareness and research efforts on the disease. As shown in Figure 1, by the year 2012, BU has been reported or suspected in at least 32 countries with Ghana recording an overall national prevalence of 20.7 per 100,000 population in the first nation-wide active case search in 2002 [9].

Equal rates of BU have been reported for both males and females [9–13]. The disease affects all age groups but the majority of cases in Africa has been recorded in children with a peak of incidence in the 10 to 14 year olds [11,14–20], whereas in Australia adults older than 60 years have accounted for most cases [21]. However by adjusting for population age structure, recent studies in Africa have shown a bimodal age-related risk of developing BU with the highest risk

in children aged between 4 and 14 years and in the elderly [22,23]. As reviewed [24], reported risk factors for the disease have varied from study to study although consistently, residence or activities within or near slow moving water bodies have been identified by most studies. Thus it appears the epidemiology and risk factors associated with occurrence of BU are geographical setting-specific, which speaks for the need for studies aimed at understanding these factors for individual BU endemic areas

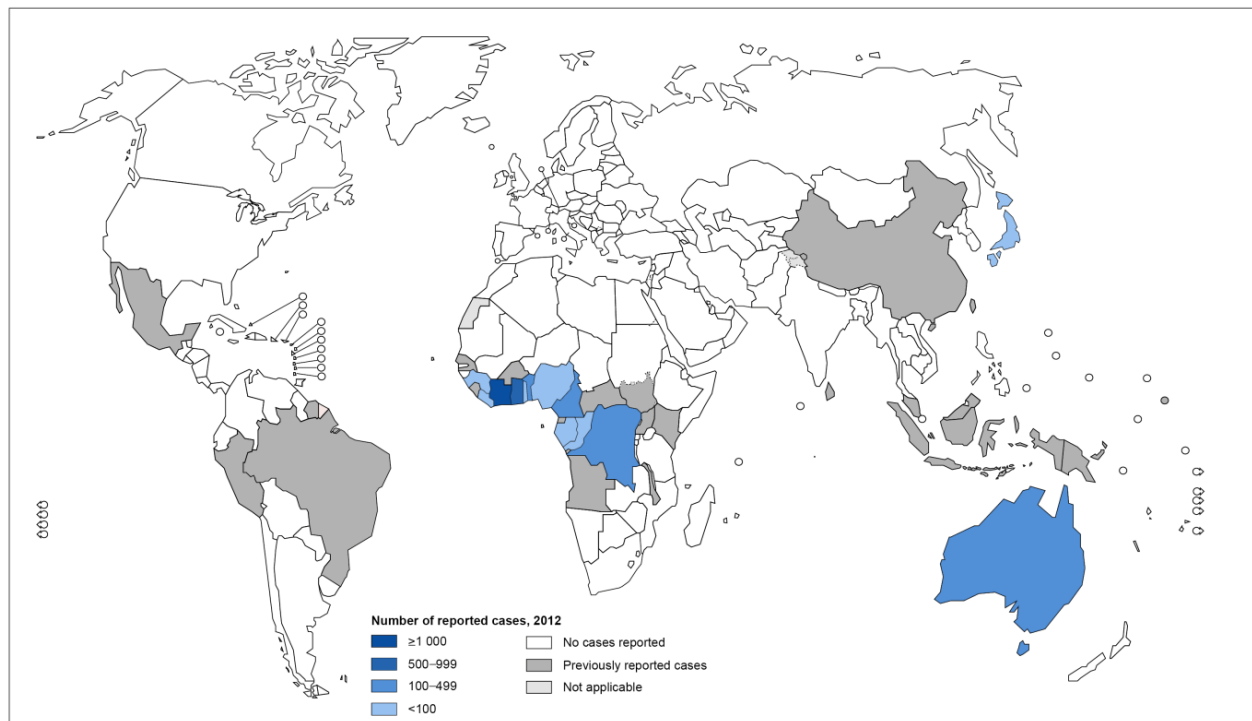


Figure 1.1. Distribution of BU worldwide by the year 2012. Map adapted from the W.H.O
http://www.who.int/buruli/Buruli_2012_global.png?ua=1

1.2 Causative agent

The pathogen that causes BU is *Mycobacterium ulcerans* and taxonomically, is a member of the phylum actinobacteria, in the order actinomycetales, suborder corynebacteriaceae and the genus mycobacterium. It has a high G+C content (65%) DNA and an unusual cell wall with a lipid-rich layer beyond the peptidoglycan layer. Due to its long generation time, which has been estimated to be around 20 hours; it is described as slow-growing mycobacterium and the optimum growth temperature is between 30 and 33°C at pH of 5.4-7.4. Though the pathogen is usually cultivated under aerobic conditions it has been reported to grow better under micro-aerophilic conditions in liquid cultures.[25]. Genome comparison of *M. ulcerans* and the fish pathogen *Mycobacterium marinum* revealed a close genetic identity (>97% nucleotide identity) between both pathogens indicating a close genetic relationship [26–28]. However, the genome of *M. ulcerans* was shown to harbor a 174kb plasmid, the pMUM001 which is absent in *M. marinum*, indicating that the acquisition of this plasmid was a key event in the evolution of *M. ulcerans* and was most likely by horizontal gene transfer [29,30]. The plasmid encodes the polyketide synthases which produce mycolactone, a macrolide toxin at the core of the pathogenesis of *M. ulcerans* infection. Through whole genome sequencing of the first ever complete genome of *M. ulcerans*, strain Agy99 isolated from a Ghanaian BU patient, a more detailed look at the evolutionary scenario was made possible [31]. The genome size of *M. ulcerans* is approximately 5.8 Mb, compared to that of *M. marinum* which is approximately 6.6 Mb. This extensive reduction in genome size is indicative of an adaptation to a protected niche environment, where genes formerly needed for survival under diverse conditions are no longer necessary. However, it is not clear to date, to which environmental niche *M. ulcerans* is adapting to, although an adaptation within the aquatic ecosystem is very likely. Detailed genome analyses of *M. ulcerans* isolates of patients from diverse geographical origin revealed extensive large sequence polymorphisms, facilitating a differentiation of *M. ulcerans* clinical isolates into two principal lineages named the ancestral and the classical lineage. While ancestral lineage strains are closely related to *M. marinum* and are only sporadically reported to cause disease in Asia and the Americas, classical lineage strains are associated with highly endemic BU foci in Africa and Australia. The reduction in copy numbers of genes encoding highly immunogenic proteins particularly in classical lineage strains suggests the need for *M. ulcerans* to evade the immune system of its new host [32]. Altogether,

M. ulcerans seems to be evolving from being an environmental pathogen and clues from genomic analysis indicate its preparing itself to be better adapted to a new niche environment.

1.3 Reservoir and transmission

Unlike tuberculosis and leprosy, person to person transmission of BU is very unlikely. Epidemiological association and occurrence of BU along slow moving and stagnant water bodies led to the hypothesis that transmission involves a reservoir in the aquatic ecosystem [33–36], from which humans are being infected via skin abrasions or through insect bites. However, several attempts at cultivating the bacteria from a myriad of environmental samples have proven futile with only one isolate in pure culture characterized so far [36]. In view of this, environmental studies aimed at understanding the ecology of *M. ulcerans* relied on the detection of *M. ulcerans* DNA by IS2404 PCR as evidence of the bacteria in the environment [35,37–41]. The use of IS2404 PCR in the analysis of environmental samples was complicated by the discovery that some other mycobacteria do possess this target. [42–44]. This necessitated the development of two multiplex real-time PCR assays targeting distinct repeated sequences in the *M. ulcerans* genome [45] and the giant plasmid. The target sequences which included IS2404, IS2606 and genes encoding the ketoreductase B domain of the mycolactone polyketide synthases, have provided a more specific and robust interpretation of environmental data over the years.

Some studies have shown that within the aquatic environment, *M. ulcerans* could be concentrated through the various feeding levels with predacious aquatic insects [38,46,47], fishes and amphibians [37,48] identified as potential reservoirs. In Australia, *M. ulcerans* DNA was detected in almost half of possum feces excreted into the environment; a significant proportion (41%) of which were detected in the BU endemic Point Lonsdale when compared to 1% detected in other non-endemic areas. This led to the implication of possums as potential reservoirs of the pathogen in this endemic setting [49]. However, to date, no similar animal reservoir has been found in African BU endemic areas [50], it is hypothesized that BU patients with chronic ulcerative lesions may play an active role in the dissemination of *M. ulcerans* in the environment in these settings [51]. During an outbreak of BU in Victoria (Australia), investigations lead to the capture of more than 11,000 mosquitoes of four different species which were pooled into 957 groups. A total of 13 pools out of the 957 were positive for *M. ulcerans* DNA based on real-time

PCR probe for all three targets suggesting that adult mosquitoes may serve as biological vectors of the pathogen [52]. Findings of some studies have however contested the role of mosquitoes in the transmission of the pathogen. One study demonstrated that while *M. ulcerans* DNA could be detected in larvae after feeding in an experimental model, the bacteria were not maintained through pupation and development into adult mosquito. Additionally, external body parts of adult mosquitoes were readily contaminated with the bacteria while feeding, whereas the guts lacked detectable *M. ulcerans* DNA [53]. A more recent field study conducted in Benin also failed to detect *M. ulcerans* DNA in a total of 7,230 flying insects which included 4,322 mosquitoes from 10 different species captured over a year [54]. Finally, analysis of the anatomical distribution of BU lesions in laboratory confirmed cases from Cameroon revealed patterns which were inconsistent with mosquito bites [23].

Taken together, these findings suggest that several modes of transmission of *M. ulcerans* should be considered.

1.4 Pathogenesis and immune response to *M. ulcerans* infection

Initial suspicion of a toxin-mediated pathogenesis of *M. ulcerans* infection was deduced from histological examination of biopsies of index patients from Australia which revealed copious amounts of bacilli clumped within a large necrotic core [2]. The toxin, later named mycolactone [55], has been shown to induce apoptosis and necrosis of many cell types including fibroblasts, leukocytes and adipocytes [56–59]. Within *in-vitro* models, the toxin was shown to inhibit the phagocytosis of the bacteria by macrophages [56,57,60,61]. In view of this, *M. ulcerans* was largely considered to be an extracellular pathogen with mycolactone being the key virulence factor.

These histological features which were consistently observed in biopsies of patients over the years are now known to be characteristic for late stage *M. ulcerans* infection. During the early phase of *M. ulcerans* infection, colonization of macrophages and to lesser extent neutrophils by the bacteria has been shown in studies of mouse [58,62] and guinea pig [63] infection models as well as in biopsies of untreated BU patients [62,64]. These observations corroborate several studies that highlight cell-mediated immune response to *M. ulcerans*. As reviewed elsewhere [65], delayed-type hypersensitivity response to the burulin skin test has been reported frequently in patients with advanced or healed lesions but rarely in patients with early staged lesions. In

addition, PBMC's from BU patients have demonstrated a significantly higher production of IFN- γ in later stages of the disease than in the early stages [66,67]. Expression of IFN- γ was also shown to be higher in pre-ulcerative lesions than in the ulcerative forms by quantitative PCR [68]. These data also indicate that protective immunity to *M. ulcerans* by Th1 response is possible.

In addition to cell mediated immunity, sero-epidemiological studies have demonstrated that individuals can mount specific humoral immune response to *M. ulcerans* infection. While investigations of humoral immune responses against mycobacteria are usually hampered by a high degree of antigenic cross-reactivity between species, investigations on exposure of populations to *M. ulcerans* were made possible by the identification and characterization of the 18 kDa small heat shock protein overexpressed by the pathogen [69,70].

1.5 Clinical presentation and Diagnosis

Typically within endemic settings in Africa, initial clinical manifestation of the disease is a firm painless nodule. Other pre-ulcerative presentations are edema, plaque and papule. While edema represents the most extensive pre-ulcerative form with ill-defined margins, papules are less than a centimeter in size and are commonly reported in Australia but absent in Africa. Plaques on the other hand have irregular edges and are more present in Africa but absent in Australia. When left untreated for some weeks, the pre-ulcerative forms progress to ulcers which are characterized by an eroded skin surface with well demarcated margins and undermined edges. The list of differential diagnosis is endless in tropical settings, where other skin conditions such as sebaceous cyst, cutaneous tuberculosis, leprosy, yaws, lipoma, cutaneous leishmaniasis, tinea, noma etc. resemble disease caused by *M. ulcerans* [71–73]. Diagnosis of BU was mainly based on clinical evidence until 2001 when the WHO recommended the inclusion of laboratory diagnostic tests in the confirmation of BU cases. Available tests are microscopic detection of Acid Fast Bacilli (AFB) in a Ziehl-Neelsen (ZN) stained smear, *M. ulcerans* culture, detection of *M. ulcerans* DNA by IS2404 PCR and histopathological analysis of skin sections. The ZN test which is already being employed at the district level for the diagnosis of TB has a very low sensitivity [74] and is also not specific for *M. ulcerans*. Culture takes an average of nine to twelve weeks to yield positive results making it an unfavorable option for timely diagnosis and treatment. Despite having high sensitivity and specificity, the IS2404 PCR and histopathological

analysis require sophisticated instrumentation and skilled operator (personnel); thereby restricting their availability and operation to reference laboratories [75–79]. Currently, a total of 17 reference laboratories assist the control programs of 10 countries with the confirmation of BU mainly by IS2404 PCR [80].

Nevertheless, BU cases are still being diagnosed solely on clinical presentation at the periphery of endemic countries in Africa. Research aimed at developing a simple point-of-care (POC) diagnostic tool has gained a lot of attention. One of such tools is the loop-mediated isothermal amplification (LAMP) which like the IS2404 PCR, is also based on the amplification of *M. ulcerans* DNA but under isothermal conditions [81–83]. It is worth noting that the LAMP assay doesn't require purified *M. ulcerans* DNA thereby cutting away time, cost and skill required for DNA isolation. In addition, higher specificity is achieved with the assay by using four to six specifically designed primers targeting different sections of the DNA sequence. Another property of *M. ulcerans* infection that has been exploited for POC assays is the ability of mycolactone to diffuse beyond infection foci, making it detectable in circulating blood [84]. Previous studies have demonstrated that detection of Mycolactone A/B by conventional thin-layer chromatography (TLC) is possible; however a minimum of 20 to 30ng concentration of the lipid is required [84–86]. An investigation conducted to address this draw-back lead to discovery that reaction of mycolactone with 2-naphthylboronic acid in a TLC flourogenic model lowers the detection limit of mycolactones from 20ng to 10ng [87]. The assay has been studied in the mouse foot pad model [75] and is currently being further evaluated within a clinical trial setting. Another noteworthy approach is the antigen capture assay which was motivated by the identification and characterization of the *M. ulcerans* protein MUL_3720 [89]. The protein, which has no orthologue in other prevalent pathogenic mycobacteria, is abundantly expressed and localized within the cell walls of *M. ulcerans*. This makes it a favorable candidate for different assay formats since the absence of orthologues in other mycobacteria will enhance its specificity and the localization within the cell wall makes it more accessible in diagnostic samples such as swabs. Work is on-going to enhance the sensitivity of this assay.

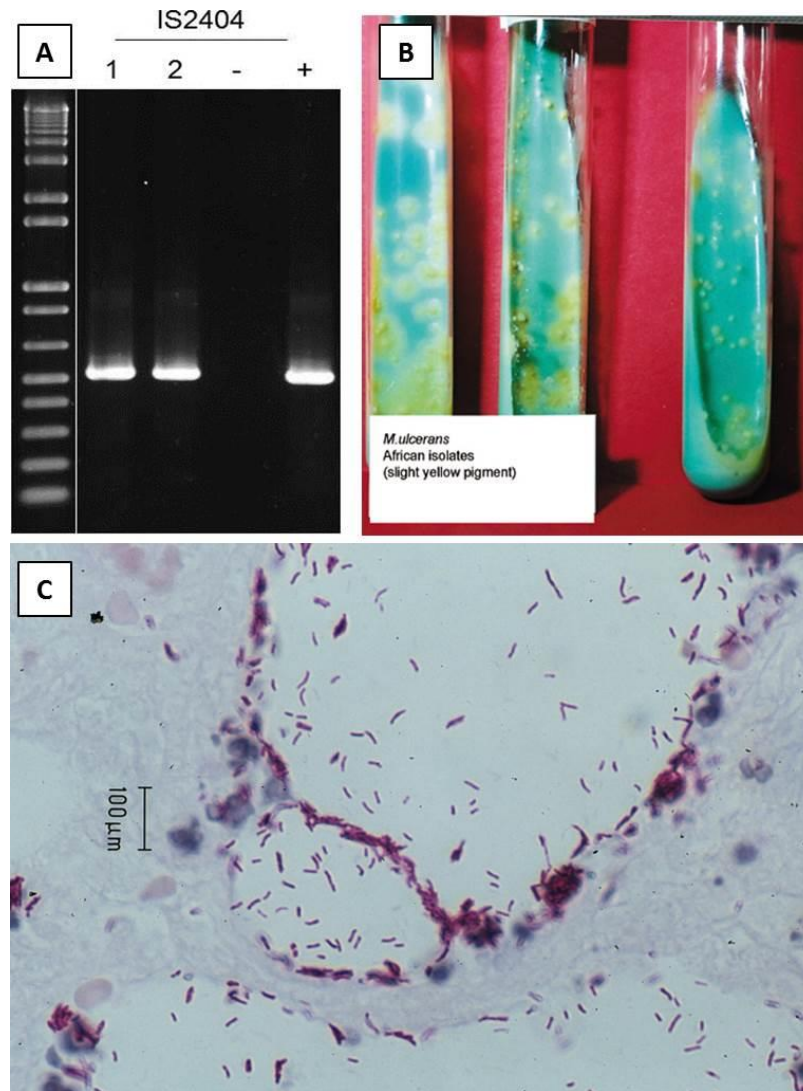


Figure 1.2. Laboratory diagnosis of BU. 2A Detection of *M. ulcerans* DNA in clinical samples by IS2404 PCR. 2B Cultivation of *M. ulcerans* on LJ media. 2C Microscopic detection of Acid fast bacilli (AFB) in a ZN stained smear. 2A and 2B were adapted from W.H.O <http://www.who.int/buruli/photos/diagnosis/en/>

1.6 Treatment and case management

Although the search for anti-mycobacterial agents for *M. ulcerans* dates as far back as the 1950's [90,91], the mainstay treatment of BU for decades was surgical excision of necrotic tissue and skin grafting for very large lesions. As it was with clinical diagnosis, treatment success depended highly on the expertise of the surgeon and recurrence rates between 6-28% were reported during this period [92–95]. Based on the activity of anti-mycobacterial agents in a mouse model [96] and a successful clinical trial in Ghana [97], the WHO in 2004 recommended a standard treatment of injectable streptomycin (STR) and oral rifampicin (RIF) for 8 weeks. Most control programs have implemented this regimen till date with diminished recurrence rates of 0-2% and surgery being employed mainly for correction of functional disabilities [97–99]. Concerns over challenges with general application of STR such as loss of hearing (side effect) [100], intramuscular administration to very young children and contraindication in pregnancy, led to the search for an all oral alternative. Some recent studies have demonstrated that substitution of STR with clarithromycin (CL) is equally efficacious in any of following combinations; RIF and CL for 8 weeks [101], RIF and STR for two weeks followed by RIF and CL for six weeks [102] or RIF and STR for four weeks followed by RIF and CL for four weeks [103]. Therapeutic application of heat (of about 40°C) on BU lesions (thermotherapy) also represents an alternative treatment approach. By exploiting the inability of *M. ulcerans* to proliferate well above 37°C [104], heat applied to lesions over a period of time have resulted in the successful treatment of some BU cases [105–107]. Although considered by the WHO as a viable alternative [108], thermotherapy is yet to be incorporated into the general treatment practice for BU.

Even with successfully treated cases, scarring deformities (cosmetic and functional) were notably associated with BU until 2006 when the WHO made a focal commitment to the prevention of disability [109]. This has resulted in the incorporation of physiotherapy in the treatment package offered to BU cases. In Ghana, well-equipped physiotherapy units have been established in health centers like Nkawie Government Hospital and Agogo Presbyterian Hospital of the Ashanti Region.



Figure 1.3. Clinical presentation of BU. 3A Nodule 3B Edema 3C Plaque 3D Ulcer
Images adapted from W.H.O http://www.who.int/buruli/photos/forms_large/en/

1.7 Control and prospects for a vaccine

With the mode of transmission of *M. ulcerans* not fully elucidated, the main approach to control has been early case detection and treatment to prevent deforming sequelae. In line with this, Buruli ulcer control programs have been established in more than 10 countries worldwide. The control programs usually operate within the framework of the National Health Service. In countries like Cameroon, BU control is integrated into that of leprosy, yaws and leishmaniasis to maximize the limited resources allocated for the control of these diseases. In Ghana, non-governmental initiatives such as *Stop Buruli* which is funded by the UBS Optimus Foundation provide support with active case search, transportation of patients to and from the health facilities as well as providing breakfast throughout the treatment period. This social intervention strengthened community participation in BU control which reflected in an increase in the number of case referrals by volunteers and school teachers in the Ga South district [110].

Ideally, vaccination would serve as the best approach to the control of BU in highly endemic settings. However, there is no effective vaccine against BU till date. As far back as 1957, the *M. bovis* BCG (Bacille Calmette Guérin) vaccine was shown to confer protection against *M. ulcerans* in a murine footpad model [111]. The vaccine also demonstrated an overall 47% efficacy in a randomized control study involving 2,500 refugees living in Uganda. This protection was however short-lived, diminishing from 72% to 0% after 6 months [112]. No evidence of a protective effect on the risk of developing BU was found in a number of case-control studies [113–116]. However, a study conducted in Benin reported a significant association of effective BCG vaccination at birth with protection against the osteomyelitic form of BU in both children and adults [19]. The evidence of a cross-reactive protective role of BCG vaccines and investigations into the underlying mechanisms led to the identification and characterization of the *M. ulcerans* homologue of the BCG antigen (Ag) 85A. Earlier experiments demonstrated that mice immunized with DNA vaccines encoding Ag85A of BCG vaccines were protected from intradermal challenge with *M. ulcerans* [117]. As expected, DNA vaccine encoding Ag85A from *M. ulcerans* demonstrated a higher protective efficacy than that encoding Ag85A from BCG when both were compared in an *M. ulcerans* footpad model [118]. An alternative vaccine strategy involves the use of a viral replicon particle as an apparatus for delivering *M. ulcerans* antigens to the immune system. The vesicular stomatitis virus (VSV) recombinant replicon particle was constructed by replacing the VSV glycoprotein G gene with

two *M. ulcerans* genes, the MUL_2232 and MUL_3720. Immunization of mice with the virus replicon particles resulted in a slight but significant decrease in bacterial load in an *M. ulcerans* footpad model [119].

In spite of the challenges faced in the development of an effective vaccine against BU, the ability of humans to mount protective immune response against *M. ulcerans* as demonstrated by only a small proportion of exposed individuals developing the disease eventually [69,120,121] and reports of some self-healing cases [122], continue to motivate research in this field.

1.8 Co-morbidity: BU and Hepatitis

Besides being the only effective antibiotic identified for BU till date, rifampicin is part of the five “first-line” antibiotic therapy for tuberculosis. As far back as 1972, a high proportion of patients being treated for tuberculosis were reported to develop abnormal liver function test (LFTs) and histological features suggestive of hepatitis. This led to the first clinical description of what was termed “Rifampicin hepatitis” in 1974 [123]. The report indicated that 10 out of 11 patients developed the symptoms within six weeks of starting rifampicin therapy.

Incidence of this condition, also known as drug-induced hepatotoxicity (DIH) has been reported in the management of other diseases like Pruritus where rifampicin is administered [124,125]. Although overt liver damage from rifampicin is rare, some studies have reported that pre-existing liver damage resulting from Hepatitis B virus (HBV) infection for example, could be aggravated [126,127]. Specifically for tuberculosis patients undergoing chemotherapy, a higher incidence of hepatotoxicity has been observed in those pre-infected with HBV (both carriers and diseased) than controls although the sole effect of rifampicin was debated in some reports [128–132]. While some studies indicate that HBV infection is highly endemic in sub-Saharan Africa, the exact burden of the disease in most countries is unknown [133]. Particularly in rural areas where other diseases like BU are also suspected to be present and underreported, co-endemicity of both diseases presents a complex challenge to clinicians.

It is also of Public Health importance that transmission of hepatitis particularly in BU endemic areas is monitored closely in order to better inform control strategies for both diseases. Furthermore, the availability of a free and effective vaccine against Hepatitis B necessitates

studies which monitor the efficiency of the vaccination program and the burden of the disease in such endemic regions.

The sixth chapter of this study assesses the burden of Hepatitis B and Delta virus infection within the BU endemic region of the Offin river valley and highlights some findings relevant for the control of the disease both within the study area as well as nationwide.

1.9 Goal

This PhD study was set up to investigate the epidemiology of BU in communities along the Offin river valley of Ghana within the context of the under-listed objectives. We anticipate that findings of this thesis will contribute to the knowledge of transmission of *M. ulcerans* within the river basin as well as in Ghana.

1.10 Objectives

1. To characterize the epidemiology of BU in 13 selected communities along the Offin river
2. To assess the age of sero-conversion to the *M. ulcerans* 18kDa shsp as a reflection of the onset of exposure to *M. ulcerans*
3. To compare the age-pattern of first humoral immune responses to the 18kDa shsp of *M. ulcerans* with those against pathogens with different modes of transmission
4. To study the dynamic population structure of *M. ulcerans* by whole genome sequencing and analysis of the bacteria isolated from cases residing within the Offin river basin
5. To study the prevalence of Hepatitis B and D virus infection and characterize the genetic diversity of the virus within the river the basin

1.11 References

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Chapter 2

Burden and Historical Trend of Buruli Ulcer Prevalence in Selected Communities along the Offin River of Ghana

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2.1 Abstract

Buruli ulcer (BU) is a neglected tropical skin disease caused by *Mycobacterium ulcerans* with more than two thirds of the global cases reported in West Africa. A nationwide active BU case search conducted in 1999 identified two health districts along the Offin River as two of the three most endemic districts in Ghana. Based on recent anecdotal accounts that transmission is unstable along the Offin River, we conducted from March to June 2013 an exhaustive household survey and active case search in 13 selected communities within a five-kilometer radius along the Offin River. The overall prevalence of BU was 2.3% among the surveyed population of 20,390 inhabitants and 477 of the total 480 cases detected (99.4%) were historical (healed) cases. By estimating the year of occurrence for each case per community and taking into account available passive surveillance records of health facilities and the District Health Directorate, we observed a general trend of continuous emergence of cases in communities located midstream the Offin River whereas downstream communities showed more sporadic patterns. We monitored the incidence of cases after the survey and recorded a cumulative incidence rate of 0.04% for the 13 communities over a 17-month active surveillance period from August 2013 to December 2014. Our data reveal an overall decline in BU incidence along the Offin River similar to the general decline in BU incidence in recent years reported by the World Health Organization for West Africa.

Keywords: Buruli ulcer, Prevalence, Incidence, Active surveillance, Ghana, Offin River

2.2 Author summary

Buruli ulcer (BU) is a tropical skin disease caused by *Mycobacterium ulcerans* and more than two thirds of the global cases reported in West Africa. The Offin has been considered the most endemic river valley in Ghana following a nationwide active case search conducted in 1999. Here, we present findings of an exhaustive household survey and case search of 13 selected communities along the Offin river aimed at addressing recent anecdotal accounts of unstable transmission of *M. ulcerans* within the river basin. We observed among the surveyed population of 20,390 inhabitants, an overall 2.3% prevalence of BU with 99.4% of the total cases detected being historical cases. We also observed a general trend of continuous and sporadic emergence of cases in mid and downstream communities, respectively. Subsequently, we detected a total of eight cases (0.04% cumulative incidence rate) in a prospective 17-month active surveillance of all 13 communities. These data confirm the recent decline in BU incidence in historically endemic communities along the Offin river basin, analogous to the observation made in recent years by the World Health Organization for West Africa.

2.3 Introduction

Buruli ulcer (BU) is a necrotizing skin disease caused by *Mycobacterium ulcerans* [1]. The disease has been reported in over 30 countries worldwide, mainly in the tropics, but the brunt of it seems to be mainly experienced in West Africa with Côte d'Ivoire, Ghana, Benin and Cameroon reporting more than 80% of the global number of cases [2]. Within the endemic countries, BU occurs in foci typically affecting inhabitants of impoverished and rural settings where access to medical care is a big challenge [3].

Control of BU is based mainly on early case detection and adequate antibiotic treatment and wound management. The current treatment regimen recommended by the World Health Organization (WHO) includes daily administration of streptomycin and rifampicin for eight weeks [4]. Advanced lesions may require debridement and/or skin grafting as an adjunct to improve healing and to prevent or correct deformities [5–7]. Nevertheless BU treatment is often associated with long hospital stays and represents a major socio-economic burden in the affected communities [8].

In Ghana, nearly 1,200 BU cases were reported between 1993 and 1998 by the first passive surveillance system established in the country and between 2004 and 2014 more than 9,000 cases have been reported. A nation-wide active case search conducted in 1999 resulted in an overall crude prevalence rate (clinically diagnosed active lesions) of 20.7 per 100,000 inhabitants [9]. The most endemic district identified within this study was the Amansie West District, which is drained by the Offin river. The river takes its source from the Mampong scarp in the Ashanti region, flows from Boanim community through Bipoa community and eventually joins the Pra river in the Central Region (Fig 2.1). With farming and alluvial gold mining being the characteristic human activities in the river basin, spatio-epidemiological studies have consistently associated these activities with BU [10,11].

From June to July 2012, we paid reconnaissance visits to all 11 Health Districts drained by the Offin River. Anecdotal accounts gathered from interactions with local health staff and leaders of some selected communities indicated the absence of recent (in the past 3 to 5 years) cases in some communities historically known to be BU endemic and the emergence of new cases in some non-endemic communities. Since BU case data at the local and district health facilities were scanty, we set up this study with the following specific objectives: i) to determine the prevalence of BU in 13 selected communities in the Offin river basin, ii) to characterize the

retrospective occurrence of BU cases for each community based on active surveillance data and available case data at the district and local health centers. We also report on active surveillance activities conducted to prospectively monitor the emergence of new BU cases in the selected communities.

2.4 Materials and Methods

Ethics statement

Ethical clearance for this study was obtained from the institutional review board of the Noguchi Memorial Institute for Medical Research, NMIMR, with a federal wide assurance number FWA00001824 and the ethics review committee of the Ghana Health Service (ethical approval ID number GHS-ERC:06/07/13). Participation in all aspects of the study was voluntary and all confirmed cases - independent of their participation- were treated according to the treatment guidelines established by the National Buruli Ulcer Control Program (NBUCP). Written informed consent was obtained from all patients before their lesions were sampled for laboratory diagnosis. Parents or guardians provided written consent on behalf of all child participants.

Study area

This study was conducted in the Offin river valley of Ghana (Fig 2.1). The Akans, who form the largest ethnic group in Ghana, are the main inhabitants of five out of the ten administrative regions (Ashanti, Central, Western, Eastern and Brong –Ahafo). The Offin river drains two of these regions namely the Ashanti and Central Regions. Due to the intense gold mining activities carried out in the river basin, anecdotal accounts suggest that this preponderance breaks down at the community level due to the influx of migrants from other parts of Ghana and neighboring West African countries.

Our study area presents major landscape differences between communities located upstream the river on one hand, and those located mid and downstream on the other hand. As illustrated in Figure 2.2, land cover of upstream communities Bedomase (A) and Krakrom (B) was mainly farmland. In contrast, the peripheries of the mid and downstream communities were generally characterized by heavy mining activities as exemplified by Ntobroso (C) and the downstream community Pokukrom (D). In addition, our assessment of the elevation patterns with respect to the Offin river course revealed that the highlands of Mampong in the Ashanti region from which the river takes its source were at least 401m (1,315 ft) high (Fig 2.3C). Thus communities A to C located upstream the river were situated in areas with altitudes of at least 256m. Conversely, the midstream to downstream region of the Offin river recorded relatively lower elevation values ranging from 173m to 98m (569-320 ft).

Following a nation-wide active BU case search [9], the NBUCP maintained a database of geographic co-ordinates of communities visited. We obtained from the NBUCP geographic co-ordinates of all communities in the 11 health districts of the Offin river valley. Using the ArcGIS 10.0 mapping software, we created a buffer of five kilometers along the river. This seeded a total of 199 communities from which we selected 10 by simple randomization using a randomization tool embedded in the software (Fig 2.3A). The selected communities were Bedomase (A) and Krakrom (B) of the Sekyere south district, Kapro (C) of the Atwima Nwabiagya district, Ntobroso (E) of the Atwima-Mponua district, Keniago (G) and Tontonkrom (H) of the Amansie West district, Dominase (I) and Nkotumso (J) of the Upper Denkyira West district, Wromanso (K) of the Amansie Central district and Pokukrom (M) of the Upper Denkyira East district (Fig 2.3A). Based on recommendations of local health staff, three additional communities known to be BU endemic were included to bring the total number to 13. These communities were; Akomfore (D) and Achiase (F) of the Atwima Mponua district, and Mfantseman (L) of the Upper Denkyira East district. Other selected communities known to be endemic based on available passive surveillance records were Ntobroso (E), Keniago (G), Tontonkrom (H), Dominase (I), Wromanso (K), Pokukrom (M) and Nkotumso (J). Passively, no BU case has ever been reported for Bedomase (A) and Krakrom (B). We were unable to substantiate the endemicity of Kapro (C) prior to the survey.

Community entry

Entry into each community was carried out by a team consisting of a research assistant from the NMIMR, a field officer from NBUCP, a local disease control officer (DCO) and a community based surveillance volunteer (CBSV). We met chiefs, traditional and opinion leaders of each community to whom we explained the structure, aims and benefits of the study. Once we received their approval, information on the study was relayed to the community members through the CBSV and community information delivery systems.

Data collection

Between February and May 2013, we conducted an exhaustive household survey and active case search for BU. We formed teams each consisting of one research assistant from NMIMR, one local health staff routinely involved in the management of BU cases and one community

volunteer. To aid in the description of the disease to participants being interviewed, each team was equipped with posters and picture charts illustrating the clinical forms of BU. Members of the teams received training on the clinical signs of BU, how to fill the survey forms and also how to take GPS co-ordinates. The teams went out to each inhabitable structure, numbered them serially and interviewed all inhabitants who were present. Households of inhabitants who were absent were noted and in two follow-up sessions on study participants (July and December 2013), we interviewed those absentees who were now available. Information on minors was provided by their parents or guardians. In addition to demographic data, clinical data were also collected for detected cases and GPS co-ordinates for every household.

Definition of variables

An inhabitant was classified as a part-time resident of a community if in the past year prior to the survey the person; a) travelled and stayed out of the community for more than 3 months and b) had his livelihood (such as occupation and education) in another community such that he spent more than 6 hours of his day-time there. A full-time resident on the other hand a) never travelled out of the community in the past year or b) travelled but stayed no longer than 3 months and c) had his livelihood in the community being surveyed and thus spent more than 6 hours of his day time there.

Each time we completed surveying a community, we extracted information on inhabitants with suspected lesions and revisited them together with a clinician of extensive experience in diagnosing BU. The clinician then examined the lesions and diagnosed them as either active or inactive (healed) BU or as BU-unrelated lesions. Generally, depressed stellate scars were anecdotally considered as healed BU lesions whereas healed lesions with cleared skin areas were considered as BU-unrelated. Thus, cases with both active and healed lesions were included in this study. Clinically diagnosed active lesions were microbiologically confirmed by the detection of *M. ulcerans* insertion sequence (IS) 2404 by diagnostic PCR. In addition, BU lesions were clinically classified according to the WHO guidelines. Category I lesion was defined as a single lesion of size less than or equal to five centimeters at the widest diameter, category II as a single lesion between five and 15 cm and category III as either a single lesion greater than 15 cm or multiple lesions or a lesion at critical areas of the body.

Estimation of the year of emergence of BU cases in each community

Using posters and charts the clinical features of BU was described to the participants after which, we asked participants if they have ever had an episode of BU at any point in their lives. This was done to determine the overall crude life time prevalence of BU in the population surveyed. However, in order to assess the historical emergence of cases for each community, we restricted our analysis of the healed cases to a 24 year prevalence period (1990 to 2013) and compared our data with passive surveillance records obtained from 2000 to 2013.

Admittedly, such retrospective analyses are prone to recall bias particularly for BU where the appearance of symptoms does not reflect the moment of contracting the disease due to a relatively unknown incubation period. For BU cases with healed lesions, we estimated the year of developing the symptoms of the disease by i) examining health records and BU 01 forms (if available) and ii) verbally interviewing the cases and confirming from at least two other household inhabitants or nearby neighbors who were present and witnessed the case having the disease. To facilitate recall in healed cases of more than one year history, estimation was done by reference to any other household member or close neighbor who was born around the period the case developed symptoms of the disease. Once this estimation was confirmed by two other inhabitants present at the time of developing the clinical symptoms of BU, the period was estimated and noted using the year of birth of the inhabitant referenced.

Active surveillance of BU

Subsequent to the exhaustive household survey, we continued to monitor the emergence of BU cases using two approaches: i) community outreach program and ii) monthly household visits by community volunteers. We conducted the community outreach once every three months in all 13 selected communities. Specifically in the months of July 2013, September 2013, December 2013 to January 2014, March 2014, July to August 2014 and November 2014. This program involved one evening of educating the community members on transmission, early case detection and treatment by showing BU documentaries and interacting with them through questions and answers. The following morning, the inhabitants were screened and those with clinically suspected BU lesions were sampled for laboratory confirmation.

For seven of the 13 communities (Achiase (F), Ntobroso (E), Akomfore (D), Keniago (G), Pokukrom (M), Wromanso (K) and Mfansteman (L)), we employed a monthly household-visit

based surveillance as an additional tool to the surveillance by the community outreach program. All seven communities were selected based on the willingness of the CBSV to voluntarily carry out the exercise. We trained and equipped one CBSV from each of the afore-mentioned communities with android phones (HTC wildfire S) pre-loaded with a BU surveillance questionnaire. The questionnaire was designed using the Epicollectplus mobile application (<http://plus.epicollect.net/>). From August 2013 to December 2014, the CBSVs were mandated to visit all households in a month and record any suspected case using the mobile application and a notebook. Suspected cases were then sampled by a local health staff and the samples were sent to the NMIMR for laboratory confirmation. We then assessed the feasibility and efficiency of this approach by evaluating the number of cases detected, the severity of lesions sampled and the community coverage (the proportion of households inventoried during the exhaustive survey that could be visited in a month by the volunteer).

Laboratory confirmation of BU cases

To confirm suspected active BU lesions, two swab specimens were collected from the undermined edges of ulcerative lesions. For pre-ulcerative lesions, one fine needle aspirate (FNA) was transferred into 500 µl phosphate buffered saline (PBS) as previously described [12] and transported to NMIMR at 4°C. Laboratory confirmation was conducted as previously described. Briefly, suspensions from pooled swabs of the same lesion containing 2 ml of PBS were concentrated by centrifuging at 3,000 x g for 15 minutes. The supernatant was then decanted and the sediment mixed with PBS to make up a final volume of 500 µl suspension from which 100 µl was used to prepare slides for Ziehl-Neelsen (ZN) microscopy. From the residual 400 µl suspension, we extracted *M. ulcerans* DNA for IS2404 PCR.

Mapping and Data Analysis

Summary data from household surveys were mapped and analyzed using the ArcMap (Economic and Social Research Institute, version 10.0). The elevation values (presented in feet) were derived from 15 meter resolution Advanced Spaceborne Thermal Emission and Reflection (ASTER) Satellite digital elevation model (DEM) data, obtained at a sun angle of 59.6 degrees.

All statistical analyses were carried out using GraphPad Prism version 6.0 (GraphPad Software, San Diego California USA) and Stata 12 (Statacorp 2011 statistical software Release 12. College Station, TX: StataCorp LP).

2.5 Results

Demographic characteristics of the surveyed population

A total of 2,822 households of three hamlets, seven villages and three peri-urban settings along the Offin river were visited to survey the population for BU.

In all, we surveyed a total population of 20,390 inhabitants, 50.08% (n=10,211) females and 49.92% (n=10,179) males (Table 2.1). The majority (90.0%) of these inhabitants were full time residents. Characteristic of a youthful population, the mean age recorded for the survey was 23.6 (S.D +/- 18.8) years and 39.7% of the surveyed population below the age of 15 years (Fig 2.4A). As expected, in all of the visited communities, the Akans represented the major ethnic group (46.9% to 96.5%). 82.8% of the population between the ages of 4 and 18 years were students. We observed that miners formed less than 1% of the population we surveyed upstream the river (0.4% from Bedomase and none from Krakrom and Kapro). On the other hand, miners were well represented in mid and downstream communities like Nkotumso and Tontonkrom where they formed the highest and second highest occupational group, respectively.

BU cases identified in the selected communities

Based on clinical signs and symptoms, we identified seven suspected active BU cases of which three were confirmed; one by both ZN and PCR and two by PCR only. All three confirmed cases (one male and two females) had ulcerative lesions. The male patient who was 6 years old presented with a category I ulcer on his thorax. One 35 year old female presented with category I on the upper left limb and the other 22 year old female had a category II ulcer on her lower left limb. Based on the clinical assessment of healed lesions by an experienced clinician, we could detect a total of 477 cases with healed BU lesions from the 13 communities. Of these 477 healed cases, 138 (28.9%) had been previously diagnosed (clinical and/or laboratory) as active BU and noted in existing passive surveillance records. We estimated 0.01% and 2.34% prevalence of active and healed BU cases respectively in the surveyed population. We observed no significant difference (P=0.12) between the proportion of historical cases in females (2.6%, 266/10209) when compared to the proportion recorded for males (2.0%, 211/10178). The age of the 477 healed cases identified during the survey at the estimated onset of the disease ranged from 0.75 to 87 years with a mean age of 31 years (SD=18). Children under 15 years accounted for 48.1% of the total number of cases. When we computed the age adjusted prevalence based on the age

distribution of the population surveyed, we observed the highest peak for children between 10 and 12 years (Fig 2.4B).

Emergence of BU cases in the community and distribution of burden along the Offin river

The BU prevalence for all surveyed communities is listed in Table 2.1. The disease burden was not uniform along the river. By grouping all 13 study communities according to their location along the Offin river, we observed that BU cases detected formed 0.1% (2 out of 2,491) of the total population surveyed upstream the river (communities A to C). This was significantly smaller ($P < 0.001$) than the proportion recorded for communities D to H located mid-stream (2.4%, 274/11,160) and I to M located down-stream the river (3.0%, 204/6,739). As illustrated in Fig 2.3B, upstream, we detected no case in Bedomase (A) but recorded a prevalence of 0.90% and 0.14% in Krakrom (B) and Kapro (C), respectively. Prevalence was higher midstream (between 1.22% and 3.89%). The highest overall prevalence (8.9%) was recorded downstream the river in the Mfantseman (L) community. In the other downstream communities we recorded a prevalence of 2.46%, 3.61%, 2.31% and 1.33% for Dominase (I), Nkotumso (J), Wromanso (K) and Pokukrom (M), respectively.

Based on the BU history data compiled for all 480 cases detected during the active case survey (ACS), we estimated the time span during which cases emerged for each community included in the survey. In a second step, we compared our results with the annual passive case surveillance (PCS) data available at the local and district health centers. We estimated a population growth rate of 3.8% per annum for communities A to H of the Ashanti region and 3.2% per annum for communities I to M of the Central region using data from the regional population census conducted in 2000 and 2010. The annual BU case estimates for both ACS and PCS were expressed per 1,000 inhabitants to facilitate the comparison of trends between communities (Fig 2.5 and Fig 2.6).

While PCS data prior to the year 2000 was not available for any of the 13 communities surveyed, our annual ACS trend corroborated reasonably well with PCS data available for the years later than 2000. Altogether, we observed slightly differing trends in BU case emergence for upstream and downstream communities. The most upstream-localized community Bedomase (A) was non-endemic, as no case has ever been detected by PCS or ACS. In upstream communities Krakrom (B) and Kapro (C), we identified only one index case each corresponding to nine cases per 1,000

inhabitants and one case per 1,000 inhabitants respectively. While no PCS data was available for both cases detected, the cases were estimated to have emerged in 2009 and 2005 for Krakrom (B) and Kapro (C) respectively. Among all the mid-stream communities (D-H), we observed a continuous emergence of BU cases with at least one case per 1,000 inhabitants being recorded each year by either ACS or PCS or both from 2000 to 2014 (Fig 2.5). The downstream communities (I to M) which incidentally represent communities located within the Central region were generally characterized by a less continuous trend (Fig 2.6). For the historically endemic community Nkotumso (J), we observed an absence of cases from 2010 to 2014 after a long period of rather consistent emergence of cases. The further downstream communities Wromanso (K) and Mfansteman (L) were characterized by sporadic emergence of cases. Additionally, the highest number of cases per 1,000 inhabitants for a single year (25 cases in 2011) was observed in Wromanso (K).

In all, 95.8% (460/480) of the total BU cases detected have resided in their respective communities for more than three months. Additionally, when we stratified the population surveyed by their length of stay in the communities, we observed that BU cases made up 3.2% (460/14,332) of the group with more than three months of residence. This was significantly higher ($P < 0.001$) when compared to the proportion recorded for those who have resided for three months or less (0.6%, 20/3613).

Active BU surveillance

By employing both community outreach and household-visit surveillance, we continued to monitor the emergence of BU cases in all 13 selected communities over the 17-month period from August 2013 to December 2014. In all, we detected 29 clinically suspected BU cases during this active surveillance period. Five of these cases were detected through the community outreach program (four from Ntobroso and one from Dominase) and 24 were detected through the monthly household visits. Eight of them (27.6%) were laboratory confirmed by IS2404 PCR. As expected for an active surveillance activity, all eight cases (five males and three females aged between 3 and 35 years, with a mean age of 18 (SD=9)) had lesions in their early stages; five were detected with pre-ulcerative lesions (four nodules and one plaque) and three presented with Category II ulcers. The 21 non-confirmed suspected BU cases were referred to the district hospital for further assessment and alternative diagnosis.

The monthly household visits resulted in the detection of seven out of the eight laboratory confirmed cases. All seven cases were detected in three communities: Achiase (four cases), Ntobroso (two cases) and Akomfore (one case). Laboratory case confirmation rate for Achiase (4 out of 6 (66.7%)) was significantly higher ($P=0.03$) than the combined rate recorded for all other communities (4 out of 23 (17.4%)). As shown in Fig 2.7, all four cases from Achiase were detected in months when the volunteer had covered more than 50% of the households.

2.6 Discussion

Limited access of the population to health facilities and the reluctance of BU patients to seek medical care have made house-to-house surveys an attractive tool in studies on the disease epidemiology [13–15]. Here, we investigated recent anecdotal accounts of unstable *M. ulcerans* transmission along the Offin river.

We observed during our survey, scanty passive surveillance data which made our annual BU case trend analysis for each community a challenge. BU patients' records preserved over time could also serve as reliable source of information for retrospective analyses which could add to the existing clinical knowledge of the disease. The importance of maintaining good patient records was demonstrated in a recent analysis of 1,227 BU case data collected from 2005 to 2011 in Benin. The study revealed a higher risk of developing osteomyelitis among male patients than female patients and a significant association between clinical presentation and development of permanent functional sequelae [16].

Inhabitants having both healed and active lesions were recorded in the study in order to account for historical trends as well as to get an overview of the current BU situation for each community. While only laboratory confirmed BU patients with active lesions were included, case definition for patients with healed lesions was based on clinical judgment. In view of the lack of passive surveillance data on BU for the study communities, findings of this study may thus serve as a reference for future longitudinal follow-up of the community.

In line with the decline in BU incidence in West Africa, Ghana recorded in 2014 nearly 50% less cases than in 2009 [2]. Along these lines, one key observation of this study is the overall decline in the prevalence of BU along the Offin river, even in known historically endemic communities. The high focal BU incidence recorded for Wromanso (K) in 2011 was perhaps due to an increase in community sensitization and awareness campaigns about BU conducted within the year. Consequently, this may have led to an increase in cases reporting to the health facilities or detection of more cases by the health staff through the awareness programs. However, some studies have reported similar upsurges in BU incidence in association with climatic and environmental changes [17–19].

In endemic communities of Africa, children have been reported as forming the majority of BU cases [20–25]. While children below 15 years of age formed nearly half of all cases detected in our study, we observed an underrepresentation of children below 5 years among cases, consistent

with the findings of a recent study conducted in Cameroon [14]. Moreover, sero-epidemiological studies in Ghana and Cameroon demonstrated that children of this age group were less exposed to *M. ulcerans* [26].

Consistent with findings of a previous study conducted along the Densu river in Ghana [13], we observed a very low prevalence of BU upstream the river whereas mid and downstream communities recorded high prevalence. Similar to the repeated association of BU with man-made modification of aquatic environments [11,17,18,23,27–29], the intense gold mining activities observed in our study area were also localized mid and downstream the river. Recently, a detailed study on land cover and its association with BU in the downstream region of the Offin demonstrated a significant association between mining and the occurrence of the disease [10]. In the same study, the distribution of alluvial gold mining patches in areas of BU foci was made evident using high resolution satellite images. The low-lying feature of the mid and downstream regions of the river also supports previously reported association of BU with landscapes of low elevation [30,31,28].

The monthly household-based surveillance conducted by the community volunteers in the course of this study resulted in the detection of seven of the eight laboratory confirmed active BU cases, all of whom had early stage lesions. This underscores the important role of community volunteers in early case detection and uncomplicated treatment of BU [32–34]. However, ideally, only category I lesions should have been detected considering that active surveillance strategies were employed to monitor the emergence of new cases after the exhaustive case search. This could be explained that some early lesions were missed since the volunteers couldn't consistently achieve 100% household coverage. Alternatively some patients may have ignored the very early clinical BU signs and may have only presented themselves to the volunteers on such visits when lesions have progressed to category II. As reviewed [35] and previously reported [36] patients with large active lesions may play a role in transmission of *M. ulcerans* by disseminating the bacteria from their active lesions into the environment which may then serve as a source of infection to others. This implies that intense continuous early case detection and timely antibiotic treatment in an endemic area may result in the interruption of this cycle leading to the gradual decline of cases, as observed for example in Ghana and Benin.

The current active surveillance approach employed by the BU control programs is mainly community based. One cost effective way to sustain the monthly household surveillance will be

to integrate the BU surveillance with other prevalent skin diseases like yaws, cutaneous leishmaniasis and leprosy [37]. As a tool, the mobile phone data collection can serve as a back-up to address the gaps in data collected at the health facility or district levels.

2.7 Acknowledgement

We are very grateful to the directors and disease control officers of all the health districts we worked in. We also thank Frank Adu-Nti, the community volunteers, local health staff and chiefs of the communities we worked in for their assistance with the fieldwork.

2.8 Figures

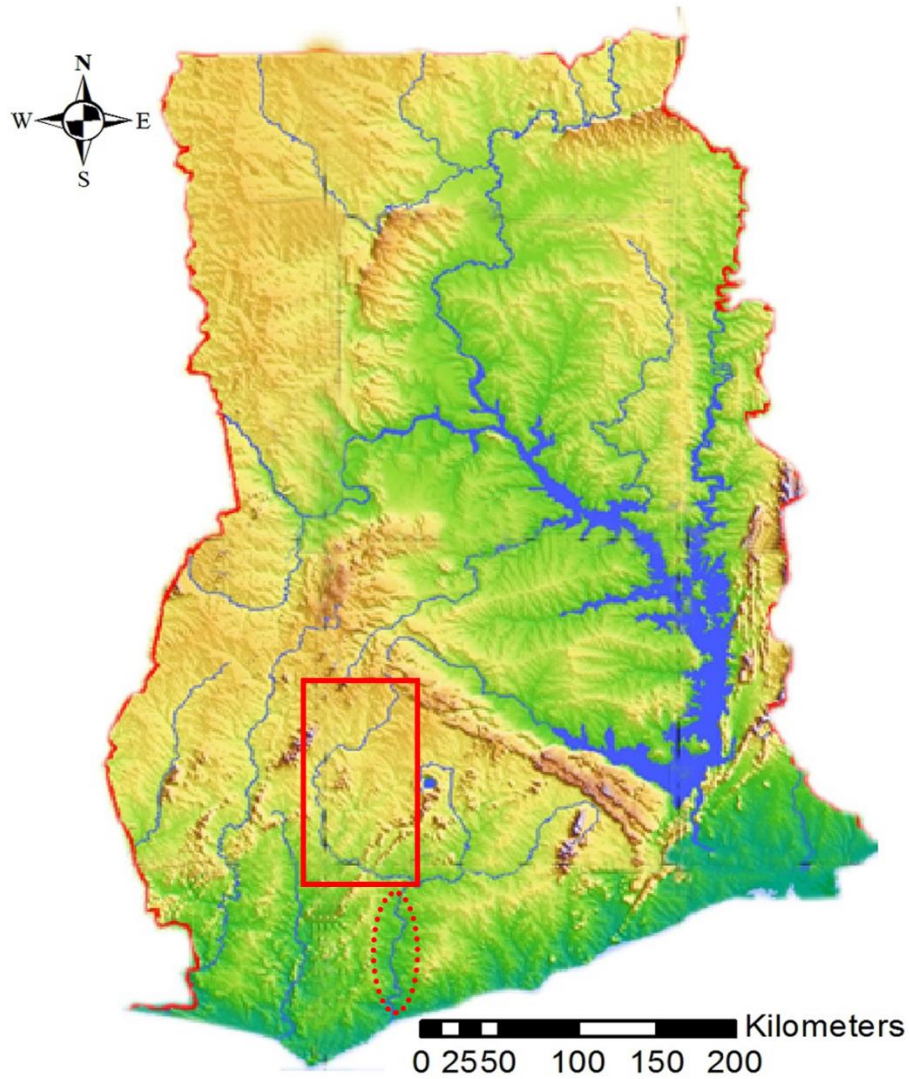


Figure 2.1. Study area. Map of Ghana showing major river systems. Our study area comprised part of the Offin river basin, shown here with a red rectangle. The Pra River is indicated by the dotted line.



Figure 2.2. Land cover and use in selected communities along the Offin river.

Upstream communities (A) Bedomase and (B) Krakrom, mid-stream community (C) Ntobroso and downstream community (D) Pokukrom

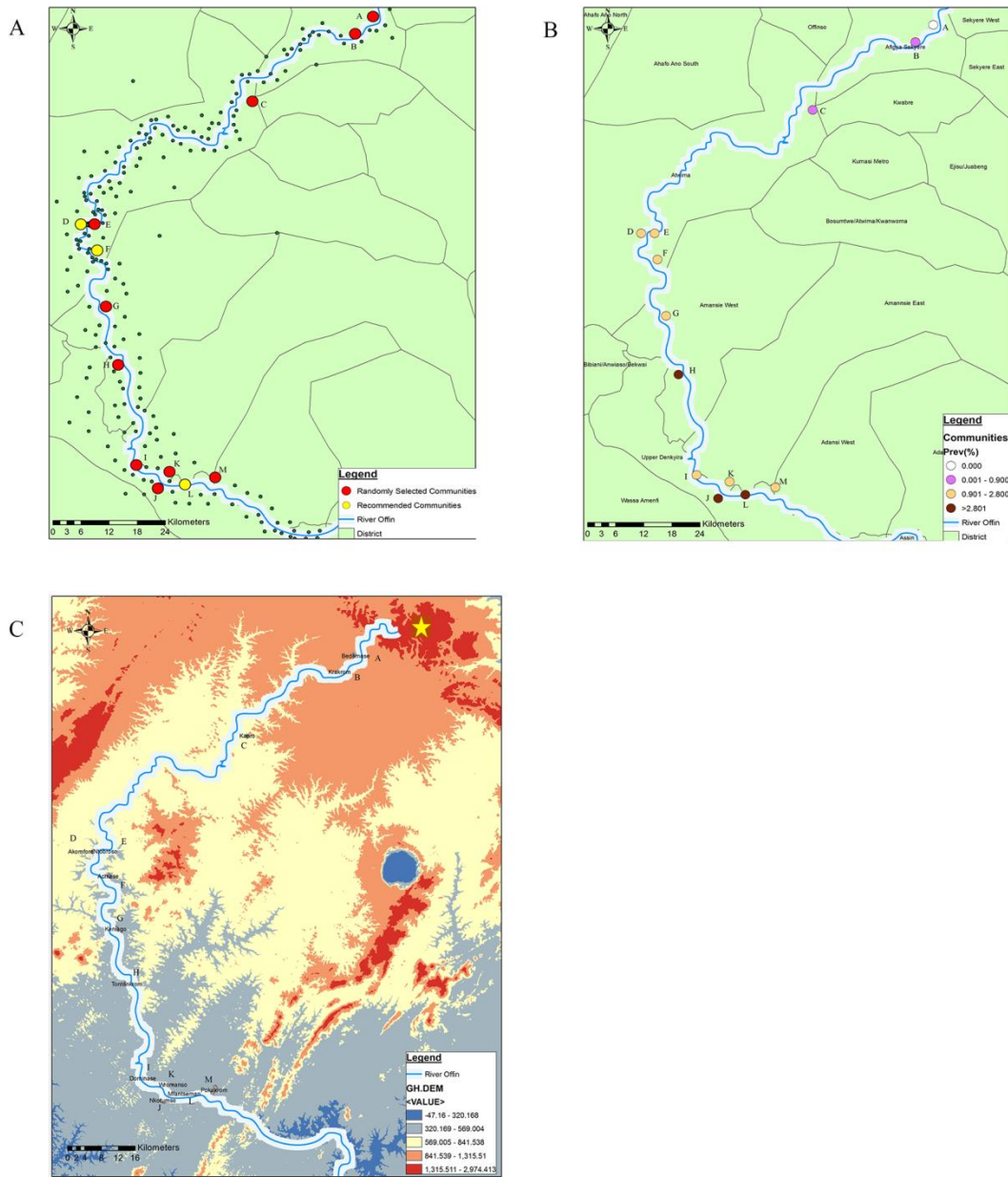


Figure 2.3. Elevation and distribution of Buruli ulcer burden along the Offin River.

(A) A map showing communities located within the 5 km buffer along the Offin river. The 10 red colored study communities were randomly selected, the three yellow colored communities were added to the study based on recommendations by local health staff. (B) Spatial distribution of Buruli ulcer prevalence along the Offin river. (C) Spatial altitudes variation (indicated in feet) along the Offin river. The Mampong highland area is indicated by the yellow star.

Community codes; Bedomase (A), Krakrom (B), Kapro (C), Akomfore (D), Ntobroso (E), Achiase (F), Keniago (G), Tontonkrom (H), Dominase (I), Nkotumso (J) Wromanso (K), Mfantseman (L) and Pokukrom (M).

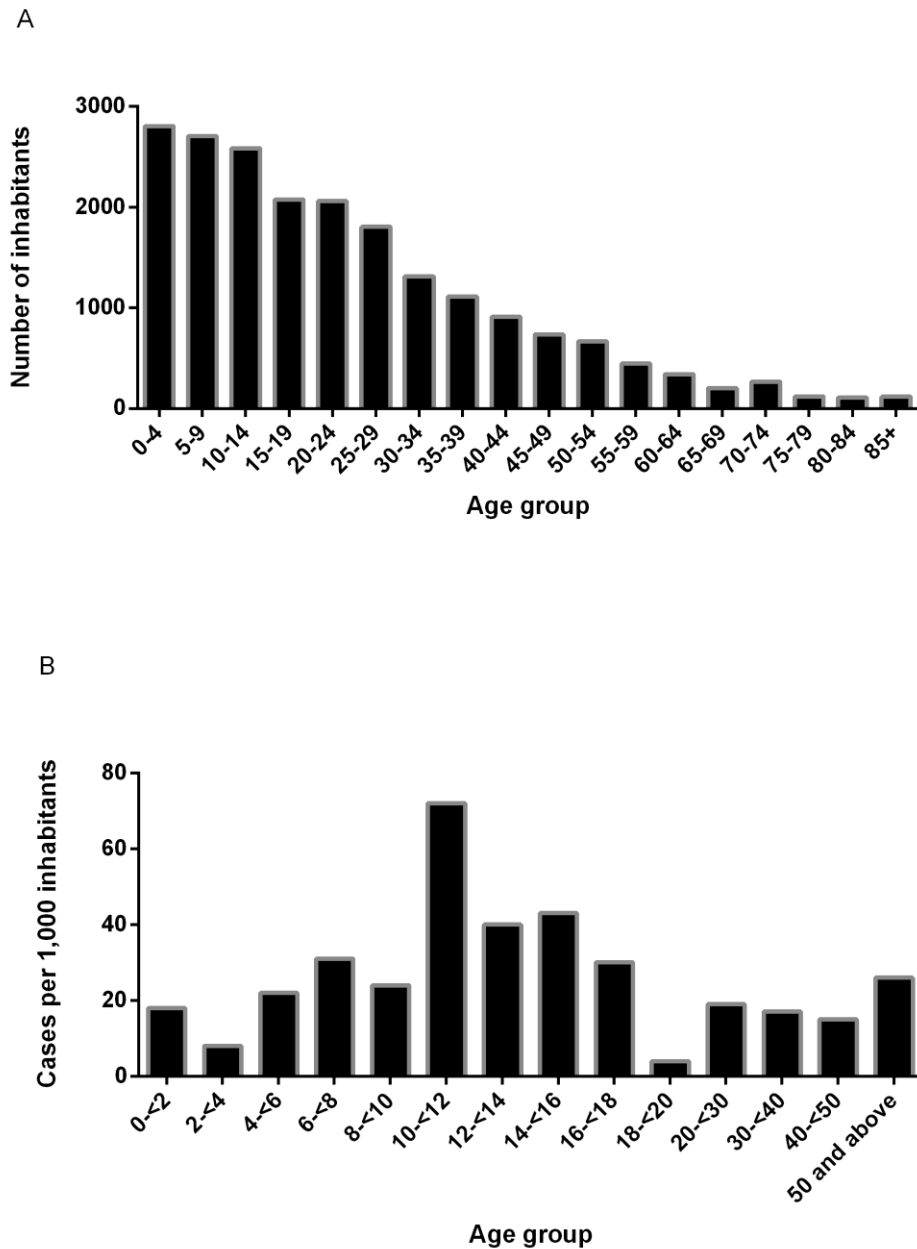


Figure 2.4. Population age distribution and age adjusted prevalence of BU in the surveyed population. (A) Age distribution of the surveyed population (n = 20,390 inhabitants) of the 13 selected study communities in the Offin river basin. (B) Age distribution of BU cases based on the age of the 480 identified cases at onset of the disease adjusted for age (per 1,000 inhabitants) using the age structure of the general surveyed population

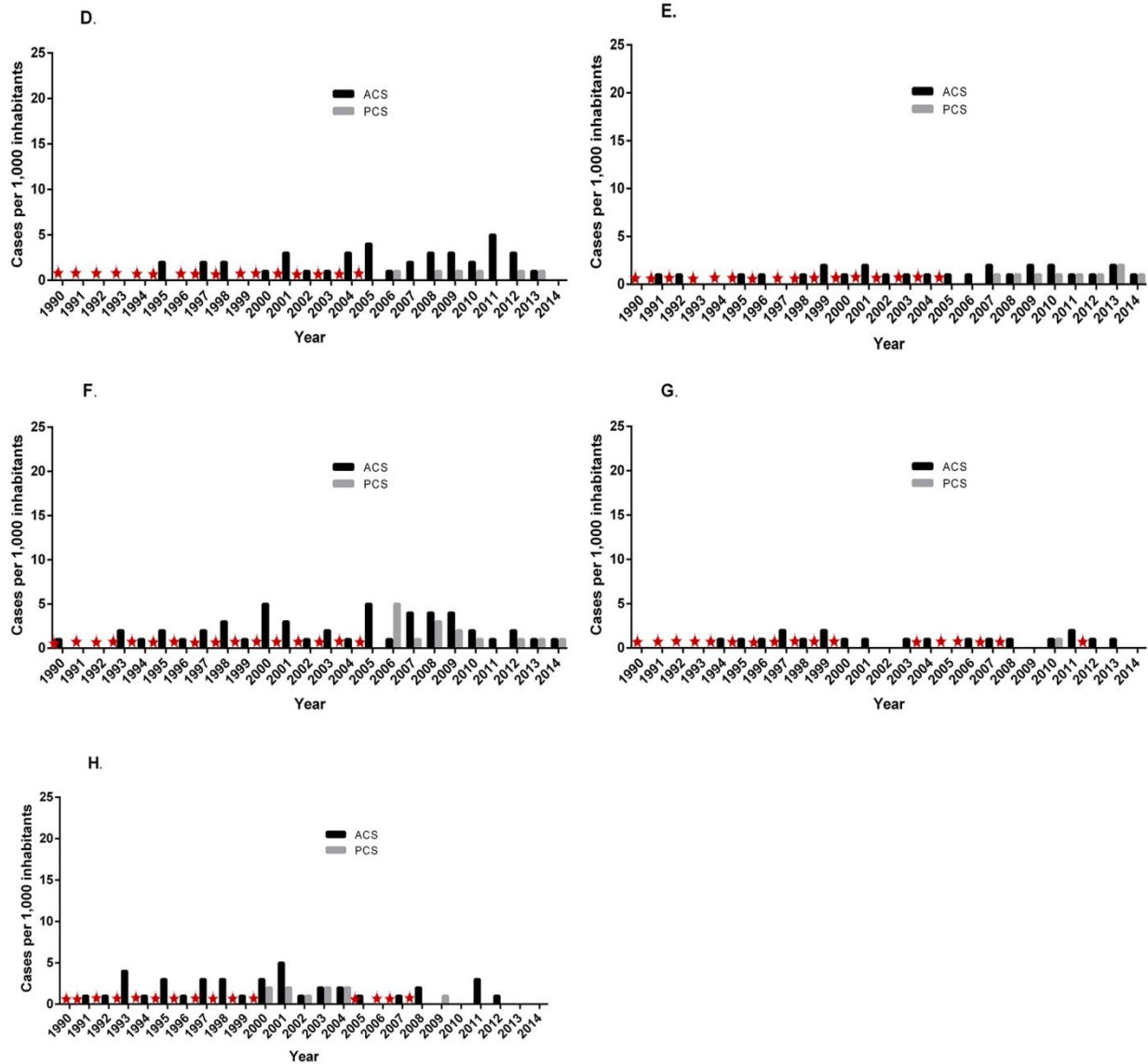


Figure 2.5. Estimated annual case trend for communities located mid-stream of the Offin river. Trends were reconstructed using data from active survey (ACS) and passive surveillance data (PCS) available at the local and district health facilities. The red star represents unavailable passive surveillance data for a particular year. Community codes: **D** (Akomfore), **E** (Ntobroso), **F**(Achiase), **G** (Keniago), **H** (Tontonkrom). No case was detected in Bedomase (**A**) and index cases were detected in Krakrom (**B**) and Kapro (**C**)

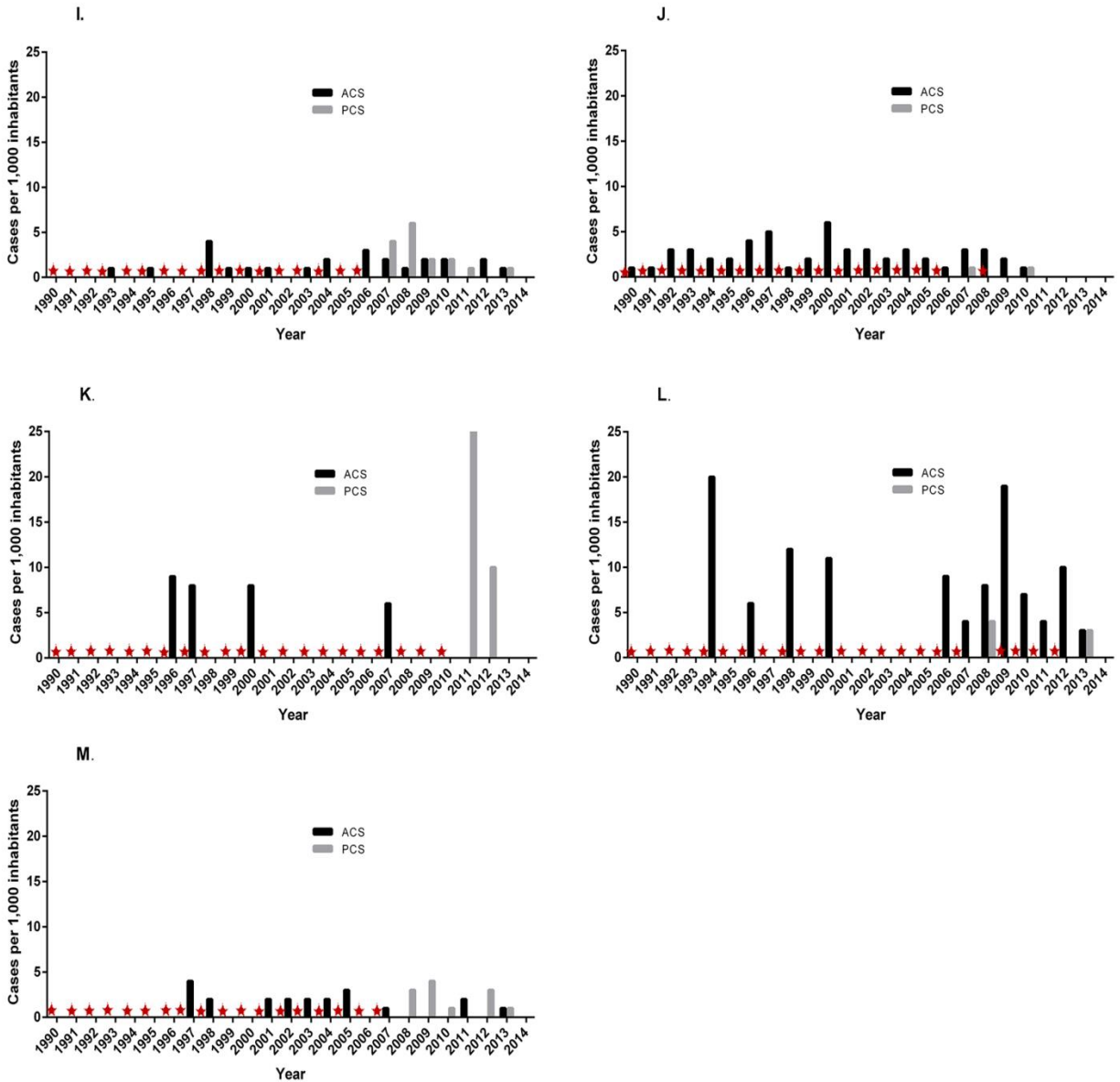


Figure 2.6. Estimated annual case trend for communities located down-stream of the Offin river. Trends were reconstructed using data from active survey (ACS) and passive surveillance data (PCS) available at the local and district health facilities. The red star represents unavailable passive surveillance data for a particular year. Community codes: **I** (Dominase), **J** (Nkotumso), **K** (Wromanso), **L** (Mfantseman), **M** (Pokukrom).

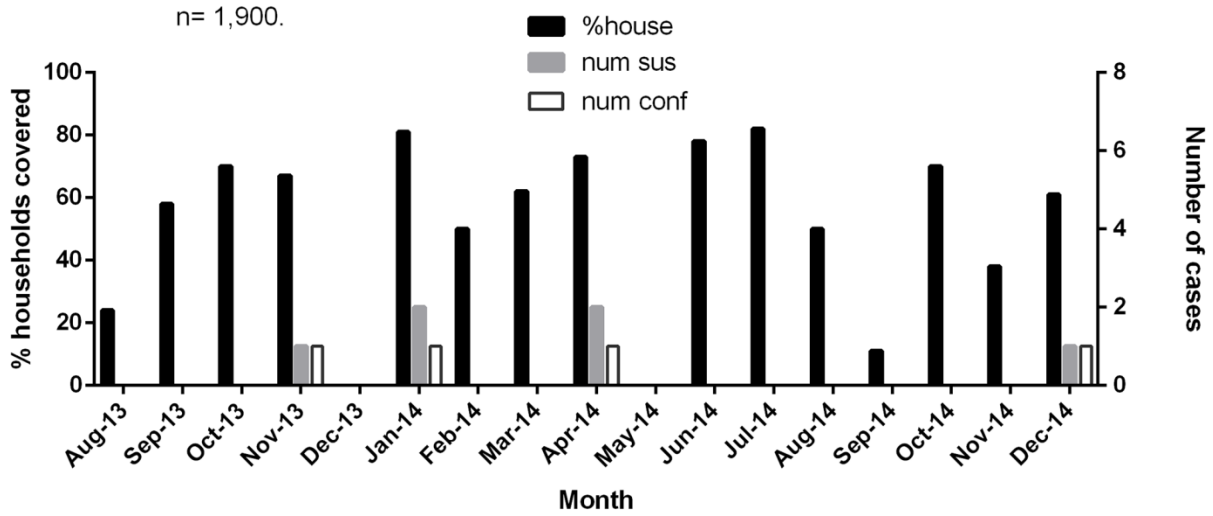


Figure 2.7. Monthly active surveillance of BU in the Achiase community. Graph shows data obtained from Achiase by monthly household visits. Black bars represent percentage of households covered by the volunteer, grey bars represent the number of suspected cases and white bars represent the number of laboratory confirmed cases.

2.9 Tables

Demographic variables	Community													
	A	B	C	D	E	F	G	H	I	J	K	L	M	Total
Population (n)	1,688	111	692	1,016	1,949	1,900	3,350	2,945	2,802	2,518	216	303	900	20,390
Type of settlement	Peri-urban	Hamlet	Village	Village	Village	Village	Village	Village	Peri-urban	Peri-urban	Hamlet	Hamlet	Village	
Households														
Inhabitants per household	1-25	2-17	2-25	1-20	1-30	1-21	1-32	1-25	1-27	1-30	1-20	1-10	1-19	1-32
Total number of households	226	16	64	157	251	295	413	399	384	370	41	85	121	2,822
Residents														
Full time (%)	87.5	84.7	93.8	89.1	84.8	92.2	86.3	93.1	92.1	93.2	74.1	90.8	91.3	90
Part time (%)	12.5	15.3	6.2	10.9	15.2	7.8	13.7	6.9	7.9	6.8	25.9	9.2	8.7	10
Total	100	100	100	100	100	100	100	100	100	100	100	100	100	
Sex														
Females, n (%)	876 (51.9)	65 (58.6)	363 (52.5)	499 (49.1)	972 (49.9)	939 (49.4)	1,719 (51.3)	1,467 (49.8)	1,423 (50.8)	1,232 (48.9)	106 (49.1)	141 (46.5)	409 (45.4)	10,211
Age range	0.2-110	0.5-72	0.2-100	0-120	0.2-115	0.1-120	0-120	0.1-114	0-110	0-100	1-85	0.3-90	0.1-99	0-120
Mean age in years (S.D)	24.3 (20.7)	22.4 (17.4)	24.0 (19.9)	23.1 (18.8)	24.4 (18.9)	25.9 (20.7)	22.9 (19.4)	22.2 (16.6)	22.9 (17.9)	23.3 (17.7)	27.0 (20.3)	23.3 (18.0)	24.3 (18.4)	23.6 (18.8)
Ethnicity (%)														
Akan	88	53.5	96.5	79	94.2	90.4	46.9	71.6	82.6	68.2	53.8	73.6	92.1	75.8
Ewe	0.3	3.3	0	8.7	0.1	1.2	0.9	6.6	9.6	14.5	30	2.1	6	5.4
Mole	0.3	0	0.1	0.1	0	0.1	0	0	0.2	0.6	0	0.3	0	0.1
Ga / Ada	0.2	1.2	0	3	0.4	0.2	0	0.7	1.4	1.6	1	0.5	0.5	0.8
Guan	0.1	0.1	0	0	0	0	0	0	0.1	0	0	0.3	0	0
Gruma	1.6	5.4	0	0	0	0.3	1.8	1	0.1	0.5	2	0.4	0	0.7
Grusi	0	0.1	0.1	0.2	0	0.4	0	0	0.7	0.9	0	2.6	0	0.3
Mande	0.1	0.1	0	0	0.1	0	0	0	0	0	0.1	0.5	0	0

Mamprusi	0	0.2	0	0.1	0.3	0.2	0	0	0	0.1	0.1	0.8	0	0.1
Kussasi	3.9	13.3	2.4	1.8	0.4	3.9	31.5	5.3	2.7	2.8	4.2	3.7	0	7.7
Others	5.5	22.8	0.9	7.1	4.5	3.3	18.9	14.8	2.6	10.8	8.8	15.2	1.4	9
Total	100	100	100	100	100	100	100	100	100	100	100	100	100	
Main Occupation (%)														
Unemployed (children and elderly)	12.6	15.3	16.5	17.1	16.7	13.8	20.9	15.1	12.8	13.4	12.1	13.2	13.8	14
Unemployed (Adult)	6.8	1.8	6.4	4.2	4.4	3.2	6.2	3	14.2	8.6	2.8	4	8.2	8
Student	42.4	38.8	36.7	32	28.7	33.1	33.8	28.5	22.7	12.8	29.6	32	28.4	28
Fisherman	0	0	0	0.1	0.2	0	0.1	0	5.2	18.4	0.5	0	0	0
Farmer	21	38.7	24.4	36.1	28.3	31.3	17.5	18	14	9	36.1	40.9	21	21
Miner	0.4	0	0	4.7	8.8	9.5	12.2	24.4	14.7	18.9	9.7	5.6	17.1	17
Skilled laborer	7.7	1.8	3	2	5.2	3.8	3.2	2.9	5.8	4	3.2	1.3	3	3
Unskilled laborer	8.8	3.6	11.7	2.5	6.3	4.6	5	7	9.4	13.5	4.6	2.3	7.2	7
Others	0.3	0	1.3	1.3	1.4	0.7	1.1	1.1	1.2	1.4	1.4	0.7	1.3	1
Total	100	100	100	100	100	100	100	100	100	100	100	100	100	
BU Prevalence(%)														
Healed cases	0	0.9	0.1	3	1.6	3.8	1.2	3.2	2.5	3.6	2.3	8.9	1.3	2.3
Active cases	0	0	0	0.1	0.1	0.1	0	0	0	0	0	0	0	0

Table 2.1. Demographic characteristics of the surveyed population.

Community codes: Bedomase (A), Krakrom (B), Kapro (C), Akomfore (D), Notbroso (E), Achiase (F), Keniago (G), Tontonkrom (H), Dominase (I), Nkotumso (J) Wromanso (K), Mfantseman (L) Pokukrom (M)

2.10 References

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Chapter 3

Late onset of the serological response against the 18 kDa small heat shock protein of *Mycobacterium ulcerans* in children

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3.1 Abstract

A previous survey for clinical cases of Buruli ulcer (BU) in the Mapé Basin of Cameroon suggested that, compared to older age groups, very young children may be less exposed to *Mycobacterium ulcerans*. Here we determined serum IgG titres against the 18 kDa small heat shock protein (shsp) of *M. ulcerans* in 875 individuals living in the BU endemic river basins of the Mapé in Cameroon and the Densu in Ghana. While none of the sera collected from children below the age of four contained significant amounts of 18 kDa shsp specific antibodies, the majority of sera had high IgG titres against the *Plasmodium falciparum* merozoite surface protein 1 (MSP-1). These data suggest that exposure to *M. ulcerans* increases at an age which coincides with the children moving further away from their homes and having more intense environmental contact, including exposure to water bodies at the periphery of their villages.

3.2 Author Summary

Although *M. ulcerans*, the causative agent of Buruli ulcer (BU), was identified in 1948, its transmission pathways and environmental reservoirs remain poorly understood. The occurrence of *M. ulcerans* infections in endemic countries in West and Central Africa is highly focal and associated with stagnant and slow flowing water bodies. BU is often described as a disease mainly affecting children <15 years of age. However, taking the population age distribution into account, our recent longitudinal survey for BU in the Mapé Dam Region of Cameroon revealed that clinical cases of BU among children <5 years are relatively rare. In accordance with these findings, data of the present sero-epidemiological study indicate that children <4 years old are less exposed to *M. ulcerans* than older children. Sero-conversion is associated with age, which may be due to age-related changes in behavioural factors, such as a wider movement radius of older children, including more frequent contact with water bodies at the periphery of their villages.

3.3 Introduction

It has been established that the chronic necrotizing skin disease BU is caused by the emerging pathogen *Mycobacterium ulcerans*, however the mode(s) of transmission and environmental reservoirs are still unknown. Comparative genetic studies have revealed that *M. ulcerans* has diverged from the fish pathogen *M. marinum*. Through the acquisition of a plasmid, *M. ulcerans* has gained the ability to produce a cytotoxic and immunosuppressive macrolide toxin, referred to as mycolactone [1,2]. In addition to *M. ulcerans* strains isolated from human lesions, which belong either to the classical or to the ancestral lineage [3], other mycolactone-producing mycobacteria (MPM) have been identified as fish and frog pathogens and given diverse species names [4-7]. However, recent comparative genomic analyses have shown that all MPM are genetically closely related and can be divided into three principal ecovars of *M. ulcerans* [8]. Extensive pseudogene formation and genome downsizing of the human *M. ulcerans* pathogen are indicative for an adaptation to a more stable ecological niche. In African endemic settings both the physical environment and organisms such as amoeba, insects, fish and frogs have been proposed as possible environmental reservoirs of the pathogen [9]. Accordingly, direct inoculation of bacteria into the skin from an environmental reservoir, but also bites from insects, such as mosquitos or water bugs have been suggested as route of infection. While possums have been identified as an animal reservoir in BU endemic areas of Southern Australia [10], no mammalian reservoir has so far been detected in Africa. The distribution pattern of lesions is not indicative for a particular route of infection [11] and a genetic fingerprinting study of *M. ulcerans* isolates has revealed a highly focal transmission pattern, which excludes certain modes of transmission [12].

While it has long been generalized that in African BU endemic areas children below the age of 15 are most affected by the disease [13], population age-stratified data from our previous survey for BU in the Mapé Basin of Cameroon showed that children less than 5 years old were underrepresented among cases [11]. One explanation for this observation may be a lower degree of exposure of very young children to *M. ulcerans*. Sero-epidemiological studies in Ghana have shown that screening blood sera of local populations for the presence of IgG specific for the 18 kDa shsp of *M. ulcerans* represents a tool to monitor exposure of populations to *M. ulcerans* [14]. However, in these investigations study participants were older than 5 years of age. Since a proportion of study participants of all age groups tested positive, it is still not known at which

age immune responses against *M. ulcerans* start to emerge and hence where and at which age exposure to the pathogen begins.

In the present sero-epidemiological study the potential association between age and exposure to *M. ulcerans* was investigated by determining anti-18 kDa shsp IgG titres in 875 individuals from BU endemic sites in the Densu River Basin of Ghana and the Mapé Basin of Cameroon. In these cross-sectional surveys we included more than 100 children less than 5 years old allowing us to estimate the age of sero-conversion, which may provide 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). Written informed consent was obtained from all individuals involved in the study. Parents or guardians provided written consent on behalf of children.

Study design

We investigated the association between age and exposure to *M. ulcerans* by determining serum antibody titres against the 18 kDa shsp in individuals living in two different BU endemic areas.

In Cameroon, serum samples were collected from inhabitants of the village of Mbandji 2. This village is located in the Bankim Rural Health Area of the Bankim Health District, where we conducted a cross-sectional house-by-house survey for BU in early 2010, including the collection of data on the population age structure. These data and the subsequent identification of BU cases until June 2012 were published in our previous study [11]. In the present study we provide updated information based on a continued monitoring of new BU cases in this area until May 2013. The age-specific incidence rates were calculated using the ages of the BU cases identified between March 2010 and May 2013 and the population age distribution as collected in the house-by-house survey in the Bankim Health District.

Sera were collected in January 2011 from all inhabitants of Mbandji 2, who agreed to participate (395 individuals with a nearly equal gender distribution). Re-sampling of 80 blood donors from Mbandji 2 was carried out one year after the first blood collection to analyze stability of anti-18 kDa shsp serum IgG levels over time.

The second study site comprised villages within the Obom sub-district of the Ga-South district in Ghana. This sub-district is one of the major BU endemic communities along the Densu River Basin. The villages from which the sera were collected, have active transmission on-going as they have continuously reported cases for the past five years. Study participants included 96 laboratory confirmed BU patients (57 females and 39 males) as well as 4 age-, sex-, and home village-matched controls for each patient (384 control individuals).

Demographic data as well as history of known previous mycobacterial infections were recorded for all participants at both sites. While the majority of individuals had no history of mycobacterial infections, eight study participants from Mbandji 2 reported to having had tuberculosis (2), leprosy (1) or BU (5). All control participants recruited in Ghana had no history of mycobacterial infection. The age distribution of study participants from Cameroon and Ghana is shown in Figure 3.1A and 3.1B, respectively.

Blood sera from the 875 individuals were tested for the presence of anti-18 kDa shsp antibodies in an ELISA format. In addition, 96 sera from children living in Mbandji 2 were tested by Western Blot analysis for the presence of antibodies against this protein, as well as against a *Plasmodium falciparum* MSP-1 protein domain in order to assess the exposure and immune responses of child study participants to this mosquito transmitted parasite.

ELISA

96-well Nunc-Immuno Maxisorp plates (Thermo Scientific) were coated with 0.25 µg recombinant *M. ulcerans* 18 kDa shsp per well in 100 µl phosphate-buffered saline (PBS) and incubated over night at 4°C. Plates were washed four times with washing buffer (dH₂O, 2.5 % Tween 20) 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 µl 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 µl of 1:8000 diluted goat anti-human IgG (γ-chain specific) antibodies 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 µl TMB Microwell Peroxidase Substrate (KPL) was added per well. The reaction was stopped after 5 minutes using 0.16 M sulfuric acid. The absorbance was measured at 450 nm in a Tecan Sunrise microplate reader.

Western Blot analysis

15 µg of recombinant *M. ulcerans* 18 kDa shsp or 5 µg of a *Plasmodium falciparum* MSP-1 protein domain (amino acids 34-469 of strain K1) were separated on NuPAGE Novex 4-12 % Bis-Tris ZOOM Gels with 1.0 mm 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 blocking buffer 3 (5 % skim milk in PBS containing 0.1 % Tween 20) and cut into thin strips. Membrane strips were then incubated with human blood sera at a 1:1000 dilution in blocking buffer 3 for 2 hours at RT. Strips were repeatedly washed with 0.3 M PBS containing 1 % Tween 20 and after that incubated with 1:20'000 diluted goat anti-human IgG (γ -chain specific) antibodies 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 version 6.0 (GraphPad Software, San Diego California USA) and R version 3.0.1 [15].

The distribution of antibody titres and the differences between two successive antibody titres are presented as box plots. These comprise 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.

We compared changes in OD between age categories in the Cameroon dataset using the Kruskal-Wallis test. Levene's test for homogeneity of variances was used to compare the degree of variation by age category. We compared the OD values for the Ghana matched cases and controls using conditional logistic regression.

The overall bias and variation between the first and second Ghanaian serum samples was estimated using the Bland-Altman method [16].

3.5 Results

Age distribution of BU incidence and *M. ulcerans* 18 kDa shsp-specific serum IgG responses among individuals living in the Mapé Basin of Cameroon

The age-specific BU incidence rates for the population in the Mapé Basin were calculated using 76 BU cases identified between March 2010 and May 2013. Based on these cases, a low incidence rate of BU was detected for children less than 4 years of age (Figure 3.2A).

The age-distribution of IgG titres against the *M. ulcerans* 18 kDa shsp for a cross-sectional survey of 395 individuals from the village Mbandji 2 is shown in Figure 3.2B. While high antibody titres were detected in individuals of all age groups over 4 years, none of the children younger than four years showed an ELISA IgG titre above the background, which was determined by Western Blot analysis as OD < 0.35. Analysis of the 96 sera sampled from children less than 7 years old by Western Blot analysis showed no specific bands representing IgG antibodies against the 18 kDa shsp for sera from children <4 years of age (Figure 3.3). In contrast, Western blot positive sera were found in all tested age groups >4 years old. Since very weak IgG titres were recorded for some of the sera from four year olds, sero-conversion may start in some children around this age.

Serum IgG responses against a domain of *Plasmodium falciparum* merozoite surface protein 1 among children living in the Mapé Basin of Cameroon

IgG titres against a recombinant fragment of MSP-1 were determined by Western Blot analysis.

In contrast to the lack of antibody responses against the 18 kDa shsp in children <4 years old, serum IgG responses against the *P. falciparum* malaria parasite MSP-1 domain were detected in all age groups tested. Strong staining of the MSP-1 band was observed for the majority of sera collected from children between one and seven years of age as well as for one of the infants (Figure 3.4).

Stability of anti-18 kDa shsp IgG titres

One year after the first serum collection in Mbandji 2, 80 of the 395 study participants were re-sampled. While only minimal changes in antibody titres against the 18 kDa shsp were recorded overall, more individuals had a decreased than an increased serum IgG level after one year (Figure 3.5A).

Increases in OD tended to be small and confined to the older children and young adults (Figure 3.5B). The most distinct changes, characterized by a marked decrease of antibody titres between the two surveys, occurred in young adults.

There was a significant association between age group and the absolute change in OD (Kruskal-Wallis test $p=0.01$) and borderline evidence of an association between the variation in changes in OD and age group (Levene's test for homogeneity of variances, $p=0.08$).

***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 titres were also determined in sera from 96 BU patients and 384 healthy matched control individuals living in a second BU endemic site in West Africa, the Densu River Valley in Ghana. Each serum sample was tested twice, once in each of two independent experiments (Figure S3.1). Negligible overall bias between experiments was observed with the mean difference (OD1-OD2) of 0.024. There was also a reasonably small variation in the individual differences with the 95 % limits of agreement from -0.0796 to 0.1278. There was no evidence of a difference in the ELISA OD values between the cases and controls ($p=0.99$) (Figure 3.6A).

While sero-responders were identified in all age groups of individuals more than 6 years old, none of the sera from children younger than 5 years exhibited a distinct anti-18 kDa shsp IgG titre (Figure 3.6B). Results of representative subsets of sera which tested negative, moderately positive or 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).

3.6 Discussion

A high degree of antigenic cross-reactivity among mycobacterial species complicates investigations on *M. ulcerans*-specific humoral immune responses. However, the immunodominant 18 kDa shsp [17], which is overexpressed in *M. ulcerans* [18], represents a suitable serological marker for exposure to *M. ulcerans* [14]. Diverse outcomes of infection with other mycobacteria, such as *M. tuberculosis* and *M. leprae* have been associated with both host and pathogen factors. While only one study has investigated a possible association between BU and host genetics [19], various behavioural factors that may lead to increased risk to develop 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 [20]. While the generalization persists that children <15 years old are most affected by the disease [13], our recent survey for BU in the Mapé Basin [11] and continued monitoring of new BU cases in this region have 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 below the age of four are underrepresented among cases when adjusting for the population age distribution. Data of our previous sero-epidemiological investigations revealed that the proportion of individuals from a BU endemic area showing serum IgG titres against the 18 kDa shsp of *M. ulcerans* is comparable for all age groups >5 years [14]. Results of the present study, including for the first time a substantial number of serum samples from children <5 years of age, showed that children of this age group have not yet sero-converted. Hence, young children appear to be considerably less exposed to *M. ulcerans*. This reduced exposure may be explained by the smaller movement radius away from the house of these very young children. Although, these small children do leave the house, they usually do so being carried by a caregiver and are therefore not in direct contact with the environment, at more distant places from their homes. No significant difference could be observed when comparing anti-*M. ulcerans* 18 kDa shsp antibody titres between BU patients and controls. This may be related to the immune-suppressive effect of mycolactone and concurs with the lack of a serological response in experimentally infected mice (unpublished data).

The 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 [21]. In African BU endemic settings, the highly focal transmission of *M. ulcerans* haplotypes [12,22,23], as well as the distribution

pattern of BU lesions on the body [11], speak against an exclusive role of mosquito vectors in transmission. Here we observed in children <5 years frequent sero-conversion for the MSP-1 antigen of the mosquito-transmitted malaria parasites in the absence of an IgG response against the *M. ulcerans* 18 kDa shsp. The age distribution of BU cases and the relatively abrupt changes in this risk of contracting BU with age do not speak for transmission of BU by mosquito species commonly found within the small movement radius of very young children.

Within the framework of our analyses, blood was collected for a second time from a limited number of participants one year after the first sample. Results of this pilot study showed that anti-18 kDa shsp IgG titres were relatively stable in older adults. Future studies of the age-related changes in behaviour of three to six year old children, monitoring of their movement radius and water contact patterns in combination with larger longitudinal serological and environmental studies may have the potential to shed further light onto the mode of transmission and relevant environmental reservoirs of *M. ulcerans*.

3.7 Figures

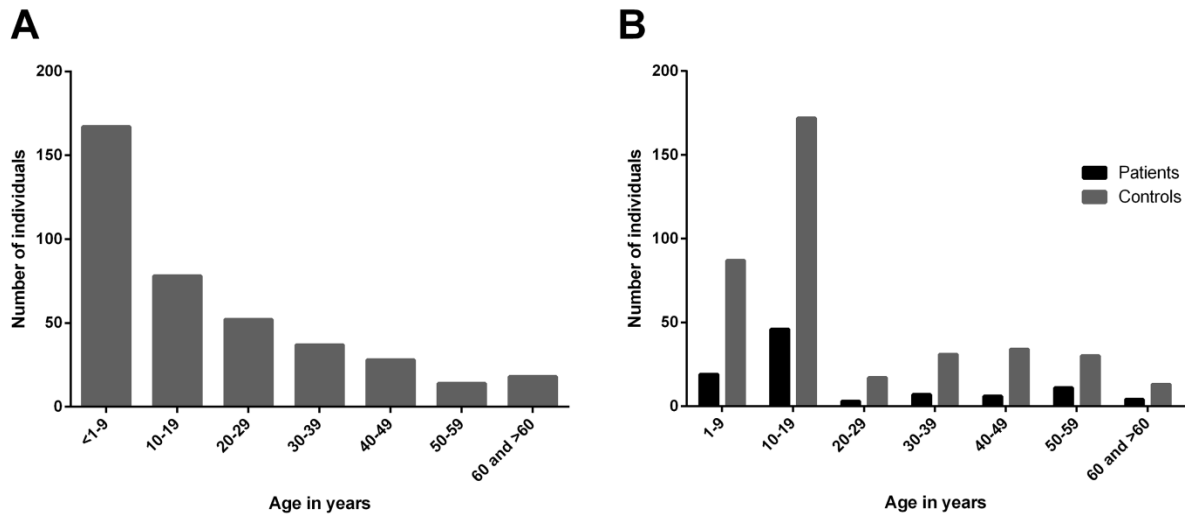


Figure 3.1. Age distribution of study participants. A In Cameroon, serum samples were collected from 395 healthy individuals from the BU endemic village of Mbandji 2. B In the Obom sub-district of the Ga-South district in Ghana, blood sera were collected from 96 BU patients (black) and 384 control individuals (grey) of the different age groups shown.

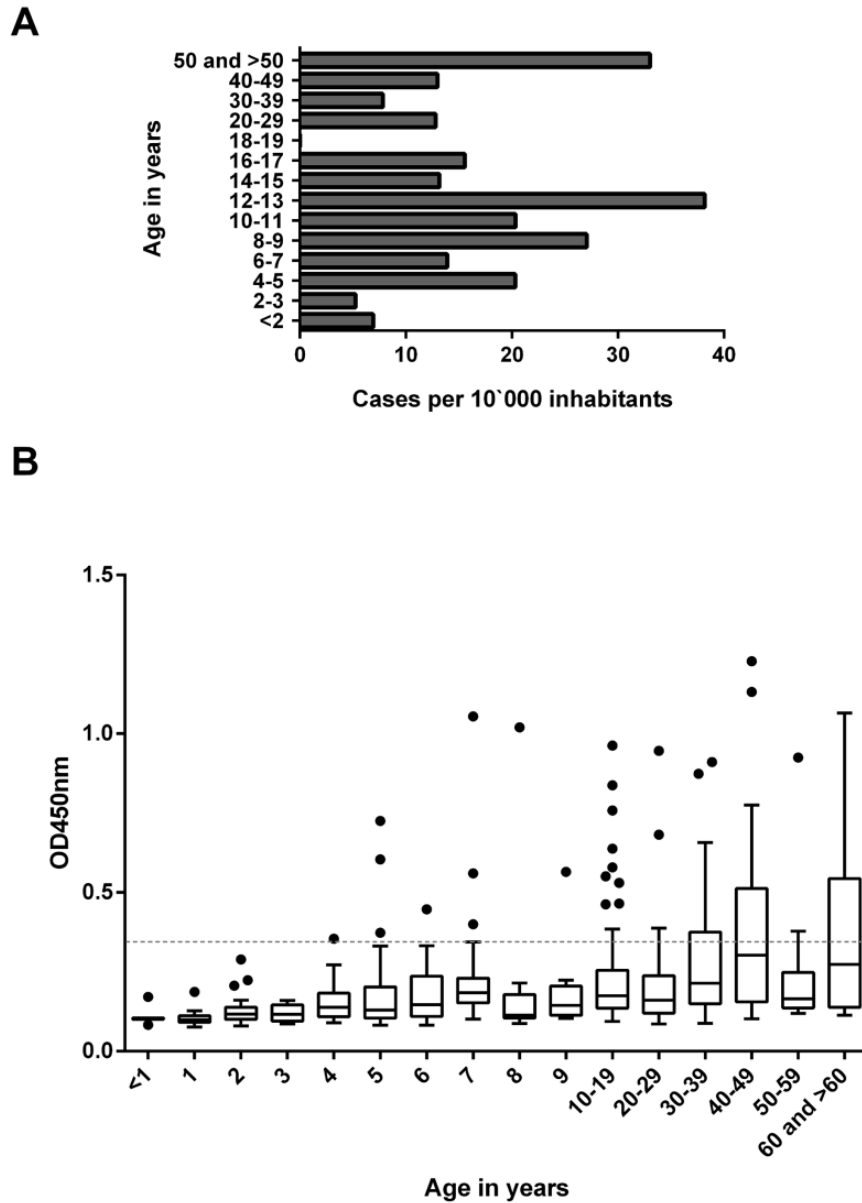


Figure 3.2. Age distribution of BU incidence and anti-18 kDa shsp IgG serum titres among healthy inhabitants of Mbandji 2. A Incidence of reported BU by age in the Bankim Health District (March 2010 – May 2013). B Boxplot of OD values of 1:100 diluted serum samples from inhabitants of Mbandji 2 tested in an anti-*M. ulcerans* 18 kDa shsp IgG specific ELISA by age group. No IgG titres above the background level were observed for children below the age of four. The background response (OD = 0.35) is indicated as a dotted line.

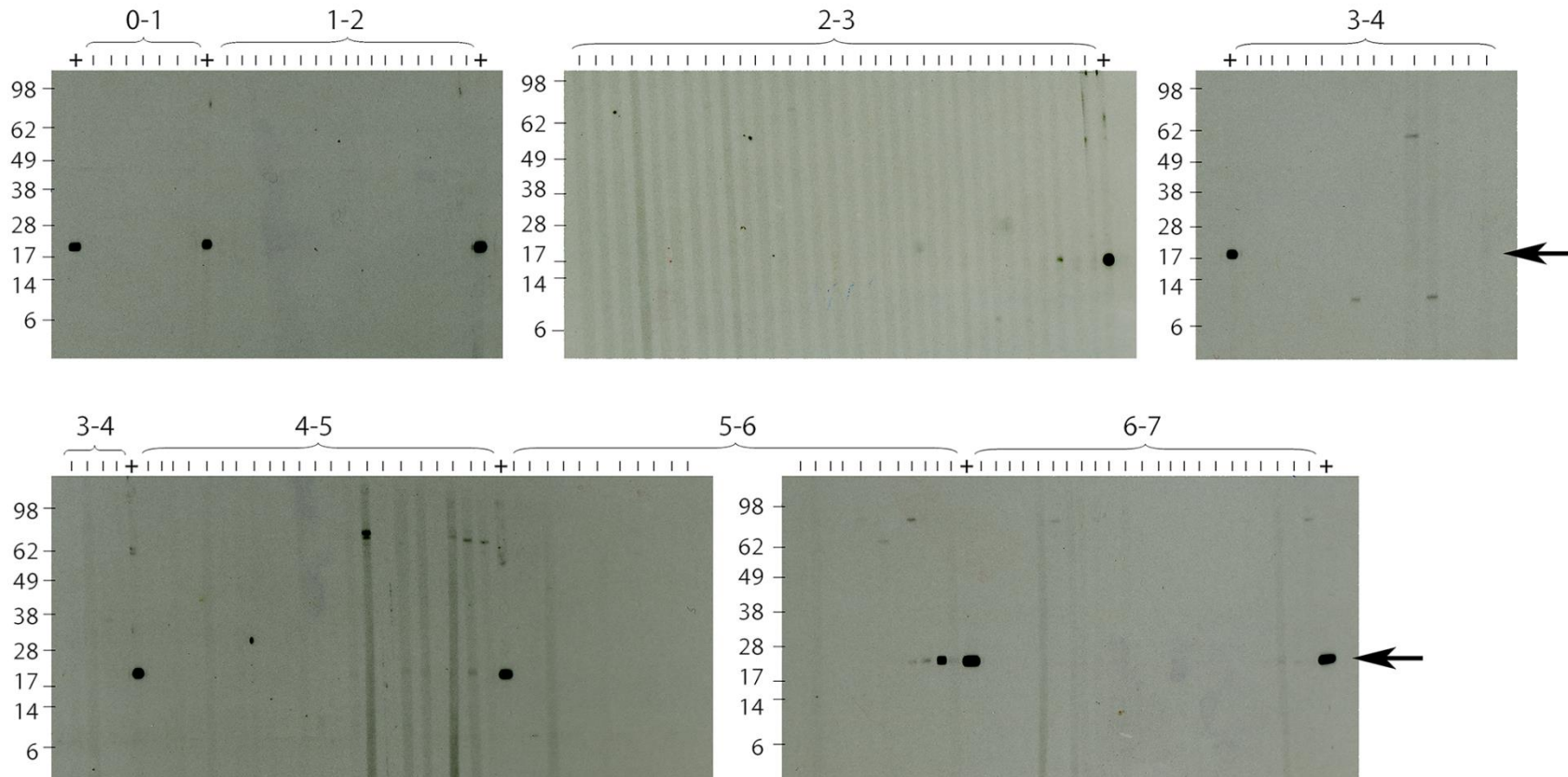


Figure 3.3. Western blot analysis of anti-18 kDa shsp IgG responses in children. Sera collected from children living in Mbandji 2 were tested for the presence of anti-*M. ulcerans* 18 kDa shsp IgG by Western Blot analysis. No specific bands were detected for very young children below the age of four. An ELISA positive control serum (OD = 0.963) was included between each of the age groups tested (+; arrow at band corresponding to size of the 18 kDa shsp).

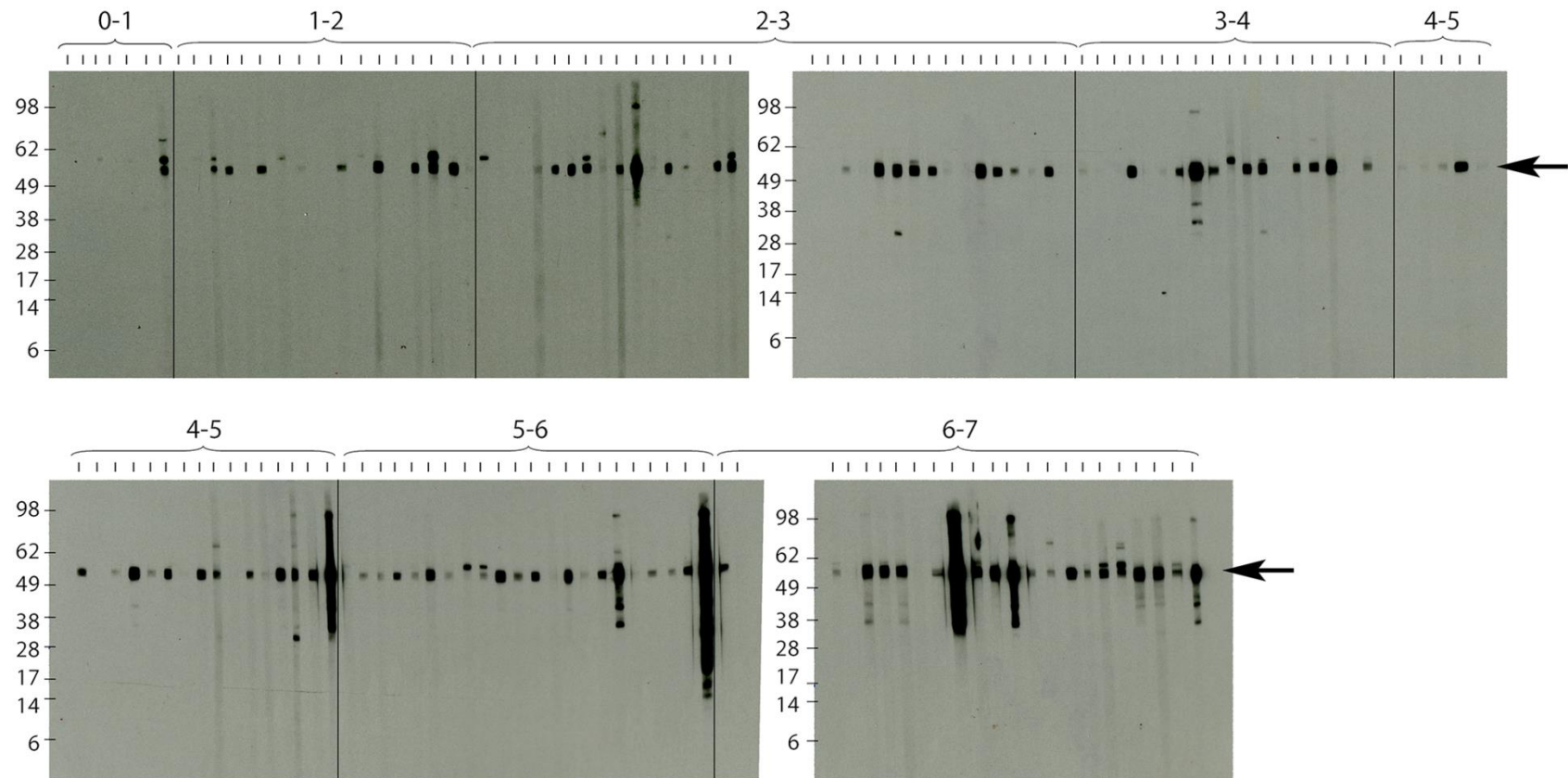


Figure 3.4. Western blot analysis of anti-*P. falciparum* MSP-1 IgG responses in children. Sera from children living in Mbandji 2 were tested for IgG responses against a domain of the *P. falciparum* MSP-1 protein by Western Blot analysis. Specific bands were detected in the majority of individuals of all tested age groups above 1 year. The band corresponding to the specific signal of the MSP-1 protein domain is indicated with an arrow.

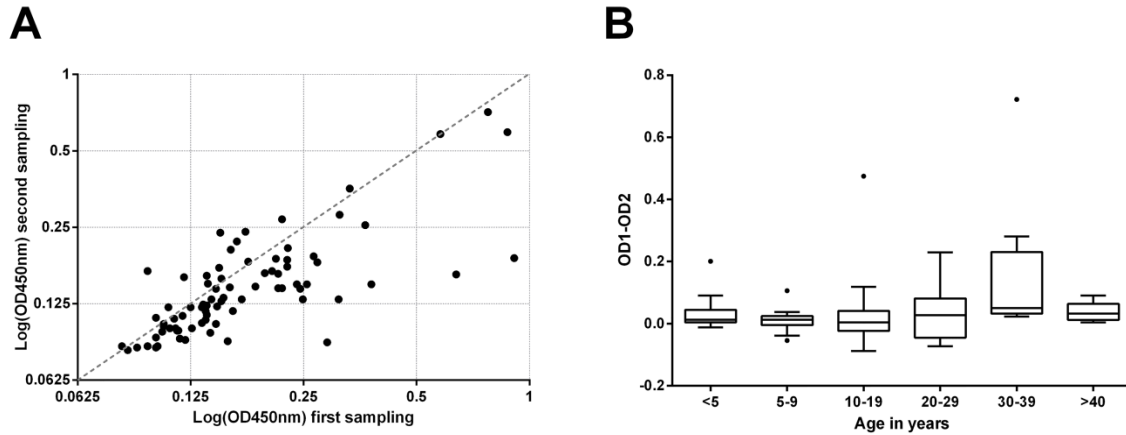


Figure 3.5. Changes in anti-*M. ulcerans* 18 kDa shsp IgG titres in sequentially collected serum samples. An IgG titres against the *M. ulcerans* 18 kDa shsp were determined in serial serum samples collected from 80 individuals. The majority of changes were small and most individuals showed a slightly decreased titre after one year. B Boxplot of differences in OD values between the two samples are shown by age group. Changes in antibody titres were most pronounced in young adults.

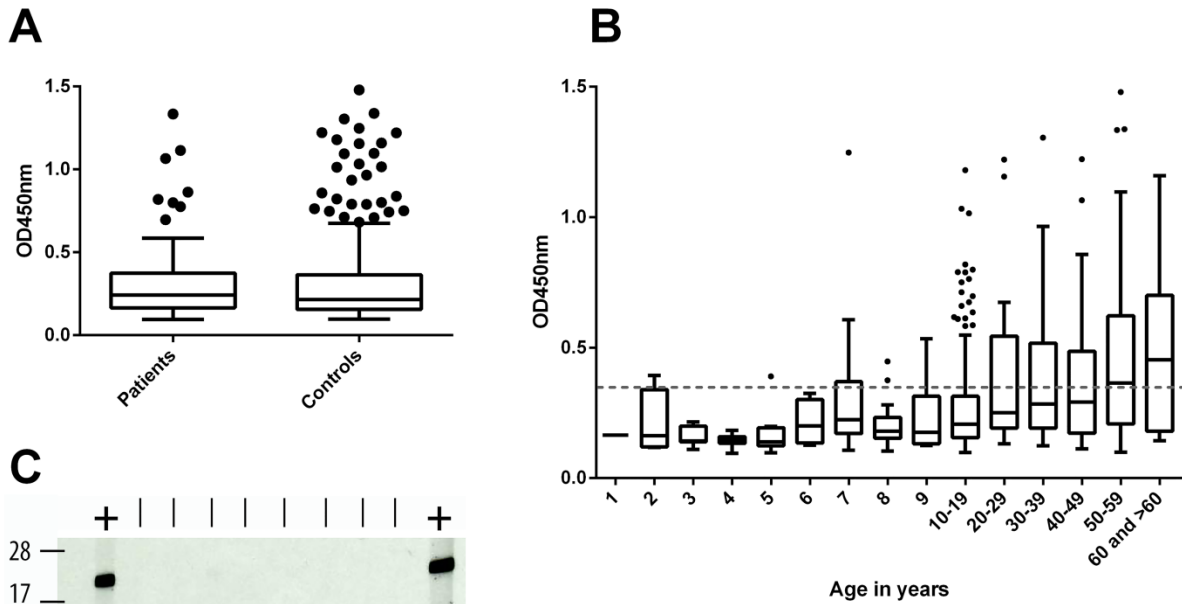


Figure 3.6. Anti-*M. ulcerans* 18 kDa shsp IgG titres in sera from Ghanaian BU patients and control individuals. A Box plots showing OD values of 1:100 diluted sera from Ghanaian BU patients (n = 96) and control individuals (n = 384) tested in an anti-*M. ulcerans* 18 kDa shsp IgG specific ELISA. B Boxplot showing the distribution of OD values for BU patients and control individuals for different age groups. No IgG titres above the background level were observed for children below the age of 5 years. The background response (OD = 0.35) is indicated as a dotted line. C Sera from eight 2-year-old children were tested by Western Blot analysis to reconfirm the absence of anti-*M. ulcerans* 18 kDa shsp IgG. An ELISA positive control serum (OD = 0.76) was included (+) flanking the tested sera.

3.7.1 Supplementary Figures

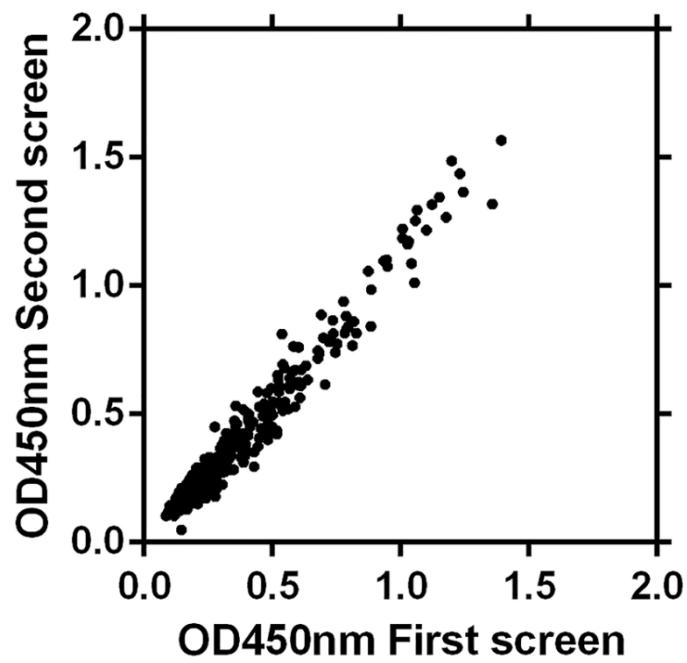


Figure S3.1. Duplicate ELISA testing of sera. All serum samples collected from individuals living in the BU endemic Densu River Basin of Ghana were tested twice (screen 1 and screen 2).

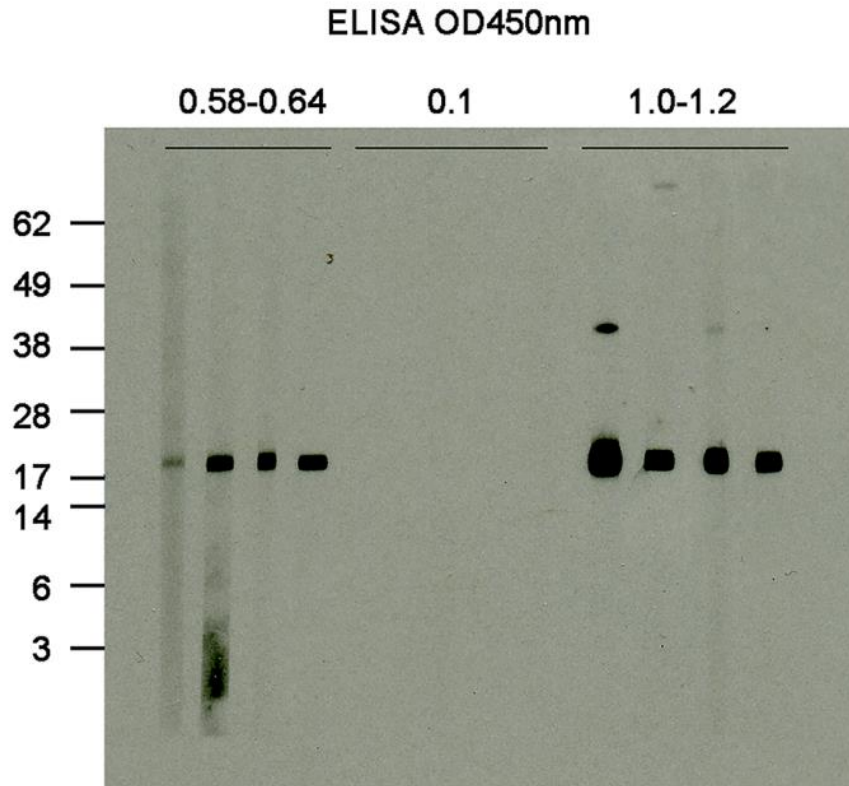


Figure S3.2. Reconfirmatory Western blot of randomly chosen Ghanaian sera. A subset of sera from Ghana which tested moderately positive (OD = 0.58–0.64), negative (OD = 0.1) and highly positive (OD = 1.0–1.2) by ELISA were tested by Western Blot analysis. Specific bands were detected for ELISA positive sera, while no signal was obtained for ELISA negative sera.

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Chapter 4

A Sero-epidemiological Approach to Explore Transmission of *Mycobacterium ulcerans*

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4.1 Abstract

The debilitating skin disease Buruli ulcer (BU) is caused by infection with *Mycobacterium ulcerans*. While various hypotheses on potential reservoirs and vectors of *M. ulcerans* exist, the mode of transmission has remained unclear. Epidemiological studies have indicated that children below the age of four are less exposed to the pathogen and at lower risk of developing BU than older children. In the present study we compared the age at which children begin to develop antibody responses against *M. ulcerans* with the age pattern of responses to other pathogens transmitted by various mechanisms. A total of 1,352 sera from individuals living in the BU endemic Offin river valley of Ghana were included in the study. While first serological responses to the mosquito transmitted malaria parasite *Plasmodium falciparum* and to soil transmitted *Strongyloides* helminths emerged around the age of one and two years, sero-conversion for *M. ulcerans* and for the water transmitted trematode *Schistosoma mansoni* occurred at around four and five years, respectively. Our data suggest that exposure to *M. ulcerans* intensifies strongly at the age when children start to have more intense contact with the environment, outside the small movement range of young children. Further results from our serological investigations in the Offin river valley also indicate ongoing transmission of *Treponema pallidum*, the causative agent of yaws.

4.2 Author Summary

Buruli ulcer is a debilitating skin disease caused by *Mycobacterium ulcerans*. Although the understanding of this enigmatic pathogen has improved after decades of research, the mode of transmission is yet to be fully elucidated. Recent epidemiological studies have shown an underrepresentation of Buruli ulcer cases in children below the age of four as compared to older children. In order to investigate the different exposure of children to *M. ulcerans* and to several other pathogens with diverse modes of transmission, we conducted a sero-epidemiological study of 1,352 residents within a five kilometer radius along the Offin River of Ghana. While our results show early exposure of children to the mosquito transmitted malaria parasite *Plasmodium falciparum* as well as to soil transmitted helminths of the genus *Strongyloides*, a later onset of immune response was observed at an age of around four and five years for *M. ulcerans* and the trematode *Schistosoma mansoni*, that is transmitted by contact with infested water. Similarities in age-dependent exposure of these two pathogens suggest that transmission may take place outside of the very young children's movement range when they come into contact with the environment at the periphery of their villages.

4.3 Introduction

Buruli ulcer (BU) is a neglected tropical skin disease presenting with a wide range of cutaneous manifestations, from non-ulcerated nodules, plaques or oedema to characteristic necrotizing ulcers [1]. While BU cases have been reported in more than 30 countries worldwide, most patients are from infection foci located in remote and rural tropical regions of West and Central Africa. BU is caused by infection with *Mycobacterium ulcerans*, a pathogen that has emerged from *M. marinum* by acquiring a plasmid conferring the capacity of producing the unique macrolide toxin mycolactone, accounting for much of the pathology of BU [2,3]. Until today, the mode of transmission of *M. ulcerans* has remained inconclusive, although proximity to aquatic habitats has long been identified as the major risk factor for contracting the disease [4]. Infection is thought to take place through either physical contact with undefined environmental reservoirs via skin abrasions or insect vectors [5–7].

It has long been recognized that in African BU endemic settings the majority of BU patients are children below 15 years of age [8]. However, a clear underrepresentation of children below the age of four becomes evident when the population age distribution is taken into account [9,10]. In line with this observation, our previous sero-epidemiological studies in Ghana and Cameroon have indicated that children below five years of age rarely develop antibody responses against the 18 kDa small heat shock protein (shsp) of *M. ulcerans* and thus seem to be considerably less exposed to the pathogen than older children [11]. While investigations of humoral immune responses against mycobacteria are complicated by a high degree of antigenic cross-reactivity between species, the immunodominant 18 kDa shsp overexpressed by *M. ulcerans* [12] represents a suitable marker for exposure to this pathogen [13]. It has no homologues in other prevalent pathogenic mycobacteria such as *M. bovis* and *M. tuberculosis* and additionally, sera from inhabitants of BU non-endemic regions generally showed no reactivity with this protein [13]. While populations living in BU non-endemic communities in proximity to the BU endemic regions seem to be similarly exposed to the 18 kDa shsp of *M. ulcerans* [14,15], we have observed for the three Ghanaian BU endemic river valleys Densu [15], Offin (this report) and Volta [15] an association between BU prevalence and the percentage of healthy individuals that have sero-converted.

Here we present a sero-epidemiological study carried out in the BU endemic Offin river valley of Ghana including 1,352 participants from 13 communities. The main objective was to compare

the age-pattern of first humoral immune responses to the 18 kDa shsp of *M. ulcerans* with those to pathogens with various modes of transmission in order to contribute to our understanding of the transmission of *M. ulcerans*.

4.4 Methods

Ethics statement

Ethical clearance for the study was obtained from the institutional review board of the Noguchi Memorial Institute for Medical Research (Federal-wide Assurance number FWA00001824). Written informed consent was received from all individuals involved in the study. Parents or guardians provided written consent on behalf of their children.

Study area

The Offin River is one of the major water bodies associated with BU in Ghana. It runs through the Ashanti and Central Regions, covering 11 health districts. In a nationwide active BU case search conducted in 1999, the Offin river valley was shown to be highly endemic for BU [16].

A total of 1,560 inhabitants of ten randomly selected communities and an additional three communities known to be BU endemic and located within a five kilometer radius along the Offin River (120 per community) (Fig. 4.1) were randomly selected for sampling. Two milliliters of whole blood were drawn from 1,352 of 1,560 (87%) selected inhabitants in July 2013. In order to reduce dropout rates due to repetitive bleeding of participants, we randomly assigned each of the sampled individuals to one of three groups (A, B, and C) with each consisting of 450, 451, and 451 individuals to be followed up after 6 (January 2014), 12 (July 2014) or 18 (January 2015) months, respectively. Blood separation was achieved by centrifugation of the whole blood at $2'000 \times g$ for 10 minutes. Serum was subsequently stored at -80°C pending serological analysis. First serological analyses were performed between January and July 2014 allowing for a more thorough follow up of individual cases, such as sero-converters or sero-reverters.

ELISA to detect anti-*M. ulcerans* 18 kDa shsp antibodies in serum samples

ELISA was performed as described previously [11]. Briefly, 96-well Nunc-Immuno Maxisorp plates (Thermo Scientific) were coated with $0.25 \mu\text{g}$ recombinant *M. ulcerans* 18 kDa shsp per well, washed with washing buffer (dH_2O , 0.01% Tween 20) and incubated with blocking buffer (5% non-fat dry milk in PBS). Subsequently, plates were incubated with human blood serum samples (1:100 diluted). After washing, horseradish peroxidase conjugated goat anti-human IgG (γ -chain specific, SouthernBiotech) was added. Plates were washed and developed with TMB Microwell Peroxidase Substrate (KPL). The reaction was stopped using 0.16 M sulfuric acid.

The absorbance was measured at 450 nm in a Tecan Sunrise microplate reader. All samples were tested in duplicates and mean values were calculated.

The cut-off value for positivity ($OD_{450} = 0.25$) was determined by testing serum samples with a range of ODs in ELISA by Western Blot analysis (Fig. S4.1). Sero-conversion/reversion was defined as a change in OD (ΔOD) between baseline and follow up samples of at least ± 0.3 .

Western Blot analysis to detect anti-*M. ulcerans* 18 kDa shsp and anti-*P. falciparum* AMA-1 antibodies

Western Blot analysis was performed as described [11]. Shortly, 15 μg of recombinant *M. ulcerans* 18 kDa shsp or *P. falciparum* AMA-1 were separated on NuPAGE Novex 4–12% Bis-Tris ZOOM Gels with 1.0 mm IPG well (Invitrogen) under reducing conditions. After electrophoresis, proteins were transferred onto nitrocellulose membranes using an iBlot Gel Transfer Device (Invitrogen). Membranes were blocked with 5% non-fat dry milk in PBS containing 0.1% Tween 20 and cut into strips. Strips were then incubated with human blood sera (1:1000 dilution), washed with 0.3 M PBS containing 1% Tween 20 and thereafter incubated with horseradish peroxidase conjugated goat anti-human IgG (γ -chain specific, Southern Biotech). After a second washing step, bands were visualized by chemiluminescence using ECL Western Blotting substrate (Pierce).

Simultaneous detection of antibodies to treponemal and non-treponemal antigens

The contagious diseases syphilis and yaws are caused by closely related *Treponema pallidum* spp. Serological diagnosis requires detection of distinct antibodies against both a treponemal antigen and a non-treponemal antigen. Non-treponemal tests become reactive during the initial stage of infection and generally revert to negative after treatment. However, treponemal antigen-based confirmation is needed, since detectable antibodies can also be due to other inflammatory conditions. Treponemal serological tests on the other hand may remain reactive for life and thus require a positive non-treponemal test result to confirm active infection [17]. Here we used the Dual Path Platform (DPP) assay manufactured by Chembio Diagnostic Systems for the simultaneous detection of antibodies to treponemal and non-treponemal antigens. This serological test, which was developed for the point-of-care diagnosis of syphilis [18], was

recently also applied to and evaluated for the screening and detection of patients with yaws [19]. We tested 5 µl of the serum samples in strict accordance with the manufacturer's instructions.

ELISA to detect antibodies to *Schistosoma* and *Strongyloides* antigens

Preparation of *S. mansoni* antigens. Soluble egg antigen (SEA) and adult worm antigen extract (AWE) were prepared as described previously [20]. Briefly, frozen *S. mansoni* eggs were homogenized in PBS (pH 7.2) on ice and subsequently extracted for 3 hours at 4°C. The extract was centrifuged at 100'000 x g for 2 hours at 4°C and the supernatant was stored in aliquots at -80°C until use. Adult *S. mansoni* worms were homogenized in PBS (pH 7.2) containing 2 mM PMSF. The extract was centrifuged at 80'000 x g for 3 hours at 4°C and the pellet was re-suspended. After overnight incubation at 4°C the suspension was centrifuged again at 80'000 x g for 3 hours at 4°C. The supernatant was concentrated and centrifuged at 15'300 x g for five minutes at 4°C before being stored in aliquots at -80°C until use.

Both *S. mansoni* antigens show cross-reactivity with antibodies elicited by other *Schistosoma* spp. (*S. haematobium*, *S. mekongi* or *S. japonicum*).

Preparation of *S. ratti* antigen. Mass production of antigens from *S. stercoralis* filariform larvae suffers the drawbacks of cost, being labor intensive and constituting a risk of infecting laboratory technicians handling the larvae. Heterologous antigen of filariform larvae of the rat parasite *S. ratti* provides comparable sensitivity and specificity, thereby making it a suitable alternative antigen for sero-diagnosis of human strongyloidiasis [21–23]. For this study, third-stage larvae (iL3) of *S. ratti* were collected as described previously [24] and frozen at -80°C. For the preparation of raw antigen, larvae were homogenized in PBS (pH 7.2) on ice. Proteins were extracted for 24 hours at 4°C on a stirrer and the suspension was centrifuged at 100'000 x g for 1 hour at 4°C. The supernatant was stored in aliquots at -80°C.

Detection of anti-*Schistosoma* and anti-*Strongyloides* antibodies by ELISA. Immulon 2HB plates (Thermo Labsystems) were coated with *S. mansoni* or *S. ratti* antigens in 0.05 M sodium carbonate buffer (pH 9.6) for 48 hours at 4°C. After washing with tap water containing 0.05% Tween 20, diluted sera (1:160 in PBS, pH 7.2, 0.05% Tween 20) were added to the plates and incubated for 15 minutes at 37°C. After additional washing steps, horseradish peroxidase conjugated goat anti-human IgG (KPL) was added. Plates were incubated for 15 minutes at 37°C, subsequently washed and o-Phenylendiamine dihydrochloride (OPD, Sigma) diluted in 0.6

M sodium phosphate buffer, pH 5.0 supplemented with 0.03% H₂O₂ was added. The reaction was stopped with 8 M H₂SO₄ and the absorption was read with a Multiscan FC reader (Thermo Scientific) at 492 nm. The presence of anti- *Schistosoma* antibodies was reconfirmed for the serum of a young child by immune fluorescence assay (IFA) carried out as described [20]. Serum samples positive in the *Strongyloides* ELISA were confirmed by re-testing in duplicates.

Cut-off values (Table 4.1) were determined previously by receiver operating characteristic (ROC) analysis of results obtained for 50 sera of healthy Swiss blood donors, 20 sera each of patients infected with *S. mansoni* or *S. stercoralis*, and 120 sera of individuals with other parasitic infections (Fig. S4.2). The sensitivity for the *S. mansoni* ELISA was 98% and 80% for AWE and SEA, respectively, while the specificity was 96% and 92% (Table S4.1). SEA can exhibit cross-reactivity with *Trichinella* and to a lesser extent with *Filaria*. The sensitivity of the *S. ratti* ELISA was 95%, while the specificity was 84% due to potential cross-reactivity with *Filaria* and *Echinococcus* ssp (Table S4.1).

Data analysis

ELISA results were analyzed using GraphPad Prism version 6.0 (GraphPad Software, San Diego California USA). The distribution of antibody titers is presented as box plots. These comprise 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. The overall difference and variation between samples tested in duplicates was estimated using the Bland-Altman method [25].

4.5 Results

Age distribution of *M. ulcerans* 18 kDa shsp specific serum IgG responses

In order to assess exposure of the population living in a five kilometer radius along the Offin River to *M. ulcerans*, serum samples taken from 1,352 study participants of 13 communities across different age groups (Table 4.2) were tested in duplicates for the presence of anti- *M. ulcerans* 18 kDa shsp IgG by ELISA. Between the two duplicate test results, a negligible overall bias with a mean difference (OD1-OD2) of 0.004 was estimated. The variation in individual differences was very small, with 95% limits of agreement from -0.095 to 0.103. At baseline, 18% of the serum samples contained IgG titers against the *M. ulcerans* protein. In all, 3% (46/1,352) of the participants were BU cases with healed or active lesions and 13% (179/1,352) were household contacts of BU patients. Amongst the BU cases, 24% (11/46) had antibodies against the *M. ulcerans* 18 kDa shsp, while 15% (26/179) of the household contacts contained anti- *M. ulcerans* 18 kDa shsp titers in their sera.

The age distribution of the mean of duplicate anti- *M. ulcerans* 18 kDa shsp IgG titers for the study participants is shown in Fig. 4.2A and Table 4.2. While in all age groups above nine years individuals with high IgG titers were detected, only three sera from children below the age of five years contained 18 kDa shsp specific antibodies and also most of the sera from children between five and seven years tested negative. The only child below four years of age with positive test result was a three year old resident of Mfantseman, a BU endemic community. We carefully followed up this particularly interesting individual case at two occasions. Before May 2013, he lived for his entire life in a BU non-endemic community (Wanpiem) not included in this study, but was transferred thereafter to the BU endemic community Mfantseman for schooling and was enrolled there in this study. Shortly after sampling his blood in July 2013, he was relocated to Wanpiem by his guardian. Scheduled for sampling after 12 months (July 2014), we followed him up at Wanpiem and observed that he had sero-reverted. His serum still tested negative for anti- *M. ulcerans* 18 kDa shsp IgG after 18 months of follow up (January 2015). Thus, his total length of stay in the BU endemic Mfantseman community was three months (May-July 2013); coinciding with the only sampling point at which we observed a high anti- *M. ulcerans* 18 kDa shsp IgG titer in his serum. Western blot results for the three time points reconfirmed the ELISA data (Fig. 4.2B). Testing of sera from all other study participants younger than five years by Western Blot analysis revealed no specific bands (representing IgG

titers) against the 18 kDa shsp for sera from children below four years of age (Fig. S4.3), while positive sera were detected in all age groups ≥ 4 years (Fig S4.3 and S4.4), indicating that sero-conversion may start in some children around the age of four years.

Stability of anti- *M. ulcerans* 18 kDa shsp IgG titres

Follow up samples were taken from 319 of 450 (71%), 329 of 451 (73%), and 356 of 451 (79%) participants assigned to groups A, B and C after 6, 12, or 18 months, respectively (Fig 4.3A). Testing of the serum samples for the presence of anti- *M. ulcerans* 18 kDa shsp IgG revealed that based on the defined value for the difference between paired samplings ($\Delta OD = 0.3$), antibody titers remained stable for 98%, 97%, and 92% of the study participants, respectively. After 6, 12, and 18 months 1%, 0.3%, and 6% of the sero-positive individuals had sero-reverted, while 1%, 3% and 1.4% of the sero-negative participants had sero-converted (Fig 4.3B-D).

Age distribution of IgG titres against antigens of other pathogens with known mechanism of transmission

***Plasmodium falciparum*.** Serum samples collected from children below the age of four years were tested for the presence of IgG titers against the AMA-1 protein of the mosquito transmitted malaria parasite *P. falciparum*. In contrast to the late onset of the serological response in children for the *M. ulcerans* 18 kDa shsp, Western Blot analysis for AMA-1 showed that a number of sera from one (1/3) and two (7/15) year old infants and almost all sera from three year old children (26/37) contained antibodies against this microneme protein of sporozoites and merozoites [26] (Fig 4.4, Table 4.2).

***Treponema pallidum*.** Serum samples from all children below the age of 11 years ($n = 402$) and representatives of older age groups ($n = 217$) were analyzed for the presence of antibodies against non-treponemal and treponemal antigens using the DPP Screen and Confirm assay (Chembio Diagnostic Systems). Antibodies against both treponemal and non-treponemal antigens were detected in sera from only a limited number of children below the age of 11 years (4/402 children, 1%). Sera from nine children below the age of 11 years (9/402, 2.2%) tested positive for the treponemal antigen only. A higher percentage of sera from older children and adults tested positive for both antigens (15/217; 6.9%) as well as for the treponemal antigen alone (13/217; 6%) (Table 4.2).

Strongyloides spp. Sera from all children below 11 years of age and from the same representatives of older age groups as tested for antibodies against *T. pallidum* antigens were also analyzed for IgG titers against raw antigens of *S. ratti* by ELISA as a marker for exposure to *S. stercoralis*, since *S. ratti* antigen is cross-reactive with antibodies elicited by infection with this soil-transmitted helminth. The overall sero-positivity was low with 41/619 (6.6%) sera yielding equivocal test result and 33/619 (5.3%) testing positive. However, 3/170 (1.8%) children aged five years and below already tested positive (Fig 4.5, Table 4.2), demonstrating early exposure to infested soil.

Schistosoma mansoni. Serum samples from all children <11 years and from the same representatives of older age groups as tested for antibodies against the other antigens were also analyzed for the presence of IgG against SEA and AWE of *S. mansoni*. Of the 619 sera, 29 (4.7%) and 32 (5.2%) tested positive in the SEA and AWE ELISA, respectively. Most of the responders (n = 18; 2.9%) tested positive in both assays. While none of the children below the age of five tested positive against *S. mansoni* SEA, seven children aged five to ten had sero-converted and 16/142 (11.3%) individuals aged between 11 and 20 had developed high anti-SEA antibody titers (Fig 4.6A, Table 4.2). A similar pattern was observed for the AWE with only one child aged below seven testing positive (Fig 4.6B, Table 4.2). The serum of this child was reconfirmed as being weakly positive by IFA (Fig 4.6C).

4.6 Discussion

Since only a minority of individuals exposed to *M. ulcerans* develops clinical BU, sero-epidemiological studies represent a valuable tool to assess the exposure of populations in BU endemic areas to the pathogen. In line with data obtained from previous sero-epidemiological investigations in BU endemic areas located in the Densu river basin of Ghana and in the Mapé river basin of Cameroon [11,15], we reconfirmed here for the population of the BU endemic Offin river valley that young children below four years of age are considerably less exposed to *M. ulcerans* than older children. In contrast, our serological data showed, as expected, that exposure to the mosquito transmitted malaria parasite *P. falciparum* and to soil-transmitted helminths of the genus *Strongyloides* takes place already in very young children, as indicated by an early development of humoral immune responses against these pathogens in some of the infants. The delay in exposure of *M. ulcerans* and the relatively abrupt onset are in stark contrast to the age-patterns for *Plasmodium* and *Strongyloides*. Contact with larvae-infested soil through faecal contamination is likely to be responsible for the observed early development of anti-*Strongyloides* serum IgG responses. Our results suggest that contact with *M. ulcerans* occurs outside the small movement range of infants, providing indirect evidence against mechanisms of transmission involving vectors or reservoirs present in the vicinity of the children's homes. However, our data do not exclude an involvement of insect vectors commonly found at the periphery of villages close to water contact sites. We recognize that the age distribution of anti-*P. falciparum* serum antibodies depends not only on the mode of transmission, but also on the transmission intensity [27,28]. While there is some evidence that mosquitos may be involved in transmission of *M. ulcerans* in south-eastern Australia [29,30], it is overall unlikely that they play a major role as vectors in African BU endemic settings. This assumption is also supported by previous molecular epidemiological studies showing that transmission of newly emerging genetic variants of *M. ulcerans* is geographically highly clustered [31,32].

In Ghana schistosomiasis is mainly caused by *S. haematobium* and *S. mansoni* [33]. The egg forms of the parasite are shed into the environment via urine or faeces of an infected person. Through a complex life cycle involving an intermediate snail host, a healthy individual can be infected by coming into contact with water sources infested with invasive larvae [34]. Therefore, the risk of infection is related to water contact patterns. Peak prevalence is usually observed in school-aged children, but may be shifted to adulthood depending on the degree of endemicity in

a setting [35]. In the present study we observed in the Offin river basin the typical age distribution of *Schistosoma* exposure with children below the age of eight being significantly less affected than older children and young adults. This pattern was similar to the age distribution of exposure to *M. ulcerans* in the same population. While the mechanisms of infection by schistosomes and *M. ulcerans* are likely to be different, similarities in the age-dependent patterns of exposure may be related to changes in water contact patterns. It remains to be elucidated whether infection with *M. ulcerans* from an environmental reservoir takes place through skin lesions or via invertebrate vectors, such as aquatic insects. In addition, our data indicate that anti-*M. ulcerans* 18 kDa shsp IgG titers are relatively stable with only 2%, 3% and 8% of individuals followed up after 6, 12 and 18 months, respectively, having sero-converted or sero-reverted. A limitation of this longitudinal study was that study participants were followed up only once after 6, 12 or 18 months. This study design was chosen to reduce dropout rates of study participants due to repeated blood drawings. Follow up of all study participants at three time points would have strengthened conclusions on the stability of antibody titers and in combination with environmental studies might have shed further light onto contact patterns of individuals with *M. ulcerans*.

As part of our study we also analyzed serological responses of children below the age of 11 years to treponemal and non-treponemal antigens in order to assess exposure to *T. pallidum* subspecies *pertenue*, the causative agent of yaws, which is transmitted by direct contact with the fluid from the lesion of an infected person. In contrast to syphilis, caused by the closely related *T. pallidum* subspecies *pallidum*, yaws mainly affects children living in poor rural areas of tropical countries [36]. Traditionally, the recommended algorithm for the serological diagnosis of treponemal diseases includes a non-treponemal test for screening, and a treponemal test for confirmation. Non-treponemal tests detect antibodies to non-treponemal antigens such as cardiolipin and lecithin released from damaged host cells or lipoprotein-like material released from the treponemes. Due to the occurrence of false-positive reactions, treponemal test results based on *T. pallidum* antigen are required for a reconfirmation of non-treponemal tests [37].

While eradication campaigns in the 1950s and 1960s by mass treatment of affected communities led to a drastic reduction of worldwide cases, yaws has lately re-emerged in Africa, Asia and the western Pacific [38] and Ghana was recently reported to be among the three most endemic

countries for yaws [39]. Official case notification rates were 32 and 383 per 100,000 population in 2010 for the Ashanti and Central regions, respectively [40], but underreporting is suspected. In this study antibodies to non-treponemal and treponemal antigens were detected in four of 402 (1%) children below the age of 11, indicating active yaws transmission in the affected communities. Antibodies to the treponemal antigen only were found in 2.2% of the children. Since yaws and syphilis are serologically indistinguishable, the interpretation of test results in adolescents and adults would require careful clinical assessment. While a recently published study in the Northern Region of Ghana has not found evidence of active yaws despite of continued case reporting [41], our data demonstrate evidence of ongoing yaws transmission in communities of the Offin river valley. There is an urgent need for more comprehensive data on the prevalence of yaws in Ghana to better implement mass drug administration programs. Comparative genome analyses, environmental studies, as well as serological and epidemiological studies of BU affected populations have in the last decades gradually broadened our knowledge of environmental reservoirs and probable infection routes of *M. ulcerans*. Future longitudinal sero-epidemiological and environmental studies over longer time periods combined with the monitoring of environmental contact patterns may be required to unravel mysteries of *M. ulcerans* transmission.

4.7 Acknowledgements

We thank Dr. Edwin Ampadu and William Opare of the National Buruli Ulcer Control Program in Ghana, the District Health Directors and Disease Control Officers of all the Health Districts we worked in, the Volunteers of all study communities for their support and also Frank Adu-Nti for coordinating and assisting with our field studies. We thank Christina Krebs and Karin Stoll-Rudin (Diagnostic Centre, Swiss TPH) for expert technical assistance.

4.8 Figures



Figure 4.1. Study area. Map of the Offin river basin in Ghana with selected study communities indicated as red dots.

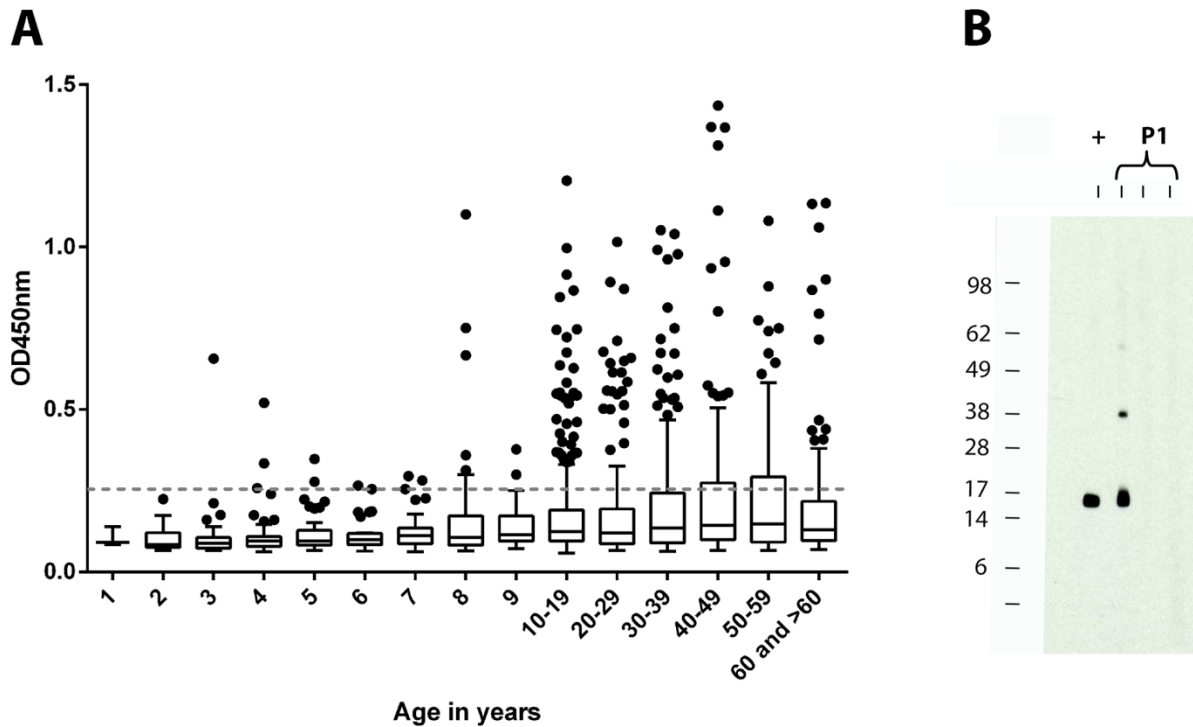


Figure 4.2. Age distribution of anti- *M. ulcerans* 18 kDa shsp serum IgG titers. (A) Boxplots of OD values of 1:100 diluted serum samples tested in the anti- *M. ulcerans* 18 kDa shsp IgG specific ELISA by age group. The background cut-off value (OD<0.25) is indicated as a dotted line. The number of study participants for each age group (right y-axis) is visualized by grey dots connected by a grey line. (B) Sera obtained from a child participant (P1) at three different time points were tested for the presence of anti- *M. ulcerans* 18 kDa shsp serum IgG by Western Blot analysis. While the serum of the child tested positive at baseline, sero-reversion was observed after it had moved to a BU non-endemic community. A positive control serum (+) was included. The molecular weight standard (in kDa) is shown on the left of the blot.

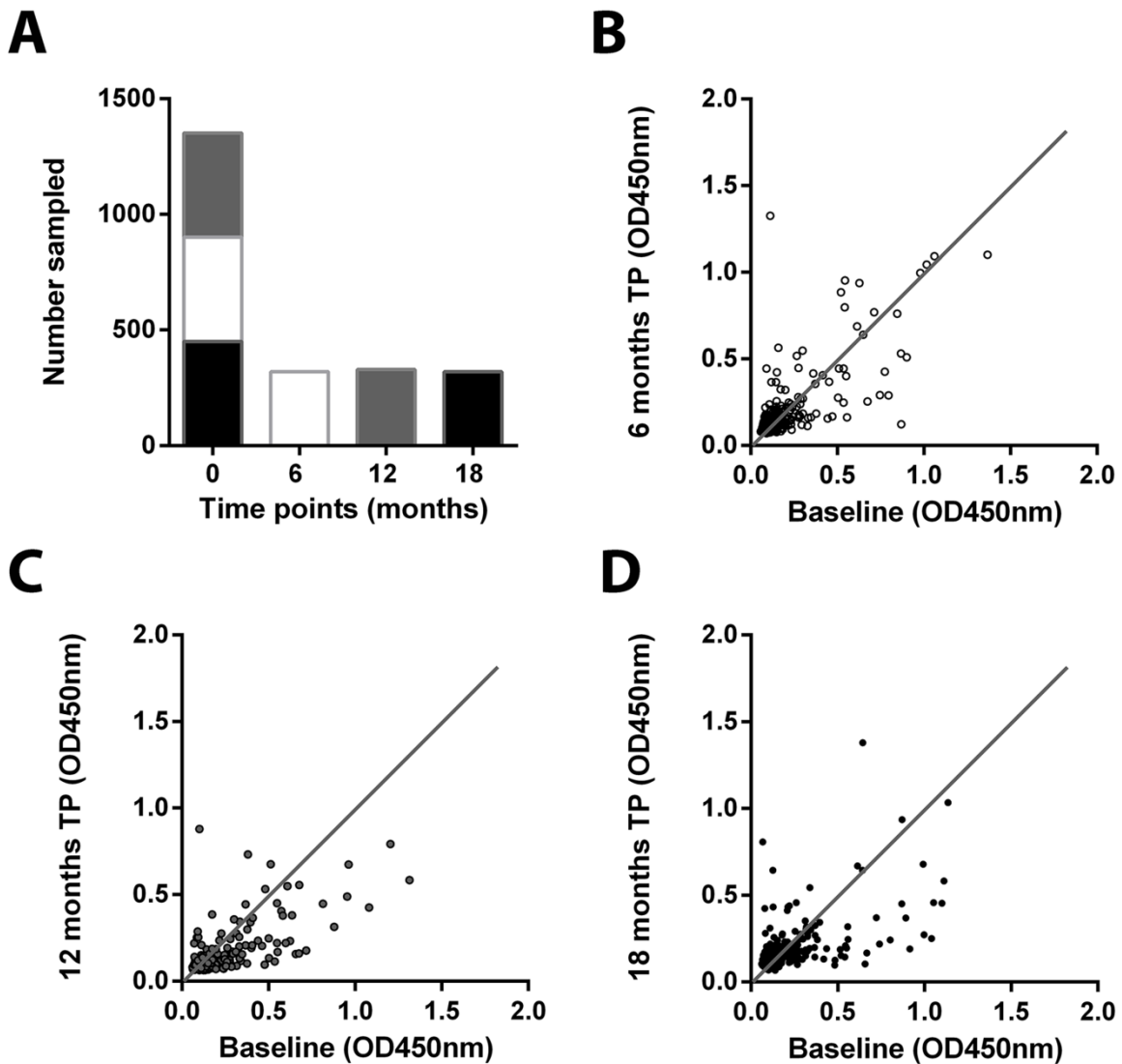


Figure 4.3. Design and results of a longitudinal sero-epidemiological study. (A) Blood samples were taken in July 2013 from 1,352 inhabitants of the communities selected. All sampled individuals were followed up after either 6 (January 2014), 12 (August 2014) or 18 (January 2015) months. IgG titers against the *M. ulcerans* 18 kDa shsp were determined in serum samples collected at baseline and compared to titers in samples collected at different time points (TP) after 6 (B), 12 (C) or 18 (D) months. A straight line was drawn to visualize deviations between time points analyzed.

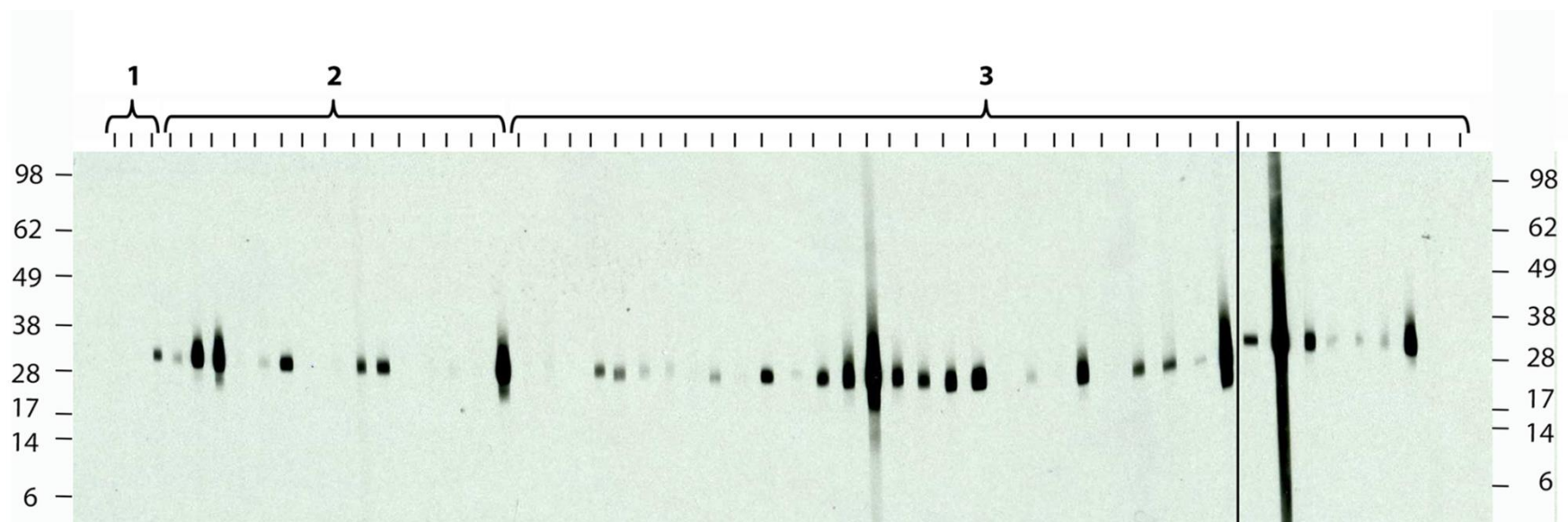


Figure 4.4. Western blot analysis of anti- *P. falciparum* AMA-1 serum IgG responses in young children aged one (1), two (2) and three (3) years. A number of sera from children aged one and two years already contained serum IgG against recombinant *P. falciparum* AMA-1 protein, as determined by Western Blot analysis. The molecular weight standard (in kDa) is shown next to the two blots that are separated by a vertical black line.

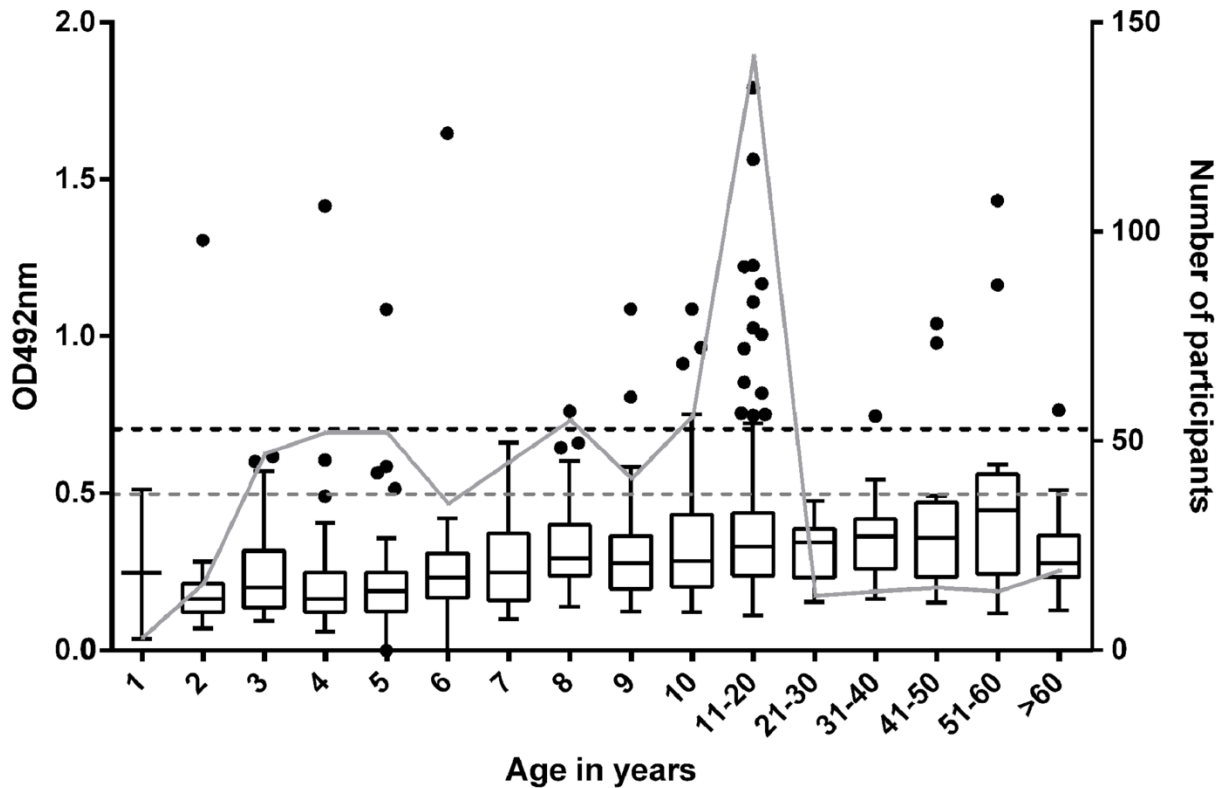


Figure 4.5. Age distribution of anti- *Strongyloides* serum IgG titers. All sera from children below the age of 11 years as well as a subset of sera from older age groups were tested for the presence of anti- *Strongyloides* antigen IgG by ELISA. The grey and the black dotted line indicate the cut-off OD values for equivocal and positive test results, respectively. The number of study participants for each age group (right y-axis) is visualized by grey dots connected by a grey line.

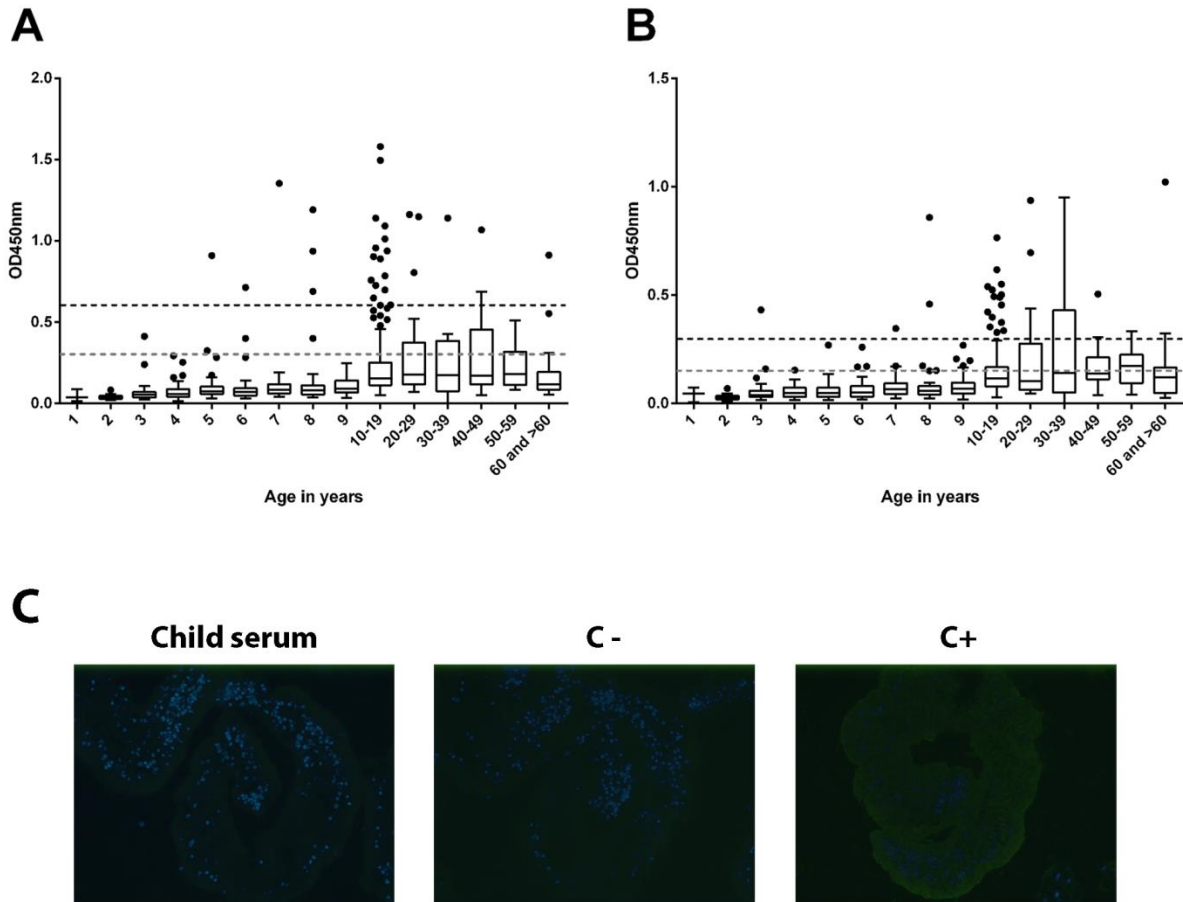


Figure 4.6. Age distribution of anti- *S. mansoni* SEA and AWE serum IgG titers. All sera from children below the age of 11 years and a subset of individuals of older age groups were tested for the presence of IgG against *S. mansoni* SEA (A) and AWE (B) by ELISA. The grey and the black dotted line indicate the cut-off OD values for equivocal and positive test results, respectively. The number of study participants for each age group (right y-axis) is visualized by grey dots connected by a grey line. The serum of the only young child testing positive in the AWE ELISA was reconfirmed to be positive by IFA. The serum of this child as well as a negative control serum labelled C- and a positive control serum labelled C+ is shown (C).

4.8.1 Supporting Figures

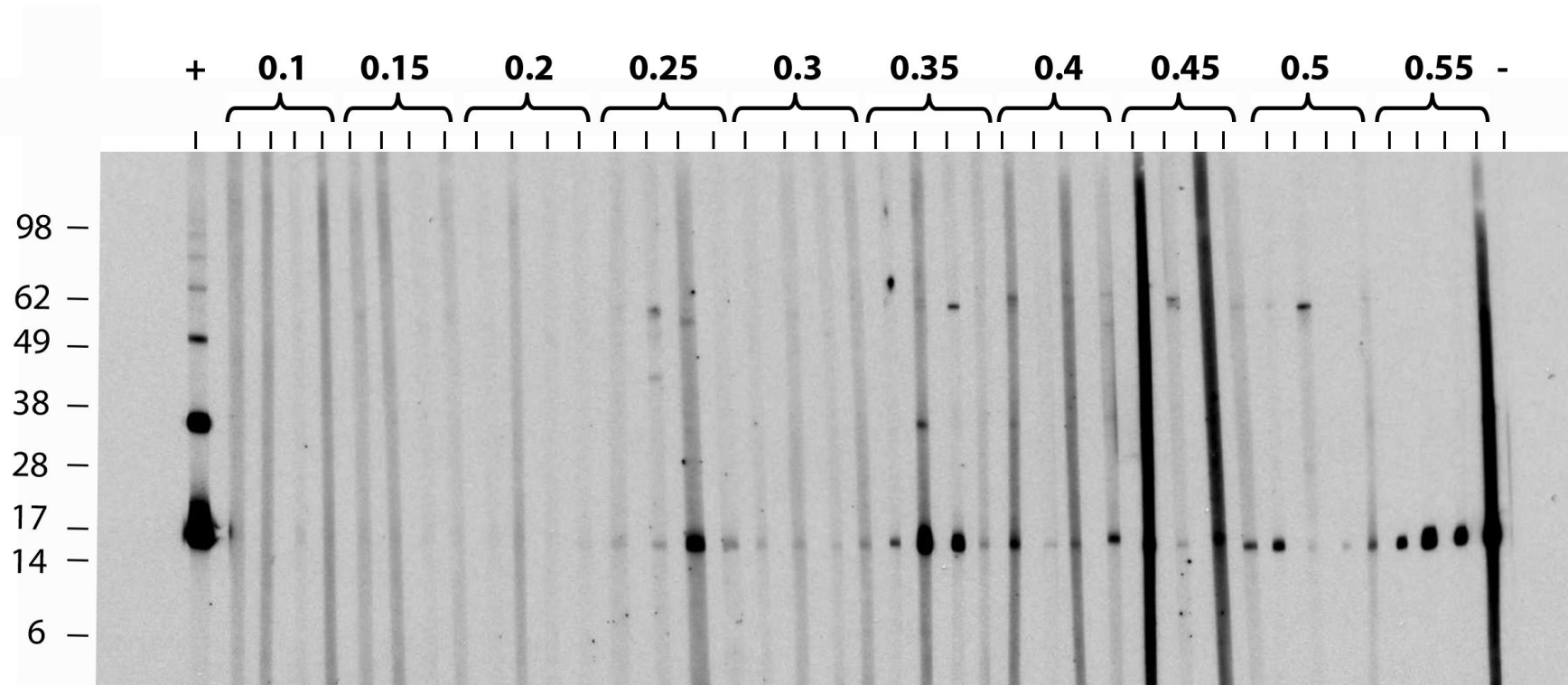


Figure S4.1. Western Blot analysis of serum samples with a range of ODs in ELISA.

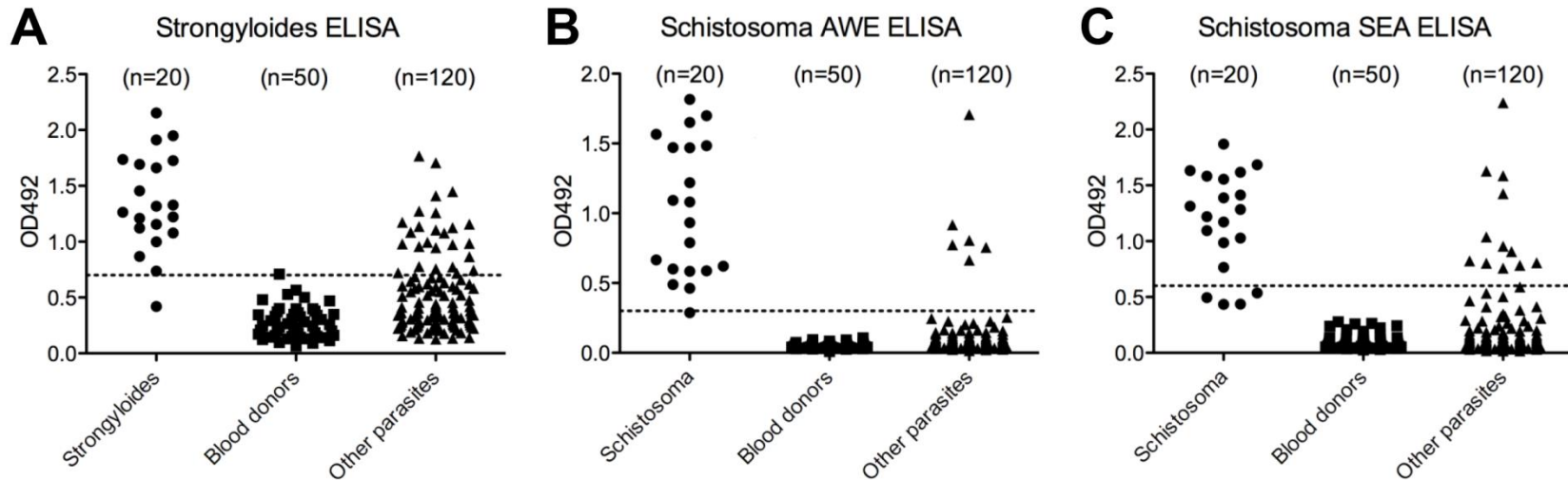


Figure S4.2. Detection of IgG against antigens of *S. ratti* iL3 (A), *S. mansoni* AWE (B) and *S. mansoni* SEA (C) in serum of parasitologically confirmed strongyloidiasis patients (A, Strongyloides), serum of parasitologically confirmed schistosomiasis patients (B and C, Schistosoma), negative control individuals (Blood donors) and patients with other parasitic infections (Other parasites). Each symbol shows a single serum sample. The corresponding cut-off is indicated in each graph by a horizontal line. The cut-off for the *Strongyloides* ELISA is 0.7, for *Schistosoma* AWE 0.30 and for *Schistosoma* SEA 0.60. n = number of samples.

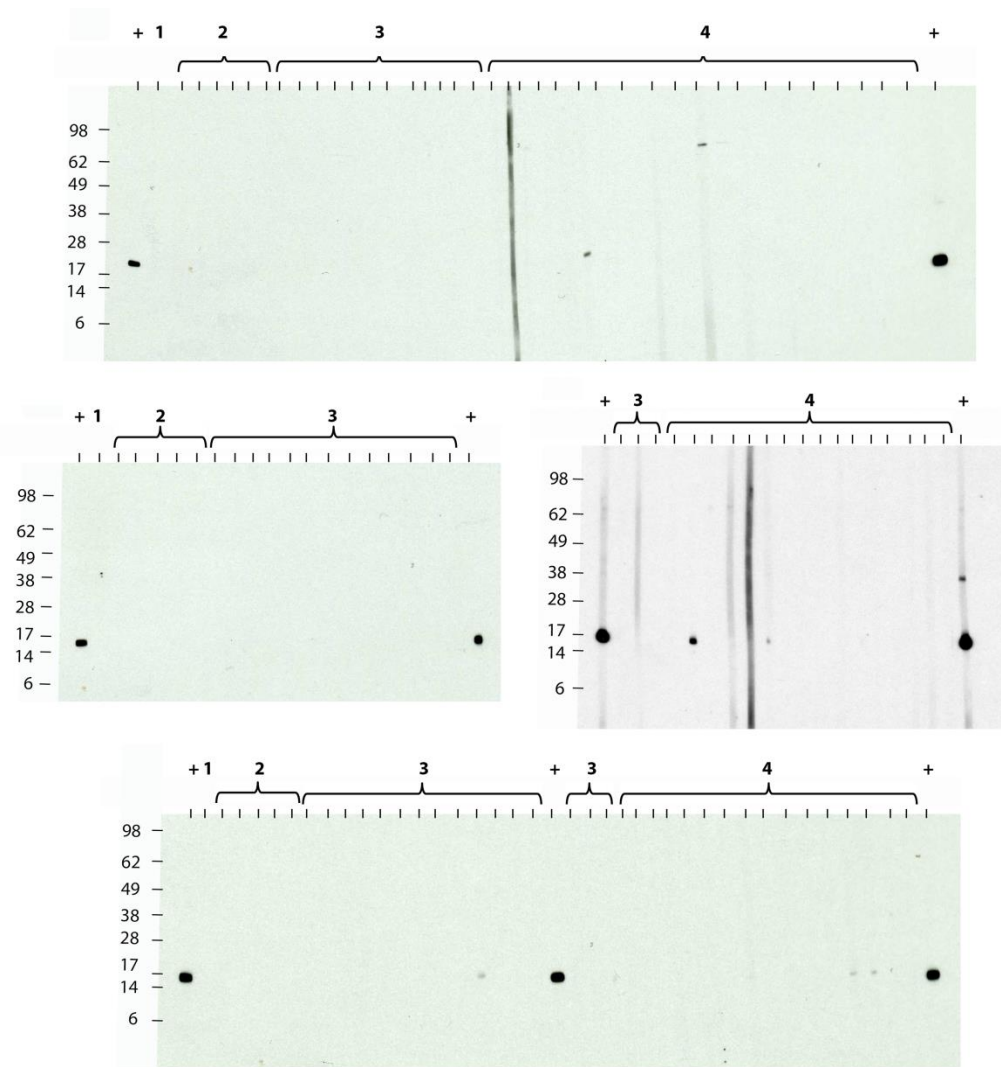


Figure S4.3. Western Blot analysis of sera from all children <5 years for anti-18 kDa shsp IgG responses.

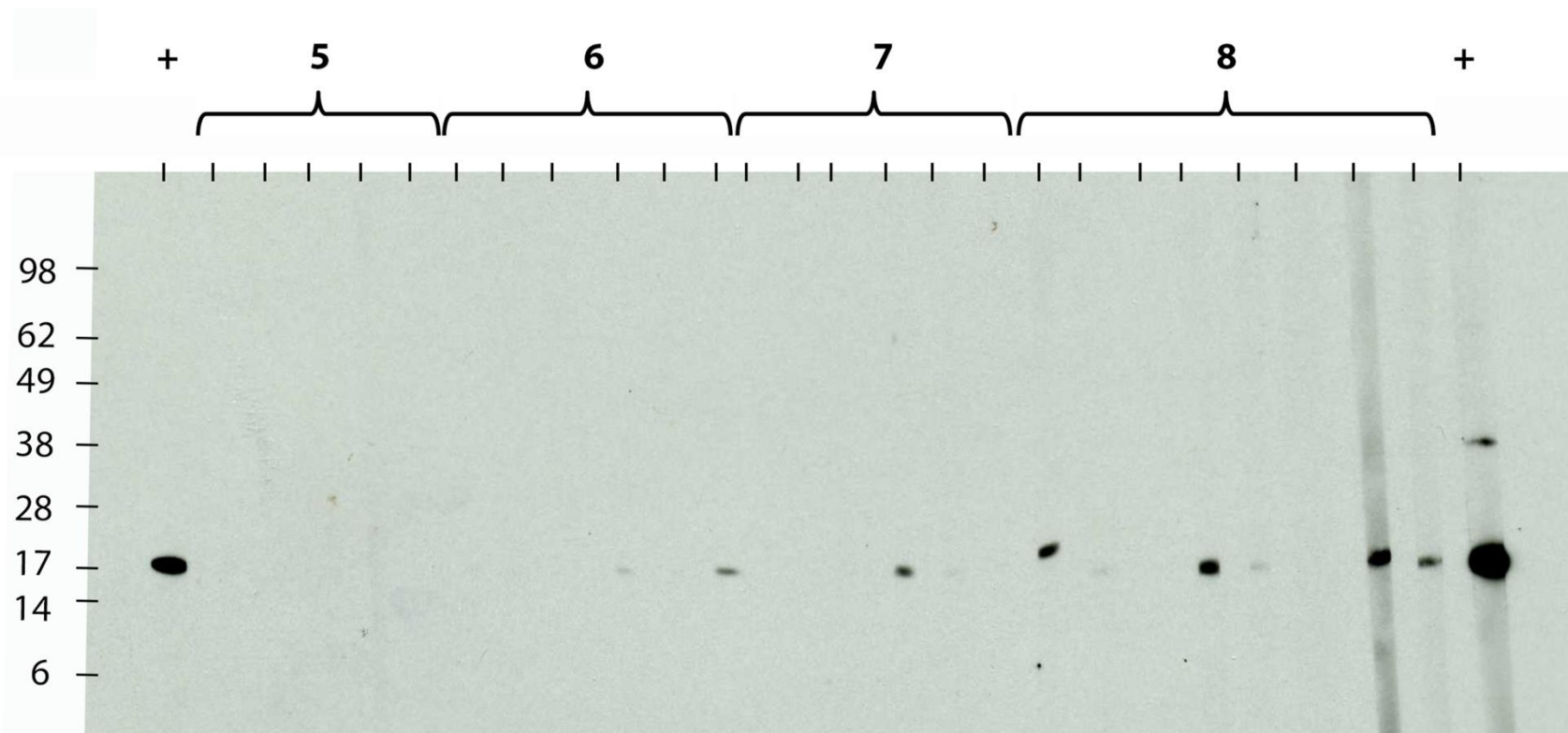


Figure S4.4. Western Blot analysis of sera from a number of children >4 years for anti- *M. ulcerans* 18 kDa shsp IgG responses.

4.9 Tables

Pathogen	Antigen	ELISA cut-off (OD)		
		Negative	Equivocal	Positive
<i>S. mansoni</i>	SEA ^a	< 0.3	0.3 - 0.59	≥ 0.6
	AWE ^b	< 0.15	0.15 - 0.29	≥ 0.3
<i>S. ratti</i>	raw antigen	< 0.50	0.50 - 0.69	≥ 0.70

^aSoluble egg antigen, ^bAdult worm antigen extract

Table 4.1. Cut-off ODs for the *S. mansoni* and *S. ratti* ELISA.

Variables	Age in years																
	1 ^a	2	3	4	5	6	7	8	9	10	11-20	21-30	31-40	41-50	51-60	>60	
n = Total population (%)	544 (2.7)	694 (3.4)	645 (3.2)	661 (3.3)	570 (2.8)	534 (2.7)	546 (2.7)	570 (2.8)	487 (2.4)	614 (3.1)	4,598 (22.8)	3,827 (19)	2,335 (11.6)	1,507 (7.5)	1,019 (5.1)	976 (4.8)	
n = Participants (% of population)	3 (0.6)	16 (2.3)	47 (7.3)	52 (7.9)	52 (9.1)	35 (6.6)	45 (8.2)	55 (9.6)	41 (8.4)	56 (9.1)	306 (6.7)	183 (4.8)	170 (7.3)	110 (7.3)	96 (9.4)	85 (8.7)	
n = anti- <i>M. ulcerans</i> 18 kDa shsp pos. (% of participants)	0 (0)	0 (0)	1 (2)	3 (6)	2 (4)	2 (6)	3 (7)	8 (15)	3 (7)	9 (16)	49 (16)	34 (19)	43 (25)	30 (27)	25 (26)	20 (24)	
n = anti- <i>P. falciparum</i> AMA-1 pos. (%)	1/3 (33)	7/15 (47)	26/37 (70)	---	---	---	---	---	---	---	---	---	---	---	---	---	
n = anti- <i>T. pallidum</i> pos. (%)	Both antigens	0 (0)	0 (0)	1 (2)	0 (0)	0 (0)	0 (0)	1 (2)	0 (0)	1 (2)	1 (2)	5/142 (4)	1/13 (8)	1/14 (7)	1/15 (7)	1/14 (7)	6/19 (32)
	Treponemal antigen only	0 (0)	0 (0)	0 (0)	0 (0)	3 (6)	0 (0)	1 (2)	1 (2)	1 (2)	3 (5)	4/142 (3)	2/13 (15)	0 (0)	2/15 (13)	0/14 (0)	5/19 (26)
n = anti- <i>Strongyloides</i> spp. pos. (%)	0 (0)	1 (6)	1 (2)	1 (2)	1 (2)	1 (3)	0 (0)	1 (2)	2 (5)	4 (7)	15/142 (11)	0/13 (0)	1/14 (7)	3/15 (20)	1/14 (7)	1/19 (5)	
n = anti- <i>S. mansoni</i> pos. (%)	SEA ^b	0 (0)	0 (0)	0 (0)	0 (0)	1 (2)	1 (3)	1 (2)	3 (5)	0 (0)	1 (2)	16/142 (11)	2/13 (15)	1/14 (7)	2/15 (13)	0/14 (0)	1/19 (5)
	AWE ^c	0 (0)	0 (0)	1 (2)	0 (0)	0 (0)	0 (0)	1 (2)	2 (4)	0 (0)	2 (4)	15/142 (11)	2/13 (15)	4/14 (29)	3/15 (20)	0/14 (0)	2/19 (11)

Table 4.2. Population, study participants and serum positivity for the different antigens tested. Total numbers and percentages of the study population, study participants and the proportions of serum samples tested positive in the different serological tests are shown. ^ano child below one year participated in the study, ^bsoluble egg antigen, ^cadult worm antigen extract

4.9.1 Supporting Tables

ELISA	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	ROC AUC
Strongyloides	95.0 (75.1-99.9)	84.3 (78.4-89.1)	0.95
Schistosoma AWE	97.5 (79.2-99.9)	95.9 (92.2-98.2)	0.98
Schistosoma SEA	80.0 (56.3-94.3)	92.4 (87.8-95.7)	0.96

Table S4.1. Sensitivity and Specificity of ELISAs. The sensitivity and specificity with 95% CI (confidence interval) and the AUC (area under the ROC curve) values for the *S. mansoni* and *S. ratti* ELISAs are shown.

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Chapter 5

Spatiotemporal Co-existence of two *Mycobacterium ulcerans* Clonal Complexes in the Offin River Valley of Ghana

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5.1 Abstract

In recent years, comparative genome sequence analysis of African *Mycobacterium ulcerans* strains isolated from Buruli ulcer (BU) lesion specimen has revealed a very limited genetic diversity of closely related isolates and a striking association between genotype and geographical origin of the patients. Here, we compared whole genome sequences of five *M. ulcerans* strains isolated in 2004 or 2013 from BU lesions of four residents of the Offin river valley with 48 strains isolated between 2002 and 2005 from BU lesions of individuals residing in the Densu river valley of Ghana. While all *M. ulcerans* isolates from the Densu river valley belonged to the same clonal complex, members of two distinct clonal complexes were found in the Offin river valley over space and time. The Offin strains were closely related to genotypes from either the Densu region or from the Asante Akim North district of Ghana. These results point towards an occasional involvement of a mobile reservoir in the transmission of *M. ulcerans*, enabling the spread of bacteria across different regions.

5.2 Author Summary

Infection with *Mycobacterium ulcerans* causes the debilitating skin disease Buruli ulcer. Until today, transmission pathways and reservoirs of this emerging pathogen are not well understood. Generally, it is assumed that infection occurs after contact with potential environmental sources of *M. ulcerans* through puncture wounds or lacerations or via invertebrate vectors, such as aquatic insects contaminated with the bacteria. Comparative genome analyses of *M. ulcerans* strains isolated from patients living in the same BU endemic areas have revealed a close relationship between the genotype detected and the geographical origin, indicating that the reservoir of the pathogen is relatively fixed in space. In the present study, we report the co-circulation of two distinct *M. ulcerans* clonal complexes in the same BU endemic area over space and time. Since members of these two clonal complexes were closely related to strains from either the Densu river valley or the Asante Akim North district of Ghana, we conclude that a mobile reservoir of *M. ulcerans* may be involved in the occasional spread of the bacteria across different regions.

5.3 Introduction

Mycobacterium ulcerans is an emerging pathogen with elusive reservoirs and transmission pathways. It causes the devastating skin disease Buruli ulcer (BU) that mainly affects rural populations in West Africa [1]. *M. ulcerans* is a descendant of the fish and occasionally human pathogen *Mycobacterium marinum* [2], from which the new species has evolved through the acquisition of a plasmid encoding the enzymatic machinery for the synthesis of the macrolide toxin mycolactone [3]. From this common ancestor at least three different lineages or ecovars have evolved through genome reduction [4]. Clinical isolates from Africa belong to the classical lineage and differ from each other only in a very limited number of single nucleotide polymorphisms (SNPs) [4, 5], indicative for a highly clonal recent expansion of the pathogen in Africa. BU is characterized by a focal distribution of cases within endemic countries. Previous studies have revealed a strong association between genotype and the geographic origin of strains [4, 6, 7], speaking for the development of local clonal *M. ulcerans* complexes following the introduction of this pathogen into a new area. The limited genomic diversity found within these local clonal complexes is however sufficient for studies on the distribution of variants at a micro-epidemiological level [8]. Since human-to-human transmission seems to be rare, findings point towards infection from a relatively localized environmental reservoir of the pathogen. In view of the association of BU outbreaks with stagnant and slow-flowing water bodies, a reservoir in the aquatic ecosystem is considered likely [9]. While in several African endemic areas a single unique clonal complex has been identified [4, 6-8], a recent comparative whole genome sequencing study of isolates from residents of the Asante Akim North district of Ghana showed for the first time the concurrent presence of two distinct clonal complexes within one BU endemic area [10].

Within the framework of a comprehensive genome analysis of clinical *M. ulcerans* isolates from Ghana, we analyzed genomes of a limited number of strains from the Offin river valley and equally observed a co-existence of two clonal complexes.

5.4 Methods

Ethics statement

Ethical approval for the study was obtained from the institutional review board of the Noguchi Memorial Institute for Medical Research (Federal-wide Assurance number FWA00001824). Written informed consent was provided by all study participants.

Study area and *M. ulcerans* isolates

In an exhaustive active BU case search conducted in 2013 and a subsequent continuous monitoring of cases over a 17-months period in 13 randomly selected communities located in the historically highly BU endemic Offin river valley, an unexpectedly low prevalence of BU was revealed with only 11 laboratory-confirmed cases identified [11]. Two *M. ulcerans* strains that could be isolated from two of the 11 patients, as well as three *M. ulcerans* strains isolated in 2004 from lesions of two BU patients residing in the valley were analyzed in this study (Table 5.1). In addition, we included 48 *M. ulcerans* isolates from patients residing in BU endemic areas located in the Densu river valley of Ghana, which were part of a previous SNP typing study [8]. All *M. ulcerans* strains were subjected to whole genome sequencing.

DNA extraction and whole genome analysis

Genomic DNA was extracted from *M. ulcerans* cultures by phenol-chlorophorm extraction and ethanol precipitation as described previously [12]. Multiplexed genomic DNA libraries were prepared and sequenced on an Illumina HiSeq 2000 on 75-bp paired-end runs [13]. Illumina reads were aligned to the complete reference genome of *M. ulcerans* strain Agy99 (GenBank accession number CP000325.1) with an insert size between 50 and 400 bp using BWA version 0.7.10. SNPs were identified using SAMtools [14] as described [15] and were filtered for a minimum mapping quality of 30 and a quality cutoff of 75%. SNPs called in repetitive regions of the *M. ulcerans* reference genome (737,280 bp) were excluded from the analysis and only the SNPs mapped in the core genome (4,894,326 bp) were used to construct the phylogenetic trees.

Phylogenetic analyses

Maximum-likelihood phylogenetic analysis was performed using RAxML [16] on the alignment of identified SNPs from across the Ghanaian genomes sequenced here, together with genomes

sequenced in previous studies [4, 10, 17]. Additional strains isolated from BU patients from other regions of Ghana and from Benin and Australia were included in the analysis to provide a comprehensive genetic context for the analysis of genetic diversity among the Offin and Densu isolates.

5.5 Results/Discussion

Phylogenetic analysis demonstrated the expansion of a single clonal complex in the Densu river valley (Fig 5.1). This complex has diversified substantially, but still forms a separate cluster, distinct from other African local clonal complexes (Fig 5.2 and 5.3). When compared to the second branch of the classical lineage of *M. ulcerans* - isolates from Australia - it is evident, that all African isolates are genomically extremely closely related (Fig 5.2). In contrast to the observation of a single clonal complex in the Densu river valley, our analysis revealed for the Offin river valley the presence of members of two distinct clonal complexes (Fig 5.3). Two Offin isolates (NM031 and NM997) were closely related to isolates from the Densu river valley of Ghana (Fig 5.1 and 5.3). The other three Offin isolates (NM022B, NM022D and NM972) – separated from the two Densu-like Offin strains by 29 SNPs – clustered with strains (belonging to a clonal complex designated Agogo-1; [10]) from the Asante Akim North district in the Ashanti region of Ghana (Fig 4.3). Intra-genotype average diversity was low with 12 and 17 identified SNPs among Densu-like and Agogo-1-like Offin isolates, respectively. Not a single SNP difference was found between the genomes of two strains (NM022B and NM022D) isolated from two different lesions of the same patient. In a next step, we combined the phylogenetic analysis of the Offin isolates with information on the residence of the patients within the river valley and the year of strain isolation (Fig 5.4). In both 2004 and 2013 one member each of the two clonal complexes, isolated from BU patients resident in different communities was found. Due to the limited number of isolates and missing details on the travel history of the BU patients from which these strains have been isolated, no firm conclusions could be drawn concerning the apparent lack of geographical clustering. However, the data revealed a co-circulation of two distinct *M. ulcerans* clonal complexes in the Offin river valley over space and time.

In contrast to the remarkably strong link between genotype and geographical origin of clinical *M. ulcerans* isolates reported in previous genotyping studies conducted in African BU endemic foci [6-8], two distinct *M. ulcerans* clonal complexes were recently found to co-exist in the Asante Akim North district of Ghana among strains isolated within the short time frame of two years and an area of only 30km² [10]. It was concluded that *M. ulcerans* genotypes might be spread across larger areas, suggesting the presence of a rather mobile reservoir of infection in addition to the postulated more focalized aquatic niche environment typically associated with the pathogen [9]. In this context, recent data indicated that *M. ulcerans* is able to persist for several

months in underwater decaying organic matter [18], possibly as a commensal in protective aquatic host environments [19-21]. While the specific factors favoring the persistence of *M. ulcerans* in the environment and its transmission are yet to be explored, a complex interplay between environmental factors as well as biotic and abiotic drivers is assumed [22, 23]. Reductive genome evolution of *M. ulcerans* speaks for niche adaptation [17].

In the present study we revealed the co-existence of both Densu-like and Agogo-1-like *M. ulcerans* genotypes in communities along the Offin river at two time points separated by ten years. Our data thus show that co-existence of clonal complexes in one BU endemic area may prevail over longer time periods. A mobile mammalian host, allowing the bacteria to replicate and to be shed to the environment, that way forming a reservoir [18] from which humans may be infected by unknown mechanisms, could be the missing link explaining the spread of *M. ulcerans* from an established BU endemic region to a new area. However, as demonstrated here by the presence of only a single clonal complex in the Densu river valley, the exchange of genetic *M. ulcerans* variants between BU endemic areas appears to be an extremely rare event.

While in Australia possums have been identified as a host for *M. ulcerans* [24], another line of evidence points to the involvement of humans with chronic ulcerative BU lesions in the spread of the bacteria in African BU environments. Extensive whole-genome sequencing studies are required to further unravel the evolutionary history and population structure of *M. ulcerans* in Africa.

5.6 Figures

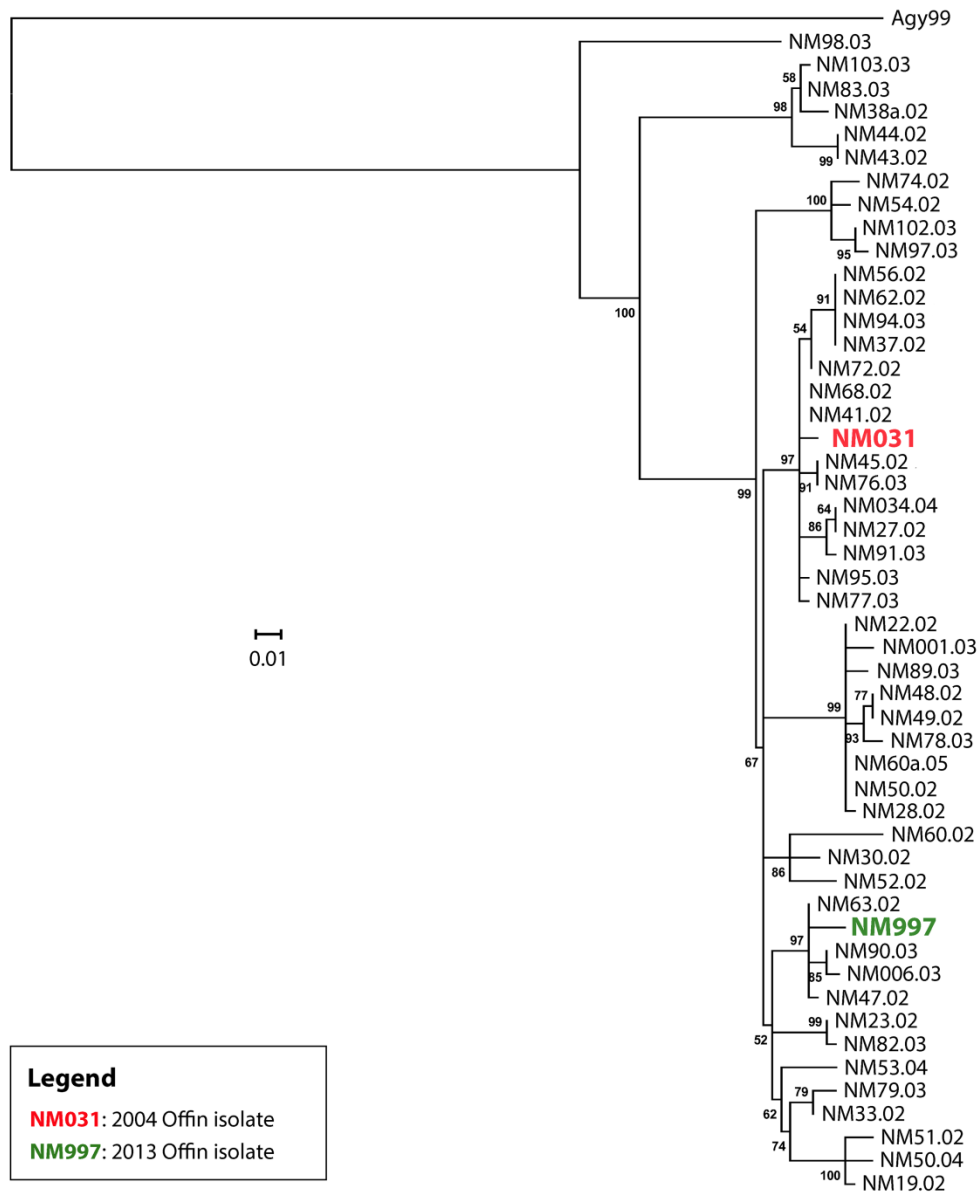


Figure 5.1. Phylogenetic reconstruction of *M. ulcerans* isolates belonging to the clonal complex circulating in the Densu river valley of Ghana. Maximum-likelihood phylogenetic tree based on 292 variable nucleotide positions across *M. ulcerans* isolates produced by RAxML. A total of 48 *M. ulcerans* isolates from the Densu river valley and two isolates from the Offin river valley were shown to belong to the same clonal complex that has diversified substantially over time. The tree was rooted using *M. ulcerans* strain Agy99 as an out-group. Bootstrap values are shown along the branches. 0.01 = scale for genetic distance.

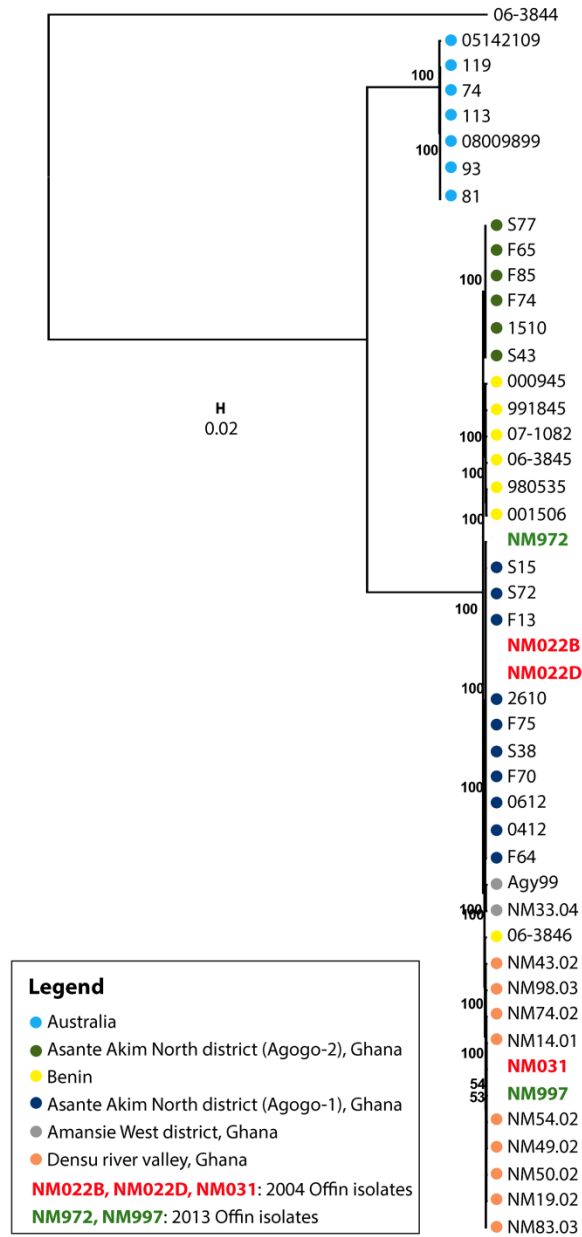


Figure 5.2. Phylogenetic reconstruction of *M. ulcerans* isolates belonging to the classical lineage (Australian and African strains). Maximum-likelihood phylogenetic tree based on 11,194 variable nucleotide positions across *M. ulcerans* isolates by RAxML. *M. ulcerans* isolates from the Offin and Densu river (one representative each of the previously identified SNP haplotypes [8]) valleys were placed in a broader genomic context. The tree was rooted using *M. ulcerans* strain Mu 06-3844 (isolate from a fish farm in Belgium) as an out-group. Bootstrap values are shown along the branches. 0.02 = scale for genetic distance.

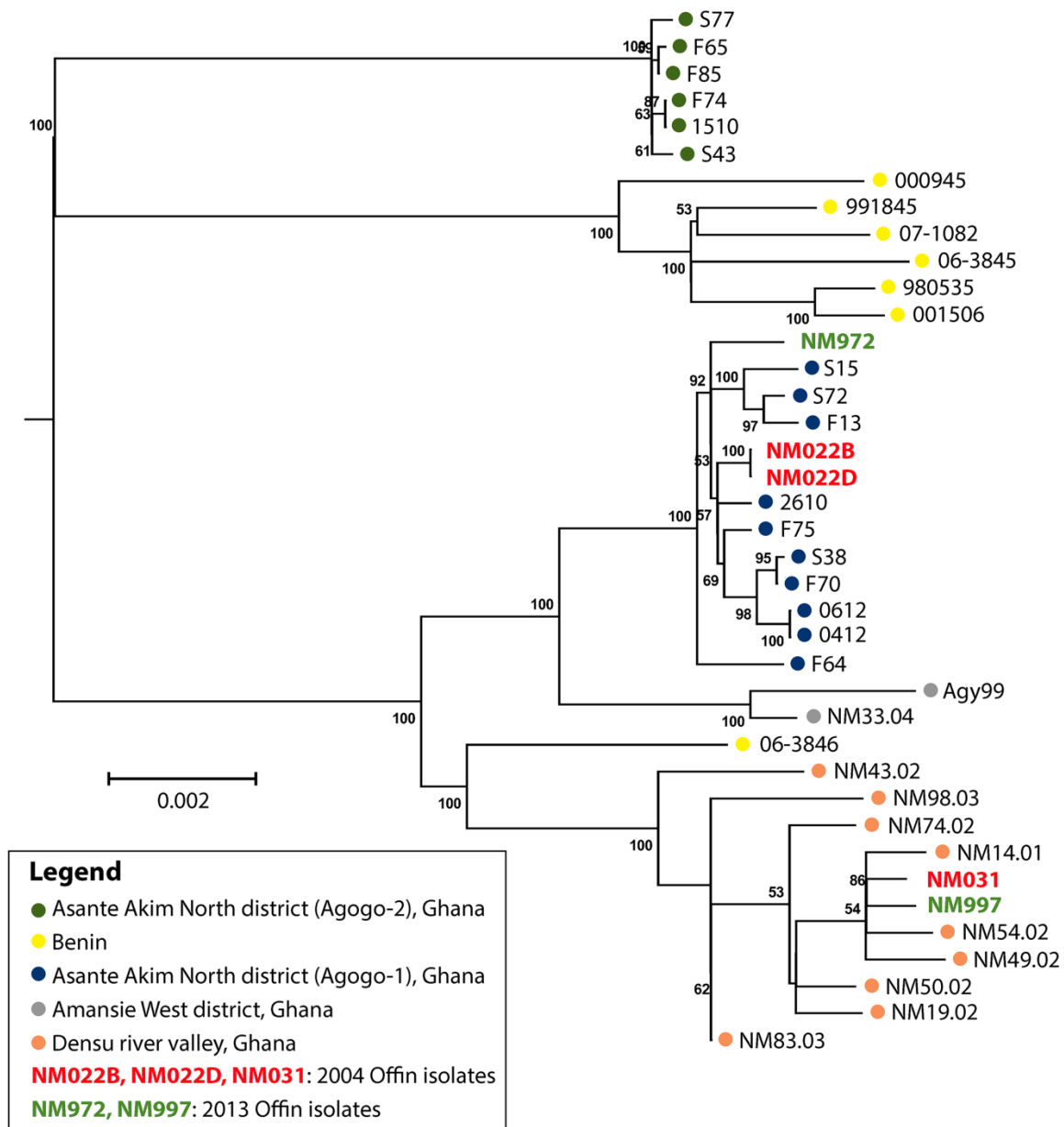


Figure 5.3. Phylogenetic reconstruction of closely related *M. ulcerans* isolates from different BU endemic areas of Ghana and from Benin. Maximum-likelihood phylogenetic tree based on 776 variable nucleotide positions showing relations between the strains at a higher resolution than in Fig. 5.2. Bootstrap values are shown along the branches. 0.002 = scale for genetic distance.

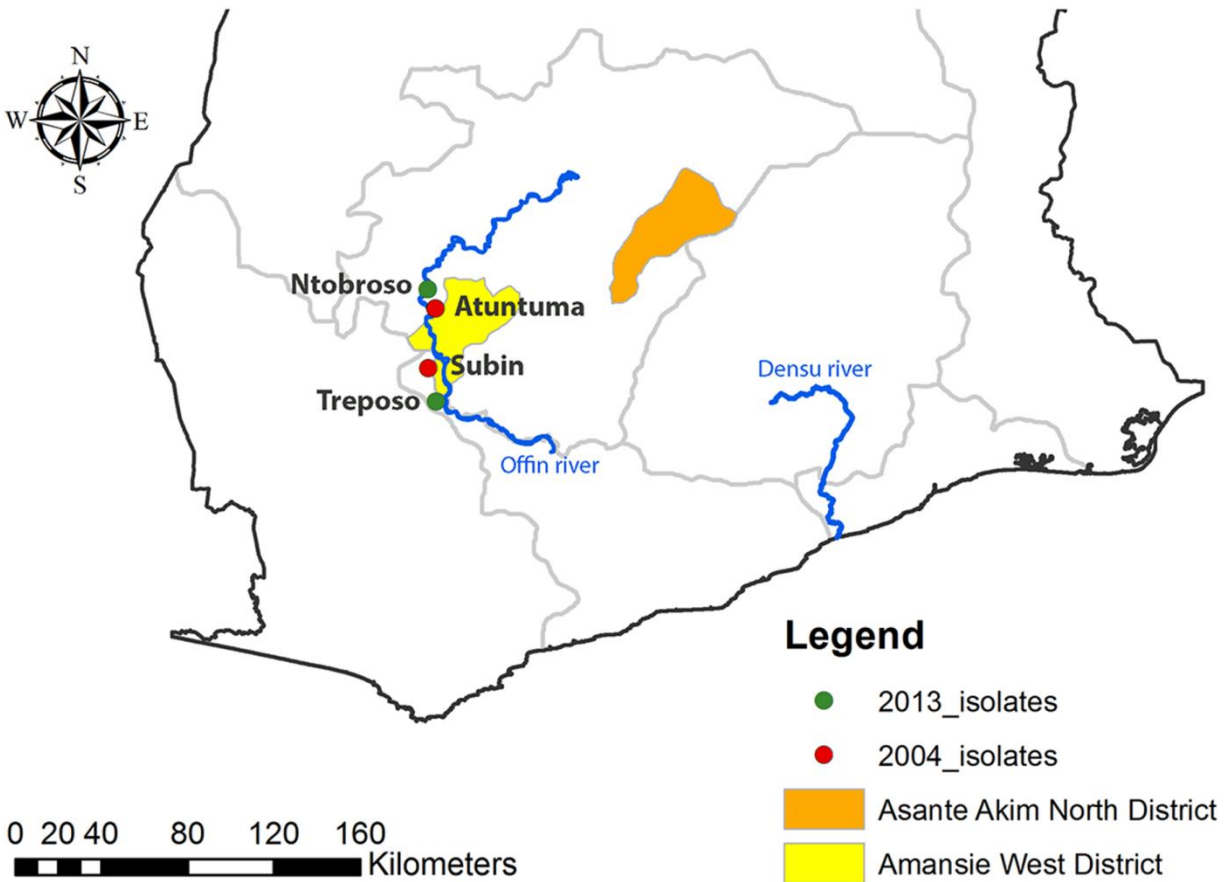


Figure 5.4. Geographical distribution of *M. ulcerans* isolates from the Offin river valley. Map of the Offin river basin, depicting the residences of BU patients from whom the strains have been isolated in 2004 (red) and 2013 (green). The background map was created using the *ArcMap* program in *ArcGIS* v.10.0 software.

5.7 Tables

Isolate ID	Year of isolation	Village of residence*	Genotype	Average sequence coverage
NM022B	2004	Atuntuma	Agogo-1	101.54
NM022D	2004	Atuntuma	Agogo-1	88.304
NM031	2004	Subin	Densu	91.743
NM972	2013	Treposo	Agogo-1	90.481
NM997	2013	Ntobroso	Densu	94.425

Table 5.1. *M. ulcerans* isolates sequenced in this study.

*see Figure 5.4. for location along the Offin river

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Chapter 6

Limited Genetic Diversity of Hepatitis B Virus in the General Population of the Offin River Valley in Ghana

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6.1 Abstract

Hepatitis B virus (HBV) infections account for approximately 780,000 deaths per year, most of which occur in the developing world. Co-infection with HBV and hepatitis delta virus (HDV) may lead to the most severe form of viral hepatitis. In Ghana, knowledge on the prevalence of HBV and HDV in the general population is scanty and the few genetic analyses of the prevailing HBV genotypes are dating back more than a decade. In the present study, 1,323 serum samples from individuals living in a rural area (Offin river valley) of Ghana were analyzed for the presence of the hepatitis B surface antigen (HBsAg). Positive sera were subsequently tested for the presence of anti-HDV antibodies. A total of 107 (8%) sera were HBsAg positive with an 8.4% prevalence of anti-HDV antibodies among the HBsAg positives. Phylogenetic analysis, based on HBV pre-S/S sequences, attributed all 52 typable samples to genotype E. All belonged to serotype *ayw4*. While 19 sequences clustered with those from a number of African countries, the other 33 formed a separate cluster distinguished by an intergroup mean distance of 1.5% from the pan-African HBV/E cluster. Successful implementation of HBV vaccination in the region was reflected by the low HBsAg carrier rate of 1.8% among children ≤ 11 years.

6.2 Introduction

Despite the availability of effective hepatitis B virus (HBV) vaccines, the global burden of hepatitis B remains high, with an estimated 240 million chronically infected individuals and about 780,000 deaths from cirrhosis and hepatocellular carcinoma each year [1]. Transmission of HBV can occur through diverse routes including perinatal, sexual and household contact, or by unsafe injections. The HBV genome is a circular, partially double-stranded DNA, approximately 3.2 Kb in length, which contains four partially overlapping open reading frames, encoding the polymerase (P), the surface proteins (pre-S1/pre-S2/S), the core antigen and the soluble antigen 'e' (preC/C), and the regulatory protein (X), respectively [2]. To date, HBV isolates have been classified into eight confirmed (A to H) [3-7] and two tentative (I and J) [8, 9] genotypes, based on a divergence of >7.5% in the whole genome, or >4% in the S gene sequence. In recent years it has become increasingly evident that a distinct global geographical distribution of HBV genotypes is a major factor responsible for differences observed in clinical manifestations and response to antiviral treatment and vaccination [10-12], emphasizing the importance of genotyping studies to identify the locally prevailing genotypes.

While hepatitis B is hyper-endemic in sub-Saharan Africa, underreporting, due to limited access to healthcare and lack of knowledge on the infection, hinder precise accounts on the actual burden, particularly in remote areas [13]. Previous studies have shown that HBV genotype E (HBV/E) is by far the most prevalent in West and Central Africa [14-17], spreading in a vast crescent with a span from Senegal to Namibia. The other two dominating genotypes circulating in Africa, are found mainly in Southern, Eastern and Central Africa (HBV/A) [13], and in Northern Africa (HBV/D) [18-20]. A recent emergence of genotype E, within the last 130 years, has been proposed, based on the fact that this genotype is restricted to the African continent and shows the lowest diversity as compared to other genotypes [14, 21, 22]. However, the origin and evolutionary history of HBV/E remain unclear. While recent studies in Ghana have investigated HBsAg prevalence rates within specific groups, such as HIV patients (13-17% [23, 24]), pregnant women (11-13% [25-27]), blood donors (7.5-15% [28-31]), or prison inmates (17-25.5% [32, 33]), knowledge of HBV prevalence in the general population is limited. Although it has been reported that HBV/E is the prevailing genotype in historical specimens from Ghana [34, 35], detailed information on the current population structure of HBV in the country is lacking.

An estimated 15-20 million individuals worldwide are co-infected with HBV and hepatitis delta virus (HDV), representing the most severe form of chronic hepatitis [36, 37]. HDV is a defective virus requiring hepatitis B surface antigen (HBsAg) to survive, and is principally transmitted by the parenteral route. Except for a recent study reporting an 11.3% seroprevalence of HDV infection in a small number of patients with HBV-related liver diseases in Accra [38], data on the prevalence of HDV in Ghana are lacking.

The objective of the present study was to investigate the prevalence and genomic diversity of HBV in the general population of the Offin river valley in Ghana.

6.3 Methods

Ethics statement

Ethical approval for the study was obtained from the institutional review board of the Noguchi Memorial Institute for Medical Research (Federal-wide Assurance number FWA00001824). Written informed consent was provided by all study participants or, in the case of children, by their parents or guardians.

Design of the study

The Offin river runs through two (Ashanti and Central) of the ten Ghanaian regions (Fig 6.1), covering a total of 11 health districts. In a preceding study, we investigated the epidemiology of the tropical skin disease Buruli ulcer in selected communities of the Offin river valley of Ghana by conducting an exhaustive household survey in 2013 [39]. Based on the demographic information collated for 20,390 residents of 13 communities located in seven health districts (Table 6.1) spread along the Offin river (Fig 6.1), we grouped the population of each community by age and randomly selected from all of the age groups a total of 1,560 residents (120 per community). Of these 1,560 individuals, 1,352 consented to donate blood for sero-epidemiological studies of multiple pathogens [40].

In the present study, we analyzed 1,323 of the 1,352 serum samples retrospectively for the presence of HBV and HDV markers. The distribution of study participants by age group and community of residence is shown in Fig S6.1. Moreover, we re-evaluated general information on the communities, as well as study questionnaires administered in 2013 to 20,390 inhabitants of the 13 study communities, to extract demographic information relevant for the study of hepatitis in this region, including road networks, social and health system infrastructure and travel habits.

Serological analysis

A total of 1,323 retrospective blood serum samples stored at -80°C were analyzed in the present study.

Detection of HBsAg

Serum samples were screened for the presence of HBsAg by immunochromatography (Advanced Quality ONE STEP HBsAg Test Strip, Intec Products Inc., China) according to the manufacturer's instructions. Briefly, we applied 100 μl of serum onto the test strips and recorded

the presence or absence of a red band on the nitrocellulose strip after 15 minutes. In order to validate the performance of this rapid test, we analyzed 88 serum samples in parallel by ELISA using the HBsAg BioAssay ELISA Kit (US Biological, Salem, USA) in strict accordance to the manufacturer's instructions. The same results were obtained in both tests, with three samples yielding a positive result.

Detection of anti-HDV antibodies

The HDV antigen (HDAg) can elicit a specific antibody response in infected individuals. We analyzed all HBsAg-positive serum samples for the presence of anti-HDV antibodies by ELISA (ETI-AB-DELTAK-2, DiaSorin, Italy) according to the manufacturer's instructions.

Nucleic acid extraction, HBV PCR amplification and nucleotide sequencing

Total nucleic acids were extracted from 200 µl of HBsAg-positive serum samples using the High Pure Viral Nucleic Acid Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Nucleic acids were re-suspended in 50 µl of TE buffer and 3 µl were applied in the PCR assays.

Amplification of the HBV pre-S/S region was attempted by a semi-nested PCR assay, in which samples negative in the first round, were subjected to a second round of PCR. In both reactions, PCR amplification of extracted DNA was performed with 2.5 units of FirePolTaq-Polymerase (Solis BioDyne, Tartu, Estonia), FirePol buffer, 2 mM MgCl₂ and 0.4 mM dNTPs with 1.8 µl of 10 µM forward and reverse primers each in a total volume of 30 µl. While primer pairs PS1 (5'-CCATATTCTTGGGAACAAGA-3') and P3 (5'-AAAGCCCAAAGACCCACAA-3') were applied in the first PCR round to generate a product of 1,405 bp, P3 was replaced by S2 (5'-GGGTTTAAATGTATACCCAAAA-3') in the second round, amplifying a product of 1,227 bp. PCR reactions were carried out in a Gene Amp PCR System 9700 PCR machine (Applied Biosystems) and thermal conditions for PCR amplifications included an initial denaturation step at 94°C for 5 min followed by 32 cycles at 94°C for 30 s, 57°C for 30 s and 72°C for 1 min and a final extension step, 72°C for 10 min. PCR products were analyzed on 1% agarose gels. Nucleotide sequencing was accomplished using primers PS1, PS8 (5'-TTCCTGAACTGGAGCCACCA-3'), PS4 (5'-ACACTCATCCTCAGGCCATGCAGTG-3'), S2, S4 (5'-TGCTGCTATGCCTCATCTTCT-3') and P3 for the larger sequence, and PS1, PS8, PS4 and S2 for the smaller sequence. We also amplified a smaller fragment spanning the S gene

by applying the same PCR procedure using primers PS1a (5'-GGAAAACATCACATCAGGAT-3') and P3 for the first round of PCR and primers PS1b (5'-AAAATTCGCAGTCCCCAACC-3') and P3 for the second round. In this case, the same primers were used for nucleotide sequencing. PCR products were purified using the Nucleo Spin Extract II Kit (Macherey-Nagel, Düren, Germany) and sequenced at Macrogen Inc., Europe (Amsterdam, the Netherlands).

Statistical analysis

All statistical analyses were carried out with GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA) and Stata 12 (Statacorp 2011 statistical software Release 12. College Station, TX: StataCorp LP). The associations between categorical variables were assessed using Pearson's Chi square test or Fisher's exact test. The level of statistical significance was set $P < 0.05$.

Phylogenetic analysis and inference of serotypes

Genotyping of HBV was performed by analyzing a partial sequence of the genome - the pre-S/S region - consistent with genotyping of the full genomic sequence. Nucleotide sequences of the pre-S/S or S genes were aligned using the ABI Prism AutoAssembler, version 1.4.0 (Perkin-Elmer, Waltham, MA). Maximum-likelihood phylogenetic analysis was performed using the Kimura 2-parameter model [41] of MEGA version 6.0 [42]. Previously published mutations in the S region associated with "escape" or with diminished antibody binding were predicted using the Geno2pheno[HBV] online tool at <http://hbv.geno2pheno.org/index.php>. The Geno2pheno[HBV] tool was also used to predict drug resistance mutations in the part of the reverse transcriptase open reading frame that overlaps with that of S. Genetic distances were estimated using MEGA 6.0 tools [42].

HBV serotypes were predicted based on the amino acids present at either three or five known positions (122, 160, 127, 159 and 140) within the S gene [43] using the web-based HBV Serotyper Tool that can be accessed at <http://hvdr.bioinf.wits.ac.za/SmallGenomeTools> [44].

Nucleotide sequence accession numbers

The HBV/E sequences determined in this work have been deposited in the DDBJ/GenBank/EMBL database (accession numbers KU522251-KU522302).

6.4 Results

Demographic characteristics of the study population and participants

In order to gain insight into the burden of hepatitis B and D infection in the general population of the Offin river valley in Ghana, we analyzed serum samples from a representative proportion of individuals living in 13 selected communities located up-, mid- and downstream along the Offin river (Fig 6.1). Of the total of 20,390 residents of these communities, 1,323 individuals (from 71 to 123 inhabitants per community) aged between 1 and 90 years (mean age 25.3 ± 19.5 years, median 19 years) participated in the study (Fig S6.1), with 59% ($n = 781$) being females and 41% ($n = 542$) being males (Table 6.1). The 13 communities belonged to seven health districts, all of which reported to have introduced the pentavalent diphtheria, tetanus, pertussis, Haemophilus influenzae type B, hepatitis B (DPTHH) vaccine in 2002 following the WHO Expanded Program on Immunization (EPI).

As listed in Table 6.1, the characteristics of the 13 selected study communities varied, ranging from small settlements located within farmlands and lacking basic social amenities and a good road network, like Krakrom, to larger communities, like Dominase, which in addition to having all the social amenities, is connected to the district capital by a good road network. Almost 40% ($7,812/20,390$) of the general population reported to have travelled out of their communities in the past three months with the majority of those ($n = 4,659$) reporting to have resided in a metropolitan town for at least one day. While 80% ($16,262/20,390$) of the inhabitants were Akans, the other 20% were composed of Ewe, Mole, Ga/Ada, Guan, Gruma, Grusi, Mande, Mamprusi, Kussasi and others [39]. There were also 37 (0.18%) nationals of other West African countries, namely Benin ($n = 2$), Burkina Faso ($n = 7$), Côte d'Ivoire ($n = 4$), Togo ($n = 4$), Niger ($n = 12$) and Nigeria ($n = 8$).

Prevalence of HBsAg and anti-HDV antibodies in the Offin River Valley population

One hundred and seven (8.1%) of the 1,323 serum samples tested were HBsAg positive, with a nearly equal gender distribution of 7.7% ($n = 60$) in females and 8.7% ($n = 47$) in males ($p = 0.516$). There was no significant association between ethnic groups and positivity for HBsAg ($p = 0.609$), but a high variation in prevalence (1.9% to 14.9%) between communities, which was however not associated with the quality of road or social infrastructure of the community (Table 6.1). The age distribution of HBsAg positive individuals in relation to the total number of study

participants is depicted in Fig 6.2. HBsAg was detected in a significantly ($p < 0.001$) higher proportion of individuals aged above 11 years (11.1%; 99/890) than in children aged 11 years or less (1.8%; 8/433). Prevalence of HBsAg-positivity was low (2%; 2/101) in the age group ≥ 60 years.

Nine (8.4%) of the 107 HBsAg carriers were tested positive for anti-HDV antibodies. All nine positive sera (ABUS036, BUDS073, NKS076, NKS089, NKS100, TNS007, TNS058, TNS088 and WMS021) were from residents of five study communities (Table 6.1) located mid- and downstream along the Offin river (Fig 6.1).

Phylogenetic analysis of HBV isolates circulating in the Offin river valley

HBV DNA could be amplified from 66/107 (62%) samples positive for HBsAg. Phylogenetic analysis was performed with pre-S/S sequences obtained from 52 of the 66 PCR products. The remaining 14 sequences showed high similarity to the other sequences obtained, but each of them contained several ambiguous nucleotide positions (suggesting mixed infections with more than one genetic HBV variant), and the sequences were therefore not included in the phylogenetic analysis.

The 52 sequences (obtained from residents of 12 of the 13 study communities) were compared with representative sequences of the HBV isolates from genotypes A-J retrieved from GenBank (see accession numbers in the legend of Fig 6.3). The resulting phylogenetic reconstruction was rooted by including the sequence of a virus isolate from a Woolly monkey (WMHBV) as an out-group (GenBank accession number AY226578). All Offin river valley sequences clustered with those of HBV/E strains from Ghana and other countries included in the alignment (Fig 6.3).

A more detailed resolution of the population structure of HBV from the Offin river valley was achieved by aligning the sequences exclusively with other HBV/E sequences. The overall mean genetic distance among the 52 sequences of this study was 1.2% based on the pre-S/S region, and 0.6% when only the S region was analyzed. Interestingly, maximum likelihood reconstruction, based on the complete pre-S/S region, revealed the presence of two separate genetic clusters, with an intergroup mean distance of 1.5%. While 19 sequences grouped with HBV/E strains from Ghana, Madagascar, Benin, Côte d'Ivoire, Nigeria, and Niger (pan-African cluster), the 33 others formed a separate cluster that did not contain any sequence other than those from the Offin river valley (Fig 6.4). Representatives of the Offin cluster were from 11 of the 13 study

communities, while sequences belonging to the pan-African cluster originated from nine communities (Table S6.1). However, no significant association was found between sequence origin and affiliation to one of the two different clusters ($p = 0.589$). The mean genetic distance of the 19 sequences attributed to the pan-African cluster was 1.6%, compared with 0.7% among the 33 belonging to the Offin cluster. Characteristics of all individuals for which sequence information is available are listed in Table S6.2.

Detection of relevant HBV signature motifs, deletions and other mutations in the pre-S/S region

The deduced amino-acid sequences of the pre-S/S region were highly conserved among the 52 HBV isolates analyzed in this study. All of them had a single amino acid deletion at the N-terminus of the pre-S1 region (residue Met12 in sequences of other genotypes), leading to a pre-S1 region of 118 amino acids in length, a feature characteristic for HBV/E strains [45]. Moreover, all sequences showed the genotype E pre-S1 amino acid signature motif Leu3SerTrpThrValProLeuGluTrp11. Amino acid residue Met83, that has been reported to introduce a new translational start codon in the pre-S1 region [45], was present in all but one (NBUS067) sequence. The emergence and selection of new pre-S variants is a common event in chronically HBV infected patients. Genetic defects are usually due to in-frame deletions of different sizes in the carboxy terminus of the pre-S1 region, or are caused by substitutions at the start codon of the pre-S2 region with complete abolishment of M protein synthesis [46]. Here, deletions of 27 and 25 amino acids (sample PKS034) and of six amino acids (PKS077) were detected in the pre-S region. Moreover, substitutions leading to the loss of the pre-S2 start codon (Met119) were detected in three sequences (NBUS058, NKS010 and WMS009).

The S region is particularly conserved. It has been reported that the majority of the HBV/E isolates from South-West Africa contain an Ile57 residue, while those from North-West Africa show a Thr57 residue [45]. In this study, 51 Ghanaian sequences had a Thr residue, while only one (TNS014), belonging to the pan-African cluster, had an Ile at that position.

Neutralizing antibodies induced by immunization against HBV are mainly directed against conformational epitopes of the major antigenic ‘a’ determinant, spanning amino acids 124-147 of the S region [47]. A number of amino acid substitutions within this region, mainly between positions 137 and 147, have been described as vaccine or immune escape mutants [48]. In two of

the HBV sequences obtained in this study (MFS048 and TNS091), we detected the mutation sS143L, previously identified in an HBsAg escape mutant [49]. No known mutations in the S-overlapping reverse transcriptase region, relevant for phenotypic resistance to five antiretroviral drugs (Lamivudine, Adefovir, Entecavir, Tenofovir and Telbivudine) were found.

Two nonsense mutations of the S gene - one at position Leu216 (sLeu216*) affecting six HBV sequences (PKS034, TNS014, WMS009, BUDS073, TNS055 and TNS065), and the other at position Trp182 (sTrp182*) found in sequence BUDS005 - were detected, resulting in a C-terminal truncation of the S protein in these strains. All deletions and substitutions described above are shown in Table 6.2.

Serotype distribution of HBV in the Offin river valley

According to the previously described amino acid sequence algorithms [43] R¹²², K¹⁶⁰, L/I¹²⁷ (found in 94% of the sequences) and R¹²², K¹⁶⁰, P¹²⁷, G¹⁵⁹, S¹⁴⁰ (detected in the remaining 6%), all the 52 HBV sequences of this study were classified as HBV serotype *ayw4*.

6.5 Discussion

Analysis of sera collected from inhabitants of 13 communities located in the Offin river basin of Ghana by a rapid strip assay revealed a high (>8%) HBsAg prevalence in the rural population under study. Since it is known that the sensitivity of rapid strip assays may be lower than that of more complex enzyme immunoassays [50], we validated the performance of the rapid test used by analyzing a subset of samples in parallel by an ELISA. Both assays yielded identical results for the 88 samples tested. Antibodies to HDV were found in the serum of more than 8% of the 107 HBsAg carriers. This frequency of co-infection is similar to that observed in a recent study conducted in Accra, where a HDV sero-prevalence of 11.3% was found among 53 patients with HBV-related liver disease [38]. HBV can be transmitted horizontally by exposure to infected blood and various other body fluids or vertically by spread from mother to child at birth, with the majority of the children infected before the age of 6 months becoming chronic carriers [1]. In our study population we observed a significantly lower HBsAg carrier rate (1.8%) among children ≤ 11 years than in the older population (11.1%), coinciding with the 11 year period since when Ghana has introduced HBV vaccination with the pentavalent DPTHH vaccine. These data tend to confirm that HBV vaccination is effectively implemented in the Offin river valley. In a recent study performed in rural areas of Ghana, an at least 95% coverage for all three doses of DPTHH by the end of the first year of life was reported. However, immunizations suffered from poor timeliness, with substantial inequity across educational and socio-economic classes, due to weak supply chain management and poor access to health services [51]. Therefore, more detailed studies are needed to assess the efficacy of HBV vaccination in Ghana. While the administration of the hepatitis B vaccine at birth or in early childhood has been effective in reducing the incidence of the disease in many endemic regions [52, 53], immunization programs will not benefit patients already chronically infected with HBV. The low HBsAg carrier rate in the age group ≥ 60 years may be related to the higher mortality rate in elderly individuals with viral hepatitis [54, 55], which has been attributed in part to a higher prevalence of co-morbidities. Since HBsAg carriage was high in adolescents and adults, our data demonstrate that public health efforts are required to screen and subsequently ensure access to treatment, particularly for persons suffering from cirrhosis or advanced stages of liver disease. Moreover, additional needs for the management of concurrent or sequential infection with HBV and other viruses such as HIV, HCV and/or HDV - often associated with more severe and progressive liver disease and a

higher incidence of cirrhosis and hepatocellular carcinoma - should be addressed. These include the identification and initial treatment of the dominant virus followed by monitoring of the co-infecting virus [56]. Another essential aspect to be considered in the management of hepatitis is that populations with high HBV prevalence live in regions also endemic for mycobacterial infections such as tuberculosis, leprosy and Buruli ulcer. Special caution has to be paid when treating individuals with advanced stage liver disease with standard anti-mycobacterial regimens in order to avoid drug-induced hepatitis [56-58]. In this context, drug-induced liver injury has been reported to be three- to six-fold higher in persons infected with HBV, HCV or HIV who are receiving anti-tuberculosis drugs, due to hepatotoxicity of isoniazid, rifampicin and pyrazinamide [59].

Until today only a limited number of HBV genotyping studies have been conducted in Ghana. Two reports have indicated the exclusive presence of genotype E in samples collected more than 15 years ago [34, 35]. Strains analyzed in these studies came from targeted groups, such as pregnant women, HIV seropositive individuals and blood donors. In the present study, we provide the first molecular study of HBV from carriers resident in rural communities of the Offin river valley of Ghana. Although more than a decade has passed, and the co-circulation of two genotypes, namely A and E, has been reported in other West African countries [35], our phylogenetic analysis, based on the pre-S/S sequence of 52 HBV samples, showed that all of them belonged to genotype E, and all were predicted to belong to serotype *ayw4*. However, two separate E *ayw4* genetic clusters were found. The Offin cluster, comprising 33/52 sequences obtained from individuals living in 11 of the 13 study communities, showed a low diversity with an intra-group mean distance of only 0.7%. With an intra-group mean distance of 1.6%, the 19 sequences belonging to the pan-African cluster were more diverse, and originated from nine different communities, including Akomfore, Krakrom and Mfantseman, which are the three most remote communities. The Offin river basin has been a focus of small scale gold mining for many centuries and has produced more gold than any other river system in Ghana [60]. Over the years, there has been up-scaling of mining activities coupled with the influx of artisans from other regions of Ghana, as well as of citizens from other countries. During our exhaustive household survey in the Offin river valley, we encountered a number of nationals from other West African countries, particularly from Niger, Nigeria and Benin, representing one potential source for the introduction of new HBV genetic variants into the river basin.

In general, the very low diversity of the 52 pre-S/S region (1.2%) and S region (0.6%) sequences of this study is in line with previously published data reporting a strikingly low genetic diversity of HBV/E [14, 21]. Analysis of 610 S region sequences has revealed a mean genetic diversity of 0.8% for HBV/E as compared with a 2.1% diversity for 167 African HBV/A sequences [21]. The apparently relatively recent emergence of genotype E in Africa is contrasted by the excessive spread of this genotype throughout West Africa. While this has led to hypotheses on a more efficient mechanism of transmission, as compared to genotype A, it has been speculated in a recent report that historical mass vaccination campaigns with unsafe injection needles may have caused the current high prevalence rates of HBV/E throughout sub-Saharan Africa [21]. As a result of the increasing selection pressure caused by widespread HBV vaccination, immunotherapy and chronic HBV infections, various escape mutations in the HBsAg gene have been reported. Neutralizing antibodies induced by immunization are targeted against the conformational epitopes of the ‘a’ determinant of the surface antigen [47]. Mutations within this determinant may affect the binding of anti-HBs antibodies, allowing for the replication of the virus in vaccinated individuals, and pose a challenge to immunoassay detection. Although only individuals testing HBsAg positive were included in the present study, we identified the previously described [49] Ser143Leu substitution in the ‘a’ determinant. The codon 143 mutation has been reported to cause false negative results in some commercial HBsAg immuno-assays [61]. These data stress the importance of using HBsAg assays with a high sensitivity, in particular for the screening of blood bank donors. In addition, we detected two nonsense mutations in several HBV sequences at positions Leu²¹⁶ (sLeu216*) and Trp¹⁸² (sTrp182*) of the S gene, for which potent oncogenic activity was recently demonstrated, suggesting a role in hepatocarcinogenesis [62]. No known resistance mutation to antiretroviral drugs was detected in our dataset, which might be explained by the lack of selection pressure due to the limited use of the drugs in this region.

In summary, we revealed a high prevalence of HBsAg carriers among adults and the occurrence of HDV co-infection in the general population of the Offin river valley of Ghana. A low genetic diversity was found among the HBV sequences, and HBV/E was the only genotype detected. We conclude that in addition to strict adherence to the EPI schedule for children, there is need for an anti-hepatitis B campaign in the form of screening and assessing the severity of liver disease in order to select individuals eligible for treatment, prioritizing patients with advanced stage liver

disease. While in resource-rich countries effective treatments for chronic hepatitis are widely available, access to antiviral therapy is restricted in developing countries and should be urgently improved. Challenges associated with this endeavor have to be faced from the perspectives of global health and social justice [63].

6.6 Acknowledgements

We thank Dr. Edwin Ampadu and William Opare of the National Buruli Ulcer Control Program in Ghana, the District Health Directors and Disease Control Officers of all the Health Districts we worked in, and the Volunteers of all study communities for their support.

6.7 Figures

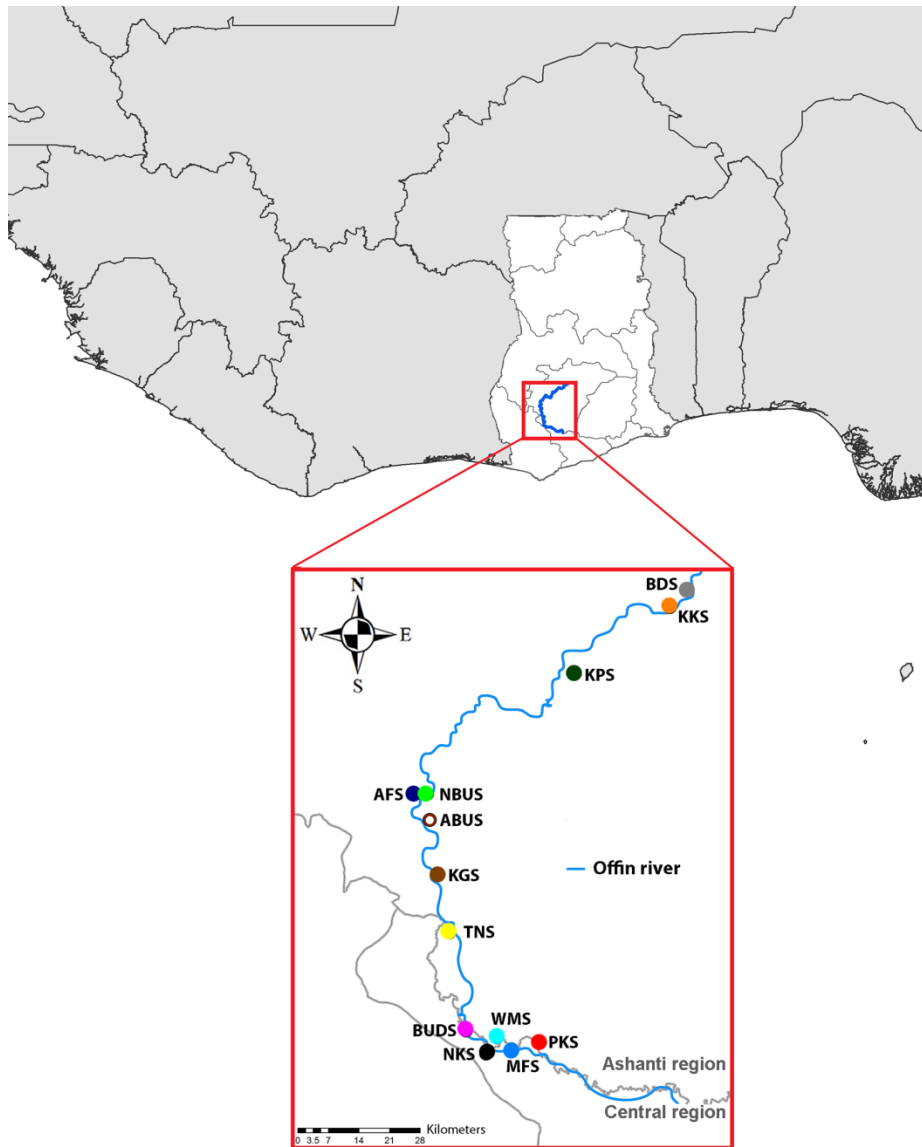


Figure 6.1. Study area. Map of Ghana with the Offin river valley and surrounding countries. The 13 study communities along the Offin River are indicated as multicolored dots: Bedomase (BDS); Krakrom (KKS); Kapro (KPS); Akomfore (AFS); Ntobroso (NBUS); Achiase (ABUS); Keniago (KGS); Tontonkrom (TNS); Dominase (BUDS); Wromanso (WMS); Nkotumso (NKS); Mfantseman (MFS); Pokukrom (PKS). The grey lines in the Ghana map indicate the borders of the Ghanaian regions. The background maps were created using the *ArcMap* program in *ArcGIS* v.10.0 and were modified with Adobe Photoshop CS6.

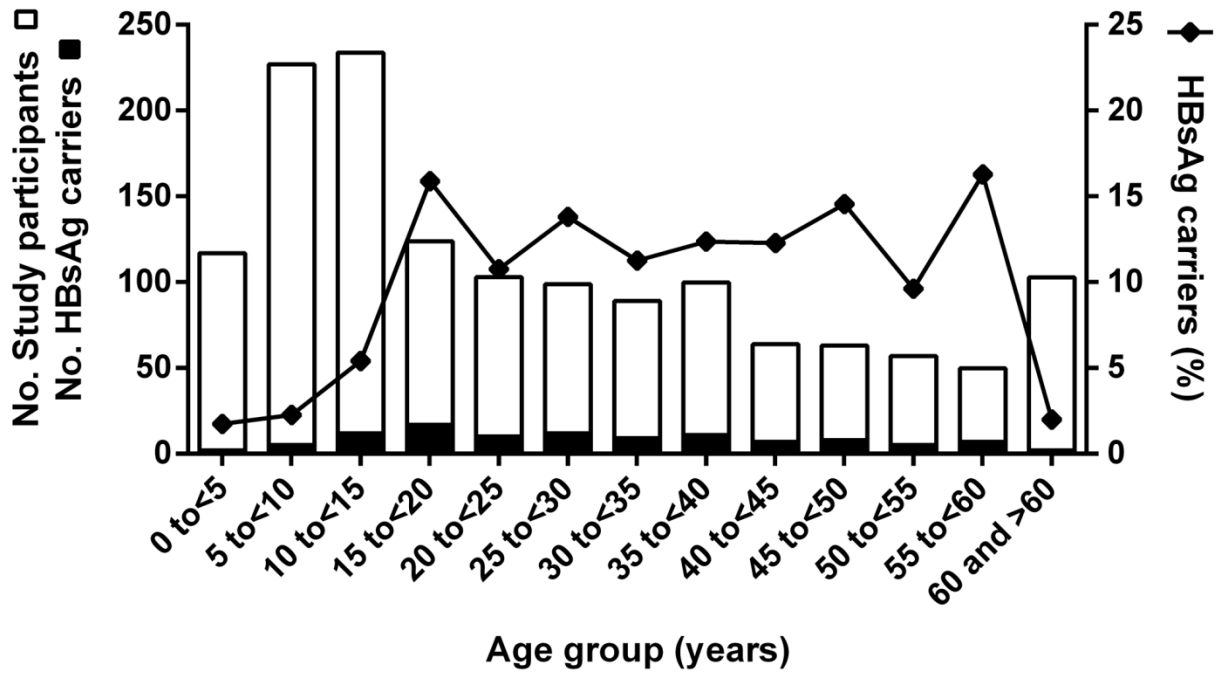


Figure 6.2. Age distribution and percentage of HBsAg carriers. A stacked graph of the total number of study participants for each age group (white bar) and of the corresponding number of HBsAg carriers (black bar) (left y-axis) is shown. The percentage of HBsAg carriers for each age group (right y-axis) is indicated by squares.

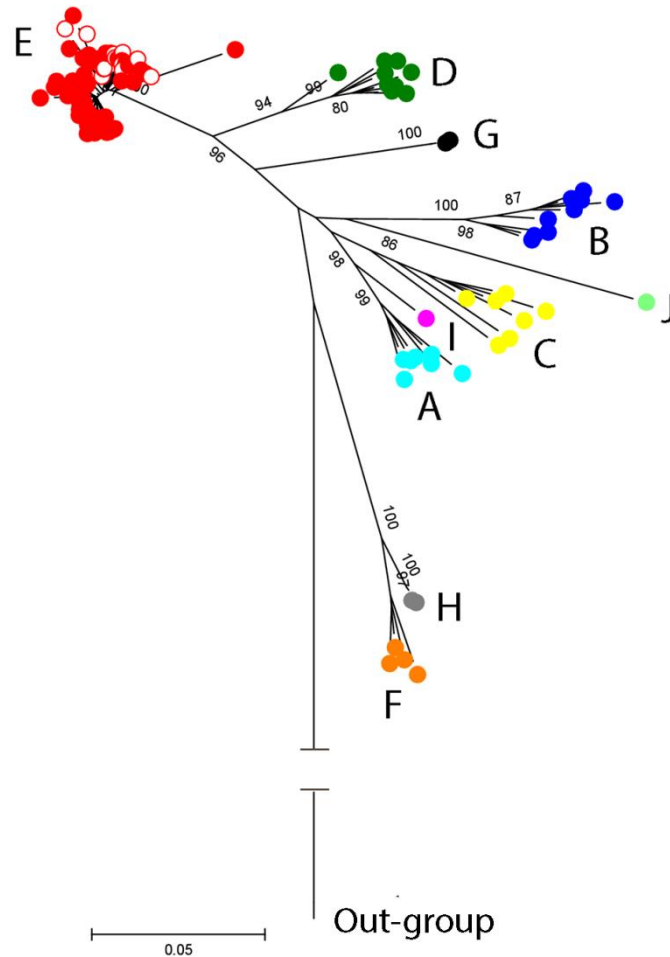


Figure 6.3. Phylogenetic reconstruction of world-wide and Offin river valley HBV sequences based on the pre-S/S region. A maximum-likelihood phylogenetic tree of HBV pre-S/S sequences obtained in this study (red dots) together with publically available sequences covering all HBV genotypes (multicolored dots) was constructed with 1000 bootstrap replicates using the Kimura 2-parameter +G +I model [41] contained in MEGA6 [42]. The tree is drawn to scale, with branch lengths corresponding to the number of substitutions per site. Reference sequences retrieved from GenBank are for genotypes A (AY233278, HE576988, AM184126, AM180623, FJ692609, GQ331047, FN545833), B (AB073842, AB073836, AB033555, AB100695, AB219427, DQ463789, EF473977, AP011093, GQ358145), C (AF223954, AB033556, X75656, AB048704, AB241109, AP011102, AP011104), D (JF754615, EU594428, AY233291, KF192838, GQ205378, AB493846, FJ904430, FN594771, JN664919), E (circles without fill: AB205192, AB106564, DQ060830, AB201290, AB205188, AB091255, AB091256, HM363611, FN594765, AB205191, AB205190), F (AF223964, X69798, AB036914, DQ823087), G (AB056513, EF634480), H (EU498228, EF157291), I (FJ023660), and J (AB486012). The sequence of a Woolly monkey HBV was included as an out-group.

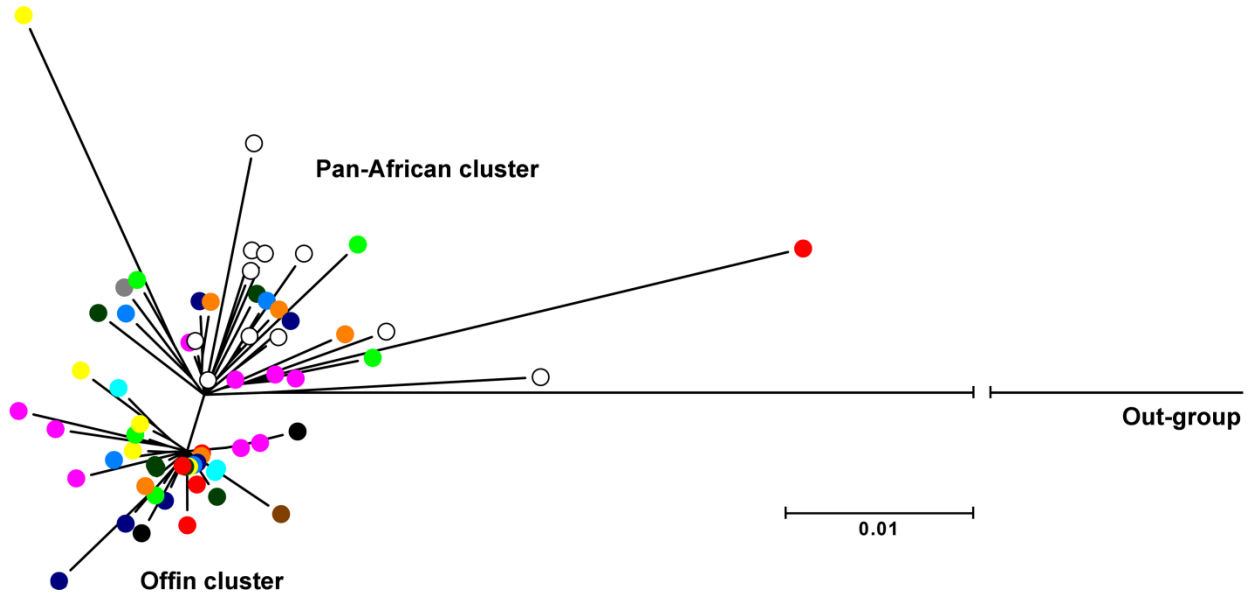


Figure 6.4. Phylogenetic reconstruction of HBV/E sequences. A maximum-likelihood phylogenetic tree based on the pre-S/S gene region sequence (1,200 bp) was constructed using the Kimura 2-parameter +G model [41] embedded in MEGA6 [42]. While HBV sequences obtained in this study are indicated as multicolored dots (according to village of residence), HBV genotype E sequences retrieved from GenBank are depicted as empty circles (accession numbers (origin): AB205192 (Ghana), AB106564 (Ghana), DQ060830 (Madagascar), AB201290 (Benin), AB205188 (Ghana), AB091255 (Côte d'Ivoire) AB091256 (Côte d'Ivoire), HM363611 (Nigeria), FN594765 (Niger), AB205191 (Ghana), AB205190 (Ghana)). The sequence of the genotype D AY233291 from South Africa was included as an out-group.

6.7.1 Supporting Figures

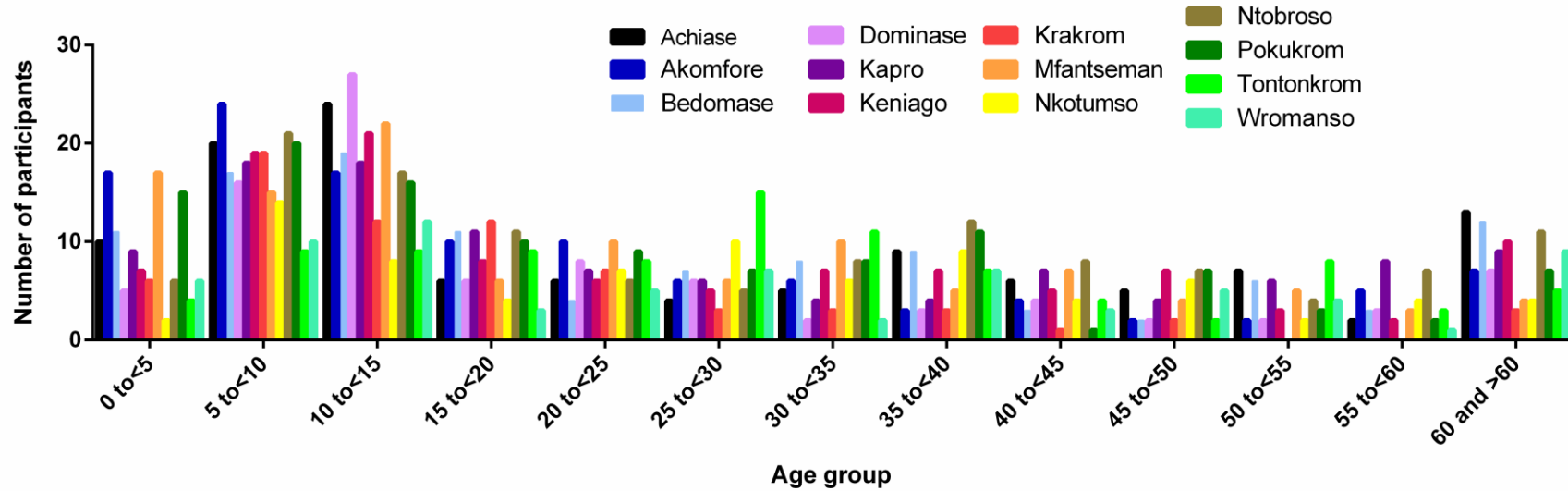


Figure S6.1. Distribution of study participants by age group and residential community.

6.8 Tables

Variables	Community*													Total
	ABUS	AFS	BDS	BUDS	KPS	KGS	KKS	MFS	NKS	NBUS	PKS	TNS	WMS	---
Health districts‡	AM	AM	SS	UDW	AN	AW	SS	UDE	UDW	AM	UDE	AW	AC	---
Community Infrastructure														
Road network§	C2	C3	C2	C1	C1	C2	C3	C3	C1	C2	C3	C2	C3	---
Social infrastructure#	SRM	SR	SRM	SRMH	SR	SRMH	-	SRM	SRMH	SRMH	SRMH	SRMH	SR	---
Surveyed Population														
Total inhabitants, n	1,900	1,016	1,688	2,802	692	3,350	111	303	2,518	1,949	900	2,945	216	20,390
Recent travel history, n (%)	518 (27.3)	350 (34.4)	546 (32.3)	1,360 (48.5)	192 (27.7)	1,156 (34.5)	67 (60.4)	78 (25.7)	1,120 (44.5)	758 (38.9)	409 (45.4)	1,117 (37.9)	141 (65.3)	7,812 (38.3)
Recent contact with major cities, n (%)	354 (18.6)	150 (14.8)	357 (21.1)	1,036 (37.0)	96 (13.9)	705 (21.0)	11 (9.9)	10 (3.3)	462 (18.3)	446 (22.9)	281 (31.2)	662 (22.5)	89 (41.2)	4,659 (22.8)
Study Participants														
Total, n	117	113	112	91	111	107	71	114	80	123	116	94	74	1,323
Females, n (%)	64 (54.7)	64 (56.6)	68 (60.7)	48 (52.7)	62 (55.9)	66 (61.7)	40 (56.3)	65 (57.0)	48 (60.0)	77 (62.6)	82 (70.7)	49 (52.1)	48 (64.9)	781 (59.0)
HBsAg sero-prevalence, n (%)	3 (2.6)	12 (10.6)	3 (2.7)	9 (9.9)	11 (9.9)	2 (1.9)	7 (9.9)	6 (5.3)	10 (12.5)	11 (8.9)	10 (8.6)	14 (14.9)	9 (12.2)	107 (8.1)
Anti-HDV antibody sero-prevalence among HBsAg pos, n (%)	1 (33.3)	0 (0)	0 (0)	1 (11.1)	0 (0)	0 (0)	0 (0)	0 (0)	3 (30)	0 (0)	0 (0)	3 (21)	1 (11.1)	9 (8.4)

Table 6.1. Characteristics of the study population and HBsAg and anti-HDV antibody seroprevalence. * ABUS = Achiase; AFS = Akomfore; BDS = Bedomase; BUDS = Dominase; KPS = Kapro; KGS = Keniagio; KKS = Krakrom; MFS = Mfantseman; NKS = Nkotumso; NBUS = Ntobroso; PKS = Pokukrom; TNS = Tontonkrom; WMS = Wromanso

‡ AM = Atwima Mponua district; SS = Sekyere south district; UDW = Upper Denkyira West district; AN = Atwima Nwabiagya district; AW = Amansie West district; UDE = Upper Denkyira East district; AC = Amansie Central district

§ C1 = asphalt road surface, major road connecting one district capital to another with relatively high traffic volume; C2 = gravel road surface, major road connecting one town to another with relatively low traffic volume; C3 = gravel road surface, minor road connecting one town to another with cars sparingly plying the road

S = school; R = religious facilities; M = central market; H = health center

	Pre-S region				S region									
	Met ⁸³	Gly ⁷¹ -Asn ⁹⁷ , Arg ¹¹² -Arg ¹³⁶	Val ¹³⁵ -Phe ¹⁴⁰	Met ¹¹⁹	Thr ⁵⁷	Gln ¹⁰¹	Ile ¹¹⁰	Ser ¹¹⁴	Leu ¹²⁷	Ser ¹⁴⁰	Ser ¹⁴³	Gly ¹⁵⁹	Trp ¹⁸²	Leu ²¹⁶
AFS097	Pro
AFS115	Pro
BUDS005	His	*	.
BUDS006	Leu	Ala
BUDS073	*
KKS062	Pro
KKS064	Ile
KPS088	Ala	.	.
MFS048	Leu	.	.	.
NBUS058	.	.	.	Thr
NBUS062
NBUS067	Lys
NBUS070	Thr	Pro
NBUS078	His
NKS010	.	.	.	Ile
PKS034	.	-----	Ala	.	*
PKS077	.	.	-----
TNS014	Ile	Leu	.	.	.	*
TNS055	*
TNS065	*
TNS091	Leu	.	.	.
WMS009	.	.	.	Thr	*

Table 6.2. Comparison of deduced amino acids of sequences obtained in this study and HBV/E consensus. Only relevant mutations and deletions described in the text are shown. *nonsense mutations

6.8.1 Supporting Tables

	Community#													Total
	ABUS	AFS	BDS	BUDS	KPS	KGS	KKS	MFS	NKS	NBUS	PKS	TNS	WMS	
Offin cluster sequences (n)	0	4	0	5	4	1	2	2	2	2	4	4	3	33
Pan-African cluster	0	2	1	4	2	0	3	2	0	3	1	1	0	19
Total	0	6	1	9	6	1	5	4	2	5	5	5	3	52

Table S6.1. Origin of HBV sequences belonging to the Offin and pan-African clusters. #Achiase (ABUS); Akomfore (AFS); Bedomase (BDS); Dominase (BUDS); Kapro (KPS); Keniago (KGS); Kakrom (KKS); Mfantseman (MFS); Nkotumso (NKS); Ntobroso (NBUS); Pokukrom (PKS); Tontonkrom (TNS); Wromanso (WMS)

Community/ ID	Gender	Age	Additional HBsAg carriers in household	Relevant mutations (S region)	Anti-HDV antibody positivity
AFS068	Female	12	NI [#]		No
AFS080	Male	18	1		No
AFS097	Female	33	NI	sL127P	No
AFS101	Female	38	NI		No
AFS113	Female	4	NI		No
AFS115	Male	27	NI	sL127P	No
BDS103	Male	27	1		No
BUDS005	Female	55	NI		No
BUDS006	Female	21	NI		No
BUDS015	Female	13	NI		No
BUDS023	Male	13	NI		No
BUDS033	Female	23	NI		No
BUDS038	Male	8	NI		No
BUDS051	Female	17	NI		No
BUDS073	Female	35	1 (BUDS081)	sL216*	Yes
BUDS081	Male	21	1 (BUDS073)		No
KGS053	Female	32	NI		No
KKS011	Male	17	NI		No
KKS013	Male	18	2 (KKS062, KKS064)		No
KKS033	Male	15	NI		No
KKS062	Female	10	2 (KKS013, KKS064)		No
KKS064	Male	15	2 (KKS013, KKS062)	sL127I	No
KPS021	Male	4	NI		No
KPS029	Male	24	NI		No
KPS078	Male	8	1		No
KPS088	Female	19	NI		No
KPS091	Male	36	1 (KPS094)		No
KPS094	Male	13	1 (KPS091)		No
MFS014	Female	31	NI		No
MFS022	Female	5	NI		No
MFS043	Male	19	NI		No
MFS048	Female	25	NI	sS143L	No
NBUS058	Female	42	NI		No
NBUS062	Female	42	1 (NBUS070)		No
NBUS067	Male	45	NI		No
NBUS070	Female	23	1 (NBUS062)	sL127P	---
NBUS078	Male	29	NI		---

NKS010	Female	42	1 (NKS020)		---
NKS076	Male	34	NI		Yes
PKS003	Male	27	NI		No
PKS034	Male	9	2	sL216*	No
PKS066	Female	13	NI		No
PKS077	Female	35	NI		No
PKS096	Female	17	NI		No
TNS014	Female	50	1	sS140L, sL216*	No
TNS055	Male	28	NI	sL216*	No
TNS065	Female	55	NI	sL216*	No
TNS088	Male	14	2 (TNS091)		Yes
TNS091	Male	16	2 (TNS088)	sS143L	No
WMS009	Female	23	2 (WMS008)	sL216*	No
WMS081	Male	25	NI		No
WMS082	Male	12	NI		No

Table S6.2. Demographic and serological data of HBsAg carriers, for which sequence information is available. # NI = not identified

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Chapter 7

General Discussion and Conclusion

7.1 General remarks

Following the first definitive clinical description of *M. ulcerans* infections in 1948 and the declaration of the disease as a considerable Public Health burden at the WHO meeting in Yamoussoukro in 1998, research on BU has gained increased attention. The intensified research and control efforts invested into BU since then have primarily reflected on the improvement of treatment and diagnosis and have also led to a considerable demystification of the disease in the affected populations. Nevertheless, several aspects of BU epidemiology and transmission have still not been fully elucidated. Through collaborative studies embedded in the Stop Buruli Initiative, the NMIMR and the Swiss TPH have shed light on the epidemiology and transmission of BU in the Densu river valley of Ghana [1–3]. The collaborative research was then extended to the Offin river basin which has been known to be the most endemic area in Ghana for many years. By this extension, we anticipated that findings from this PhD study will add on to what we currently know from the studies along the Densu river and in the bigger picture, contribute to the knowledge of BU epidemiology and *M. ulcerans* transmission in Ghana and globally.

7.2 Control of BU and other neglected tropical diseases in the Offin river basin

7.2.1 Impact of early case detection and treatment of BU on disease control

An important aspect of infectious disease control is the identification of risk factors that increase the probability that the host will come into contact with the pathogen. Understanding of risk factors makes it possible to identify preventable risks and to implement effective public health measures. This has not been possible with BU, as the definite mode of transmission is not known. Moreover, there is no effective vaccine. BU control has therefore relied solely on early case detection and treatment with a combination of anti-mycobacterial agents. Reports from the most recent WHO meeting on BU indicated a reduction by more than 50% in the number of globally reported cases in 2014 when compared to 2009 [4]. For example in Ghana, there has been a steady decline in the number of new cases reported from 1,048 in 2010 to 443 in 2014 [5]. Since 2009, Prof. Yeboah-Manu's group at NMIMR under the Stop BU Initiative has consistently conducted together with the national BU control program active case search followed by laboratory confirmation of cases in endemic communities along the Densu river. While in the first three years the number of detected cases increased, the trend reversed within the subsequent three years. In recent years, there has been an increase in suspected lesions within the communities pointing towards an enhanced awareness among populations and health workers. However, the percentage of suspected cases confirmed by PCR declined steeply and other conditions were rather diagnosed using a differential diagnosis system (Yeboah-Manu et al. 2015, manuscript in preparation). This downward trend seems to occur also in the BU endemic Offin river basin, since we detected only three PCR-confirmed cases after an exhaustive active case search of 20,390 inhabitants (chapter 2 of this thesis). Moreover, after employing a number of active surveillance strategies which included monthly household visits, we could only detect eight more cases post the exhaustive survey. The recent decline in reported BU cases could be due to the intense active case search activities conducted in preceding years. Following the establishment of the first BU surveillance system in Ghana in 1993, the then high disease burden attracted a lot of research and control activities particularly within the Amansie West District, which in 1999 was identified as the most endemic district in Ghana [6]. In addition to the

observed decline of the number of BU cases, we did not find the large late stage ulcers that the district was noted for, but rather, early stage lesions (Figure 7.1). We assume that these trends reflect the intensified continuous control activities, supported by organizations such as WHO, ANESVAD and Safe water under the umbrella of the BU control program of Ghana health service.

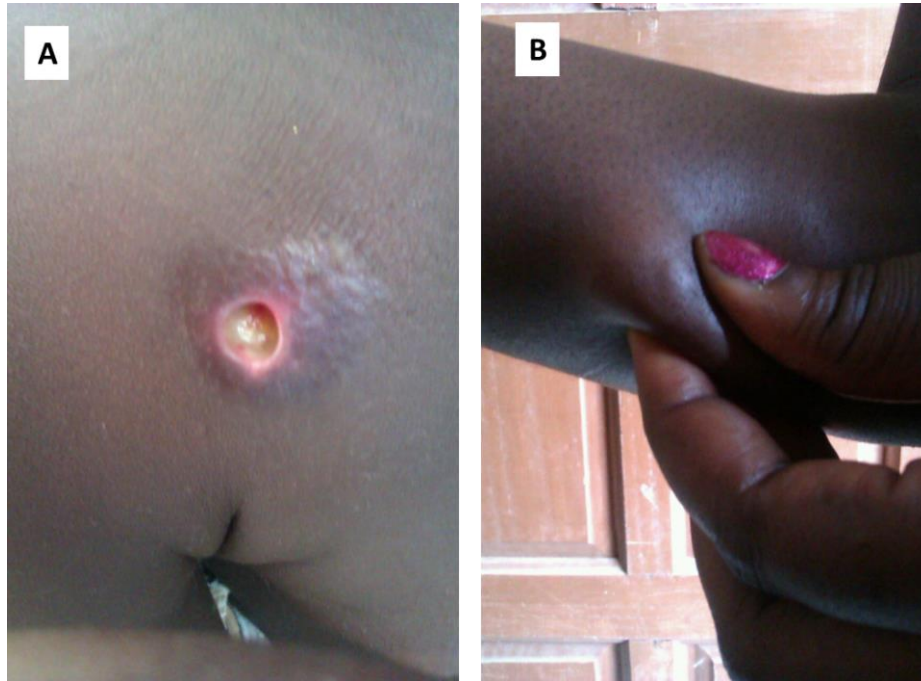


Figure 7.1. Identification of cases with early staged lesions. All cases detected during our study of the BU epidemiology in the Offin river basin presented with early staged lesions. 1A Typical Cat I ulcer on the shoulder of one case detected in the active case search. 1B Typical Cat I nodule presented by a case detected by a volunteer in Achiase during a household visit.

The decline in BU case numbers after case detection and successful treatment support the view [7] that patients with chronic active lesions may play a role in the transmission of *M. ulcerans* by shedding the bacteria from active lesions into the environment, which then becomes a source of infection for others. There are similarity with *M. leprae* infection (leprosy), where humans have been established as reservoirs of the pathogen and some studies have suggested that transmission from an environmental source to man is also likely [8–12]. However, in contrast to *M. leprae*, infections, direct person-to-person transmission of *M. ulcerans* seems to be rare. The widespread introduction of multi drug therapy for leprosy (which included rifampicin) in 1981 resulted in an 85% reduction in globally reported leprosy cases from 4 -5 million cases in the mid 1980's to

800,000 by the year 2000 [13,14]. These parallel scenarios demonstrate that early case detection and treatment can facilitate an interruption of the infection cycle, thereby resulting in a decline of cases which eventually reflects at the national and global scale. While this positive development for BU is encouraging, the global research agenda that includes understanding the mode of transmission and the ecology of *M. ulcerans* should be pursued. Furthermore, these successful activities should be continuous, as an interruption of the disease control efforts may lead to an upsurge of cases as is being seen with leprosy, where countries including Bangladesh and Madagascar were declared by the WHO as leprosy-free some decade ago, but the disease has bounced back to remote and poor areas as well as the capital cities [15,16].

7.2.2 Opportunities for an Integrated Disease Surveillance and Control Response

For many low-resourced countries, there is extensive geographic overlap and co-endemicity of neglected tropical diseases [17,18], which has prompted global calls for an integration of control strategies [19,20]. However, for many years, the link between disease surveillance and response in most countries within the sub-Saharan Africa region was only weak; a sign of inadequate preparedness to handle disease outbreaks should they occur. This led to a proposal by the WHO's Regional Office for Africa (WHO AFRO) for an Integrated Disease Surveillance and Response (IDSR) strategy in 1998 [21,22]. In its entirety, the IDSR strategy was meant to marshal the limited human and financial resources available for the different disease control programs (which includes the National Buruli Ulcer Control Programme) to operate a system that monitors disease outbreaks (at both community and district levels), conduct laboratory diagnoses and report outbreaks in a timely fashion. Since the implementation of the IDSR, some global successes have been recorded for countries like Uganda where in 2000, an outbreak of Ebola Haemorrhagic Fever was managed within the framework of the IDSR which eventually led to the identification of important means of transmission of the virus [23]. In Ghana, the IDSR has played similar roles in cholera outbreaks and has contributed to the decline in the incidence of polio, measles and yellow fever which have been recorded over the years by means of its vertical system of operation.

Within the framework of our BU case detection activities in the Offin river basin (Chapter 2) we equipped community volunteers with mobile phones to conduct monthly surveillance of BU at a household level. We observed that during such home visits, the volunteers seized the opportunity

to execute other responsibilities such as distribution of insecticide treated nets (for malaria control) and follow up on children who missed out on health outreach programs such as mass drug administration (MDA). This served as a primary indication that integration of disease control was feasible at least at the level of early case detection and treatment which forms the core of strategies mapped out for most neglected tropical diseases. In addition, with one round of exhaustive active BU case search, we could gather case data for other diseases such as tuberculosis and leprosy, demonstrating an opportunity to cut cost by using the same number of staff and logistics primarily intended for investigation into the burden of BU. Furthermore, by collaborating with the local health staff, we screened and treated participants being followed up on for other endemic diseases like malaria and syphilis.

A core component of the IDSR strategy included gathering disease data which better informs health policy and helps in decision making. To this end, the District Health Information Management System (DHIMS) in 2012 was restructured to an internet-based system of reporting (DHIMS II) with the overall objective of improving data quality and reliability across the various strata of the health system [25]. During our investigations in 2013, we observed that some records were still maintained on paper based platforms with a widespread loss of information pertaining to annual cases of BU reported for most districts. Our current mobile phone based surveillance system presents an opportunity to preserve the case notification records since these data are backed-up automatically and can be accessed at any time. By including the surveillance of other diseases on this platform, disease control officers can effectively monitor outbreaks of diseases and better co-ordinate intervention activities from the district office. For diseases like polio, Guinea worm infection and leprosy, where national incidence rates are close to the threshold set for elimination by the WHO, their individual control programs face an imminent threat of collapse. In view of this, the most effective way to sustain surveillance of all diseases with very low incidence (which currently includes BU) would be to integrate all the surveillance activities.

When taken together, our surveillance system that was well developed for BU has helped to and may act as driving force for strengthening other surveillance activities by offering possible synergies and common resources to improve various aspects of public health within the river basin.

7.3 Ecology and Transmission of *M. ulcerans*

7.3.1 Potential reservoirs of *M. ulcerans*

The success of micro-epidemiological studies in tracking transmission routes, identifying infection sources as well as reservoirs of several bacterial pathogens has chiefly depended on the genetic delineation of isolates. The extreme lack of genetic diversity of *M. ulcerans* reflected by the inadequate resolution offered by conventional genotyping tools posed a major setback to micro-epidemiological studies on BU for several years [26–30]. Molecular tools employed have however evolved over the years with each new approach shedding more light on the epidemiology of the pathogen by strain delineation. For example, variable number of tandem repeat (VNTR) typing offered a limited resolution by differentiating a collection of strains from Ghana into three VNTR types [31]. Beyond the district level, the VNTR typing however failed to discriminate the strains; a setback which was overcome with the advent of whole genome sequencing. Based on the identification of informative single nucleotide polymorphisms (SNPs) through whole genome analysis of a collection of isolates from BU patients in Ghana, which included representatives of the three previously identified VNTR types [31,32], a real-time PCR SNP typing method was developed [3]. Subsequent SNP typing of a collection of strains from residents of the Densu river basin resulted in the discrimination of these strains into 10 different SNP haplotypes [3]. Linking of the strains to the residential villages of the patients revealed geographical clustering of several SNP haplotypes with one haplotype (founder haplotype) being widely distributed. This led to the hypothesis that the “founder haplotype” was first introduced to the endemic area and with time accumulated some mutations causing it to evolve into the other (clustered) haplotypes. In addition, this spoke for a reservoir with very limited mobility.

A more recent study [33] was based on the whole genome analysis of a collection of 18 isolates from the Agogo community in the Ashanti region of Ghana. Based on their SNP profiles, 10 of the 18 isolates clustered with other strains from the neighboring Amansie West district. This implied that within the broader context of the Ashanti region, the “Amansie West genotype” (Agogo-1) is a local clonal complex which had spread and persisted within the region. Within the same collection of strains, a second clonal complex, “Agogo-2” was also identified. This complex, which clustered with a Nigerian isolate, was however randomly distributed within the endemic region implying co-existence of both clonal complexes. Consistent with these findings, we observed by whole genome and phylogeographic analysis of a limited number of strains, the

presence of two distinct clonal complexes within the Offin river valley (Chapter 5). Both were represented by strains isolated from patients in 2004 and 2013 and neither clustered geographically. While in the Densu river basin [3] and in two BU endemic areas of Cameroon [34] single local clonal complexes were found – indicative for *M. ulcerans* reservoirs with limited mobility - our current findings along with the recent study from the Agogo community [33] suggest that members of local clonal complexes of *M. ulcerans* may be carried occasionally over wide distances to other areas and establish there. However, the nature of this mobile reservoir in African endemic settings is yet to be explored.

In this context, environmental studies inspired by outbreaks of BU in Australia led to the detection of *M. ulcerans* DNA in mosquitoes and feces of possums. A number of possums also had BU-like lesions containing *M. ulcerans* bacteria, which led to a hypothesized mode of transmission with the possums acting as probable reservoirs and mosquitos as potential vectors [35]. The search for similar mammalian reservoirs in African BU endemic settings has however yielded futile results. A field capture of over 700 rodents in high and low endemic regions of Uganda in 1972 led to identification of some rodents with mycobacterial infections, none of which was caused by *M. ulcerans* [36]. A similar study also involving the capture of 348 small mammals in six villages in Benin found no *M. ulcerans* DNA in pooled samples of the captured animal body parts or feces [37]. Within the context of this PhD we conducted an extensive environmental sampling in communities along the Offin River (Aboagye *et al.*, 2015 manuscript in preparation) and did not encounter any domestic or wild mammals with BU-like lesions. Furthermore, the natives of the communities we worked in denied knowledge of the existence of such animals. The analysis, which was supported by a colleague PhD student, however found *M. ulcerans* DNA positivity among soil and vegetation samples. This observation together with the downward trend of new cases speaks more for humans as likely reservoirs of *M. ulcerans* in the African endemic settings.

Movements of patients with active lesions into new geographical regions on a county-, region- or community-scale in West Africa, may lead to an introduction of new variants. Subsequently, by means of activities that facilitate the dissemination of the bacteria into the environment, other inhabitants that come into contact with the contaminated source may get infected.

7.3.2 Evidence for the presence of *M. ulcerans* in the environment.

Efforts at elucidating the ecology of *M. ulcerans* in the environment was inspired by several epidemiological studies that have consistently associated activities or residency near water bodies with BU [38]. Ideally, successful isolation and characterization of the pathogen from the environment could have served as definite proof of which specific part of the environment is responsible for transmission of *M. ulcerans* to man. However, *M. ulcerans* has an extremely slow growth rate, making it very likely to be outgrown by other faster growing environmental organisms (including decontamination-resistant other environmental mycobacterial species) on artificial media. In view of this, several attempts at cultivating the bacteria from environmental source has been unsuccessful with the description of only one environmental isolate published so far [39]. While detection of the *M. ulcerans* DNA sequences IS404, IS2606 and KR by PCR [40,41] is not a definitive proof of viability, it has been utilized to demonstrate the presence and spread of the bacteria in several environmental studies. Based on the high positivity rate of detection of *M. ulcerans* DNA in a village along the Densu river valley, a recent isolate from the environment has been obtained and is currently being further characterized (personal communication, Prof. Dorothy Yeboah-Manu).

Within the framework of this PhD study, we conducted an extensive environmental sampling and analysis of 1166 samples from communities along the Offin. We demonstrated that in accordance with the low prevalence of BU cases detected within the river basin (Chapter 2), the proportion of environmental samples positive for *M. ulcerans* was extremely low (3.9%) when compared with the 22.5% recorded for the Densu river valley (Aboagye *et al.*, 2015 manuscript in preparation). In addition to recording a higher number of BU cases when compared to the Offin river, communities along the Densu had higher sero-positivity rates of 33% for the *M. ulcerans* 18KDa small heat shock protein (shsp) [2], when compared to 18% for those along the Offin (Chapter 4).

Altogether, we conclude that *M. ulcerans* is contracted through environmental exposure. Furthermore, we hypothesize that there is a correlation between sero-positivity, incidence of BU cases and the rate of detection of *M. ulcerans* DNA in the environment, if all three parameters are monitored within a study site.

7.3.3 Exploring the relationship between exposure to and transmission of *M. ulcerans*

The *M. ulcerans* 18KDa shsp has been identified and characterized as a suitable antigen for assessing the exposure of populations to the pathogen [42]. In a previous sero-epidemiological study conducted within the Densu river valley, individuals with antibodies against the *M. ulcerans* 18KDa shsp were identified in nearly all age groups under study [2]. However, none of the participants within that study was below 5 years of age, making it impossible to determine the age of onset of sero-conversion. Within the framework of this PhD study (Chapter 3), we analyzed a collection of sera which included more than 100 children below 5 years of age from the BU endemic Densu river valley (Ghana) and Bankim health district (Cameroon). We observed that children below 5 years of age were less exposed to the pathogen when compared to older children. None of the study participants <4 years old contained antibodies against the pathogen in their sera. In accordance with this observation, we found that by estimating the age at which patients within the Offin basin contracted BU, children below the age of 5 years were under-represented although the majority of our cases fell below 15 years of age (chapter 2 of this thesis). We therefore conclude that exposure to *M. ulcerans* starts at around 5 years of age which coincides with the age when children use to acquire new activities and expand their range of movement. Typically within our study communities along the Offin, these activities may include schooling, expansion of social networks (particularly making and visiting new friends) and execution of duties, such as the collection of water and fire wood, which eventually increases the likelihood of them coming into contact with the environment contaminated with *M. ulcerans* at the periphery of the community.

Within the last decades researchers have postulated different modes of *M. ulcerans* transmission. Theories that have been proposed to explain the mechanism of *M. ulcerans* infection include 1) inhalation of aerosolized *M. ulcerans* from contaminated water [43,44], 2) acquisition of *M. ulcerans* through bite of an insect or other vector, and 3) contamination of existing wounds or sites of trauma by environmental reservoirs such as soil, vegetation and water among others [45]. However none of these theories have been confirmed.

To further shed light on the possible transmission routes of *M. ulcerans*, we collected blood serum samples from a total of 1,352 individuals living along the Offin river and compared the first humoral immune response to *M. ulcerans* with pathogens with different modes of transmission. Contrary to what we observed for exposure to *M. ulcerans*, nearly all children

below 4 years had antibodies against the mosquito-transmitted *Plasmodium falciparum* AMA-1 antigen in their sera, which casted doubt on the involvement of mosquitos in the transmission of *M. ulcerans*. Some case control studies have identified using bed nets [46] and repellants [47] as protective factors against *M. ulcerans* infection. Based on our finding, we infer that potential insect vectors localized within the homes are not likely to play a role in the transmission of the pathogen. Since we observed an early serological response to antigens of soil-transmitted *Strongyloides*, an involvement of the soil around the houses in the transmission of *M. ulcerans* is not likely. In contrast, we found that the serological response to both egg and adult worm antigens of the water-related *Schistosoma* parasite sets in as late as the response to *M. ulcerans*, strongly inferring that transmission of *M. ulcerans* is most likely connected to an environmental reservoir in the aquatic ecosystem outside of the limited movement range of small children. Still unclear is the mechanism of entry of the bacteria into the skin; inhalation of aerosolized *M. ulcerans* from contaminated water is now considered to be very unlikely. When placed within the context of the remaining two hypotheses explaining the entry of *M. ulcerans* into humans [46,48], our findings indicate that infection may occur through both contact between an existing wound and an environmental source (potentially mud [49]) contaminated with *M. ulcerans* and inoculation of the pathogen into the skin via an unknown vector associated with stagnant water bodies.

Overall, we conclude that exposure to *M. ulcerans* starts at around five years of age, coinciding with the age at which children take on new activities and increase their movement range to include going out of their households and having more intense contact with the periphery of the community. In addition, by comparing the age pattern of the first humoral immune response to *M. ulcerans* with that of pathogens with different modes of transmission, we are of the opinion that the exposure is most likely by contact with *M. ulcerans* present in the aquatic ecosystem at the periphery of the communities.

7.4 Hepatitis B virus infection: burden, genetic diversity and implications for

BU treatment

Management of patients with co-infection of two or more diseases represents generally a complex challenge for clinicians and public health staff. With the exception of HIV co-infections, which have gained a lot of research attention due to the aggressive acceleration of clinical BU symptoms [50–57], studies on the co-infection of BU with other diseases are scanty. To the best of our knowledge, no study has been conducted on the co-infection of BU with HBV, which should be of public health concern. Rifampicin is the only highly effective antimycobacterial drug against BU and it should be of major concern, that, overt liver damage has been associated with TB cases with pre-existing HBV infection (both carriers and diseased) undergoing rifampicin chemotherapy [58–61].

While the highest burden of HBV infection occurs in sub-Saharan Africa and Asia, most of the populations are unaware of their HBV infection status [62]. Within the framework of this PhD thesis, we analyzed the prevalence of the hepatitis B surface antigen (HBsAg) in sera of individuals living in BU endemic communities of the Offin river valley as an indication of chronic HBV carriage. We observed that the HBsAg carrier rate among children below 12 years of age was low with 1.8% when compared to older participants >12 years, where the carrier rate was as high as 11.1% (Chapter 6). There has been an effective vaccine against HBV since the late 1970's [63], however, it was not until 2002 that vaccination against the virus was incorporated into Ghana's immunization schedules following the WHO Expanded Program on Immunization (EPI). Specifically for all seven health districts of the Offin river in which we worked, the vaccination of infants against HBV started around 2002 (personal communication with all disease control officers). While the low infection rate recorded for children <12 years reflects the 11 year period of running the EPI with inclusion of the HBV vaccine, periodic studies are needed to assess the effectiveness of the program.

While all HBV isolates from the Offin river basin were characterized as genotype E, phylogenetic analysis based on the sequence analysis of the pre-S/S region revealed clustering of a number of the Offin strains with those of other neighboring West African countries, particularly Niger, Nigeria, Benin and Cote D'Ivoire. The Offin river which has been a place of small scale gold mining for many centuries, has produced more gold than any other river system

in Ghana [64]. Over the years, there has been upscaling of small scale mining activities coupled with the influx of artisans from other regions in Ghana as well as citizens of other countries. During our exhaustive survey of the Offin river population (Chapter 2), we observed a number of West African nationals (particularly from Niger, Nigeria and Benin) resident within the river basin for several years. While none of the samples we analyzed was drawn from other nationals, the possibility that a foreign variant was introduced into the river basin cannot be ignored.

Recently, a new BU infection focus has been identified within the Dunkwa Offin municipality (personal communication, Mr George Amofa of the District health directorate, Upper Denkyira East). Given the high HBV positivity rates (>10%) recorded here for some communities, we recommend that new BU cases should be screened for HBV infection before commencing rifampicin treatment in all areas where co-infection of BU and HBV is likely. Given the evidence of potential hepatotoxicity associated with the use of rifampicin, there is a challenge of managing BU patients pre-infected with HBV. To address this we recommend the re-consideration of thermotherapy [65–67] as an alternative treatment for BU. As mentioned in Chapter 2, early case detection would potentially guarantee that cases detected will present with early stage lesions which can be managed relatively easily by thermotherapy [68]. In this context it should also be mentioned that major research efforts have been invested into the search for a replacement of streptomycin by another antibiotic, because of the severe side effects and numerous challenges associated with streptomycin administration.

Altogether, we conclude that transmission of HBV along the Offin river is mainly horizontal. Additionally, there are preliminary indications that the expanded EPI program which includes vaccination of infants against HBV infection is effective. We recommend based on our findings that screening and vaccination of the adult population should be considered.

7.5 Outlook

Based on the multidisciplinary approach to investigate the various arms of this PhD study, we have identified key findings which add to the existing knowledge of the epidemiology and transmission of *M. ulcerans* in Ghana and globally. As an outlook, we highlight findings, achievements, conclusions and areas of future research priorities below:

- i) We have established a field study site along the Offin river and demonstrated the decline in BU cases over the years. The study site subsequently served as a well characterized epidemiological setting for the investigation of other aspects of this thesis as well as for follow up of study participants.
- ii) We have established an active BU surveillance system which can be integrated with surveillance of other neglected tropical diseases to save cost and maximize resources directed and allocated for control of neglected tropical diseases within the Offin basin. Furthermore the electronic platform for data collection on the incidence of BU can be incorporated to address the gaps in data storage at the district level.
- iii) We have identified the spatiotemporal co-existence of two *M. ulcerans* clonal complexes by whole genome sequencing analysis of a limited number of clinical isolates from the Offin river. This implies that *M. ulcerans* strains are occasionally carried from a BU endemic site to a distant site and can subsequently establish as pathogen at the new site. Whole genome sequencing of larger strain collections of different geographic origin will help shedding more light on this finding.
- iv) We have demonstrated that exposure of populations to *M. ulcerans* generally starts at around 5 years of age. This may be associated with movement of children away from their homes and having contact with the periphery of the community, including water bodies or other sources potentially contaminated with *M. ulcerans*. Longitudinal follow up of children coupled with environmental surveillance and behavioral studies may shed more light on reservoirs and transmission routes of *M. ulcerans*.
- v) We found strong evidence that vaccination against HBV with the current DTPHH vaccine is effective. While strict adherence to the vaccination protocol is encouraged for newly-born infants, there is a need for a health campaign to test and vaccinate or treat the adult population. Detailed studies are required to assess the efficiency of the vaccination program periodically.

7.6 References

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