Bacteriological and immunological studies towards effective control of
***Mycobacterium ulcerans* disease (Buruli ulcer)**

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Dorothy Kyerewah Yeboah-Manu

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Herren Prof. Dr N. Weiss, Prof. Fred Binka, PD Dr. Claudia Daubenberger, Dr Thomas Bodmer und Prof. Dr Gerd Pluschke

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Prof. Dr Hans-Jakob Wirz

Dekan

Dedicated to the memory of my loving mother, Maame Animah with love. Thank you for all that you gave to me.

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Summary

After tuberculosis and leprosy, Buruli ulcer (BU) which is caused by *Mycobacterium ulcerans*, is the most common mycobacterial infection in immuno-competent humans. Since the 1980s BU has gained significant public health importance in the tropics especially in West Africa, including Ghana. The establishment of control measures is hampered as a result of the scarcity of understanding of many features of the disease. Priority areas for research defined by WHO include: understanding the mode of transmission, development of simpler methods for early diagnosis, development of effective antibiotic treatment, and the understanding of protective immune responses to support vaccine development.

The availability of *M. ulcerans* isolates from endemic areas is necessary for detailed transmission studies and the analysis of efficacy of antibiotics for the treatment of BU. However, cultivation of *M. ulcerans* from clinical specimens is burdensome; reported recovery rates are as low as 20%. We evaluated four different decontamination methods and one non-decontamination procedure in combination with four egg-based media for the primary isolation of *M. ulcerans* from tissue specimens excised from BU lesions. Oxalic acid decontamination and culture on LJ medium supplemented with glycerol was the most efficient procedure and achieved a recovery rate of 75.6%. The success of cultivation depended also on a good sampling procedure. The use of the optimised cultivation method has allowed the production of a large isolate collection.

For efficient case management and confirmation of epidemiological data, it is necessary to reconfirm clinical diagnosis by laboratory procedures. We used culture together with PCR and direct AFB staining to establish a system of reconfirming cases clinically diagnosed at the Amasaman Health Centre, Ghana. All three methods showed a comparable sensitivity and the laboratory analysis demonstrated a high accuracy of clinical judgment by an experienced clinician.

Current recommendation by the WHO requires that BU patients be treated with a combination of rifampicin and streptomycin for 8 weeks before surgical excision. In many infectious diseases, the development of drug resistance has a serious impact on patient management. It is therefore essential to monitor the drug susceptibility of *M. ulcerans*. We analysed the susceptibility of 28 isolates to rifampicin, streptomycin

isoniazid and ethambutol and identified both streptomycin and rifampicin resistant strains in Ghana. Findings from this study call for reconsideration of the current treatment guidelines.

Currently, micro-epidemiological studies aiming to reveal transmission chains cannot be done in BU. This is due to the low degree of genetic polymorphism in *M. ulcerans* revealed by routinely used genetic fingerprinting procedures. We used VNTR typing based on a newly identified polymorphic locus designated ST1 and the previously described locus MIRU 1 to detect genetic diversity in isolates from Ghana. Analysis revealed three different genotypes in isolates from Ghana, demonstrating for the first time genetic diversity among *M. ulcerans* isolates in an African country.

Ex vivo ELISpot analysis of IFN-γ secreting cells was carried out by stimulating PBMCs from BU patients with PPD, IPP and IRIV. Data from the study demonstrated for the first time that *M. ulcerans* infection-associated systemic reduction in IFN-γ responses is not confined to stimulation with live or dead mycobacteria and their products but extends to other antigens. We also showed that the immune suppression reversed after surgical treatment and that the suppression is not related to reduction in IL-12 secretions. This indicates that the observed systemic immunosuppression was not the consequence of a genetic defect in T cell function predisposing for BU but is rather related to the presence of *M. ulcerans* bacteria.

In a longitudinal study, we compared recovery of immediate effector function of Vγ2Vδ2 T cells in surgically treated BU patients to that of TB patients under chemotherapy. At the time of diagnosis, systemic production of IFN-γ after IPP stimulation was suppressed in both disease states but reverses after treatment. Restoration of Vγ2Vδ2 reactivity was slow such that an optimum response was not yet achieved by two months in both populations. Our result demonstrates that immunosuppression in BU may not be caused by the terpenoid toxin of *M. ulcerans* (mycolactone) alone.

Zusammenfassung

Nach Tuberkulose und Lepra ist Buruli Ulkus (BU), verursacht durch *Mycobacterium ulcerans,* die häufigste mycobakterielle Infektion bei immunkompetenten Menschen. In den letzten dreissig Jahren hat BU in den Tropen, insbesondere in West Afrika, einschliesslich Ghana, an Bedeutung gewonnen. Die effiziente Kontrolle diser Infektion ist durch fehlendes Verständnis vieler Aspekte der Krankheit erschwert. Die von der WHO aufgestellten Forschungsprioritäten beinhalten: Übertragungswege zu verstehen, einfachere Methoden für eine Frühdiagnostik und eine effektive Antibiotikabehandlung zu entwickeln und für eine Imfstoffentwicklung zu einem besseren Verständnis der schützenden Immunantworten zu kommen.

Die Etablierung einer Sammlung von *M. ulcerans* Isolaten aus endemischen Gebieten ist für detaillierte Transmissionstudien und die Analyse der Effektivität von Antibiotika gegen *M. ulcerans* erforderlich. Die Kultivation von *M. ulcerans* ist langwierig und beschrieben Ausbeuten liegen teilweise unter 20%. Für die Primär-Isolation von *M. ulcerans* aus Gewebeproben von BU Läsionen evaluierten wir vier Dekontaminations-Methoden und ein Nicht-Dekontaminations Verfahren mit vier Medien auf Ei-Basis. Oxalsäure-Dekontamination und Kultivation auf LJ Medium mit Zusatz von Glycerin war das effektivste Verfahren mit einer Aubeute von 75.6%. Der Erfolg der Kultivation hing jedoch auch von einer guten Probennahme ab. Die Verwendung eines optimierten Kultivationsprotokolls emöglichte uns den Aufbau einer grossen Probensammlung.

Für effizientes Patientenmanagement und die Bestätigung epidemiologischer Daten ist eine Rückbestätigung der klinischen Diagnose durch Labormethoden unerlässlich. Für die Etablierung einer Routine-Labordiagnostik der Fälle, die im Amasaman Health Center in Ghana klinisch diagnostiziert wurden, setzten wir die Kultivation zusammen mit einer PCR-Analyse und direkter AFB Färbung der Mycobakterien ein. Alle drei Methoden zeigten vergleichbare Sensitvität. Ferner erwies sich die durch einen erfahrenen Kliniker durchgeführte klinische Diagnose als sehr zuverlässig.

Derzeitige empfiehlt die WHO zur Behandlung von BU eine achtwöchige Antibiotikatherapie mit einer Kombination aus Rifampicin und Streptomycin mit eventuell nachfolgender chirurgischer Entfernung der Läsionen. Bei vielen Infektionskrankheiten hat Antibiotikaresistenz ernsthafte negative Konsequenzen für die

therapeutischen Optionen. Es ist somit unerlässlich, die potentielle Entwicklung von Antibiotikaresistenzen bei *M. ulcerans* zu überwachen. Wir analysierten 28 Isolate aus Ghana auf ihre Empfindlichkeit gegenüber Rifampicin, Streptomycin, Isoniazid und Ethambutol und identifizierten sowohl Streptomycin-, als auch Rifampicin- resistente Stämme. Die Resultate dieser Studie sprechen dafür, die derzeitigen Behandlungsrichtlinies zu überdenken.

Molekular-epidemiologische Studien zur Identfizierung möglicher Übertragungswege sind im Fall von BU wegen des geringen genetischen Polymorphismus des Erregers und der daraus resultierenden geringen Auflösungskraft der gängigen Genotypisierungsmethoden nicht möglich. Wir haben VNTR-Typisierungmethoden entwickelt und eingesetzt, um die genetische Diversität der ghanaischen Isolaten zu untersuchen und haben bei einem neu identifizierten polymorphen Genlocus (ST1) und bei einem bereits beschriebenen Locus (MIRU1) unterschiedliche Allele gefunden. Bei den Stämmen aus Ghana wurden drei verschiedene Genotypen gefunden. Damit wurde zum ersten Male genetische Diversität bei *M. ulcerans* Isolaten innerhalb eines afrikanischen Landes nachgewiesen.

Die Frequenz von IFN-γ sekretierenden T Zellen wurde durch *ex vivo* ELISpot Analysen mit PBMC von BU Patienten durchgeführt. Die PBMC wurden mit PPD, IPP und Virosmen stimuliert. Die Resultate zeigten erstmals, dass die systemische Reduktion der IFN-γ Antwort, die mit der *M. ulcerans* Infektion assoziiert ist, nicht nur mit lebenden oder abgetöteten Mycobakterien und ihren Zellprodukten in Verbindung steht, sondern auch andere Antigene betrifft. Weiterhin zeigten wir, dass die Immunsuppression nach chirurgischer Behandlung wieder aufgehoben wurde und dass sie nicht mit einer Reduktion der IL-12 Sekretion assoziiert ist. Dies deutet darauf hin, dass die systemische Immmunsuppression keine Konsequenz eines genetischen Defekts der T-Zell Funktion ist, sondern in Zusammenhang mit der Anwesenheit von *M. ulcerans* Bakterien steht.

In einer Longitudinal-Studie verglichen wir die Wiederherstellung der Effektorfunktion von Vγ2Vδ2 T Zellen in chirugisch behandelten BU Patienten und TB Patienten unter Chemotherapie. Zum Zeitpunkt der Diagnose war bei beiden Krankheiten die systemische Produktion von IFN-γ nach IPP Stimulation unterdrückt. Im Verlauf der Therapie normalisierte sich diese Reaktivität wieder, jedoch sehr langsam, so dass in

beiden Patienten-Populationen auch nach zwei Monaten die Normalwerte noch nicht völlig erreicht waren. Unsere Resultate weisen darauf hin, dass die bei BU zu beobachtende Immunsuppression nicht allein von dem von *M. ulcerans* produzierten immunsuppressiven Toxin, Mycolacton, verursacht ist.

Abbreviations

AFB	Acid Fast Bacilli
BCG	Bacille Calmette & Guerin
BU	Buruli Ulcer
CD4, 8	Cluster of Differentiation 4, 8
CTL	Cytotoxic T Lymphocyte
DNA	Deoxyribonucleic
ELISPOT	Enzyme Linked Immunospot
HIV	Human Immunodeficiency Virus
IFN-γ	Interferon gamma
IL-4, 5	Interleukin 4, 5
IPP	isopentenyl-pyrophosphate
IRIV	Immunopotentiating Reconstituted Influenza Virosome
IS	Insertion Sequence
KDa	Kilodalton
LPS	Lipopolysaccharide
MHC	Major Histocompatibility Complex
MIRU	Mycobacterial Interspersed Repetitive Unit
MLST	Multilocus Sequence Typing
mRNA	Messenger Ribonucleic Acid
PCR	Polymerase Chain Reaction
PPD	Purified Protein Derivative
RFLP	Restriction Fragment Length Polymorphism
rRNA	Ribosomal Ribonucleic Acid
Th	Helper T cell
TNF-α	Tumour Necrosis Factor alpha
VNTR	Variable Number Tandem Repeats
WHO	World Health Organisation
ZN	Ziehl-Neelsen

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Chapter 1: Introduction

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1.1 *Historical facts and epidemiology*

Buruli ulcer (BU) affects mainly the skin and it is caused by the environmental pathogen *Mycobacterium ulcerans*. It is the third most common mycobacterial disease, after tuberculosis and leprosy that occur in immuno-competent individuals (1). Historically, the disease was first discovered in 1897 by Sir Robert Cook, a British physician working in Uganda and later by Kleinschmidt in northeast Congo during the 1920s (1,2), but the first definitive description of cases and the etiologic agent was published in 1948 by Professor Peter MacCallum and his colleagues in Australia (3). Between the 1960s through the 1970s new endemic foci were identified in several tropical countries including Uganda, the Democratic Republic of the Congo, and Papua New Guinea. Some of the reports were by Oye and Ballion, P. G. Janssens and Meyers *et al* who made the claim that, traumatic lesions might be involved in the transmission of *M. ulcerans*. By 1974, more than four hundred cases had been described in Zaire (2,4). In the 1960s, many patients in refugee camps in an area near the Nile River in Uganda, called Buruli, had ulcers which were caused by *M. ulcerans* (4). The disease has since become to be known as BU.

The global burden of BU is not clear, because of lack of efficient reporting system in most endemic countries (5). Nevertheless, it is now known that BU is endemic in at least thirty-two tropical countries of Africa, Western Pacific, Asia, the Indian Ocean and Latin America (figure 1) (5). The worst affected region is within countries lying along the Gulf of Guinea in West Africa, where BU has replaced leprosy as the second most common mycobacterial disease, after tuberculosis. Cases have been detected in all the countries with Ghana, Ivory-Coast, Togo, Cameroon and Benin recording the highest number of cases (6-11). The prevalence of BU in some of the villages in this area is higher than that of tuberculosis and can affect more than 20% of the inhabitants. In Ivory-Coast, more than 15,000 (12) cases were reported between 1978 and 1999 while nearly 2,000 cases were reported within a 4-year period in one hospital in Benin (13). Very few cases have been reported in non-endemic areas in Europe and North America. Although, BU affects all age groups in both sexes, it has been reported to affect mainly children 15 years of age

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and below in Africa (12). Most of the lesions are located on the legs, feet, arms and hands.
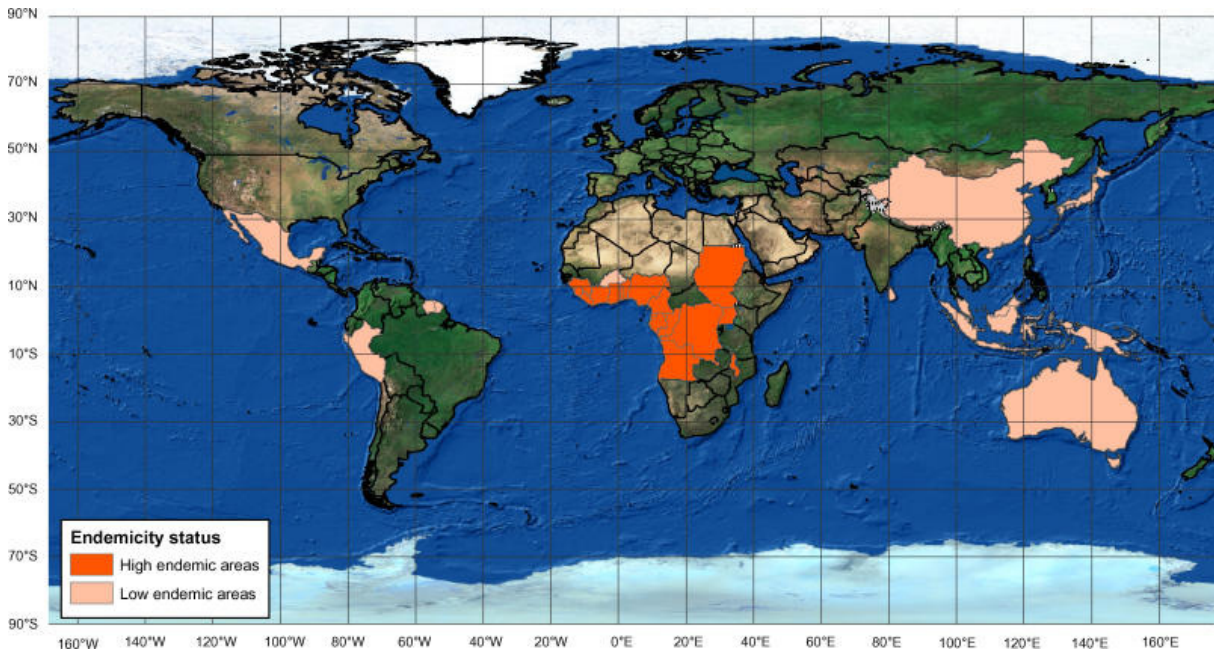
Figure 1: Countries reporting Buruli ulcer. (Source: Johnson *et al*., *PLoS Med*, 2005)

At an international meeting in July 1998 in Côte d'Ivoire, the Yamoussoukro Declaration on Buruli Ulcer was made, expressing the concern that little is known about this disease, and called on the international community to support control and research efforts (14).

1.2 *Buruli ulcer in Ghana*

The first documented case of Buruli ulcer in Ghana was a patient from Amasaman at the Korle-Bu Teaching Hospital in 1971 (15). The presence of additional cases along the Densu River in the area was considered a possibility. In 1989, van der Werf *et al*. published a series of 96 cases in the Afram valley at Agogo, in the Ashanti Akim North District in the Ashanti Region (16). Amofa *et al* also in 1993 described a major endemic focus in the Amansie West district in the same region (17). Since then, there have been a number of reports of scattered endemic foci in various parts of the country, particularly in the Ashanti Region.

Currently the disease represents a significant proportion of all disease cases registered in some endemic district health facilities. BU is spreading very quickly in Ghana. It was previously believed that the disease exists only in areas around swampy vegetation and tropical rain forest in the country. A national survey that was conducted in 1999 however established that the disease could be found in all ten administrative regions of the country. During the national survey, over 6000 BU cases were identified across the country (18). The overall prevalence of BU in Ghana was estimated to be 20.7 per 100,000 populations making it the second most prevalent mycobacterial disease after tuberculosis.

Efforts been made to control the disease in Ghana include offering of free treatment to BU patients and training of health workers to improve diagnosis. However diagnosis is usually delayed as a result of socio-cultural beliefs and distance to treatment centres (19). Diagnosis of BU is usually made on the basis of clinical case definition without laboratory confirmation.

1.3 *Pathogenesis and clinical Presentation*

1.3.1 *Causative organism*
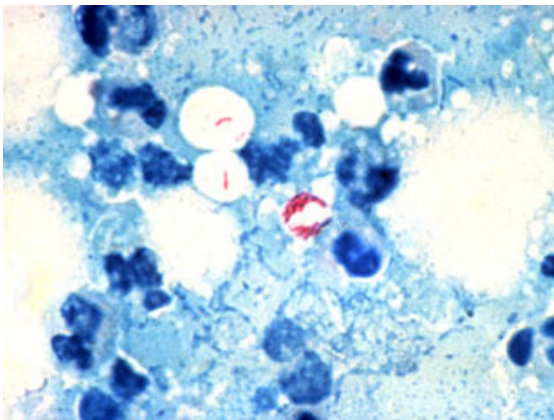
Figure 2: Ziehl Neelsen stained smear of *M. ulcerans* from Buruli ulcer lesion observed under oil immersion (x1000). Note the acid fast bacilli in clumps.

Taxonomically, *M. ulcerans* is a member of the phylum actinobacteria, in the order actinomycetales, suborder corynebacteriaceae and the genus mycobacterium. Like other

mycobacterium species *M. ulcerans* has a high G+C content (65%) DNA and an unusual cell wall with a lipid-rich layer beyond the peptodiglycan layer. It has a long generation time, which has been estimated to be around 20 hours; it is therefore described as slow-growing mycobacterium and the optimum growth temperature is between 31 and 33°C at pH of 5.4-7.4 (20). The organism usually grows under aerobic conditions but it grow better under micro-aerophillic conditions in liquid cultures (21). *M. ulcerans* belongs to a group of mycobacteria commonly referred to as opportunistic or occasional pathogens (22) and it is the only that does not have an extracellular infection and the pathology is mediated by toxin secretion (23).

1.3.2 *Clinical Presentation*

M. ulcerans may enter the skin by traumatic inoculation and that some biting insects may be involved (24). After successful entry, the organism confines itself to the subcutaneous tissues and the overlaying skin, where it multiplies. The incubation period is extraordinarily variable, and has been estimated to range from 2 weeks to 3 years, with an average of 2 to 3 months. The disease begins typically as a painless nodule under the skin at the site of the trauma. In some geographical areas the first manifestation is a papule rather than the firm, painless nodule. After a few weeks, the nodule gradually enlarges and erodes through the skin surface, leaving a well-demarcated ulcer with a necrotic slough in the base and widely undermined edges (20,25). This form of disease presentation is termed as localised. Analysis of a large number of cases by Meyers and colleagues suggested that in some cases, infections spread rapidly and bypass the localized nodular-ulcerative stage. This disseminated form results in oedematous plaques that, if untreated, lead to ragged ulcers (figure 3) (26). Like the other steps in pathogenesis, the mode of spread is not apparent. *M. ulcerans* may spread to distant foci through the lymphatic and haematogenous pathway. Severe osteomyelitis is well-known and this may lead to amputation and other disabilities (27).

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<div style="text-align:center">Nodule (a)</div>

<div style="text-align:center">Papule (b)</div>

<div style="text-align:center">Oedema (c)</div>

<div style="text-align:center">Ulcer (d)</div>

<div style="text-align:center">Plaque</div>

<div style="text-align:center">Plaque (e)</div>

Figure 3: Clinical forms of Buruli ulcer (Source: Portaels F, Johnson P, Meyer WM editors, 2001. WHO

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1.3.3 *M. ulcerans Toxin and Histopathology*

The pathological manifestation of *M. ulcerans* infection is thought to be mediated by toxin(s) production. A polyketide derived macrolide toxin, named mycolactone, with a potent tissue necrotizing activity, is deemed to be the major effector molecule. The genes that encode for the synthetic machinery of mycolactone are located on a circular giant plasmid with a molecular size of 174kb named pMUM001. More than half of the plasmid consist of genes that encode the enzymes required for the synthesis of mycolactone (28). This toxin produced a necrotising effect in guinea pigs, which was histologically similar to that seen in human patients (23). In addition it has *in vitro* activity against a number of immune cells, including those important for the control of mycobacterial infection(29) (30). Hence it has been postulated that secretion of the toxin by the invading microbe causes extensive necrotic damage to the host tissues particularly the dermis, panniculus, and fascia and the suppression of immune response. Histological analysis of early lesions reveals extensive cutaneous tissue necrosis with large numbers of extracellular bacilli in clumps and scanty inflammatory cells, which may be the result of the immunosuppressive action of the toxin. Analysis of tissue shows central necrosis of subcutaneous fat surrounded by granulation tissue with giant cells which lacks the typical caseation or tubercles seen in tuberculosis (31).

1.4 *Diagnosis*

In endemic areas, most cases are diagnosed on clinical evidence (32) and an experienced clinician can often make accurate clinical diagnoses of BU. Nevertheless, a number of diseases can be confused with Buruli ulcer disease in each of its clinical stages; thus, laboratory tests can help to confirm the diagnosis. The World Health Organisation (WHO) recommendations require positive results with at least two diagnostic test systems to reconfirm clinically diagnosed BU disease (20) These diagnostic systems are: 1) detection of acid-fast bacilli (AFB) in a smear stained by the Ziehl-Neelsen technique 2) positive culture of *M. ulcerans* 3) positive polymerase chain reaction (PCR) for the detection of *M. ulcerans* DNA and 4) histopathological analysis of excisional biopsy specimen. These methods vary in sensitivity, specificity, speed and cost. Specimen for

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the first three diagnostic methods can be either surgically excised material or smears from ulcerative lesion.

1.4.1 *Direct Smear Examination*

The direct observation under the microscope for AFB after staining with carbol fucshin has been demonstrated to have a low sensitivity. It requires a concentration of 10^4 bacilli per millilitre suspension to give a positive smear test. In addition this method lacks specificity and as a number of other mycobacterial species can cause skin lesions, the detection of AFB alone does not establish *M. ulcerans* as the cause of the illness (33). However it is good for its rapidity and does not require sophisticated equipment, making it suitable for endemic regions in Africa where resources are scarce.

1.4.2 *Culturing of M. ulcerans*

Isolation of the causative agent is the final proof laboratory diagnostic method, which in addition offers the advantage of obtaining isolates that can be used for in-depth studies aiming to understand open questions, like the mode of transmission and drug susceptibility. Culturing of *M. ulcerans* is however difficult and slow and a number of studies have indicated that the sensitivity of culture is very low, coming up to only 35% (24). A major contributing factor is that samples sent to microbiology laboratories for the isolation of *M. ulcerans* are usually contaminated with faster growing microorganisms; hence there is need for selective decontamination to remove these contaminants prior to inoculation of the growth medium. At the same time *M. ulcerans* itself is to some extent susceptible to these harsh treatments, and even after decontamination, *M. ulcerans* cultures are frequently contaminated by faster growing bacteria and fungi (34). It takes 6 weeks to 5 months for *M. ulcerans* cultures to be positive on solid medium.

1.4.3 *Detection of M. ulcerans DNA by PCR*

PCR assay is good for its rapidity and results can be obtained the same day. It is also sensitive and in theory requires only a very few cells to give a positive result. However it is expensive and therefore may not be used routinely in endemic countries in Africa. PCR

requires expensive equipments and elaborate infrastructures to prevent false results. On the other hand it will be useful for central or research institutes in endemic countries in Africa to establish PCR assays. This can be used for rapid differential diagnosis of cases that prove to be difficult to diagnose on clinical grounds alone. Several PCR-based assays detecting different genomic targets have been developed. *M. ulcerans* specific DNA targets include the 16s rRNA gene (35), the 65-kDa heat shock protein gene (36) and the insertion DNA sequence *IS2404* (37). Presently, the recommended target for the diagnosis of BU is the insertion sequence *IS2404*, which is present in about 250 copies in the *M. ulcerans* genome (20), therefore improving the sensitivity of the assay.

1.4.4 *Histopathological Analysis*

"Characteristic" histopathologic changes are considered one of the confirmatory laboratory methods for Buruli ulcer disease; however, the features are non-specific and change as the lesion evolves from a nodule to an ulcer (20,38) . Several authors have described the histopathologic changes of Buruli ulcer disease as different patients progress through the different clinical stages (39). Necrosis of subcutaneous tissues and dermal collagen accompanied by minimal inflammation and AFB are considered the most reliable histopathologic features for the diagnosis of Buruli ulcer disease. Furthermore, the selection of specimen is very critical and quality of specimen varies for the different stages. Whilst biopsy specimen that include necrotic subcutaneous tissue and the undermined edge of ulcerated lesions is good for the diagnosis of ulcers, specimens from skin and subcutaneous tissue are suitable for the diagnosis of non-ulcerated lesions (20).

1.5 *Treatment*

1.5.1 *Surgery*

Presently, the standard method of treating patients with BU is the surgical excision of infected tissue followed by skin grafting. This procedure apart from being invasive and very expensive, costing around $780 per treatment as reported in Ghana (40), has varying degree of success which depends on a number of factors including the experience of the

clinician. This is because there are no strict guidelines as to the extent of excision of lesions, thus the surgeon has to make a very good judgement between healthy and infected tissue. Recurrence rates ranging from 5-47% have been report in different studies. More importantly not all patients in rural endemic areas have access to health institutions that offer surgical services (41). As a result patients first try to solve their health problem within their communities by self- treatment or visiting local health providers, who treat them with herbal preparations (19). Such individuals may later present with very extensive lesions that requires a long post-operative care and restorative physiotherapy, which increases the cost of treatment. Some of them even end up with amputation and varying degrees of disability. A study conducted by Martson et al (8) reported that almost 30% of persons with healed lesions had chronic functional disabilities, including loss of eye and limbs.

1.5.2 *Drug Treatment*

Treatment with antimycobacterial agents has been considered disappointing especially at the advanced stage of the disease. Reports of human trials have been very discouraging; while clofazimine (42) and cotrimoxazole (43) was found to be ineffective, a combination of dapsone and rifampin was found to have limited efficacy for ulcers (44). On the contrary, *M. ulcerans* is susceptible to rifampicin, some aminoglycosides, macrolides and quinolones *in vitro* (45,46). The failure of these drugs to effectively inhibit *M. ulcerans* growth in humans has been hypothesised to be due to the inability of the drugs to penetrate the necrotic lesions and or the ongoing necrotic activity of the persistent toxin (47). Findings from mice model studies suggest that a combination of rifampicin with either streptomycin or amikacin have strong bactericidal activity (48,49). Treatment of mouse footpad with a combination of rifampicin and amikacin for 12 weeks decreased progressively, the number of viable counts reduced and treated mice did not relapse after 17 weeks. A clinical trial conducted by Etuaful and colleagues reported that a minimum of 4 weeks treatment with rifampin and streptomycin combined, inhibits the growth of *M. ulcerans* in pre-ulcerative lesions, as confirmed by at least one of the following; direct AFB staining, PCR and culture (41). However, they could not confirm that this combination could replace surgery and suggested it to be used as an adjunct to

surgery. Based on this successful report and other observations, a recent WHO guidelines have been developed that required an eight weeks course of treatment with rifampicin and streptomycin (47). First clinical experience indicates that this treatment leads to healing without subsequent surgery in about 50% of cases.

1.6 *Transmission*

Currently the exact mode of transmission of *M. ulcerans* is still not clarified. However BU affects people in scattered foci and endemic foci are usually associated with wetlands with hot and humid climates (50). In Uganda, hundreds of cases occurred among refugee populations camping close to the Nile River and the incidence of cases subsided when the refugees were moved out of the area (4). Upsurge of cases have also being reported in areas that the environment has been disturbed; examples include, flooding, damming of rivers, introduction of rice swamp fields and irrigation systems (1). In Nigeria, increased incidence occurred when a small stream was dammed to make an artificial lake (51). So also in Philip Island, the formation of a small swamp led to increased cases, which declined when the irrigation was improved (52).

M. ulcerans is an environmental mycobacterium and the involvement of aquatic species in endemic areas as either environmental reservoirs and/or vectors for transmission seems likely. *M. ulcerans* has been detected in aquatic bugs (53), mollusc (54), fish (55)and biofilm on vegetation (56). These have been determined mainly using PCR based on the detection of *IS2404* DNA sequence (57), which is now known not to be very specific for *M. ulcerans* (58). Only two pure cultures of *M. ulcerans* have been obtained from environmental sources so far (47). In a laboratory experimental model, an aquatic insect was able to infect the tail of laboratory mice by biting (59). Thus infected insects may accumulate *M. ulcerans* in their salivary glands and pass on to man through biting. This hypothesis is strengthened by the detection of *M. ulcerans* in the salivary gland of an aquatic insect, *Naucoris* spp (60). The extent of man to man transmission is not proven but evidence of developing BU after a human bite is known; if man to man transmission is a natural occurrence needs to be established (61).

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There is no available instituted measure for prevention of BU due to the inadequate knowledge on transmission and the lack of an effective vaccine against BU. A study conducted in Ivory-Coast however revealed that covering of the exposed body sites by wearing of long trousers in endemic communities is protective (8). Also the *M. bovis* BCG vaccines seems to offer some degree of protection, especially against systemic infections (62).

1.7 Genetic Diversity in M. ulcerans Isolates

M. ulcerans seems to be one of the most extreme examples of bacterial homogeneity. This low degree of genetic polymorphism in the organism's DNA sequence has restricted the expected discriminating power of a number of markers and methods that are routinely used for genetic fingerprinting of other bacterial species. Global analyses of isolates have resulted in the sub-grouping of *M. ulcerans* according to their geographical origin, at continental level. African isolates are usually grouped together as one genotype.   Some of the markers and procedures employed include:

Restriction Fragment Length Polymorphism (RFLP): This technique uses variability in the nucleotide sequence and frequency of certain DNA sequences in bacterial chromosomes. These differences are revealed by using rare cutting restriction enzymes (RE) that cut DNA at specific recognition sites (63). The resulting genomic DNA fragments are subsequently analysed by gel electrophoresis. Differences in fragment sizes and numbers occur due to base substitutions, additions, deletions or sequence rearrangements within RE recognition sequences. Usually the analysed DNA sequence is used as a probe in a southern hybridization procedure to reduce the number of fragments and also determine the polymorphism of the marker in the genome. RFLP is most suited for studies at the intra-specifies level or among closely related taxa. The pioneering work of Jackson *et al* used an RFLP-based method with the plasmid pTBN12 as a probe for typing isolates from Africa and Australia (64). This study identified 11 RFLPs distinguishing the African strains from the Australian isolates. Furthermore this study was able to differentiate the isolates from Victoria into three subgroups and was able to distinguish isolates from Zaire and Benin. A PCR based genotyping assay, using the

IS2404 and *IS2606* insertion sequences, developed by Stinear *et al* recognised nine distinct profiles that also differentiated *M. ulcerans* strains according to the geographical origin (65) . Chemlal *et al* used only the insertion sequence *IS2404* as a probe in an RFLP based fingerprinting assay and divided the isolates into 6 types: African, Australian, Mexican, South American, Asian and South-east Asian types (66).

Sequencing of the 16SrRNA gene: This method employed the slow evolution of this gene over time to identify the relatedness of different organisms. This method showed that *M. ulcerans* is very closely related to *M. marinum* and just differ in a single base pair at position 1248. Portaels *et al* sequenced the 3'end of the 16SrRNA gene of 17 different isolates and were able to differentiate them into three types: the African, Australian and American type (67).

PCR restriction profile analysis: This method employed a triple restriction of the amplified product of the 3' end region of the 16SrRNA gene and gave three different profiles according to the geographic origin. It could not differentiate *M. marinum* from *M. ulcerans* isolates originating from Southeast Asia and South America (68).

Amplified Fragment Length Polymorphism: In this technique whole genomic DNA is restricted by two enzymes, addition of double stranded oligo-nucleotide adaptors to the ends of the fragments followed by selective amplification of the modified fragments with primers specific to the adaptors. Huys *et al* evaluated this procedure for inter and intra-specific differentiation of *M. bovis*, *M. tuberculosis* and *M. ulcerans* and reported that AFLP it is good for inter-species differentiation but not intra-species differentiation; clearly differentiate *M. ulcerans* from the *M. tuberculosis* complex and classified the 12 *M. ulcerans* isolates into two continental types (69).

Multi-locus sequencing typing (MLST): This method employs the variability in the base sequence in a set of housekeeping genes. MLST was used to type *M. ulcerans* isolates by sequencing eight genes and this resulted in the identification of six genotypes according to their geographical origin; that is Africa, Australia, Mexico, South America, Asia and South-east Asia (70).

Analysis of the polymorphism in the *IS2404* and the frequently GC rich region in mycobacterium species by amplification of the genomic regions between these genomic markers yielded ten different band patterns. All the African isolates produced the same band pattern, and isolates from Papua New-Guinea produced two different band patterns (71).

MIRU-VNTR: This is a PCR-based method that determines genomic polymorphism based on differences in the copy number of repetitive units of 46-100 bp. It is faster than most of the methods described above and more importantly has been found to be reproducible between different laboratories. In addition, it has been shown to have a high discriminating power in the members of *M. tuberculosis* complex. Two independent studies that employed this method also could not differentiate African strains from both west and central Africa. In the first study 39 different isolates analysed with four polymohphic MIRUs identified 7 genotypes worldwide (72). In the second study nine VNTRs sequences obtained from the genome sequence of the related species *M. marinum* also gave similar findings (73).

These findings are indicative for a clonal population structure of *M. ulcerans*. Thus there is no method that can be used currently for micro-epidemiological investigations like tracing the transmission pathways of this pathogen.

1.8 *Immune responses to M. ulcerans*

Immune response to *M. ulcerans* infection is characterised by low inflammatory response and lack of granuloma formation in early lesions. Gooding *et al* found that infection with *M. ulcerans* is associated with T cell anergy as PBMCs from individuals with BU exhibited reduced lymphoproliferation and production of IFNγ follwing stimulation with live *M. ulcerans* or *M. bovis* (74). A follow-up study by the same group analysed the Th1 and Th2 response of subjects with active and healed Buruli ulcer and household contacts. Following stimulation with *M. ulcerans* or *Mycobacterium bovis* BCG it was shown that Th1 anergy persist even upon healing (75). In Guyana, Prevot *et al* demonstrated that in

active BU, in vitro production of IL-10 in PBMCs after stimulation with *M. ulcerans* was significantly increased compared to tuberculin positive controls and the reverse was true for IFNγ (76). This systemic finding paralleled in vivo mRNA levels of these cytokines. In resected tissues, the level of IFN-γ mRNA was higher, and IL-10 mRNA was lower in nodular lesions than ulcerative lesions after stimulation with heat-killed *M. ulcerans*. Westernbrink *et al* in a whole blood assay demonstrated a systemic reduction in IFNγ production in response to PPD antigens in patients with early lesions compared to those with later stage lesions (77). These findings from different studies suggest that Th1 response is down regulated early in BU disease partly by IL-10 secretion or by immune modulation activities of *M. ulcerans*.

Mycolactone toxin described in association with the pathology of BU has profound effects on immune cells in vitro(23). An investigation by Pahlevan *et al* on the activity of partially purified *M. ulcerans* toxin on different human immune-competent cells found that the toxin produced greater than 95% inhibition of LPS-induced release of TNF-α and IL-10 from human monocytes. It also causes loss of adherence of monocytes without cell death (30). Addition of mycolactone to macrophages and fibroblast affected the organisation of the cytoskeleton that leads to growth arrest and apoptosis (78). Furthermore, IL-2 production from activated T lymphocyte was blocked by the toxin (30,78,79) This was after Pimsler *et al* had already demonstrated that *M. ulcerans* culture filtrate causes suppression of T cell response to *Concanavalin A* and inhibited phagocytosis of latex beads by macrophages (80). In mouse model studies, it appears upon infection, some *M. ulcerans* is initially internalised and transported to lymph nodes for initiation of adaptive immune response by professional APCs, however, expression of mycolactone inhibits further phagocytosis, enhance phagocyte necrosis and apoptosis and inhibits expression of pro-inflammatory cytokines such as TNF-α (29,81).

In spite of this local immune suppression, there is evidence that sensitivity to *M. ulcerans* antigen develops during infection (82). BU patients have been shown to response to a crude antigen preparation, Burulin, from *M. ulcerans*. This positive skin response was found mainly in patients at the late stages of disease or when healed (83). This suggests

that a delayed type hypersensitivity response may be important in healing. In addition, spontaneous healing has been observed confirming the pivotal role of the immune system in the control of BU (22). The histological appearance of later lesions is also found to be similar to that of other mycobacterial diseases with small numbers of extracellular organisms and the formation of granulomas (84,85). The importance of Th1 in protective immune response against MU is confirmed recently by a report of extensive multifocal lesions associated with HIV infection (86).

BU patients do mount humoral immune responses to *M. ulcerans*. Work carried out by Dobos et al in Ivory–Coast, demonstrated that BU patients produce antibodies against *M. ulcerans* independent of the disease stage (87). This was evident by 43 out of 61 BU patients testing positive to *M. ulcerans* culture filtrate. This was confirmed in Australian patients where 9 out of 11 patients had antibodies to *M. ulcerans* (74).

1.9 *Immune Response to intracellular mycobacteria*

Infection with *M. tuberculosis* (Mtb) is mainly by inhaling bacilli containing droplet nuclei. Inside the host they are engulfed by alveolar macrophages/dendritic cells (88). These cells may kill, process and present mycobacterial antigen at the regional lymph node to initiate adaptive immune response. Only 5-10% of individuals who are infected with the bacilli progress to disease and the remaining 90% even though may not progress to disease are unable to completely eradicate the pathogen (89). This persistent infection has been termed latent Mtb infection and reflects successful immune-mediated containment of Mtb. Control of infection with mycobacteria relies heavily on the cellular immune system; that is the interaction of lymphocytes and *M. tuberculosis*-infected macrophages and dendritic cells to form granulomas (90). In addition to walling of the infected site, granulomas provide a microenvironment to facilitate interactions between the infected macrophages and other participating immune cells (90). In fact, granuloma formation is considered as hallmark of protective immuno-pathological response of the host following infection with Mtb. A range of experiments in animal models and humans support crucial roles of CD4+, CD8+ and γδ T cells in immune protection (90-92).

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1.9.1 *Role of the different T cell subsets in immune protection against intracellular mycobacteria*

CD4$^+$ T cells play a central role in immune control of *M. tuberculosis* infection. Mtb-derived peptide antigens degraded in the phagolysosomal compartment of infected macrophages are presented together with MHC II to CD4$^+$ T cells, resulting in their activation (93). The importance of this T cell subset is convincingly demonstrated by the loss of CD4 T cells in HIV infection and the corresponding susceptibility to TB. These observations have been confirmed in mice model studies using adoptive transfer and knock out models in CD4-/- and MHC II-/- (94). The main function of the activated CD4$^+$ T cells is the release of cytokines, like INF-γ and TNF-α, which limit intracellular Mtb growth by the up-regulation of microbicidal mechanisms in macrophages (95). The critical role of these cytokines in mycobacterial infections have been revealed by studies in humans subjects with either mutations in the INF-γ receptor (96,97) or receiving anti-TNF-α therapy with increased vulnerability to mycobacterial infections (98). CD4+ T cells may also contribute to the control of mycobacterial infections in non IFNγ dependent mechanisms (99), possibly by interactions such as CD40-CD40L and OX40-OX40L (91). Finally, antigen specific CD4+ T cells may be involved in lysis of infected macrophages through the Fas/Fas-ligand interactions and exocytosis of cytolytic granules (91).

CD8+ T cells are present in mycobacterial granulomas, where they have access to and are poised to prevent dissemination of infected cells (100). There are two subsets of CD8+ T cells that participate in immune response against mycobacterial infection. One subset is restricted by MHC class I molecules and recognise bound peptide antigens. The other subset is restricted by CD1 molecules, which have been shown to present mycobacterially-derived lipids, glycolipids and lipopeptides (100,101). Functionally, these T cell subsets have been found to lyse infected macrophages and dendritic cells, reducing levels of intracellular bacterial load (102). Lysis of infected cells by both subsets is mediated by the peforin/ granzyme pathway and Fas/FasL interactions (103).

γδ T cells play an important role in host response to TB, especially at the early phase. Vγ9Vδ2+ T cells are activated by *Mycobacterium tuberculosis* and recognise

mycobacterial nonpeptide phosphoantigens (92). Mycobacterial responsive Vγ9Vδ2 T cells are usually found in disease lesions. Vδ1 T cells are the predominant γδ T cells in the cerebro-spinal fluid of normal individuals, however, Vγ9Vδ2 T cells become the major subset in the CSF of tuberculosis meningitis patients (104). Vγ9Vδ2 T cells from children with tuberculosis have reduced IFN-γ production and granulysin expression, which reversed after successful chemotherapy (105). Functionally, mature Vγ9Vδ2 T cells display a potent natural killer (NK)-like cytotoxic activity (106) and produce secreted cytokines that may be important for cellular traffic and granuloma formation (104). Nothing has been reported to date on the role of these cells in immunity to *M. ulcerans*.

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Reference List

1. Asiedu K, Scherpbier R & Raviglione M. Buruli ulcer, Mycobacterium ulcerans infection. 2000. WHO.

2. **Meyers WM**. 1994. Tropical dermatology, p. 291-377. Springler-Verlag, Heidelberg.

3. **MacCallum P, T. J. B. G. &. S. H.** 1948. A new mycobacterial infection in man. J.Pathol Bacteriol 93-122.

4. **Clancey, J., R. Dodge, and H. F. & Lunn**. 1962. Study of a mycobacterium causing skin ulceration in Uganda. Ann.Soc.Belg.Med.Trop. **42**:585-590.

5. **Johnson, P. D., T. Stinear, P. L. Small, G. Pluschke, R. W. Merritt, F. Portaels, K. Huygen, J. A. Hayman, and K. Asiedu**. 2005. Buruli ulcer (M. ulcerans infection): new insights, new hope for disease control. PLoS.Med. **2**:e108.

6. **Aguiar, J. and C. Stenou**. 1997. [Buruli ulcers in rural areas of Benin: management of 635 cases]. Med.Trop.(Mars.) **57**:83-90.

7. **Fukunishi, Y.** 1999. [Present status of Buruli ulcer in Ghana, West Africa]. Nihon Hansenbyo.Gakkai Zasshi **68**:175-184.

8. **Marston, B. J., M. O. Diallo, C. R. Horsburgh, Jr., I. Diomande, M. Z. Saki, J. M. Kanga, G. Patrice, H. B. Lipman, S. M. Ostroff, and R. C. Good**. 1995. Emergence of Buruli ulcer disease in the Daloa region of Cote d'Ivoire. Am.J.Trop.Med.Hyg. **52**:219-224.

9. **Meyers, W. M., N. Tignokpa, G. B. Priuli, and F. Portaels**. 1996. Mycobacterium ulcerans infection (Buruli ulcer): first reported patients in Togo. Br.J.Dermatol. **134**:1116-1121.

10. **Noeske, J., C. Kuaban, S. Rondini, P. Sorlin, L. Ciaffi, J. Mbuagbaw, F. Portaels, and G. Pluschke**. 2004. Buruli ulcer disease in Cameroon rediscovered. Am.J.Trop.Med.Hyg. **70**:520-526.

11. **Oluwasanmi, J. O., T. F. Solankee, E. O. Olurin, S. O. Itayemi, G. O. Alabi, and A. O. Lucas**. 1976. Mycobacterium ulcerans (Buruli) skin ulceration in Nigeria. Am.J.Trop.Med.Hyg. **25**:122-128.

12. **Kanga, J. M. and E. D. Kacou**. 2001. [Epidemiologicl aspects of Buruli ulcer in Cote d'Ivoire: results of a national survey]. Bull.Soc.Pathol.Exot. **94**:46-51.

13. **Debacker, M., J. Aguiar, C. Steunou, C. Zinsou, W. M. Meyers, A. Guedenon, J. T. Scott, M. Dramaix, and F. Portaels**. 2004. Mycobacterium

ulcerans disease (Buruli ulcer) in rural hospital, Southern Benin, 1997-2001. Emerg.Infect.Dis. **10**:1391-1398.

14. **van der Graaf, W. T., R. W. Scherpbier, and T. S. van der Werf**. 1999. [Buruli ulcer (Mycobacterium ulcerans infection); report from the International Congress in Yamoussoukro, Ivory Coast]. Ned.Tijdschr.Geneeskd. **143**:312-316.

15. **Bayley, A. C.** 1971. Buruli ulcer in Ghana. Br.Med.J. **2**:401-402.

16. **van der Werf, T. S., W. T. van der Graaf, D. G. Groothuis, and A. J. Knell**. 1989. Mycobacterium ulcerans infection in Ashanti region, Ghana. Trans.R.Soc.Trop.Med.Hyg. **83**:410-413.

17. **Amofah, G. K., C. Sagoe-Moses, C. Adjei-Acquah, and E. H. Frimpong**. 1993. Epidemiology of Buruli ulcer in Amansie West district, Ghana. Trans.R.Soc.Trop.Med.Hyg. **87**:644-645.

18. **Amofah, G., F. Bonsu, C. Tetteh, J. Okrah, K. Asamoa, K. Asiedu, and J. Addy**. 2002. Buruli ulcer in Ghana: results of a national case search. Emerg.Infect.Dis. **8**:167-170.

19. **Stienstra, Y., W. T. van der Graaf, K. Asamoa, and T. S. van der Werf**. 2002. Beliefs and attitudes toward Buruli ulcer in Ghana. Am.J.Trop.Med.Hyg. **67**:207-213.

20. WHO. Buruli ulcer: Diagnosis of Mycobacterium ulcerans disease. Portaels, F., Johnson, P., and Meyers, W. M. 2001.
Ref Type: Serial (Book,Monograph)

21. **Palomino, J. C., A. M. Obiang, L. Realini, W. M. Meyers, and F. Portaels**. 1998. Effect of oxygen on growth of Mycobacterium ulcerans in the BACTEC system. J.Clin.Microbiol. **36**:3420-3422.

22. **Portaels, F.** 1995. Epidemiology of mycobacterial diseases. Clin.Dermatol **13**:207-227.

23. **George, K. M., D. Chatterjee, G. Gunawardana, D. Welty, J. Hayman, R. Lee, and P. L. Small**. 1999. Mycolactone: a polyketide toxin from Mycobacterium ulcerans required for virulence. Science **283**:854-857.

24. **Meyers, W. M., W. M. Shelly, D. H. Connor, and E. K. Meyers**. 1974. Human Mycobacterium ulcerans infections developing at sites of trauma to skin. Am.J.Trop.Med.Hyg. **23**:919-923.

25. Portaels, F. Diagnosis of mycobacterium ulcerans disease. 2000. Geneva, WHO.
Ref Type: Serial (Book,Monograph)

_____

26. **Abalos, F. M., J. Aguiar, Sr., A. Guedenon, F. Portaels, and W. M. Meyers**.
2000. Mycobacterium ulcerans infection (Buruli ulcer): a case report of the
disseminated nonulcerative form. Ann.Diagn.Pathol. **4**:386-390.

27. **Pszolla, N., M. R. Sarkar, W. Strecker, P. Kern, L. Kinzl, W. M. Meyers, and
F. Portaels**. 2003. Buruli ulcer: a systemic disease. Clin.Infect.Dis. **37**:e78-e82.

28. **Stinear, T. P., A. Mve-Obiang, P. L. Small, W. Frigui, M. J. Pryor, R.
Brosch, G. A. Jenkin, P. D. Johnson, J. K. Davies, R. E. Lee, S. Adusumilli,
T. Garnier, S. F. Haydock, P. F. Leadlay, and S. T. Cole**. 2004. Giant plasmid-
encoded polyketide synthases produce the macrolide toxin of Mycobacterium
ulcerans. Proc.Natl.Acad.Sci.U.S.A **101**:1345-1349.

29. **Adusumilli, S., A. Mve-Obiang, T. Sparer, W. Meyers, J. Hayman, and P. L.
Small**. 2005. Mycobacterium ulcerans toxic macrolide, mycolactone modulates
the host immune response and cellular location of M. ulcerans in vitro and in
vivo. Cell Microbiol. **7**:1295-1304.

30. **Pahlevan, A. A., D. J. Wright, C. Andrews, K. M. George, P. L. Small, and B.
M. Foxwell**. 1999. The inhibitory action of Mycobacterium ulcerans soluble
factor on monocyte/T cell cytokine production and NF-kappa B function.
J.Immunol. **163**:3928-3935.

31. **Dobos, K. M., F. D. Quinn, D. A. Ashford, C. R. Horsburgh, and C. H. King**.
1999. Emergence of a unique group of necrotizing mycobacterial diseases.
Emerg.Infect.Dis. **5**:367-378.

32. **van der Werf, T. S., W. T. van der Graaf, J. W. Tappero, and K. Asiedu**.
1999. Mycobacterium ulcerans infection. Lancet **354**:1013-1018.

33. **Allen, B. W. and D. A. Mitchison**. 1992. Counts of viable tubercle bacilli in
sputum related and culture gradings. Med.Lab.Sci **49**:94-98.

34. **Palomino, J. C. and F. Portaels**. 1998. Effects of decontamination methods and
culture conditions on viability of Mycobacterium ulcerans in the BACTEC
system. J.Clin.Microbiol. **36**:402-408.

35. **Portaels, F., J. Agular, K. Fissette, P. A. Fonteyne, H. De Beenhouwer, P. de
Rijk, A. Guedenon, R. Lemans, C. Steunou, C. Zinsou, J. M. Dumonceau,
and W. M. Meyers**. 1997. Direct detection and identification of Mycobacterium
ulcerans in clinical specimens by PCR and oligonucleotide-specific capture plate
hybridization. J.Clin.Microbiol. **35**:1097-1100.

36. **Roberts, B. and R. Hirst**. 1997. Immunomagnetic separation and PCR for
detection of Mycobacterium ulcerans. J.Clin.Microbiol. **35**:2709-2711.

_____

37. **Ross, B. C., L. Marino, F. Oppedisano, R. Edwards, R. M. Robins-Browne, and P. D. Johnson**. 1997. Development of a PCR assay for rapid diagnosis of Mycobacterium ulcerans infection. J.Clin.Microbiol. **35**:1696-1700.

38. **Stienstra, Y., T. S. van der Werf, J. Guarner, P. L. Raghunathan, E. A. Spotts Whitney, W. T. van der Graaf, K. Asamoa, J. W. Tappero, D. A. Ashford, and C. H. King**. 2003. Analysis of an IS2404-based nested PCR for diagnosis of Buruli ulcer disease in regions of Ghana where the disease is endemic. J.Clin.Microbiol. **41**:794-797.

39. **Guarner, J., J. Bartlett, E. A. Whitney, P. L. Raghunathan, Y. Stienstra, K. Asamoa, S. Etuaful, E. Klutse, E. Quarshie, T. S. van der Werf, W. T. van der Graaf, C. H. King, and D. A. Ashford**. 2003. Histopathologic features of Mycobacterium ulcerans infection. Emerg.Infect.Dis. **9**:651-656.

40. **Asiedu, K. and S. Etuaful**. 1998. Socioeconomic implications of Buruli ulcer in Ghana: a three-year review. Am.J.Trop.Med.Hyg. **59**:1015-1022.

41. **Etuaful, S., B. Carbonnelle, J. Grosset, S. Lucas, C. Horsfield, R. Phillips, M. Evans, D. Ofori-Adjei, E. Klustse, J. Owusu-Boateng, G. K. Amedofu, P. Awuah, E. Ampadu, G. Amofah, K. Asiedu, and M. Wansbrough-Jones**. 2005. Efficacy of the combination rifampin-streptomycin in preventing growth of Mycobacterium ulcerans in early lesions of Buruli ulcer in humans. Antimicrob.Agents Chemother. **49**:3182-3186.

42. **Revill, W. D., M. C. Morrow, M. C. Pike, and J. Ateng**. 1973. A controlled trial of the treatment of Mycobacterium ulcerans infection with clofazimine. Lancet **2**:873-877.

43. **Fehr, H., M. Egger, and I. Senn**. 1994. Cotrimoxazol in the treatment of Mycobacterium ulcerans infection (Buruli ulcer) in west Africa. Trop.Doct. **24**:61-63.

44. **Espey, D. K., G. Djomand, I. Diomande, M. Dosso, M. Z. Saki, J. M. Kanga, R. A. Spiegel, B. J. Marston, L. Gorelkin, W. M. Meyers, F. Portaels, M. S. Deming, and C. R. Horsburgh, Jr.** 2002. A pilot study of treatment of Buruli ulcer with rifampin and dapsone. Int.J.Infect.Dis. **6**:60-65.

45. **Portaels, F., H. Traore, K. De Ridder, and W. M. Meyers**. 1998. In vitro susceptibility of Mycobacterium ulcerans to clarithromycin. Antimicrob.Agents Chemother. **42**:2070-2073.

46. **Thangaraj, H. S., O. Adjei, B. W. Allen, F. Portaels, M. R. Evans, D. K. Banerjee, and M. H. Wansbrough-Jones**. 2000. In vitro activity of ciprofloxacin, sparfloxacin, ofloxacin, amikacin and rifampicin against Ghanaian isolates of Mycobacterium ulcerans. J.Antimicrob.Chemother. **45**:231-233.

_____

47. **Sizaire, V., F. Nackers, E. Comte, and F. Portaels**. 2006. Mycobacterium ulcerans infection: control, diagnosis, and treatment. Lancet Infect.Dis **6**:288-296.

48. **Dega, H., J. Robert, P. Bonnafous, V. Jarlier, and J. Grosset**. 2000. Activities of several antimicrobials against Mycobacterium ulcerans infection in mice. Antimicrob.Agents Chemother. **44**:2367-2372.

49. **Dega, H., A. Bentoucha, J. Robert, V. Jarlier, and J. Grosset**. 2002. Bactericidal activity of rifampin-amikacin against Mycobacterium ulcerans in mice. Antimicrob.Agents Chemother. **46**:3193-3196.

50. **van der Werf, T. S., Y. Stienstra, R. C. Johnson, R. Phillips, O. Adjei, B. Fleischer, M. H. Wansbrough-Jones, P. D. Johnson, F. Portaels, W. T. van der Graaf, and K. Asiedu**. 2005. Mycobacterium ulcerans disease. Bull.World Health Organ **83**:785-791.

51. **Oluwasanmi, J. O., T. F. Solankee, E. O. Olurin, S. O. Itayemi, G. O. Alabi, and A. O. Lucas**. 1976. Mycobacterium ulcerans (Buruli) skin ulceration in Nigeria. Am.J.Trop.Med.Hyg. **25**:122-128.

52. **Veitch, M. G., P. D. Johnson, P. E. Flood, D. E. Leslie, A. C. Street, and J. A. Hayman**. 1997. A large localized outbreak of Mycobacterium ulcerans infection on a temperate southern Australian island. Epidemiol.Infect. **119**:313-318.

53. **Marsollier, L., R. Robert, J. Aubry, J. P. Saint Andre, H. Kouakou, P. Legras, A. L. Manceau, C. Mahaza, and B. Carbonnelle**. 2002. Aquatic insects as a vector for Mycobacterium ulcerans. Appl.Environ.Microbiol. **68**:4623-4628.

54. **Marsollier, L., T. Severin, J. Aubry, R. W. Merritt, J. P. Saint Andre, P. Legras, A. L. Manceau, A. Chauty, B. Carbonnelle, and S. T. Cole**. 2004. Aquatic snails, passive hosts of Mycobacterium ulcerans. Appl.Environ.Microbiol. **70**:6296-6298.

55. **Eddyani, M., D. Ofori-Adjei, G. Teugels, D. De Weirdt, D. Boakye, W. M. Meyers, and F. Portaels**. 2004. Potential role for fish in transmission of Mycobacterium ulcerans disease (Buruli ulcer): an environmental study. Appl.Environ.Microbiol. **70**:5679-5681.

56. **Marsollier, L., T. Stinear, J. Aubry, J. P. Saint Andre, R. Robert, P. Legras, A. L. Manceau, C. Audrain, S. Bourdon, H. Kouakou, and B. Carbonnelle**. 2004. Aquatic plants stimulate the growth of and biofilm formation by Mycobacterium ulcerans in axenic culture and harbor these bacteria in the environment. Appl.Environ.Microbiol. **70**:1097-1103.

57. **Ross, B. C., P. D. Johnson, F. Oppedisano, L. Marino, A. Sievers, T. Stinear, J. A. Hayman, M. G. Veitch, and R. M. Robins-Browne**. 1997. Detection of Mycobacterium ulcerans in environmental samples during an outbreak of ulcerative disease. Appl.Environ.Microbiol. **63**:4135-4138.

58. **Mve-Obiang, A., R. E. Lee, E. S. Umstot, K. A. Trott, T. C. Grammer, J. M. Parker, B. S. Ranger, R. Grainger, E. A. Mahrous, and P. L. Small**. 2005. A newly discovered mycobacterial pathogen isolated from laboratory colonies of Xenopus species with lethal infections produces a novel form of mycolactone, the Mycobacterium ulcerans macrolide toxin. Infect.Immun. **73**:3307-3312.

59. **Marsollier, L., J. Aubry, J. P. Saint-Andre, R. Robert, P. Legras, A. L. Manceau, S. Bourdon, C. Audrain, and B. Carbonnelle**. 2003. [Ecology and transmission of Mycobacterium ulcerans]. Pathol.Biol.(Paris) **51**:490-495.

60. **Marsollier, L., J. Aubry, E. Coutanceau, J. P. Andre, P. L. Small, G. Milon, P. Legras, S. Guadagnini, B. Carbonnelle, and S. T. Cole**. 2005. Colonization of the salivary glands of Naucoris cimicoides by Mycobacterium ulcerans requires host plasmatocytes and a macrolide toxin, mycolactone. Cell Microbiol. **7**:935-943.

61. **Debacker, M., C. Zinsou, J. Aguiar, W. M. Meyers, and F. Portaels**. 2003. First case of Mycobacterium ulcerans disease (Buruli ulcer) following a human bite. Clin.Infect.Dis. **36**:e67-e68.

62. **Portaels, F., J. Aguiar, M. Debacker, C. Steunou, C. Zinsou, A. Guedenon, and W. M. Meyers**. 2002. Prophylactic effect of mycobacterium bovis BCG vaccination against osteomyelitis in children with Mycobacterium ulcerans disease (Buruli Ulcer). Clin.Diagn.Lab Immunol. **9**:1389-1391.

63. **Dowling, T. E., C.Moritz, and J.D.Palmer**. 1990. Nucleic acids. II: Restriction site analysis, p. 250-317. *In* D.M.Hills and C.Moritz (eds.), Molecular systematics. Sinauer, Sunderland, Mass.

64. **Jackson, K., R. Edwards, D. E. Leslie, and J. Hayman**. 1995. Molecular method for typing Mycobacterium ulcerans. J.Clin.Microbiol. **33**:2250-2253.

65. **Stinear, T., J. K. Davies, G. A. Jenkin, F. Portaels, B. C. Ross, F. Oppedisano, M. Purcell, J. A. Hayman, and P. D. Johnson**. 2000. A simple PCR method for rapid genotype analysis of Mycobacterium ulcerans. J.Clin.Microbiol. **38**:1482-1487.

66. **Chemlal, K., K. De Ridder, P. A. Fonteyne, W. M. Meyers, J. Swings, and F. Portaels**. 2001. The use of IS2404 restriction fragment length polymorphisms suggests the diversity of Mycobacterium ulcerans from different geographical areas. Am.J.Trop.Med.Hyg. **64**:270-273.

67. **Portaels, F., P. A. Fonteyene, H. De Beenhouwer, P. de Rijk, A. Guedenon, J. Hayman, and M. W. Meyers**. 1996. Variability in 3' end of 16S rRNA sequence of Mycobacterium ulcerans is related to geographic origin of isolates. J.Clin.Microbiol. **34**:962-965.

_____

68. **Chemlal, K., G. Huys, P. A. Fonteyne, V. Vincent, A. G. Lopez, L. Rigouts, J. Swings, W. M. Meyers, and F. Portaels**. 2001. Evaluation of PCR-restriction profile analysis and IS2404 restriction fragment length polymorphism and amplified fragment length polymorphism fingerprinting for identification and typing of Mycobacterium ulcerans and M. marinum. J.Clin.Microbiol. **39**:3272-3278.

69. **Huys, G., L. Rigouts, K. Chemlal, F. Portaels, and J. Swings**. 2000. Evaluation of amplified fragment length polymorphism analysis for inter- and intraspecific differentiation of Mycobacterium bovis, M. tuberculosis, and M. ulcerans. J.Clin.Microbiol. **38**:3675-3680.

70. **Stinear, T. P., G. A. Jenkin, P. D. Johnson, and J. K. Davies**. 2000. Comparative genetic analysis of Mycobacterium ulcerans and Mycobacterium marinum reveals evidence of recent divergence. J.Bacteriol. **182**:6322-6330.

71. **Ablordey, A., R. Kotlowski, J. Swings, and F. Portaels**. 2005. PCR amplification with primers based on IS2404 and GC-rich repeated sequence reveals polymorphism in Mycobacterium ulcerans. J.Clin.Microbiol. **43**:448-451.

72. **Stragier, P., A. Ablordey, W. M. Meyers, and F. Portaels**. 2005. Genotyping Mycobacterium ulcerans and Mycobacterium marinum by using mycobacterial interspersed repetitive units. J.Bacteriol. **187**:1639-1647.

73. **Ablordey, A., J. Swings, C. Hubans, K. Chemlal, C. Locht, F. Portaels, and P. Supply**. 2005. Multilocus variable-number tandem repeat typing of Mycobacterium ulcerans. J.Clin.Microbiol. **43**:1546-1551.

74. **Gooding, T. M., P. D. Johnson, D. E. Campbell, J. A. Hayman, E. L. Hartland, A. S. Kemp, and R. M. Robins-Browne**. 2001. Immune response to infection with Mycobacterium ulcerans. Infect.Immun. **69**:1704-1707.

75. **Gooding, T. M., P. D. Johnson, M. Smith, A. S. Kemp, and R. M. Robins-Browne**. 2002. Cytokine profiles of patients infected with Mycobacterium ulcerans and unaffected household contacts. Infect.Immun. **70**:5562-5567.

76. **Prevot, G., E. Bourreau, H. Pascalis, R. Pradinaud, A. Tanghe, K. Huygen, and P. Launois**. 2004. Differential production of systemic and intralesional gamma interferon and interleukin-10 in nodular and ulcerative forms of Buruli disease. Infect.Immun. **72**:958-965.

77. **Westenbrink, B. D., Y. Stienstra, M. G. Huitema, W. A. Thompson, E. O. Klutse, E. O. Ampadu, H. M. Boezen, P. C. Limburg, and T. S. van der Werf**. 2005. Cytokine responses to stimulation of whole blood from patients with Buruli ulcer disease in Ghana. Clin.Diagn.Lab Immunol. **12**:125-129.

78. **George, K. M., L. P. Barker, D. M. Welty, and P. L. Small**. 1998. Partial purification and characterization of biological effects of a lipid toxin produced by Mycobacterium ulcerans. Infect.Immun. **66**:587-593.

79. **George, K. M., L. Pascopella, D. M. Welty, and P. L. Small**. 2000. A Mycobacterium ulcerans toxin, mycolactone, causes apoptosis in guinea pig ulcers and tissue culture cells. Infect.Immun. **68**:877-883.

80. **Pimsler, M., T. A. Sponsler, and W. M. Meyers**. 1988. Immunosuppressive properties of the soluble toxin from Mycobacterium ulcerans. J.Infect.Dis **157**:577-580.

81. **Coutanceau, E., L. Marsollier, R. Brosch, E. Perret, P. Goossens, M. Tanguy, S. T. Cole, P. L. Small, and C. Demangel**. 2005. Modulation of the host immune response by a transient intracellular stage of Mycobacterium ulcerans: the contribution of endogenous mycolactone toxin. Cell Microbiol. **7**:1187-1196.

82. **Huygen, K.** 2003. Prospects for vaccine development against Buruli disease. Expert.Rev.Vaccines. **2**:561-569.

83. **Stanford, J. L., W. D. Revill, W. J. Gunthorpe, and J. M. Grange**. 1975. The production and preliminary investigation of Burulin, a new skin test reagent for Mycobacterium ulcerans infection. J.Hyg.(Lond) **74**:7-16.

84. **Kiszewski, A. E., E. Becerril, L. D. Aguilar, I. T. Kader, W. Myers, F. Portaels, and P. R. Hernandez**. 2006. The local immune response in ulcerative lesions of Buruli disease. Clin.Exp.Immunol. **143**:445-451.

85. **Rondini, S., C. Horsfield, E. Mensah-Quainoo, T. Junghanss, S. Lucas, and G. Pluschke**. 2005. Contiguous spread of Mycobacterium ulcerans in Buruli ulcer lesions analysed by histopathology and real-time PCR quantification of mycobacterial DNA. J.Pathol .

86. **Toll, A., F. Gallardo, M. Ferran, M. Gilaberte, M. Iglesias, J. L. Gimeno, S. Rondini, and R. M. Pujol**. 2005. Aggressive multifocal Buruli ulcer with associated osteomyelitis in an HIV-positive patient. Clin.Exp.Dermatol **30**:649-651.

87. **Dobos, K. M., E. A. Spotts, B. J. Marston, C. R. Horsburgh, Jr., and C. H. King**. 2000. Serologic response to culture filtrate antigens of Mycobacterium ulcerans during Buruli ulcer disease. Emerg.Infect.Dis. **6**:158-164.

88. **Raja, A.** 2004. Immunology of tuberculosis. Indian J.Med.Res **120**:213-232.

89. **Flynn, J. L.** 2004. Immunology of tuberculosis and implications in vaccine development. Tuberculosis.(Edinb.) **84**:93-101.

90. **Kaufmann, S. H. E.** 2002. Protection against tuberculosis: cytokines, T cells, and macrophages. Ann.Rheum.Dis **61**:54-58.

91. **Flynn, J. L. and Chan J**. 2001. Immunolgy of tuberculosis. Annu.Rev.Immunol. **19**:93-129.

92. **Kabelitz, D., A. Bender, S. Schondelmaier, B. Schoel, and S. H. Kaufmann**. 1990. A large fraction of human peripheral blood gamma/delta + T cells is activated by Mycobacterium tuberculosis but not by its 65-kD heat shock protein. J.Exp.Med. **171**:667-679.

93. **Boom, W. H., D. H. Canaday, S. A. Fulton, A. J. Gehring, R. E. Rojas, and M. Torres**. 2003. Human immunity to M. tuberculosis: T cell subsets and antigen processing. Tuberculosis.(Edinb.) **83**:98-106.

94. **Caruso, A. M., N. Serbina, E. Klein, K. Triebold, B. R. Bloom, and J. L. Flynn**. 1999. Mice deficient in CD4 T cells have only transiently diminished levels of IFN-° , yet succumb to tuberculosis. J.Immunol. **162**:5407-5416.

95. **Flynn, J. L., J. Chan, K. J. Triebold, D. K. Dalton, T. A. Stewart, and B. R. Bloom**. 1993. An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection. J.Exp.Med. **178**:2249-2254.

96. **van, d., V, M. A. Hoeve, and T. H. Ottenhoff**. 2004. Human genetics of intracellular infectious diseases: molecular and cellular immunity against mycobacteria and salmonellae. Lancet Infect.Dis. **4**:739-749.

97. **Rosenzweig, S. D. and S. M. Holland**. 2005. Defects in the interferon-gamma and interleukin-12 pathways. Immunol.Rev. **203**:38-47.

98. **Gomez-Reino, J. J., L. Carmona, V. R. Valverde, E. M. Mola, and M. D. Montero**. 2003. Treatment of rheumatoid arthritis with tumor necrosis factor inhibitors may predispose to significant increase in tuberculosis risk: a multicenter active-surveillance report. Arthritis Rheum. **48**:2122-2127.

99. **Scanga, C. A., V. P. Mohan, K. Yu, H. Joseph, K. Tanaka, Chan J, and J. L. Flynn**. 2000. Depletionof CD4C T cells causes reactivationof murine persistent tuberculosis despitecontinued expression of IFN-° andNOS2. J.Exp.Med. **192**:347-358.

100. **Grotzke, J. E. and D. M. Lewinsohn**. 2005. Role of CD8+ T lymphocytes in control of Mycobacterium tuberculosis infection. Microbes.Infect. **7**:776-788.

101. **Rosat, J. P., E. P. Grant, E. M. Beckman, C. C. Dascher, P. A. Sieling, D. Frederique, R. L. Modlin, S. A. Porcelli, S. T. Furlong, and M. B. Brenner**. 1999. CD1-restricted microbial lipid antigen-specific recognition found in the CD8+ alpha beta T cell pool. J.Immunol. **162**:366-371.

102. **Stenger, S., R. J. Mazzaccaro, K. Uyemura, S. Cho, P. F. Barnes, J. P. Rosat, A. Sette, M. B. Brenner, S. A. Porcelli, B. R. Bloom, and R. L. Modlin**. 1997. Differential effects of cytolytic T cell subsets on intracellular infection. Science **276**:1684-1687.

103. **Stenger, S.** 2001. Cytolytic T cells in the immune response to mycobacterium tuberculosis. Scand.J.Infect.Dis **33**:483-487.

104. **Dieli, F., G. Sireci, C. Di Sano, E. Champagne, J. J. Fournie, and J. I. Salerno**. 1999. Predominance of Vgamma9/Vdelta2 T lymphocytes in the cerebrospinal fluid of children with tuberculous meningitis: reversal after chemotherapy. Mol.Med. **5**:301-312.

105. **Dieli, F., G. Sireci, N. Caccamo, C. Di Sano, L. Titone, A. Romano, P. Di Carlo, A. Barera, A. Accardo-Palumbo, A. M. Krensky, and A. Salerno**. 2002. Selective depression of interferon-gamma and granulysin production with increase of proliferative response by Vgamma9/Vdelta2 T cells in children with tuberculosis. J.Infect.Dis **186**:1835-1839.

106. **Olin, M. R., C. K. Hwa, J. Lee, and T. W. Molitor**. 2005. Gammadelta T-lymphocyte cytotoxic activity against Mycobacterium bovis analyzed by flow cytometry. J.Immunol.Methods **297**:1-11.

Chapter 2: Goal and objectives

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2.1 Goal

The purpose of this study was to contribute toward the understanding of the bacteriology of *M. ulcerans* and host immune response to *M. ulcerans* infection for effective management and control of Buruli ulcer (BU).

2.2 objectives

1. To optimise primary isolation of *M. ulcerans* from clinical samples by evaluating different culturing methods.

2. To confirm clinical diagnosis by culture, microscopy and the polymerase chain reaction.

3. To analyse the susceptibility pattern of *M. ulcerans* isolates to antimycobacterial drugs.

4. To develop and characterise *M. ulcerans* isolates by Variable Number of Tandem Repeats (VNTR) sequence typing.

5. To study cell mediated immune responses in BU by upon antigen stimulation of PBMCs from patients using ex vivo ELISpot analysis, T cell proliferation assays and cytokine measurements by ELISA.

6. To determine the kinetics of recovery of γδ T cell effector function in BU patients after surgery.

7. To analyse the serum cytokine levels of BU patients and compare to that of household contacts and non-exposed controls.

Chapter 3: Evaluation of decontamination methods and growth media for the primary isolation of Mycobacterium ulcerans from surgical specimens

Dorothy Yeboah-Manu[1], Thomas Bodmer[2], Ernestina Mensah-Quainoo[3], Samuel Owusu[1], David Ofori-Adjei[1] and Gerd Pluschke[4]

Noguchi Memorial Institute for Medical Research, Legon, Ghana[1], Institute for Infectious Diseases, University of Bern, Bern, Switzerland[2], Ga District Health Services, Amasaman, Ghana[3] and Swiss Tropical Institute, Basel, Switzerland[4]

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Abstract

We evaluated four decontamination methods and one non-decontamination procedure in combination with four egg-based media for the primary isolation of *M. ulcerans* from tissue specimens. With mycobacterial recovery and contamination rates of 75.6% and 2.4%, respectively, the combination of oxalic acid decontamination with LJ-glycerol medium yielded the best results.

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Currently laboratory diagnosis of Buruli ulcer (6) caused by *Mycobacterium ulcerans* relies on the detection of acid-fast bacilli in stained smears, the isolation of the pathogen from infected tissue or tissue exudates by culture, the identification of characteristic histopathological changes and/or the detection of mycobacterial DNA by the *IS2404* PCR. A number of studies have indicated that the sensitivity of culturing of the slow growing *M. ulcerans* is <40 % when compared with the *IS2404* PCR (1, 7 & 8). The purpose of this study was to optimize the cultivation procedure of *M. ulcerans* for surgical specimens.

Tissue specimens, collected from suspected active Buruli ulcer cases diagnosed by the WHO clinical definition (6) at the Amasaman Health Centre in the Ga district of Ghana between September 2002 and April 2003 were analyzed. Cultures were set up with a total of 41 surgical specimens collected from 36 patients. Patients usually sought medical treatment late as more than half of them presented with ulcerative lesions. Care was taken not to contaminate the excised specimens, which were stored at 4°C in 7 ml modified Dubos transport medium supplemented with 10% oleic acid-albumin-dextrose-catalase (Lenexa, KS), 2% PANTA Plus (Becton Dickinson, Franklin Lakes, NY) and 0.5 % agar-agar (Difco). All samples were processed within one week after surgical excision. Specimens were cut into smaller pieces, homogenized and suspended in 8 ml of Dulbecco's phosphate buffered saline (PBS). 100 µl of the resulting suspension was inoculated directly, i.e. without prior decontamination, onto selective Lowenstein-Jensen (LJ) medium supplemented with 0.75% glycerol and 2% PANTA Plus. The remaining suspension was divided equally into four aliquots (about 2 ml in volume). Aliquots were decontaminated with the Petroff (2), reversed Petroff (RP; 5), NALC (2) or Oxalic acid method (OA; 5). After decontamination, the homogenates were concentrated by centrifuging, and 100 µl aliquots of the 1 ml suspensions were inoculated in duplicate on the four solid media (9): LJ with 0.75% glycerol (LJG) or 0.5% pyruvate (LJP) and Ogawa medium with 1.3% glycerol (OG) or 0.5% pyruvate (OP). Inoculated tubes were incubated at 33ºC and the number of tubes that yielded *M. ulcerans*, mycobacterial colony counts per tube and the time to the appearance of visible colonies were determined. All suspected *M. ulcerans* isolates were confirmed by sequencing the first 500 base pair of the 16S rRNA gene using the MicroSeq 500 16S rDNA Bacterial Sequencing Kit (PE Applied Biosystem) as described earlier (4).

LJG gave the best (p<0.001) overall mycobacteria recovery rate when results obtained with the different decontamination methods used were cumulated (Table 1). It supported the

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growth of *M. ulcerans* from all 32 specimens from which a positive culture could be obtained by any of the conditions tested (n=41). When the results of the four different media used were cumulated (n=144 tubes per decontamination method), 4 (2.4%), 5 (3%), 24 (14.6%) and 25 (15.2%) tubes were found to be contaminated with OA, RP, P and NALC, respectively (Table 2). In combination with the best performing medium, LJG, the recovery rates were 75.6%, 68.3%, 65.8% and 63.4% for OA, P, RP and NALC, respectively. Of the 41 PANTA-supplemented LJG culture tubes inoculated with non-contaminated samples, 25 (61 %) grew *M. ulcerans*, 12 (29.2 %) got contaminated and 4 tubes (9.7%) had no growth. Macroscopically detectable *M. ulcerans* colonies appeared faster ($p < 0.05$) with the non-decontamination method (six weeks) than with the four decontamination procedures (median time with LJG: eight weeks) and the mean number of mycobacterial colony forming units obtained by this procedure was also found to be slightly higher than with the decontamination procedures. However, due to the high contamination rate, the number of positive cultures was slightly lower (odds ratio of 2.7; 95% confidence interval, 0.8-8.9) than that with the best performing decontamination method, OA in combination with LJG.

32 of 41 (78%) samples analyzed were AFB positive by microscopy after Ziehl-Neelsen staining (1) and the same number was culture positive (31 of these 32 were positive by the best performing cultivation methodology, LJG in combination with OA). Altogether, pure cultures were obtained from 30 of the 41 samples. Three of the culture negative samples were microscopy positive (for one of these, all culture tubes gave rise to non mycobacterial overgrowth) and three of the microscopy negative samples were culture positive. Out of the six cultures and microscopy negative samples only one was positive by *IS2404* PCR (8). We assume that the lesions from which the remaining five samples had been collected were not related to Buruli ulcer. Only slight differences in the performance of microscopy and culture in relation to the type of lesion was observed (Table 3). Culture positivity was slightly higher with ulcerative lesions than with nodules and plaque lesions (86 versus 70%); however this was not statistically significant.

In the present study we followed strict sampling procedure: i. great care was taken to avoid exogenous contamination when recovering samples from the surgically excised tissue; ii. the collected tissue samples were immediately suspended in a medium containing antibiotics, kept refrigerated and transported on ice; iii. samples were processed within seven days after excision in a cultivation facility in Ghana. Adherence to these strict rules resulted in a

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comparatively low contamination rate; only one nodule specimen was lost due to bacterial overgrowth of all culture tubes.

We conclude that successful primary isolation of *M. ulcerans* depends on the emphasis put on optimum sampling procedures and specimen handling prior to cultivation. The direct inoculation of specimen onto a selective LJG medium containing PANTA in addition to the use of oxalic acid for decontamination in combination with LJG medium may further increase recovery rates, particularly for specimens with a low mycobacterial load, and reduce time to recovery of *M. ulcerans*.

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Table 1:

Effect of the growth medium on the recovery of *M. ulcerans.*

Medium	Number of culture positive samples (n=164)	Mean CFU per culture tube	Time (weeks) after which macroscopic growth was observed (mean in brackets)
LJG*	112 (68.3%)	215	5-14 w (7.4 w)
LJP*	78 (47.6%)	100	6-13 w (9.4 w)
OG*	88 (53.6%)	145	8-19 w (12.5 w)
OP*	52 (31.7%)	80	8-18 w (11.8 w)

*Results with the four different decontamination methods are cumulated

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Table 2:

Effect of the decontamination method on the recovery of *M. ulcerans*.

Decontamination method	Number of culture positive samples (n=164)	Number of cultures with contamination
P*	81 (49.4%)	24 (14.6%)
RP*	86 (53.8%)	5 (3.0%)
NALC*	78 (47.6%)	25(15.2%)
OA*	86 (53.8%)	4(2.4%)

*Results with the four different media are cumulated

Table 3.

Performance of diagnostic microbiology in relation to the type of BU lesion.

Disease stage	Number of microscopy positive specimens	Number of culture positive specimens (all methods)
Nodules and plaques	16/20 (80%)	14/20 (70%)
Ulcerative lesions	16/21 (76%)	18/21 (86%)
Total	32/41 (78%)	32/41 (78%)

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Reference List

1. Asiedu, K., R. Scherpbier and M. Raviglione. 2000. Buruli ulcer, *Mycobacterium ulcerans* infection. Geneva: World Health Organisation.

2. Kent, P. T. and P. G. Kubica. 1985. Public health bacteriology, a guide to the level III laboratory. Centers for disease control, Atlanta, GA

3. Noeske, J., C. Kuaban, S. Rondini, P. Sorlin, L, Ciaffi, J. Mbuagbaw, F. Portaels and G. Pluschke. 2004. Buruli ulcer disease in Cameroon rediscovered. *Am J Trop Med Hyg*. 70:520-6.

4. Palca, A., C. Aebi, R. Weimann and T Bodmer. 2002. *Mycobacterium bohemicum* cervical lymphadenitis. *Pediatr Infect Dis J* 21:982-4

5. Palomino, J. C. and F. Portaels. 1998. Effects of decontamination methods and culture conditions on viability of *Mycobacterium ulcerans* in the BACTEC system. *J Clin Microbiol*. 36:402-8

6. Portaels, F., P. Johnson and W. M, Meyers. 2001. Diagnosis of *Mycobacterium ulcerans* disease. Geneva: World Health Organisation

7. Portaels. F., J. Agular, K. Fissette, P. A. Fonteyne, H. De Beenhouwer, P. de Rijk, A. Guedenon, R. Lemans, C. Steunou, C. Zinsou, J. M. Dumonceau and W. M. Meyers. 1997. Direct detection and identification of *Mycobacterium ulcerans* in clinical specimens by PCR and oligonucleotide-specific capture plate hybridization. J Clin Microbiol. 35:1097-100

8. Ross, B. C., L. Marino, F. Oppedisano, R. Edwards, R. M. Robins-Browne and P. D. Johnson. 1997. Development of a PCR assay for rapid diagnosis of *Mycobacterium ulcerans* infection. *J Clin Microbiol*. 35:1696-700.

9. WHO. 1998. Laboratory services in tuberculosis control, Part III: Culture

Chapter 4: Diagnosis of *Mycobacterium ulcerans* disease: an assessment of the accuracy of clinical judgement at a treatment centre in Ghana

Ernestina Mensah-Quainoo[1], Dorothy Yeboah-Manu [2], Caroline Asebi[1*], Francis Patafuor[1] David Ofori-Adjei[2], Thomas Junghanss[3], Gerd Pluschke[4]

[1]Ghana Health Service, Ministry of Health, Ghana, * Now retired, [2]Noguchi Memorial Institute for Medical Research, Legon, Ghana, [3] Department of Tropical Hygiene and Public Health, University hospital Heidelberg, Germany and [4]Swiss Tropical Institute, Basel, Switzerland

Abstract

Clinical diagnosis of M. ulcerans infection is currently accepted as sufficient basis for treating the disease. Inadequate laboratory resources in the highly endemic areas of Africa often limit possibilities for in-country confirmation of clinical judgment. However, accurate pre-treatment diagnosis is highly desirable to prevent unnecessary surgical and/or anti-mycobacterial drug treatment of patients with non-Buruli ulcer conditions. Here we analyzed records of 99 Buruli ulcer patients diagnosed clinically and treated surgically at Amasaman Health Centre in Ghana, for whom post-treatment diagnostic laboratory tests were performed. Comparison of clinical diagnoses with test results obtained by an in-country laboratory on samples of excised tissue showed a high accuracy of clinical judgment. Among lesions with three laboratory tests (microscopy for acid fast bacilli, culture and IS2404 polymerase chain reaction) done, 94% had at least one test positive and 83% had at least two tests positive. We conclude that correct clinical diagnosis of Buruli ulcer by well trained health workers is achievable. Quality of clinical diagnosis should be monitored by intermittent testing in national reference laboratories. We also advocate further research to develop highly predictive but simple diagnostic tests to aid fast pre-treatment confirmation of M. ulcerans infection at peripheral treatment centers in the highly endemic areas of Africa.

Introduction

Currently, the diagnosis of Buruli ulcer (BU), caused by *Mycobacterium ulcerans,* is mainly clinical, and this is considered sufficient basis for treatment decision (1,2). The brunt of the global burden of BU falls on West Africa, where cases occur in thousands in remote areas (3). These countries lack the resources to meet the cost of treatment, which is high (4). In particular, laboratories are inadequate or absent at peripheral centers where the majority of cases are treated. Pre-treatment diagnosis of non-ulcerated lesions is further limited. Punch biopsy is suggested to obtain specimens, but this carries risks such as dissemination of *M. ulcerans*, bleeding and precipitate ulceration. Clinical judgment will thus likely remain the mainstay of pre-treatment diagnosis – especially in endemic developing countries – until these constraints are resolved. Accuracy of this method of diagnosis is therefore of interest.

Although clinical diagnosis is considered unchallenging (5), incidents of misdiagnosis have been reported. BU cases have been missed initially (6-8). Conversely, presumed BU lesions may prove to be other conditions (9-13). Even with careful assessment, nearly a third of the cases in a study on the efficacy of antibiotic treatment (14) turned out to be other conditions, indicating that in particular the clinical diagnosis of pre-ulcerative lesions can be error-prone. The World Health Organization (WHO) requires confirmation of suspected cases with positive results of at least two tests (15) from among microscopy with staining for acid fast bacilli (AFB), culture, histopathology and IS2404 polymerase chain reaction (PCR).

Ga West Health District (GWD) in Ghana is highly endemic for BU. A survey in 1997 which found 340 active cases showed the disease clustered in rural communities in the lowland basin of the Densu River. (Mensah-Quainoo E, unpublished). A national case search in 1999 recorded 1113 cases in the district, (467 being active) constituting 20% of the total national figure of 5619 (16). In the endemic communities BU is recognized well, with several local names, such as *Aboa gbonyo, Aboa fon, Odonti fla, Odonti hela, Detifu dor* and *Tsina asane,* which aptly describe its features. As reported also for other BU endemic areas, many of the patients commonly seek medical care only after healing fails with traditional treatment. BU cases in GWD are treated at a rural health facility, Amasaman Health Centre (AHC), where surgical services were established to counter difficulties in referring cases for treatment elsewhere. Treatment decision at this centre relies exclusively on clinical diagnosis. In this study, cases diagnosed clinically over a three year period were validated with results of analysis of specimens of the excised tissue in an in-country research laboratory.

Materials and Methods

Patients

All patients in this study were reviewed by one clinician who made the final diagnostic decision. Cases/lesions were classified according to the standard WHO definitions (2). Patients clinically diagnosed as having BU and surgically treated at AHC during the 3-year period beginning of July 2001 to end of July 2004, who had single lesions on first presentation, and at least two diagnostic tests done, and whose complete clinical records were available, were included in the study. Only one patient (N3 in Table 5) received anti-mycobacterial therapy prior to surgical excision. We reviewed patients' clinical notes, Bu 01 forms (1) and laboratory records.

Laboratory diagnostic methods

Tissue samples of about 2 cm^3 were taken for laboratory analysis from the centre of pre-ulcerative lesions, and from the active edge of ulcers during surgical treatment. Samples were cut to include dermis and obviously diseased subcutaneous fatty tissue, with care to avoid contamination. Samples were placed in modified Dubos transport medium as previously described (17), kept in the cold and transported within one week to the Noguchi Memorial Institute for Medical Research (NMIMR) in Legon, Ghana, where culture, Ziehl-Neelsen staining and IS2404 PCR were performed. For each patient, all tests done in each instance were performed on the same sample.

Cultivation was done as described previously (17). Specifically, half of the diagnostic tissue specimen (about 1cm^3) was cut into smaller pieces, homogenized and suspended in Dulbecco's phosphate buffered saline (PBS). The suspension was decontaminated by the oxalic acid method. After decontamination, the homogenate was concentrated by centrifugation, and 100 µl aliquots of the 1 ml suspensions was used to inoculate in duplicate LJ tubes. These were incubated at 32ºC and all suspected *M. ulcerans* isolates were confirmed by *IS2404* PCR. Two drops of the remaining suspension was used to prepare two slides per sample for Ziehl-Neelsen (ZN) staining. Stained smears were read as previously described (17).

The remaining half of the tissue specimen was used for *IS2404* PCR. DNA was extracted by heating the remaining sample for 1 h at 95°C in 500 µl of an extraction mixture (50 mM Tris-

HCl, 25 mM EDTA, and 5% monosodium glutamate). After cooling 100µl of a 50 mg/ml lysozyme solution was added and incubated for two hours at 37°C. 70 µl of proteinase K-10x buffer (100 mM Tris-HCl, 50 mM EDTA, 5% sodium dodecyl sulphate [pH 7.8]) and 10 µl of a 20 mg/ml proteinase K solution was then added and incubated at 45°C overnight. The bacterial cell wall was fully disrupted by adding 200 µl of 0.1 mm-diameter zirconia beads (BioSpec Products) to each sample and vortexed at full speed for 4 min. Beads and undigested tissue fragments were removed by brief centrifugation, and the supernatants were transferred to fresh tubes for phenol-chloroform (Fluka) extraction. The DNA contained in the upper phase was precipitated with ethanol and re-suspended in 100 µl of water. The *IS2404* sequence was amplified in a 50µl reaction volume using the Qiagen Taq DNA polymerase kit. The primer sets used for the amplification were as described before (Ross et al, 1997). The reaction mixture contained a 1.0 µM concentration of each primer, 200 µM concentration of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 1x PCR buffer, 1 U of Taq DNA polymerase (Qiagen, Hilden, Germany), and approximately 50 ng of DNA. Thermocycling parameters were 95°C for 3mins; 35 cycles of 1min at 94°C, 1min at 60°C, and 1min at 72°C; and 7min at 72°C. Ten microliters of amplified DNA was subjected to electrophoresis in a 2% agarose gel and detected by ethidium bromide staining and UV transillumination. The amplicons were sized by comparing to a 1-kb ladder.

The results of the laboratory tests of treated patients were compared with the clinical diagnosis retrospectively, to assess the validity of the latter. The levels of agreement between different tests were quantified using Kappa statistics; where k = 0 is equivalent to the level of agreement expected by chance and k = 1 indicates perfect agreement. The NMIMR ethical review board gave permission for performing the study.

Results

Patient population and clinical features

Between July 2001 and July 2004 a total of 131 BU patients were clinically diagnosed and treated at AHC. Administrative difficulties prevented tests to be done for 20 of the 131 cases. Of the remaining 110 patients, one patient with incomplete notes and 10 who had multiple lesions were excluded. Ninety-two of the 99 remaining patients had one sample each tested; the rest 7 had additional samples from recurrent lesions tested. Forty-two males aged 3 to 70 years and 57 females aged 2 to 75 were included in the study. Median age was 11 years and 62.6 % were aged 14 years and below (Figure 1) Altogether, 91.7% of the 108 lesions included in the study were on the limbs (Table 1). The left leg and right arm were more often affected than the corresponding opposite limbs. There was a statistically significant difference between upper limbs and lower limbs in the distribution of lesions between the left and right sides (chi square = 6.3, p = 0.01) Forty-eight per cent (52/108) of lesions were pre-ulcerative, 33.3% (36/108) in earlier ulcer stages and 18.5% (20/108) were chronic ulcers. Clinical records were compared with corresponding laboratory test results.

Performance of laboratory tests

As the history, clinical features and course of illness left no doubt that the primary and recurrent lesions in the 7 patients with recurrences were caused by *M. ulcerans* infection, results of these are presented separately (Table 2). The culture for one sample was contaminated and two samples had no PCR done. Thus, 17 AFB and 15 PCR tests were carried out, and 16 cultures were evaluable. Thirty-eight (79.2%) of these 48 evaluable tests were positive. Each test method yielded at least one false negative result.

Next, we compared the clinical diagnoses at first presentation for all 99 patients with their laboratory test results. For the seven patients who had recurrences, only the test results of their primary lesions, which posed a true challenge for clinical decision, were included in this assessment. For the 99 lesions, AFB and culture were performed in all cases, whereas PCR was done for 85. The cultures for 2 of the 85 lesions were contaminated. This resulted in 83 patients with all 3 tests done and evaluable; 14 patients with only AFB and culture done and evaluable; and 2 patients with AFB and PCR evaluable. Of the evaluable tests, 78.8% (78/99), 79.4% (77/97) and 72.9% (62/85) were positive for AFB, culture or PCR, respectively (Table 3). In 36.4% (36/99) of patients, negative results with one test were associated with positive results for one or both of the two other tests; an indication of false negative results. Among

the 83 patients for whom all three tests were carried out, 94.0% (78/ 83) had at least one test positive, 83.1% (69/83) had at least two tests positive, and 53.0% (44/83) had all three tests positive (Table 3). Among the 14 cases with only AFB and culture performed, 10 had both tests positive, two had culture alone positive and two had both tests negative. The two patients with contaminated cultures tested positive for both AFB and PCR.

Nine cases had only one test positive: two had only AFB, two had only culture and five only PCR positive. The two patients with AFB-only positive both had very extensive ulcers, with one needing repeat excisions for secondary lesions that developed beyond the excised margins subsequently. One of the culture-only positive patients had an ulcer and the other a nodule. Of the five PCR-only positive cases, one was a large plaque in the popliteal fossa and 4 were ulcers; one of which – a 7-year-old lesion – was devoid of active disease. The clinically easier to diagnose ulcerative lesions thus were over- rather than under-represented among the cases that had only one test positive. Seven (7.1%) of the 99 patients tested negative for all tests done; five had all 3 tests done and two (#N2 and #N3) had only AFB and culture done. All 7 patients were living in BU endemic communities and their lesion features, as detailed in Table 5, were consistent with BU.

Agreement between the AFB/culture test-pair was statistically significantly higher than what can expected by chance (Kappa coefficient = 0.47 [standard error 0.10] and p = 0.000001). The coefficient was equally high for pre-ulcerative and ulcerated lesions (k = 0.42 [s.e. = 0.13] p = 0.00008) and (k = 0.50 [s.e. = 0.13] p = 0.0001) respectively. In contrast, agreement for the test-pairs AFB/PCR and culture/PCR for both pre-ulcerative and ulcerated lesion groups were not statistically significant. We assessed the impact of lesion stage on test results, and found a difference in test outcomes between early and late stage lesions for culture, but not for AFB and PCR (Table 4). The culture positivity rate was statistically significantly higher for pre-ulcerative lesions than for ulcers (chi square = 4.04, p = 0.04). Two-tests-positive rate for pre-ulcerative lesions was significantly higher than for ulcerated lesions (Chi square = 4.50, p = 0.03), whereas there was no difference for one- and three-tests-positive rates.

Discussion

The clinical diagnosis of BU is considered easy in endemic settings (1,18,19). Of cases clinically diagnosed in routine practice in the GWD, 93% could be reconfirmed by at least one of three tests performed by an in-country laboratory. The 72.3% positivity rate observed in our study for the *IS2404* PCR was comparable to a rate of 74.8% reported by Stienstra et al. (12) for excised tissue samples but relatively low compared to 98% reported by Phillips et al. (20) for needle biopsies. The observed 78.7% culture positivity rate was comparable to our previous findings (17) and high, compared to previously reported rates of 39 – 55% (12,20,21). The AFB positivity rate of 78.1% was higher than those reported in a number of other studies (11,20,22).

Our results with a set of samples from seven patients who had recurrences are in agreement with reported findings, indicating that all three laboratory tests yield false negative results (6,11,21,23,24). *M. ulcerans* bacilli are distributed unevenly in tissues (13,20,25-27) and inadequacy in size or site of specimen may therefore result in false negative test outcomes. In our study concordance between AFB/culture was statistically significant whereas concordance for AFB/PCR and culture/PCR was not. This may be a reflection of the different factors that account for false negatives of PCR as opposed to AFB and culture. In the case of the more sensitive *IS2404* PCR test, false negative results may primarily be caused by enzyme inhibitory substances co-purified with the template DNA. In the case of both AFB and culture, false negative results are most likely to be caused by a low density of mycobacteria in the tissue specimen analyzed. The seven patients with all tests negative fulfilled the standard criteria for clinical diagnosis the same as those lesions confirmed as BU. It has to be left open whether clinical or laboratory diagnosis was false in these cases. Also the likelihood of false positive results must be considered, in particular among lesions with only one test positive. False positive PCRs are particularly likely to occur, due to contamination of template DNA with minute amounts of PCR products (11,12). Among our nine single-test-positive cases at least the two which were culture-only positive were unquestionably BU cases. For culture, we found a statistically significantly higher test positive rate for pre-ulcerative than ulcerated lesions. This may reflect the presence of the total load of *M. ulcerans* bacilli in the pre-ulcerative situation, whereas in ulcers the bulk of bacilli may be lost along with sloughing necrotic tissues (21,25).

Laboratory diagnosis for confirmation of BU cases can either support pre-treatment decision making or assess the accuracy of clinical diagnosis after treatment. Both aspects are of increasing importance in the current situation, where antibiotic treatment is introduced and its efficacy needs to be assessed. For infectious diseases, culture of the causative organism is the most definite laboratory test. However, in the case of *M. ulcerans* culture takes several weeks and is thus impractical to aid pre-treatment diagnosis. Besides it requires a specialized laboratory and therefore inaccessible at the periphery. Ideally post-treatment tests should be done in reference laboratories in the respective endemic countries. We advocate accepting one positive test result in conjunction with clinical diagnosis done by well-trained clinicians as confirmation of BU cases. With this approach, the trade-off fraction of non-BU cases that would be counted as BU due to false single-test-positive results would probably be minimal, whereas a higher number of true BU cases would be identified. Given these considerations, we recommend training of peripheral level health workers to optimize clinical diagnosis of BU. For this, the predictive value of objective clinical features of BU must be further investigated. To sustain and monitor clinical diagnostic accuracy at the periphery, intermittent quality-control testing by in-country reference laboratories should be performed.

The recent WHO recommendation to use a combination of rifampicin and streptomycin to support or replace surgical treatment introduces further dimensions to the dilemma of laboratory diagnosis of BU. The reported cure of early lesions with this treatment (14) makes pre-treatment confirmation imperative, as healing with antibiotics alone makes post-treatment diagnosis with surgically excised tissue impossible. Furthermore, accurate pre-treatment diagnosis is highly desirable to prevent unnecessary surgical and/or anti-mycobacterial drug treatment of patients with non-BU conditions. While time consuming and/or technically demanding methods such as culture, histopathology or PCR may be used for post-treatment reconfirmation at reference laboratories, rapid, inexpensive and simple laboratory tests are required for routine pre-treatment diagnosis at the periphery, where apart from inadequate resources for workspace, equipment and reagents, qualified laboratory personnel is often lacking. Microscopic examination of samples for AFB represents currently the most suitable method for rapid pre-treatment diagnosis at centers with basic local laboratory access. If only the AFB test is applied, it appears necessary to analyze more than one sample per lesion to reduce the proportion of false negative results. In cases with strong clinical diagnosis, false negative test results would hinder rather than assist the clinician. In the case of ulcerated lesions samples for AFB staining can be collected by swabbing of the undermined edges of

the lesion (21). In the case of pre-ulcerative lesions it is being discussed to analyze three needle biopsy samples per lesion for AFB. The possible complications of biopsies include dissemination of *M. ulcerans*, bleeding, secondary bacterial infection and precipitate ulceration of closed lesions. In addition, for small children, the procedure may be difficult and risky without anesthesia. Further, cultural acceptability may be problematic in areas like GWD with popular belief that "the needle" (injection) is contra-indicated for "boils". Considering that hospital treatment is already regarded by some patients as potentially destructive (28), this may further reduce utilization of treatment services. Therefore development of diagnostic methods for BU that can be performed with blood or urine samples would be very helpful. Assay formats suitable for health facilities in the periphery, such as an agglutination or dipstick test would be preferable. Such assays are available for many infectious diseases and test formats could be easily adopted once a test principle suitable for BU has been identified. Potential test principles include the identification of *M. ulcerans* specific antigens and the detection of *M. ulcerans* specific immune responses. Ideally, the assays developed should have a high predictive value and non-technical personnel should be able to perform them under field conditions. In conclusion, our findings validate the perception that clinical diagnosis is reliable and give an indication of the degree of accuracy of clinical judgment achievable in routine practice in an endemic country of Africa.

.Table 1: Anatomical site distribution of the 108 BU lesions in the 99 patients enrolled

Location of Lesion	Male	Female	Total	%
Left Arm	6	10	16	14.8
Right Arm	11	17	28	25.9
Left Leg	15	19	34	31.5
Right Leg	10	11	21	19.4
Trunk	4	5	9	8.3
Total	46	62	108	100.0

Table 2: Laboratory test results in seven patients with multiple recurrent lesions

Case ID‡	Date	Classification and location of lesion	AFB	Culture	PCR
#R1	May 8, 02	primary; plaque at abdomen	+	+	n. d.
	Nov 12, 02	same-site recurrence; plaque	+	-	n. d.
	Jan 14, 03	different -site recurrence; papule at left thigh	+	n. e.	+
#R2	Oct 15, 02	Primary; plaque at anterior left knee	+	+	+
	Nov 08, 02	different - site recurrence; nodule at posterior right thigh	-	+	-
	Jan 15, 03	different - site recurrence; nodule at postero-lateral aspect of left thigh	+	+	+
#R3	Nov 13, 03	Primary; nodule at right leg	+	+	+
	Feb 11, 04	different - site recurrence; nodule at left foot	-	-	+
#R4	Apr 12, 02	Primary; plaque at right knee	+	+	+
	Mar 14, 03	same-site recurrence; ulcer (sample 1)	+	-	-
	Nov 13, 03	same-site recurrence; ulcer (sample 2)	+	+	_
#R5	Mar 23, 02	Primary; plaque at left thigh	+	+	+
	Oct 17, 02	same-site recurrence; plaque	+	+	+
#R6	Nov 18, 02	primary; plaque at right arm	+	+	+
	Apr 8, 03	same-site recurrence; plaque	+	+	+
#R7	Apr 24, 02	primary; plaque at right arm	+	+	-
	Dec 5, 02	same-site recurrence; plaque	+	+	+

‡ Sex and age: #R1: f, 4 y; #R2: m, 3 y; #R3: m, 10 y; #R4: f, 10 y; #R5: f, 8 y; #R6: m, 23 y;

#R7: f, 26 y; n. d. = not done; n. e. = not evaluable (contaminated culture)

Table 3: Laboratory test results of 99 clinically diagnosed primary BU lesions

Laboratory tests	Number of lesions	% of lesions
AFB +	78/99	78.8
†Culture +	77/97	79.4
*PCR +	62/85	72.9
AFB + and culture +	68/97	70.1
AFB + and culture -	8/97	8.2
AFB – and culture +	9/97	9.3
AFB – and culture -	12/97	12.4
Agreement# AFB/culture	80/97	82.5
AFB + and PCR +	52/85	61.2
AFB + and PCR -	16/85	18.8
AFB - and PCR +	10/85	11.8
AFB - and PCR -	7/85	8.2
Agreement # AFB/PCR	59/85	69.4
Culture + and PCR +	49/83	59.0
Culture + and PCR -	16/83	19.3
Culture – and PCR +	11/83	13.3
Culture – and PCR -	7/83	8.4
Agreement # culture/PCR	56/83	67.5
‡any one test pos	78/83	94.0
‡any two tests pos	69/83	83.1
‡all 3 tests pos	43/83	51.8

† 2/99 cultures were contaminated; *PCR was not done in the case of 14/99 samples;

#Agreement: Both tests positive, or both negative; ‡ lesions for which all three tests are

available

Table 4: Effect of BU lesion stage on laboratory test results (109 tests in 99 clinically diagnosed patients)

Laboratory tests	Number pre-ulcerative lesions	% of pre-ulcerative lesions	Number ulcerated lesions	% of ulcerated lesions	Total number of lesions	%
AFB +	40/52	76.9	45/57	78.9	85/109	78.0
†Culture +	43/50	86.0§	39/56	69.6	82/106	77.4
*PCR +	32/42	76.2	36/52	69.2	68/94	72.3
AFB+ and culture+	36/50	72.0	36/56	64.3	72/106	67.9
AFB+ and culture -	2/50	4.0	7/56	12.5	9/106	8.5
AFB - and culture+	7/50	14.0	3/56	5.4	10/106	9.4
AFB - and culture -	5/50	10.0	8/56	14.3	13/106	12.3
Agreement#						80.2
AFB/culture‡	41/50	82.0‡2	44/56	78.6‡3	85/106	‡
AFB+ and PCR+	26/42	61.9	31/52	59.6	52/94	55.3
AFB+ and PCR -	7/42	16.7	9/52	17.3	16/94	17.0
AFB - and PCR+	6/42	14.3	5/52	9.6	11/94	11.7
AFB - and PCR -	3/42	7.1	5/52	9.6	8/94	8.5
Agreement# AFB/PCR	29/42	69.0	36/52	69.2	60/94	63.8
Culture+ and PCR+	27/40	67.5	26/51	51.0	53/91	52.8
Culture+ and PCR -	9/40	22.5	8/51	15.7	17/91	18.7
Culture - and PCR+	3/40	7.5	9/51	17.6	12/91	13.2
Culture- and PCR-	1/40	2.5	6/51	11.8	7/91	7.7
Agreement# culture/PCR	28/40	70.0	32/51	62.7	60/91	65.9
$any one test pos,	39/40	97.5	47/51	92.2	86/91	94.5
$any two tests pos	35/40	87.5Φ	39/51	76.5	74/91	81.3
$all 3 tests pos	23/40	57.5	25/51	49.0	48/91	52.7

† 3/109 cultures were contaminated; *PCR was not done in the case of 15/109 samples;
#Agreement: Both tests positive, or both negative; $ lesions for which all three tests are available;‡ Kappa coefficient : ‡[1]k = 0.47 [standard error 0.10] and p = 0.000001;‡[2] k = 0.42 [s.e. = 0.13] p = 0.00008;‡[3] (k = 0.50 [s.e. = 0.13] p = 0.0001; **§**Culture positive rate pre-ulcerative lesions compared to ulcers: chi square = 4.04, p = 0.04; **Φ** Two-tests-positive rate for pre-ulcerative lesions compared to ulcers: Chi square = 4.50, p = 0.03

Table 5 : Features of clinically diagnosed BU patients with all laboratory tests negative

| Case ID	Age	Sex	Clinical form of lesion	Key features of lesion
#N1	22	M	Nodule	• Patient lives in endemic community • Firm lesion attached to skin, but not to underlying tissues • Lesion near characteristic previous BU scars
#N2	15	M	Nodule	• Patient lives in endemic community • Firm lesion attached to skin, but not to underlying tissues
#N3	19	M	Plaque	• Patient lives in endemic community • Firm lesion attached to skin, not attached to underlying tissues initially, but later attached • Discoloration of skin overlying lesion (purplish) • On referral to the Korle Bu Teaching Hospital, placed on anti-TB drug treatment
#N4	9	F	Ulcer	• Patient lives in endemic community • Lesion at first a small discharging sinus, later enlarged into a typical BU • Necrotic slough in situ • Lesion near characteristic previous BU scars
#N5	65	F	Ulcer	• Patient lives in endemic community • Large ulcer with undermined edges • Necrotic sloughing base • Indurated surrounding tissues and darkened surrounding skin
#N6	2	F	Ulcer	• Patient lives in endemic community • Extensive ulcer with undermined edges • Necrotic slough in situ • Indurated surrounding tissues and darkened surrounding skin
#N7	9	M	Ulcer	• Patient lives in endemic community • Large 'old-looking' ulcer with signs of active disease on edges • Indurated surrounding tissues and darkened surrounding skin
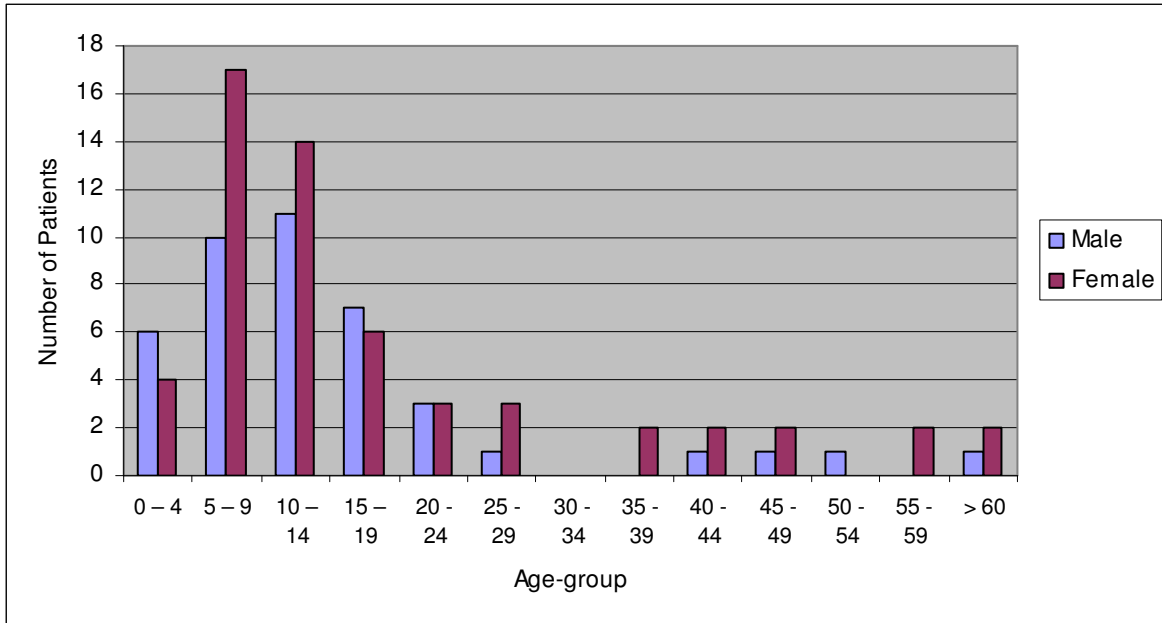
Figure 1 Age-sex distribution

Reference List

1. **Asiedu, K., Scherpbier, R, and Raviglione,** M. Buruli ulcer, Mycobacterium ulcerans infection. 2000. WHO.

2. **Buntine, J and Crofts, k**. Buruli ulcer; management of Mycobacterium ulcerans disease; a manual for health care providers. 2001. World Health Organization, Geneva, Switzerland.

3. **Johnson, P. D., T. Stinear, P. L. Small, G. Pluschke, R. W. Merritt, F. Portaels, K. Huygen, J. A. Hayman, and K. Asiedu**. 2005. Buruli ulcer (M. ulcerans infection): new insights, new hope for disease control. PLoS.Med. **2**:e108.

4. **Asiedu, K. and S. Etuaful**. 1998. Socioeconomic implications of Buruli ulcer in Ghana: a three-year review. Am.J.Trop.Med.Hyg. **59**:1015-1022.

5. **van der Werf, T. S., W. T. van der Graaf, J. W. Tappero, and K. Asiedu**. 1999. Mycobacterium ulcerans infection. Lancet **354**:1013-1018.

6. **Semret, M., G. Koromihis, J. D. MacLean, M. Libman, and B. J. Ward**. 1999. Mycobacterium ulcerans infection (Buruli ulcer): first reported case in a traveler. Am.J.Trop.Med.Hyg. **61**:689-693.

7. **Coloma, J. N., G. Navarrete-Franco, P. Iribe, and L. D. Lopez-Cepeda**. 2005. Ulcerative Cutaneous Mycobacteriosis Due to Mycobacterium ulcerans: Report of Two Mexican Cases. Int.J.Lepr.Other Mycobact.Dis **73**:5-12.

8. **Evans, M. R., J. Mawdsley, R. Bull, D. N. Lockwood, H. Thangaraj, D. Shanahan, and K. Rajakulasingam**. 2003. Buruli ulcer in a visitor to London. Br.J.Dermatol. **149**:907-909.

9. **Revill, W. D., M. C. Morrow, M. C. Pike, and J. Ateng**. 1973. A controlled trial of the treatment of Mycobacterium ulcerans infection with clofazimine. Lancet **2**:873-877.

10. **Mwanatambwe, M., M. Yajima, S. Etuaful, K. Suzuki, K. Nishigai, K. Asiedu, N. Yamada, and G. Asano**. 2002. Malignant melanoma of the buttock presenting as a Buruli ulcer. J.Dermatol. **29**:357-361.

11. **Guarner, J., J. Bartlett, E. A. Whitney, P. L. Raghunathan, Y. Stienstra, K. Asamoa, S. Etuaful, E. Klutse, E. Quarshie, T. S. van der Werf, W. T. van der Graaf, C. H. King, and D. A. Ashford**. 2003. Histopathologic features of Mycobacterium ulcerans infection. Emerg.Infect.Dis. **9**:651-656.

12. **Stienstra, Y., T. S. van der Werf, J. Guarner, P. L. Raghunathan, E. A. Spotts Whitney, W. T. van der Graaf, K. Asamoa, J. W. Tappero, D. A. Ashford, and C. H. King**. 2003. Analysis of an IS2404-based nested PCR for diagnosis of Buruli

ulcer disease in regions of Ghana where the disease is endemic. J.Clin.Microbiol. **41**:794-797.

13. **Siegmund, V., O. Adjei, P. Racz, C. Berberich, E. Klutse, F. van Vloten, T. Kruppa, B. Fleischer, and G. Bretzel**. 2005. Dry-reagent-based PCR as a novel tool for laboratory confirmation of clinically diagnosed Mycobacterium ulcerans-associated disease in areas in the tropics where M. ulcerans is endemic. J.Clin.Microbiol. **43**:271-276.

14. **Etuaful, S., B. Carbonnelle, J. Grosset, S. Lucas, C. Horsfield, R. Phillips, M. Evans, D. Ofori-Adjei, E. Klustse, J. Owusu-Boateng, G. K. Amedofu, P. Awuah, E. Ampadu, G. Amofah, K. Asiedu, and M. Wansbrough-Jones**. 2005. Efficacy of the combination rifampin-streptomycin in preventing growth of Mycobacterium ulcerans in early lesions of Buruli ulcer in humans. Antimicrob.Agents Chemother. **49**:3182-3186.

15. WHO. Buruli ulcer: Diagnosis of Mycobacterium ulcerans disease. Portaels, F., Johnson, P., and Meyers, W. M. 2001.
Ref Type: Serial (Book,Monograph)

16. **Amofah, G., F. Bonsu, C. Tetteh, J. Okrah, K. Asamoa, K. Asiedu, and J. Addy**. 2002. Buruli ulcer in Ghana: results of a national case search. Emerg.Infect.Dis. **8**:167-170.

17. **Yeboah-Manu, D., T. Bodmer, E. Mensah-Quainoo, S. Owusu, D. Ofori-Adjei, and G. Pluschke**. 2004. Evaluation of decontamination methods and growth media for primary isolation of Mycobacterium ulcerans from surgical specimens. J.Clin.Microbiol. **42**:5875-5876.

18. **Muelder, K.** 1992. Wounds that will not heal: the Buruli ulcer. Int.J.Dermatol **31**:25-26.

19. **van der Werf, T. S. and W. T. van der Graaf**. 1990. Buruli ulcer in West Africa. Lancet **336**:1440.

20. **Phillips, R., C. Horsfield, S. Kuijper, A. Lartey, I. Tetteh, S. Etuaful, B. Nyamekye, P. Awuah, K. M. Nyarko, F. Osei-Sarpong, S. Lucas, A. H. Kolk, and M. Wansbrough-Jones**. 2005. Sensitivity of PCR Targeting the IS2404 Insertion Sequence of Mycobacterium ulcerans in an Assay Using Punch Biopsy Specimens for Diagnosis of Buruli Ulcer. J.Clin.Microbiol. **43**:3650-3656.

21. **Portaels, F., J. Agular, K. Fissette, P. A. Fonteyne, H. De Beenhouwer, P. de Rijk, A. Guedenon, R. Lemans, C. Steunou, C. Zinsou, J. M. Dumonceau, and W. M. Meyers**. 1997. Direct detection and identification of Mycobacterium ulcerans in clinical specimens by PCR and oligonucleotide-specific capture plate hybridization. J.Clin.Microbiol. **35**:1097-1100.

22. **Darie, H., T. Le Guyadec, and J. E. Touze**. 1993. [Epidemiological and clinical aspects of Buruli ulcer in Ivory Coast. 124 recent cases]. Bull.Soc.Pathol Exot. **86**:272-276.

23. **Goutzamanis, J. J. and G. L. Gilbert**. 1995. Mycobacterium ulcerans infection in Australian children: report of eight cases and review. Clin.Infect.Dis. **21**:1186-1192.

24. **Phillips, R., O. Adjei, S. Lucas, N. Benjamin, and M. Wansbrough-Jones**. 2004. Pilot randomized double-blind trial of treatment of Mycobacterium ulcerans disease (Buruli ulcer) with topical nitrogen oxides. Antimicrob.Agents Chemother. **48**:2866-2870.

25. **Rondini, S., C. Horsfield, E. Mensah-Quainoo, T. Junghanss, S. Lucas, and G. Pluschke**. 2006. Contiguous spread of Mycobacterium ulcerans in Buruli ulcer lesions analysed by histopathology and real-time PCR quantification of mycobacterial DNA. J.Pathol **208**:119-128.

26. **Rondini, S., E. Mensah-Quainoo, H. Troll, T. Bodmer, and G. Pluschke**. 2003. Development and application of real-time PCR assay for quantification of Mycobacterium ulcerans DNA. J.Clin.Microbiol. **41**:4231-4237.

27. **Josse, R., A. Guedenon, H. Darie, S. Anagonou, F. Portaels, and W. M. Meyers**. 1995. [Mycobacterium ulcerans cutaneous infections: Buruli ulcers]. Med.Trop.(Mars.) **55**:363-373.

28. **Aujoulat, I., C. Johnson, C. Zinsou, A. Guedenon, and F. Portaels**. 2003. Psychosocial aspects of health seeking behaviours of patients with Buruli ulcer in southern Benin. Trop.Med.Int.Health **8**:750-759.

Chapter 5: Genetic diversity in *Mycobacterium ulcerans* isolates from Ghana revealed by a newly identified locus containing a variable number of tandem repeats

Markus Hilty,[1+] Dorothy Yeboah-Manu,[1,2+] Daniel Boakye,[2] Ernestina-Mensah-Quainoo,[3] Simona Rondini,[1] Esther Schelling,[1] David Ofori-Adjei,[2] Françoise Portaels[4], Jakob Zinsstag[1] and Gerd Pluschke[1]

Swiss Tropical Institute[1], 4002 Basel, Noguchi Memorial Institute for Medical Research[2], Legon, Ghana, Tema Muncipal Health Directorate, Tema, Ghana[3], and Institute of Tropical Medicine[4], 2000 Antwerp, Belgium

[+] contributed equally

Abstract

Molecular typing methods applied so far for *Mycobacterium ulcerans* isolates have not been able to identify genetic differences among isolates from Africa. This apparent lack of genetic diversity among *M. ulcerans* isolates is indicative for a clonal population structure. We analysed the genetic diversity of 71 African isolates, including 57 strains from Ghana, by variable number of tandem repeats (VNTR) typing based on a newly identified polymorphic locus designated ST1 and the previously described locus, MIRU 1. Three different genotypes were found in Ghana, demonstrating for the first time genetic diversity of *M. ulcerans* in an African country. While the ST1/MIRU 1 allele combination BD/BAA seems to dominate in Africa, it was only rarely found in isolates from Ghana, where the combination BD/B was dominating and observed in all districts analysed. A third variant genotype (C/BAA) was found only in the Amansie-West district. Results are indicative for the emergence and spreading of new genetic variants of *M. ulcerans* within Ghana and support the potential of VNTR-based typing for genotyping of *M. ulcerans*.

Introduction

Mycobacterium ulcerans, the causative agent of Buruli ulcer (BU) is an emerging pathogen particularly in Sub-Saharan African countries, and is also found in tropical and sub-tropical regions of Asia, the Western Pacific and Latin America (4). BU is characterized by chronic, necrotic lesions of subcutaneous tissues. Due to the lack of an established effective antimicrobial therapy, surgical excision and skin grafting is currently the recommended treatment (27).

While it is known that proximity to slow flowing or stagnant water bodies is a risk factor for *M. ulcerans* infection, the exact mode of transmission has remained an enigma (4). This is partly because no molecular typing method is available that has sufficiently high resolution for micro-epidemiological analyses. The apparent lack of genetic diversity of *M. ulcerans* within individual geographical regions (7, 8, 16, 19-21) is indicative for a clonal population structure. Genetic analyses suggest the recent divergence of *M. ulcerans* from *M. marinum* (5, 26), which is well known as fish pathogen and can cause limited granulomatous skin infections in humans (10, 11, 13). One of the hallmarks of the emergence of *M. ulcerans* as a more severe pathogen is the acquisition of a 174-kb plasmid bearing a cluster of genes necessary for the synthesis of the macrolide toxin mycolactone responsible for the massive tissue destruction seen in BU (22).

Variable number of tandem repeat (VNTR) typing is a PCR-based technique identifying alleles of defined regions of DNA that contain a variable number of copies of short sequence stretches. Resolution of the method is cumulative and can be increased by inclusion of additional loci. Tandem repeats are easily identified from genome sequence data, measurement of PCR fragment sizes is relatively straightforward and VNTR typing data can be digitalized and compared between different laboratories. Availability of complete genomic sequences has facilitated identification of repetitive genetic elements of *M. tuberculosis* (12, 15, 24, 25), *M. bovis* (14, 18) and *M. avium* (6), including short tandem repeats designated exact tandem repeats (ETRs) and mycobacterial interspersed repetitive units (MIRUs). Strain typing with these sets of polymorphic loci is developing into an important tool in the epidemiological analysis of tuberculosis (9) and ordinary

agarose gel electrophoretic separation of PCR products is usually sufficient to estimate the number of repeat units in an allele. In a study of *M. bovis* strains from Chad, VNTR-typing of a distinct number of loci is most discriminative for strains of the same clone (14).

More recently, MIRUs and other VNTRs have also been described for *M. ulcerans* and *M. marinum* (3, 23) typing. Most of the described sequences are orthologues of the *M. tuberculosis* genome database and their resolution seems to be comparable to that of the currently most discriminatory methods, the 2426 PCR analysis (19) and IS2404-Mtb2 PCR (2) which discriminate among isolates from different geographical origin, but not among strains from different endemic regions of Africa.

We describe in this report a new *M. ulcerans* specific VNTR locus (ST1) which together with the previously described MIRU 1 (23) differentiated clinical isolates from Ghana into three VNTR allele combinations.

Methods

Identification of the VNTR locus ST1

 A tandem repeat finder software (http://www.c3.biomath.mssm.edu/trf.thml) was used to screen the *M. marinum* sequence data bank (www.sanger.ac.uk/projects/M_marinum) and identified a tandem repeat containing locus which was designated ST1. This locus is present both in *M. marinum* and in *M. ulcerans* (http://genopole.pasteur.fr/Mulc/BuruList.html), but not in *M. tuberculosis* (http://www.sanger.ac.uk/Projects/M_tuberculosis). A forward (ctgaggggatttcacgaccag) and a reverse primer (cgccacccgcggacacagtcg) located in the sequences flanking the identified locus and yielding a PCR product of 423 bp was designed. Genomic sequences corresponding to the primers were 100 % identical for *M. marinum* and *M. ulcerans*.

Bacterial strains

 A panel of 11 *M. ulcerans* clinical isolates of human origin from diverse geographical origin and of 6 *M. marinum* clinical isolates of human origin from Switzerland was used to assess the polymorphism of ST1 (Table 1). The *M. marinum* isolates were from patients living in the agglomeration of Zurich, except for *M. marinum* N119 that was isolated from a patient living in Biel. The year of isolation was 1995 for strains 853 and 894, 1997 for strains 8972 and 946 and 1998 for strains N119 and 3023. In order to analyse the diversity of African *M. ulcerans* strains, 66 additional clinical isolates (12 from Benin and 54 from Ghana) were included in this study. The Ghanaian strains were isolated (28) between 2001 and 2003 from patients being treated at the Amasaman Health Centre in the Greater Accra Region of Ghana (48 isolates) or the Saint Martin Hospital Agroyesum in the Ashanti Region of Ghana (6 isolates). The residential origin of the isolates is as indicated in the supporting table.

DNA Extraction

DNA was extracted as described (17). Briefly, small bacterial pellets were heated for 1 h at 95°C in 500 µl of an extraction mixture (50 mM Tris-HCl, 25 mM EDTA, and 5% monosodium glutamate). One hundred microliters of a 50-mg/ml lysozyme solution was then added and incubated for two hours at 37°C. 70 µl of proteinase K-10x buffer (100

mM Tris-HCl, 50 mM EDTA, 5% sodium dodecyl sulphate [pH 7.8]) and 10 µl of a 20-mg/ml proteinase K solution was added. After incubation at 45°C overnight, 300 µl of 0.1-mm-diameter zirconia beads (BioSpec Products) were added to each sample and vortexed at full speed for 4 min. Beads and large debris were removed by brief centrifugation, and the supernatants were transferred to fresh tubes for phenol-chloroform (Fluka) extraction. The DNA contained in the upper phase was precipitated with ethanol and re-suspended in 100 µl of water.

PCR analysis and sequencing of PCR products

PCR reaction mixtures contained 1x *Taq* PCR buffer, deoxynucleoside triphosphates (0.2 mM each), 1 U of AmpliTaq Gold DNA polymerase (Perkin-Elmer Applied Biosystems), a 0.5 µM concentration of the primer pair and mycobacterial DNA in a final volume of 20 µl. Addition of 5 % DMSO to the reaction mix improved the yield of PCR products. The reaction was carried out using a Perkin-Elmer 9600 cycler starting with a denaturing step of 10 min at 95°C. After denaturation, the PCR was performed for 40 cycles of 0.5 min at 94°C, 0.5 min at 65°C and 1 min at 72°C. The reactions were terminated by an incubation of 10 min at 72°C. PCR fragments were analysed by agarose gel electrophoresis using 2% NuSieve agarose. The size of the amplicons was estimated by comparison with Size Marker VIII (Roche). PCR products were directly sequenced with an ABI Prism 310 Genetic Analysis System. PCR products for ST1 of 423 bp and 369 bp corresponded to a copy number of 2 and 1, respectively. For MIRU 1, copy numbers were assigned as described (23).

Results

Identification and characterization of a new VNTR locus found in *M. ulcerans* and *M. marinum*

A new VNTR locus designated ST1, was identified by screening of the *M. marinum* sequence data bank with a tandem repeat finder software. Orthologues of ST1 were found in *M. ulcerans*, but not in *M. tuberculosis*. Its repeat length of 54 bp is suitable for size analysis by standard agarose gel electrophoresis. In contrast to MIRUs (23), ST1 is not intergenic, but part of a pseudogene and therefore of no known functional interest (personal communication). When eleven *M. ulcerans* isolates of geographically diverse origin were typed by agarose gel electrophoresis of PCR products, two different alleles of ST1 were identified (Table 1). While eight strains had two repeats, two isolates, one from French Guyana and one of the two analysed isolates from Ghana had only one repeat. Of seven *M. marinum* clinical isolates tested, all five strains from patients in the agglomeration of Zurich (strains 8972, 946, 3023, 853 and 894) had three repeats, whereas strain N119 isolated from a different part of Switzerland and the reference strain used for the *M. marinum* genome sequencing project, both had two repeats. Sequence analysis of PCR products reconfirmed the size differences observed by agarose gel electrophoresis and identified six sequence variants of the repeat unit (designated A – F; Fig 1). Unlike many other VNTRs (1), ST1 showed no micro-deletions, but only single nucleotide polymorphisms (SNPs) within the sequence variants. The *M. ulcerans* strains from China and Japan turned out to have a different allele (CF; Table 1) than the other *M. ulcerans* strains with two repeats (BD). A third allele with two repeats (AC) and an allele with three repeats (ACE) was found in *M. marinum* (Table 1). Thus sequencing of ST1 improved the discrimination power of *M. ulcerans* and *M. marinum* strains compared to gel electrophoresis analysis alone and revealed distinctive genotypes for *M. marinum* compared to *M. ulcerans*.

Diversity of *M. ulcerans* isolates from Ghana

Evidence for diversity of the ST1 locus in African isolates (Table 1) prompted us to analyse additional collections of 12 disease isolates from Benin and 54 isolates from Ghana (Table 2). All strains from Benin and the majority of Ghanaian strains had an ST1

allele (BD) with two repeats. However, in most strains from the Amansie West district (including ITM-970359; Table 1), a second allele with only one repeat (C) was identified (Table 2). When the Ghanaian isolates were tested also for diversity in the loci MIRU 1 (23), VNTR 8, 9 and 19, previously described as polymorphic within *M. ulcerans* strains of different geographical origin (3), diversity was also found in locus MIRU 1. Sequence analysis of PCR products of selected strains reconfirmed the size differences observed by agarose gel electrophoresis and identified two sequence variants of the MIRU 1 repeat unit (designated A and B; Fig 1).

Altogether three VNTR allele combinations were found among the clinical isolates from Ghana (Table 2, Fig. 2 and 3). While all isolates from the Ga, Akwapim South, Ahafo-Ano North and Akim Abuakwa districts had the ST1/MIRU 1 allele combination 1 (BD/B), two allele combinations, i.e. 1 and 2 (C/BAA) were found among the five isolates from the Amansie-West district. A third allele combination (BD/BAA) was found in two strains (Agy99 from the Ga district and ITM 97-0359 from the Ashanti region) isolated before 2000 in Ghana and in all other African isolates.

Discussion

Molecular typing methods such as multi-locus sequence typing, 16S rRNA sequencing, restriction fragment length polymorphism and variable number of tandem repeats typing have revealed a remarkable lack of genetic diversity of *M. ulcerans* and a clonal population structure within given geographical regions. The discriminatory power of all these methods is particularly insufficient to differentiate between African isolates. Innovative molecular genetic fingerprinting methods are therefore required for local epidemiological studies aiming to reveal transmission pathways and environmental reservoirs of *M. ulcerans*. First attempts to use VNTR typing for *M. ulcerans* (3, 23) have identified variable loci suitable for discrimination of disease isolates at continental level. In this study we used a newly identified (ST1) and four previously described VNTRs to analyse genetic diversity within a collection of 71 *M. ulcerans* strains from Africa, including 57 isolates from Ghana. Three of the previously described VNTRs i.e. VNTR 8, 9 and 19 (3) were not able to discriminate among the African strains. Yet MIRU 1 (23) and the newly identified locus (ST1) defined three subgroups within the Ghanaian strains.

The fact that two allele combinations (BD/B and C/BAA) differing from the common African combination (BD/BAA) were found within a recent (2001-2003) collection of Ghanaian isolates is indicative for an ongoing microevolution of *M. ulcerans* and for the spreading of new variants within Ghana. It is tempting to hypothesize that allele combination 3 (BD/BAA) represents an ancestral like genotype and that the others evolved by reduction in the repeat unit numbers in the ST1 locus (from BD to C) or in the MIRU 1 locus (from BAA to B), respectively. While conversion of the MIRU 1 locus from BAA to B could be explained by deletion of the two A repeat units, conversion of the ST1 locus from BD to C by a deletional mechanism would require that a central sequence stretch of the BD repeat region comprising portions of both the B and the D repeat unit would have been lost, yielding the hybrid repeat unit C.

While allele combination 3 seems to be the most common in Africa, most of the *M. ulcerans* strains from Ghana analysed, had the allele combination 1. This genotype was found in all Ghanaian districts included in this study. Allele combination 2 dominated in

the Amansie West district, but was found exclusively there. Follow up of the temporal and spatial patterns of emergence and spreading of genotypes may contribute in future to our understanding of the transmission and epidemiology of Buruli ulcer. From the present data, we cannot draw any conclusions why certain variant appear to be the more successful than others. The fact, that we were not able to sub-group the 47 isolates from the Ga district by VNTR (with the only exception of the 'older' isolate Agy99) reconfirms that *M. ulcerans* has a clonal population structure associated with a low rate of genomic drift. Availability of the fully assembled and annotated genome sequence of *M. ulcerans* in the near future will facilitate identification of further polymorphic VNTR loci potentially contributing to further refinement of genetic fingerprinting of *M. ulcerans* isolates.

Acknowledgements

Species	Isolate	Country of origin	Number of repeats	Arrangement of the variant repeat DNA sequences
M. ulcerans	ITM 8756	Japan	2	C F
	ITM 980912	China	2	C F
	ITM 941328	Malaysia	2	B D
	ITM 884	Australia	2	B D
	ITM 9357	PNG	2	B D
	ITM 7922	French Guyana	1	C
	ITM 970359[+]	Ghana	1	C
	ITM 970321	Ghana	2	B D
	ITM 940886	Benin	2	B D
	ITM 940662	Côte d'Ivoire	2	B D
	ITM 960658	Angola	2	B D
M. marinum	894/1995	Switzerland	3	A C E
	853/1995	Switzerland	3	NOT DONE
	8972/1997	Switzerland	3	NOT DONE
	946/1997	Switzerland	3	NOT DONE
	3023/1998	Switzerland	3	NOT DONE
	N119/1998	Switzerland	2	NOT DONE
	*M. marinum**	unknown	2	A C

Table 1: ST1 alleles of *M. ulcerans* and *M. marinum* disease isolates

*isolate used for the *M. marinum* genome sequencing project

[+]isolated in 1997 from a patient living in the Amansie West district

Country of origin	District	Number of strains	ST1 allele	MIRU 1 allele	Allele combination
	Ga district	47	2 (BD)	1 (B)	1
	Ga district	1	2 (BD)	3 (BAA)	3
	Akwapim South	1	2 (BD)	1 (B)	1
Ghana	Akim Abuakwa	1	2 (BD)	1 (B)	1
	Ahafo-Ano	1	2 (BD)	1 (B)	1
	Amansie West	1	2 (BD)	1 (B)	1
	Amansie West	4	1 (C)	3 (BAA)	2
	unknown°	1	2 (BD)	3 (BAA)	3
Benin		13	2 (BD)[+]	3* (BAA)[+]	3
Côte d'Ivoire		1	2 (BD)	3*	3
Angola		1	2 (BD)	3*	3

Table 2: ST1 and MIRU 1 allele combinations of *M. ulcerans* strains from Africa

VNTR copy numbers for ST1 and MIRU 1 were determined and allele combinations assigned (1-3). Sequence profiles of *M. ulcerans* strains are shown in brackets. °isolated at the Saint Martin's hospital in the Ashanti region; * MIRU 1 copy numbers as previously described (23); [+]only one strain (ITM 94-0886) was analysed by sequencing (23).

Allele	ST1 repeat unit sequences
A	CCGGTTCTGTTTCGTCCGGTGCGACCGCTGGCACTGTCTCGACCGGTGCGACGA
B	....G....C..........
C	....G...........G
D	.......G...G.C...................................G...........G
E	– T.....G...G.C..............................G............G
F	.......G...G.C....A..............................G...........G

Allele	MIRU 1 repeat unit sequences
A*	ATGAGCCAGCCGGCGACGATGCAGAGCGAAGCGATGAGGAGGAGCGGCGCCAG
B	G...A..C..T..........................

Figure 1: Sequence variation of ST1 and MIRU 1 tandem repeat units

(-): Base deletions; (.): identical sequence positions.

*Allele A of MIRU 1 corresponds to variant A2 (23).
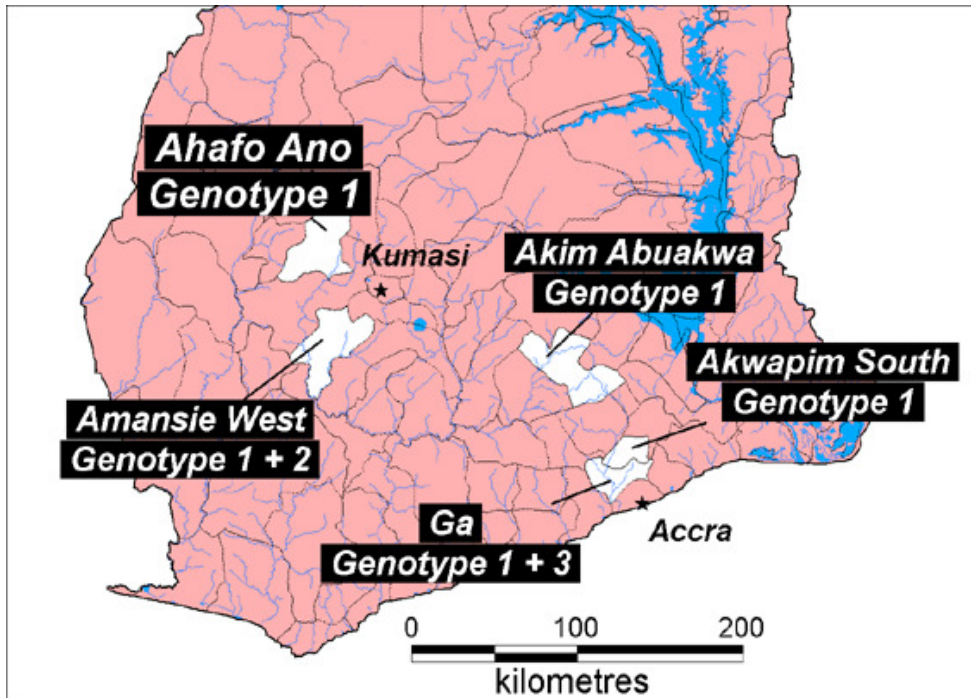
Figure 2: Map of southern Ghana showing the residential districts of patients from whom the Ghanaian isolates analysed in this study were obtained

ST1/MIRU 1 allele combinations are genotype 1: BD/B, genotype 2: C/BAA and genotype 3: BD/BAA.
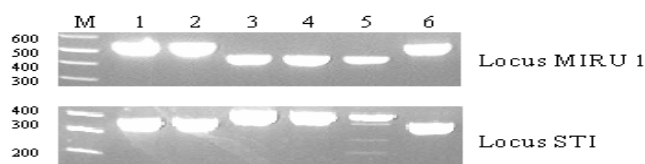
Figure 3: Agarose gel electrophoretic analysis of PCR products from amplifications with MIRU 1 primers (upper Panel) and STI primers (lower panel)

Results with selected isolates are shown. 1: strain (Amansie-West district); 2: strain (Amansie-West district); 3: strain (Ga district); 4: strain (Ga district); 5: strain (Ga district); 6: strain (Amansie-West district);

Reference List

1. **Ablordey, A., M. Hilty, P. Stragier, J. Swings, and F. Portaels**. 2005. Comparative Nucleotide Sequence Analysis of Polymorphic Variable-Number Tandem-Repeat Loci in Mycobacterium ulcerans. J.Clin.Microbiol. **43**:5281-5284.

2. **Ablordey, A., R. Kotlowski, J. Swings, and F. Portaels**. 2005. PCR amplification with primers based on IS2404 and GC-rich repeated sequence reveals polymorphism in Mycobacterium ulcerans. J.Clin.Microbiol. **43**:448-451.

3. **Ablordey, A., J. Swings, C. Hubans, K. Chemlal, C. Locht, F. Portaels, and P. Supply**. 2005. Multilocus variable-number tandem repeat typing of Mycobacterium ulcerans. J.Clin.Microbiol. **43**:1546-1551.

4. **Asiedu, K., R. Scherpbier, and M. Raviglione**. 2000. Buruli ulcer - *Mycobacterium ulcerans* infection. WHO document WHO/CDS/CPE/GBUI/2000.1.

5. **Boddinghaus, B., T. Rogall, T. Flohr, H. Blocker, and E. C. Bottger**. 1990. Detection and Identification of Mycobacteria by Amplification of Ribosomal-Rna. Journal of Clinical Microbiology **28**:1751-1759.

6. **Bull, T. J., K. Sidi-Boumedine, E. J. Mcminn, K. Stevenson, R. Pickup, and J. Hermon-Taylor**. 2003. Mycobacterial interspersed repetitive units (MIRU) differentiate Mycobacterium avium subspecies paratuberculosis from other species of the Mycobacterium avium complex. Molecular and Cellular Probes **17**:157-164.

7. **Chemlal, K., K. De Ridder, P. A. Fonteyne, W. M. Meyers, J. Swings, and E. Portaels**. 2001. The use of IS2404 restriction fragment length polymorphisms suggests the diversity of Mycobacterium ulcerans from different geographical areas. American Journal of Tropical Medicine and Hygiene **64**:270-273.

8. **Chemlal, K., G. Huys, P. A. Fonteyne, V. Vincent, A. G. Lopez, L. Rigouts, J. Swings, W. M. Meyers, and F. Portaels**. 2001. Evaluation of PCR-restriction profile analysis and IS2404 restriction fragment length polymorphism and amplified fragment length polymorphism fingerprinting for identification and typing of Mycobacterium ulcerans and M-marinum. Journal of Clinical Microbiology **39**:3272-3278.

9. **Crawford, J. T.** 2003. Genotyping in contact investigations: a CDC perspective. International Journal of Tuberculosis and Lung Disease **7**:S453-S457.

10. **Dailloux, M., C. Laurain, R. Weber, and P. Hartemann**. 1999. Water and nontuberculous mycobacteria. Water Research **33**:2219-2228.

11. **Dobos, K. M., F. D. Quinn, D. A. Ashford, C. R. Horsburgh, and C. H. King**. 1999. Emergence of a unique group of necrotizing mycobacterial diseases. Emerging Infectious Diseases **5**:367-378.

12. **Frothingham, R. and W. A. Meeker-O'Connell**. 1998. Genetic diversity in the Mycobacterium tuberculosis complex based on variable numbers of tandem DNA repeats. Microbiology-Uk **144**:1189-1196.

13. **Gluckman, S. J.** 1995. Mycobacterium-Marinum. Clinics in Dermatology **13**:273-276.

14. **Hilty, M., C. Diguimbaye, E. Schelling, F. Baggi, M. Tanner, and J. Zinsstag**. 2005. Evaluation of the discriminatory power of variable number tandem repeat (VNTR) typing of Mycobacterium bovis strains. Vet.Microbiol. **109**:217-222.

15. **Mazars, E., S. Lesjean, A. L. Banuls, M. Gilbert, V. Vincent, B. Gicquel, M. Tibayrenc, C. Locht, and P. Supply**. 2001. High-resolution minisatellite-based typing as a portable approach to global analysis of Mycobacterium tuberculosis molecular epidemiology. Proceedings of the National Academy of Sciences of the United States of America **98**:1901-1906.

16. **Portaels, F., P. A. Fonteyne, H. DeBeenhouwer, P. DeRijk, A. Guedenon, J. Hayman, and W. M. Meyers**. 1996. Variability in 3' end of 16S rRNA sequence of Mycobacterium ulcerans is related to geographic origin of isolates. Journal of Clinical Microbiology **34**:962-965.

17. **Rondini, S., E. Mensah-Quainoo, H. Troll, T. Bodmer, and G. Pluschke**. 2003. Development and application of real-time PCR assay for quantification of Mycobacterium ulcerans DNA. Journal of Clinical Microbiology **41**:4231-4237.

18. **Skuce, R. A., T. P. McCorry, J. F. McCarroll, S. M. M. Roring, A. N. Scott, D. Brittain, S. L. Hughes, R. G. Hewinson, and S. D. Neill**. 2002. Discrimination of Mycobacterium tuberculosis complex bacteria using novel VNTR-PCR targets. Microbiology-Sgm **148**:519-528.

19. **Stinear, T., J. K. Davies, G. A. Jenkin, F. Portaels, B. C. Ross, F. Oppedisano, M. Purcell, J. A. Hayman, and P. D. R. Johnson**. 2000. A simple PCR method for rapid genotype analysis of Mycobacterium ulcerans. Journal of Clinical Microbiology **38**:1482-1487.

20. **Stinear, T., B. C. Ross, J. K. Davies, L. Marino, R. M. Robins-Browne, F. Oppedisano, A. Sievers, and P. D. R. Johnson**. 1999. Identification and characterization of IS2404 and IS2606: Two distinct repeated sequences for detection of Mycobacterium ulcerans by PCR. Journal of Clinical Microbiology **37**:1018-1023.

21. **Stinear, T. P., G. A. Jenkin, P. D. R. Johnson, and J. K. Davies**. 2000. Comparative genetic analysis of Mycobacterium ulcerans and Mycobacterium

marinum reveals evidence of recent divergence. Journal of Bacteriology **182**:6322-6330.

22. **Stinear, T. P., A. Mve-Obiang, P. L. C. Small, W. Frigui, M. J. Pryor, R. Brosch, G. A. Jenkin, P. D. R. Johnson, J. K. Davies, R. E. Lee, S. Adusumilli, T. Garnier, S. F. Haydock, P. F. Leadlay, and S. T. Cole**. 2004. Giant plasmid-encoded polyketide synthases produce the macrolide toxin of Mycobacterium ulcerans. Proceedings of the National Academy of Sciences of the United States of America **101**:1345-1349.

23. **Stragier, P., A. Ablordey, W. M. Meyers, and F. Portaels**. 2005. Genotyping Mycobacterium ulcerans and Mycobacterium marinum by using mycobacterial interspersed repetitive units. J.Bacteriol. **187**:1639-1647.

24. **Supply, P., J. Magdalena, S. Himpens, and C. Locht**. 1997. Identification of novel intergenic repetitive units in a mycobacterial two-component system operon. Molecular Microbiology **26**:991-1003.

25. **Supply, P., E. Mazars, S. Lesjean, V. Vincent, B. Gicquel, and C. Locht**. 2000. Variable human minisatellite-like regions in the Mycobacterium tuberculosis genome. Molecular Microbiology **36**:762-771.

26. **Tonjum, T., D. B. Welty, E. Jantzen, and P. L. Small**. 1998. Differentiation of Mycobacterium ulcerans, M-marinum, and M-haemophilum: Mapping of their relationships to M-tuberculosis by fatty acid profile analysis, DNA-DNA hybridization, and 16S rRNA gene sequence analysis. Journal of Clinical Microbiology **36**:918-925.

27. **van der Werf, T. S., T. Stinear, Y. Stienstra, W. T. A. van der Graaf, and P. L. Small**. 2003. Mycolactones and Mycobacterium ulcerans disease. Lancet **362**:1062-1064.

28. **Yeboah-Manu, D., T. Bodmer, E. Mensah-Quainoo, S. Owusu, D. Ofori-Adjei, and G. Pluschke**. 2004. Evaluation of decontamination methods and growth media for primary isolation of Mycobacterium ulcerans from surgical specimens. J.Clin.Microbiol. **42**:5875-5876.

Chapter 6: Identification of Streptomycin and Rifampicin Resistance *M. ulcerans* isolates in Ghana

Dorothy Yeboah-Manu[1], Adwoa Asante-Poku[1], Kwaku Owusu-Darko[1], Ernestina Mensah-Quainoo[2], Markus Hilty[3], David Ofori-Adjei[1], Gerd Pluschke[3]

[1] *Noguchi Memorial Institute for Medical Research, University of Ghana, Legon,*

[2] *Ghana Health Service, Amasaman, Ga District, Ghana*

[3] *Swiss Tropical Institute, Molecular Immunology, CH 4002 Basel, Switzerland*

Abstract

Buruli ulcer caused by *Mycobacterium ulcerans* is of important public health problem especially in West-Africa. It is the third most common mycobacterial disease, after tuberculosis and leprosy. The World Health Organisation currently recommends the use of a combination of streptomycin and rifampicin for eight weeks before surgical excision. Stepwise implementation of this recommendation has started in some of the worst affected West African countries, including Ghana. Therefore this study was initiated to assess the susceptibility of *M. ulcerans* isolates from Ghana to isoniazid, rifampicin, ethambutol and streptomycin, using a microplate Alamar blue assay. Out of the 28 isolates tested, one was resistant to all four drugs analysed. All isolates with the exception of two (7.1%) and three (10.7%) were resistant to isoniazid and ethambutol respectively. On the contrary all isolates, except two (7.1%) and three (10.7%), were susceptible to rifampicin and streptomycin, respectively. This first report of rifampicin resistance in clinical *M. ulcerans* isolates raises major concern about antibiotic treatment modalities for Buruli ulcer.

Introduction

Buruli ulcer, caused by *M. ulcerans* disease is increasingly becoming an important public health problem in the tropics especially in West-Africa with thousands of reported cases (1). While it is the third most common mycobacterial disease in the world, in some of the worst affected communities in this region, it is the leading mycobacterial disease. In some villages in West Africa, the disease affects more than 20% of the inhabitants (2). Most of the patients are poor rural dwellers and about 70% of the affected are children under the age of 15 years with limited access to health services (2,3). A number of social issues such as fear of anaesthesia and surgery, distance to health centre with surgical facilities, economic burden on families due to the long stay in hospitals delays health seeking (3,4). Due to this majority of patients in endemic communities in Africa presents with extensive lesions that cause severe disabilities (3). Various studies estimates that between 25 and 66% persons healed of ulcers had chronic functional disabilities (2,5).

The standard treatment of BU is surgical excision followed by skin grafting, because the available antimycobacterial compounds have been considered ineffective. The main objective of treatment in Buruli ulcer is to stop the progression of infection and healing of lesions (2). Surgical treatment requires wide excision including margins of healthy tissue to prevent relapse due to subcutaneous infection from left over bacilli. In addition, it is not always successful, and relapse rates between 6-47% have been reported (2,6,7).

A combinational treatment with aminoglycosides and rifampicin has shown good efficacy in *M. ulcerans* infection animal model studies (8,9). Supported by results of a clinical trial in Ghana (10) and ongoing clinical studies in several countries, the current WHO guidelines, require that a patient is treated with a combination of rifampicin and streptomycin for 8 weeks before surgery. This is expected to reduce the indication for surgery, extent of surgery and relapse rates. Surveillance of drug resistance in endemic countries is essential to determine the existing level and nature of the drug resistance problem.

The commonly used agar proportion method for mycobacterial antibiotic susceptibility testing requires 8 weeks of incubation before a pattern of susceptibility is established for *M. ulcerans* (11). The duration of the assay can be shortened by the use of the BACTEC radiometric system to around 10 days (12). However the BACTEC method is expensive and uses radioactivity, which makes is out reach for endemic countries with limited resources. The ability to detect mycobacterial growth by identification of the colour changes associated with the reduction of nitrate to nitrite is the basis for the microplate Alamar Blue drug susceptibility assay (MABA). MABA has been used previously in drug-susceptibility testing of *M. tuberculosis* to antituberculosis drugs. Very good correlations with the proportional and BACTEC methods have been described (13,14). Alamar blue is a resarzurin-based oxidation-reduction indicator which measures colorimetric drug MICs for *M. tuberculosis* for up to 7 days. MICs are determined by using serial dilutions of anti-TB drugs in 96-well microtiter plates, broth, and *M. tuberculosis* isolates. It is also less expensive, simpler to execute than the proportion method, and has a high through-put. This study had two objectives: i) to adapt the MABA assay for *M. ulcerans* and ii) to use this method to evaluate the susceptibility of *M. ulcerans* isolates to the recommended WHO antimycobacterial agents for treatment.

Materials and Methods

Mycobacterial isolates: Seven mycobacterial isolates obtained from pulmonary TB patients attending a teaching hospital in Ghana were used for the establishment of the MABA assay in our laboratory. Of the seven isolates, six were identified as belonging to the *M. tuberculosis* complex (MTC) and one was MOTT by conventional biochemical assays-niacin production, nitrate reduction and sensitivity to thiophen-2-carboxylic acid hydrazide and p-nitrobenzoic acid. The drug susceptibility patterns of all seven isolates to rifampicin, isoniazid, streptomycin and ethambutol have already been tested with the standard proportional method (15). This was used as the gold standard procedure for the establishment of the microplate Alamar blue assay (MABA).

Twenty-eight strains of *M. ulcerans* analyzed in this study came from a collection of *M. ulcerans* isolates generated in our laboratory between 2001 and 2005 (table 1). Preliminary identification of isolates was done by analyzing the rate of growth, colonial morphology and acid-fastness by the ZN staining. All isolates were confirmed by the presence of the insertion sequence *IS2404* in a PCR reaction (16).

Preparation of bacterial suspension for MABA: Mycobacterial isolates used were first subcultured on LJ medium and incubated at 37°C and 31°C for *M tuberculosis* and *M. ulcerans* respectively. Mycobacterial suspensions were prepared by emulsify 2-3 loops of logarithmic growing mycobacteria with 0.5ml 7H9 medium supplemented with OADC and 0.05% tween 80 (7H9-T) in a screw capped tube containing eight glass beads. After dispersing the bacteria by minimal vortexing, the clumps in the suspension were allowed to settle for a while and the supernatant was transferred to a new tube. The turbidity of the supernatant was adjusted to McFarland 1 with 7H9 broth supplemented with casitone and OADC (7H9GC). In the case of *M. ulcerans*, the supernatant was passed through a filter of 0.8µm pore size to obtain a uniform suspension. This was diluted further by 1:25 for the MABA assay.

Drug Solutions: Rifampicin (RIF), isoniazid (INH) and streptomycin (STR) were purchased from Sigma Chemicals whilst ethambutol (EMB) was obtained from Aldrich-Fluka. Stock solutions of INH, EMB and STR were made by dissolving the powder in

deionised water, whilst that of rifampin was in dimethyl sulphoxide. After sterilising by filtration, the stocks were then divided into 1-ml aliquots and frozen at -70C. The stock solutions were thawed, and working dilutions were made by diluting the stock solution in 7H9GC medium. Fresh dilutions were made on each day that testing was performed.

Susceptibility testing with MABA: The MICs of each antibiotic were determined for each isolate. MABA susceptibility testing was performed according to the method of Franzblau *et al* (17) and Luna-Herrera *et al* (14) with slight modifications. Briefly serial dilutions of the tested drugs were made in a 96 well plate (falcon 3072), using the culturing broth as the diluent. We compared the performance of 7H9GC to 7H9-T, results of assays with 7H9GC were always a day or two ahead of 7H9-T and hence we used the former through out this study. The final drug concentration ranges were 2-0.062µg/ml; 1-0.031 µg/ml; 4-0.125µg/ml & 16-0.5µg/ml for RIF, INH, STR and EMB respectively. Then each test well was inoculated with 100µl of bacterial suspension of about 10^4 colony forming units. The plate was then covered with its cover and placed in sealable polyethylene bag before incubating at appropriate temperatures (37°C for TB isolates and 31°C for *M. ulcerans* isolates). For the TB isolates assays were read each morning on days 6-9 while for the *M. ulcerans*, it was read on day 7 and each other day, until day 17. The assays were read for colour change by visual examination by at least two people blindly for each assay. The colour change of the test sample was compared to that of a 1% mycobacterial suspension control well and the MIC was defined as the lowest concentration with a comparable blue colour as this control well.

Proportion Method: We repeated the susceptibility testing for all *M. ulcerans* isolates which showed resistance to rifampicin and or streptomycin by the indirect proportion method using the critical concentrations of 40 mg/ml for rifampin, 0.2 mg/ml for isoniazid (INH), 4 mg/ml for streptomycin, and 2 mg/ml for ethambutol. An isolate was considered susceptible to an anti-mycobacterial agent if the number of colonies that grew on the drug containing plate was < 1% of the number of colonies that grew on the drug-free control, intermediate resistance if the proportion is between 1 and 10%, and resistant if the proportion is > than 10% (18,19).

Results

Agreement between MABA and agar proportion method: Table 2 summarizes the results of the in vitro susceptibility of seven TB isolates using the proportion and MABA procedures. Of the seven isolates from TB patients, all six MTC strains were susceptible to rifampicin, while the MOTT strain was resistant in the proportion method. All the seven isolates were susceptible by the MABA procedure and the MIC was ≤0.062µg/ml. We repeated both procedures for the isolate (K91) with the discordant result; the result remained the same for both methodologies. It was again resistant by the proportion method and the MIC determined by MABA became 0.125µg/ml, higher compared to the previous value of ≤ 0.062µg/ml. Thus from our study the agreement between the two methods for the determination of susceptibility to rifampicin was 85.7%.

Four of the TB isolates were susceptible and the remaining three were found to be resistant by the agar proportion method to isoniazid. When we analyzed the isolates by MABA, all four susceptible strains had a MIC ≤ 0.031µg/ml. For the resistant isolates two had MIC>1µg/ml and the remaining had 0.5µg/ml. We repeated the isolate with the MIC of 0.5µg/ml and obtained the same result. Using the parameters by Yajko *et al*, (20) this was described as intermediate. For five of the streptomycin susceptible isolates by the proportion method, the MIC using MABA was between 0.125µg/ml and 0.25µg/ml. The MIC of the two resistant isolates was >4µg/ml. The agreement between the two methods for the analysis of in vitro activity of streptomycin against the TB isolates was 100%. All isolates tested were susceptible to ethambutol using the two methods. MICs as determined by MABA ranged between <0.5 and 4µg/ml. Five of the isolates had MIC of 1µg/ml, one had 0.5µg/ml and the remaining had 4µg/ml. Thus among the 28 individual susceptibility assays done with the seven isolates only two discordant result were found (Table 2). Hence the overall agreement between these methods was 92.8%.

Drug susceptibility pattern of *M. ulcerans* isolates: All 28 clinical *M. ulcerans* isolates from Ghana were susceptible to rifampicin with the exception of two isolates (NM050/05 and NM038/04). One of the strains was from Ga district and the other from the Amansie-West district respectively. The later was resistant to all the drugs tested. Most of the

isolates were resistant to INH (82.1%); two were susceptible whilst three had intermediate susceptibility. Out of the 28 isolates, 3 (10.7%) were susceptible, 1 (3.6) intermediate and 24 (85.7%) were resistant to ethambutol. 25 (89.2%) were susceptible, 2 (7.2%) were intermediate and 1(3.6%) was resistant to streptomycin. Thus out of the 28 isolates, 1 was resistant (3.6%) to all drugs, 1 susceptible (3.6%) to all drugs and the remaining were resistant to at least one drug. Majority (71.4%) were not susceptible to both isoniazid and ethambutol, and this we considered as having the normal pattern. When we compared the spectrum of antibiotics-resistance to VNTR genotypes (26), all the STR and RIF resistance strains had genotype 1.

Discussion

Conventionally the drug susceptibility pattern of mycobacterium species is determined by comparing the number of colonies that grow on a plate/tube of drug containing medium with the number of colonies that grow on a drug free control medium (21). Unfortunately this method is limited as a result of the time required to obtain the end result. With the slow growing *M. ulcerans*, it takes about eight weeks to establish a susceptibility pattern (11). In addition it is labour intensive. Several rapid methods have been established for susceptibility testing in *M. tuberculosis*. These methods include: 1) detection of mutations in specific genetic markers (22) 2) flow cytometric analysis (23) and 3) BACTEC radiometric analysis (12). These methods are also limited for use in developing countries as the reagents are expensive and also require elaborate infrastructure. In this study we adapted the microplate Alamar blue assay for the determination of drug susceptibility of *M. ulcerans* isolates. Findings from the assays confirmed the rapidity of the assay and had a good correlation with the agar proportion method (14). It took 10 days to obtain the results of 70%, 14 days for almost 90% and 21 days for 100% of the 28 strains tested. The advantage of this assay is its simplicity in terms of infrastructure requirement as MICs were established visually.

With the exception of two and three isolates, all the isolates tested were resistant to isoniazid and ethambutol respectively and this finding is comparable with previous data. The phenotypic analysis of *M. ulcerans* isolates from different endemic regions revealed that about 80% of the African isolates were resistant to isoniazid (24). Also *in vitro* susceptibility studies showed that *M. ulcerans* is highly resistant to ethambutol (25). In contrast only two and three isolates were not susceptible to rifampicin and streptomycin respectively. This finding raises major concern regarding the recommended combination treatment of BU patients with rifampicin and streptomycin for eight weeks before surgical excision. This recommendation has not yet been established fully in most of the endemic countries. The identification of resistant strains in Ghana, which is one of the worst affected countries, represents a great threat to this recommendation. A major consideration is, that rifampicin and streptomycin are effective antituberculosis drugs. Incidentally, all the worst affected countries are endemic for tuberculosis and the above

antibiotics are essential for their respective DOTS programs. Most BU patients carry high bacterial burden at some stage of the disease, which is very crucial for the selection of drug resistant mutants. Furthermore, BU is a tropical disease that occurs normally in children and streptomycin has side effects in growing children when exposed to the sun. With the limited number of strains and different BU foci included in this study, the analysis must be expanded with isolates from other foci to establish the depth of the problem.

Facility	ID	Year Isolates	Age	Sex	District Of Origin	Patient Class	Site Of Lesion	Type Of Lesion
AHC	NM012/01	2001	19	M	Ga	Recurrent	Lower limb	Nodule
AHC	NM014/01	2001	21	M	Ga	New	Abdomen	Ulcer
AHC	NM018/02	2002	39	F	Ga	New	Upper limb	Nodule
AHC	NM023/02	2002	10	F	Ga	New	Lower limb	Plaque
AHC	NM027/02	2002	26	F	Ga	New	Upper limb	Plaque
AHC	NM028/02	2002	4	M	Ga	New	Upper limb	Nodule
AHC	NM030/02	2002	10	F	Ga	New	Upper limb	Plaque
AHC	NM031/02	2002	16	F	Ga	New	Upper limb	Plaque
AHC	NM033/02	2002	10	F	Ga	New	Lower limb	Nodule
AHC	NM037/02	2002	38	F	Ga	New	Upper limb	plaque
AHC	NM038/02	2002	11	F	Ga	Recurrent	Upper limb	Nodule
AHC	NM040/02	2002	75	M	Ga	New	Upper limb	Plaque
AHC	NM050/02	2002	8	M	Ga	New	Lower limb	Ulcer
AHC	NM051/02	2002	9	F	Ga	Recurrent	Lower limb	nodule
AHC	NM049/05	2005	40	M	Ga	New	Lower limb	nodule
AHC	NM050/05	2005	10	F	Ga	New	Lower limb	Ulcer
AHC	NM051/05	2005	4	M	Ga	New	Upper limb	ulcer
AHC	NM053/05	2005	4	M	Ga	New	Lower limb	plaque
AHC	NM054/05	2005	17	M	Ga	New	Upper limb	ulcer
AHC	NM057/05	2005	16	F	Ga	new	Lower limb	ulcer
AHC	NMO59/05	2005	4	M	Ga	New	Upper limb	ulcer
AHC	NM061/05	2005	4	M	Ga	New	Abdomen	Nodule
AHC	NM063/05	2005	3	M	Ga	New	Upper limb	Ulcer
AHC	NM065/05	2005	41	F	Ga	New	Lower limb	Ulcer
SMH	NM022/04	2004	7	F	AW	New	Neck	plaque
SMH	NM031/04	2004	12	F	AW	New	Upper limb	Nodule
SMH	NM037/04	2004	17	F	AAN	New	Lower limb	Nodule
SMH	NM038/04	2004	5	F	AW	new	Lower limb	Plaque

Table 1: the clinical isolates we used in this study. AHC=Amasaman Health Centre, SMH= Saint Martin Hospital, Agroyesum, AW=Amansie West and AAN=Ahafo-Ano North

Isolate	MIC (µg/ml)				MABA				Proportional Method			
	RIF	INH	STR	EMB	RIF	INH	STR	EMB	RIF	INH	STR	EMB
KB1	≤0.062	>1	>4	1	S	R	R	S	S	R	R	S
K3	≤0.062	≤0.031	≤0.125	1	S	S	S	S	S	S	S	S
K4	≤0.062	≤0.031	≤0.125	0.5	S	S	S	S	S	S	S	S
K6	≤0.062	≤0.031	≤0.125	1	S	S	S	S	S	S	S	S
K68	≤0.062	≤0.031	0.25	1	S	S	S	S	S	S	S	S
K91	≤0.062	>1	>4	4	S	R	R	S	R	R	R	S
K97	≤0.062	0.5	0.25	1	S	I	S	S	S	R	S	S

Table 2: Susceptibility pattern of clinical pulmonary tuberculosis' isolates using the microplate alamar blue assay (MABA) and the indirect proportional method. The MIC values were obtained from the MABA assay.

S= Sensitive, R= Resistance

ID	SUSCEPTIBILITY			
	RIF	INH	STR	EMB
NM012/01	S	R	I	R
NM014/01	S	R	S	R
NM018/02	S	R	S	R
NM023/02	S	R	S	R
NM027/02	S	R	S	R
NM028/02	S	R	S	R
NM030/02	S	R	S	R
NM031/02	S	R	S	R
NM033/02	S	R	S	R
NM037/02	S	R	S	R
NM038/02	S	R	S	R
NM040/02	S	R	S	S
NM050/02	S	I	S	I
NM051/02	S	R	S	R
NM049/04	S	R	S	R
NM050/05	R	I	S	S
NM051/05	S	I	I	R
NM053/05	S	R	S	R
NM054/05	S	R	S	R
NM057/05	S	S	S	R
NMO59/05	S	R	S	R
NM061/05	S	R	S	R
NM063/05	S	R	S	R
NM065/05	S	S	S	S
NM022/04	S	R	S	R
NM031/04	S	R	S	R
NM037/04	S	R	S	R
NM038/04	R	R	R	R

Table 3: Results of susceptibility tests for 26 isolates of Mycobacterium tuberculosis exposed to isoniazid (INH), rifampicin (RIF) and ethambutol (EMB) by microplate alamar blue assay.

Reference List

1. **Sizaire, V., F. Nackers, E. Comte, and F. Portaels**. 2006. Mycobacterium ulcerans infection: control, diagnosis, and treatment. Lancet Infect.Dis **6**:288-296.

2. **Johnson, P. D., T. Stinear, P. L. Small, G. Pluschke, R. W. Merritt, F. Portaels, K. Huygen, J. A. Hayman, and K. Asiedu**. 2005. Buruli ulcer (M. ulcerans infection): new insights, new hope for disease control. PLoS.Med. **2**:e108.

3. **Stienstra, Y., W. T. van der Graaf, K. Asamoa, and T. S. van der Werf**. 2002. Beliefs and attitudes toward Buruli ulcer in Ghana. Am.J.Trop.Med.Hyg. **67**:207-213.

4. **Sizaire, V., F. Nackers, E. Comte, and F. Portaels**. 2006. Mycobacterium ulcerans infection: control, diagnosis, and treatment. Lancet Infect.Dis **6**:288-296.

5. **Asiedu, K. and S. Etuaful**. 1998. Socioeconomic implications of Buruli ulcer in Ghana: a three-year review. Am.J.Trop.Med.Hyg. **59**:1015-1022.

6. **Bretzel, G., V. Siegmund, P. Racz, F. Vloten, F. Ngos, W. Thompson, P. Biason, O. Adjei, B. Fleischer, and J. Nitschke**. 2005. Post-surgical assessment of excised tissue from patients with Buruli ulcer disease: progression of infection in macroscopically healthy tissue. Tropical Medicine and International Health **10**:1199-1206.

7. **Kanga, J. M., D. E. Kacou, A. Sangare, Y. Dabila, N. H. Asse, and S. Djakeaux**. 2003. [Recurrence after surgical treatment of Buruli ulcer in Cote d'Ivoire]. Bull.Soc.Pathol.Exot. **96**:406-409.

8. **Dega, H., J. Robert, P. Bonnafous, V. Jarlier, and J. Grosset**. 2000. Activities of several antimicrobials against Mycobacterium ulcerans infection in mice. Antimicrob.Agents Chemother. **44**:2367-2372.

9. **Dega, H., A. Bentoucha, J. Robert, V. Jarlier, and J. Grosset**. 2002. Bactericidal activity of rifampin-amikacin against Mycobacterium ulcerans in mice. Antimicrob.Agents Chemother. **46**:3193-3196.

10. **Etuaful, S., B. Carbonnelle, J. Grosset, S. Lucas, C. Horsfield, R. Phillips, M. Evans, D. Ofori-Adjei, E. Klustse, J. Owusu-Boateng, G. K. Amedofu, P. Awuah, E. Ampadu, G. Amofah, K. Asiedu, and M. Wansbrough-Jones**. 2005. Efficacy of the combination rifampin-streptomycin in preventing growth of Mycobacterium ulcerans in early lesions of Buruli ulcer in humans. Antimicrob.Agents Chemother. **49**:3182-3186.

11. **Thangaraj, H. S., O. Adjei, B. W. Allen, F. Portaels, M. R. Evans, D. K. Banerjee, and M. H. Wansbrough-Jones**. 2000. In vitro activity of ciprofloxacin, sparfloxacin, ofloxacin, amikacin and rifampicin against Ghanaian isolates of Mycobacterium ulcerans. J.Antimicrob.Chemother. **45**:231-233.

12. **Dhople, A. M. and K. Namba**. 2002. In vitro activity of sitafloxacin (DU-6859a) alone, or in combination with rifampicin, against Mycobacterium ulcerans. J.Antimicrob.Chemother. **50**:727-729.

13. **Collins, L. and S. G. Franzblau**. 1997. Microplate alamar blue assay versus BACTEC 460 system for high-throughput screening of compounds against Mycobacterium tuberculosis and Mycobacterium avium. Antimicrob.Agents Chemother. **41**:1004-1009.

14. **Luna-Herrera, J., G. Martinez-Cabrera, R. Parra-Maldonado, J. A. Enciso-Moreno, J. Torres-Lopez, F. Quesada-Pascual, R. Delgadillo-Polanco, and S. G. Franzblau**. 2003. Use of receiver operating characteristic curves to assess the performance of a microdilution assay for determination of drug susceptibility of clinical isolates of Mycobacterium tuberculosis. Eur.J.Clin.Microbiol.Infect.Dis. **22**:21-27.

15. **CANETTI, G., S. FROMAN, J. Grosset, P. HAUDUROY, M. LANGEROVA, H. T. MAHLER, G. MEISSNER, D. A. MITCHISON, and L. SULA**. 1963. MYCOBACTERIA: LABORATORY METHODS FOR TESTING DRUG SENSITIVITY AND RESISTANCE. Bull.World Health Organ **29**:565-578.

16. **Ross, B. C., L. Marino, F. Oppedisano, R. Edwards, R. M. Robins-Browne, and P. D. Johnson**. 1997. Development of a PCR assay for rapid diagnosis of Mycobacterium ulcerans infection. J.Clin.Microbiol. **35**:1696-1700.

17. **Franzblau, S. G., R. S. Witzig, J. C. McLaughlin, P. Torres, G. Madico, A. Hernandez, M. T. Degnan, M. B. Cook, V. K. Quenzer, R. M. Ferguson, and R. H. Gilman**. 1998. Rapid, low-technology MIC determination with clinical Mycobacterium tuberculosis isolates by using the microplate Alamar Blue assay. J.Clin.Microbiol. **36**:362-366.

18. **CANETTI, G., N. RIST, and J. Grosset**. 1963. [Measurement of sensitivity of the tuberculous bacillus to antibacillary drugs by the method of proportions. Methodology, resistance criteria, results and interpretation.]. Rev.Tuberc.Pneumol.(Paris) **27**:217-272.

19. **Yajko, D. M., J. J. Madej, M. V. Lancaster, C. A. Sanders, V. L. Cawthon, B. Gee, A. Babst, and W. K. Hadley**. 1995. Colorimetric method for determining MICs of antimicrobial agents for Mycobacterium tuberculosis. J.Clin.Microbiol. **33**:2324-2327.

20. **Yajko, D. M., J. J. Madej, M. V. Lancaster, C. A. Sanders, V. L. Cawthon, B. Gee, A. Babst, and W. K. Hadley**. 1995. Colorimetric method for determining MICs of antimicrobial agents for Mycobacterium tuberculosis. J.Clin.Microbiol. **33**:2324-2327.

21. **CANETTI, G., N. RIST, and J. Grosset**. 1964. PRIMARY DRUG RESISTANCE IN TUBERCULOSIS. Am.Rev.Respir Dis **90**:792-799.

22. **Drobniewski, F. A. and S. M. Wilson**. 1998. The rapid diagnosis of isoniazid and rifampicin resistance in Mycobacterium tuberculosis--a molecular story. J.Med.Microbiol. **47**:189-196.

23. **Reis, R. S., I. Neves, Jr., S. L. Lourenco, L. S. Fonseca, and M. C. Lourenco**. 2004. Comparison of flow cytometric and Alamar Blue tests with the proportional method for testing susceptibility of Mycobacterium tuberculosis to rifampin and isoniazid. J.Clin.Microbiol. **42**:2247-2248.

24. **Portaels, F., P. A. Fonteyene, H. De Beenhouwer, P. de Rijk, A. Guedenon, J. Hayman, and M. W. Meyers**. 1996. Variability in 3' end of 16S rRNA sequence of Mycobacterium ulcerans is related to geographic origin of isolates. J.Clin.Microbiol. **34**:962-965.

25. **Portaels, F., H. Traore, K. De Ridder, and W. M. Meyers**. 1998. In vitro susceptibility of Mycobacterium ulcerans to clarithromycin. Antimicrob.Agents Chemother. **42**:2070-2073.

26. **Hilty, M., D. Yeboah-Manu, D. Boakye, E. Mensah-Quainoo, S. Rondini, E. Schelling, D. Ofori-Adjei, F. Portaels, J. Zinsstag, and G. Pluschke**. 2006. Genetic diversity in Mycobacterium ulcerans isolates from Ghana revealed by a newly identified locus containing a variable number of tandem repeats. J.Bacteriol **188**:1462-1465.

Chapter 7: Systemic suppression of interferon-gamma responses in Buruli ulcer patients resolves after surgical excision of the lesions caused by the extracellular pathogen *Mycobacterium ulcerans*

Dorothy Yeboah-Manu[†,*] Elisabetta Peduzzi,[†] Ernestina Mensah-Quainoo,[‡] Adwoa Asante-Poku,* David Ofori-Adjei,* Gerd Pluschke,[†] and Claudia A. Daubenberger[†, 1]

Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, Ghana

[†] *Swiss Tropical Institute, Molecular Immunology, CH 4002 Basel, Switzerland*

[‡] *Ghana Health Service, Amasaman, Ga District, Ghana*

Abstract

Buruli ulcer (BU), caused by *Mycobacterium ulcerans*, is the third most common mycobacterial infection in immuno-competent humans besides tuberculosis and leprosy. We have compared by *ex vivo* ELISpot analysis interferon-gamma (INF-γ) responses in peripheral blood mononuclear cells (PBMC) from BU patients, house-hold contacts and individuals living in an adjacent *M. ulcerans* non-endemic region. PBMC were stimulated with PPD and non-mycobacterial antigens like reconstituted influenza virus particles and isopentenyl-pyrophosphate. With all three antigens the number of INF-γ spot forming units was significantly reduced in BU patients compared to the controls from a non-endemic area. This demonstrates for the first time that *M. ulcerans* infection-associated systemic reduction in INF-γ responses is not confined to stimulation with live or dead mycobacteria and their products, but extends to other antigens. IL-12 secretion by PPD stimulated PBMC was not reduced in BU patients, indicating that reduction in INF-γ responses was not caused by diminished IL-12 production. Several months after surgical excision of BU lesions, INF-γ responses of BU patients against all antigens used for stimulation recovered significantly, indicating that the measured systemic immuno-suppression was not the consequence of a genetic defect in T cell function predisposing for BU but is rather related to the presence of *M. ulcerans* bacteria.

Introduction

BU caused by *M. ulcerans* is an infectious disease characterized by chronic, necrotizing ulceration of subcutaneous tissues and the overlying skin. The disease starts as a subcutaneous nodule, papule or plaque that eventually ulcerates and progresses over weeks to months until surgical excision or spontaneous healing occurs [1]. After tuberculosis and leprosy BU is the third most common mycobacterial infection in immuno-competent humans [2]. The main burden of disease falls on children living in sub-Saharan Africa but healthy people of all ages, races and socio-economic class are susceptible [3]. The effectiveness of anti-mycobacterial drug therapy has not been proven [3]. Consequently, surgery is presently the recommended treatment option [4]. In BU lesions clumps of extra-cellular acid-fast organisms surrounded by areas of necrosis are found particularly in subcutaneous fat tissue [5]. *M. ulcerans* produces a family of macrolide toxin molecules, the mycolactones, which are associated with tissue destruction and local immunosuppression [6]. In cell culture experiments mycolactones produce apoptosis and necrosis in many human cell types [7;8]. The toxin appears to play a role in inhibiting the recruitment of inflammatory cells to the site of infection, which explains at least in part why inflammatory responses are poor in BU lesions [5]. However, intra-lesional influx of leukocytes and granulomatous responses in the dermis and panniculus has been reported in late stages of the disease [9] [10]. Spontaneous healing can occur and is often accompanied by a conversion of the Burulin (*M. ulcerans* sonicate) skin test from negative to positive. However, the immune mechanisms involved in protection against BU are largely unknown.

The importance of INF-γ for immunity against mycobacterial infections in humans is demonstrated by the increased susceptibility of children carrying complete INF-γR1 chain deficiency to environmental mycobacterial infection [11]. Apart from CD4^{+} T cells, γδ T cells, natural killer cells and CD8^{+} T cells are potent sources of IFN-γ. CD4 T cells can be differentiated into Th1 and Th2, distinguished by their patterns of cytokine production after antigen activation. Apart from other cytokines, Th1 cells preferentially secrete INF-γ, while Th2 cells preferentially secrete IL-4 and IL-5. Th1 or Th2 development is determined by the cytokine environment during T cell activation in the

primary response to antigen with IL-12 and INF-γ implicated in the decision to adopt a Th1 phenotype [12]. INF-γ binds to the INF-γR1/INF-γR2 receptor complex and stimulates innate cell-mediated immunity through NK cells and activation of bactericidal mechanisms in macrophages. The central role of INF-γ in MHC class I and class II restricted antigen processing and presentation is well documented [13]. At present, the contribution of INF-γ in immunity to extra-cellular *M. ulcerans* remains to be established.

Peripheral blood mononuclear cells (PBMC) of BU patients with active disease showed significantly reduced lympho-proliferation and INF-γ secretion in response to stimulation with live or killed preparations of *M. bovis*, *M. ulcerans*, *M. tuberculosis* and the recombinant protein Ag85 of *M. tuberculosis* [14-18]. Here we have determined in a cross-sectional study the frequency of INF-γ secreting cells in PBMC from BU patients, their household contacts and controls from BU non-endemic areas by *ex vivo* ELISpot analysis. The antigens used for stimulation included isopentenyl-pyrophosphate (IPP), reconstituted influenza virus particles (virosomes) and tuberculin purified protein derivative (PPD) of *M. tuberculosis*, which stimulate distinct T cell subsets, like Vγ2Vδ2 T cells [19], CD4 T cells [20], CD4 and Vγ2Vδ2 T cells [21], respectively. Results demonstrate that BU associated reductions in INF-γ responses are not confined to stimulation with live or dead mycobacteria and mycobacterial antigens. Furthermore, it is shown for the first time that in individual BU patients this suppression in INF-γ secretion improved in a time interval of 5 to 10 months.

Materials and Methods

Study population

Thirteen BU patients, 19 clinically healthy household contacts that never had clinical BU and 18 healthy persons living in a *M. ulcerans* non-endemic districts in the Greater Accra region of Ghana were enrolled for the study (Table I). All BU patients enrolled were residents of the BU-endemic Ga District and presented with pre-ulcerative or ulcerative lesions at the Amasaman Health Centre. Informed consent was obtained from study participants or their parents or guardians before enrollment. For ethical reasons, the age range of the non-exposed controls that were included into the analysis was higher than that of the BU patients. Ethical approval for the study was obtained from the local ethical review board of the Noguchi Memorial Institute for Medical Research, University of Ghana, Legon. Clinical diagnosis of BU was reconfirmed as described [22;23] by one or more laboratory verification tests (Table II) including culture of *M. ulcerans*, microscopic detection of Acid Fast Bacilli (AFB) or IS*2404* PCR. The clinical pictures of the patients ranged from nodules or plaques to severe ulcerative forms (Table II). In the BU group nine persons and in the other two groups each 10 persons with a BCG scar were recruited (Table I). PBMC were isolated from venous peripheral blood using Ficoll-Hypaque gradient centrifugation following standard procedures and cryo-preserved prior to the analysis.

Antigens

After thawing PBMC were directly stimulated with 50 μM isopentenyl pyrophosphate (IPP; Sigma), 10 μg/ml tuberculin purified protein derivative of *M. tuberculosis* (PPD; Statens Seruminstitut, Denmark), 10 μg/ml PHA (Sigma) or 20 μg/ml reconstituted influenza virus particles (IRIV, Berna Biotech). These influenza virosomes are spherical uni-lamellar vesicles prepared by detergent removal from influenza surface glycoproteins and mixtures of natural and synthetic phospholipids containing H1N1 from influenza virus strain A/Singapore/6/86 [24]. Cell culture medium consisted of RPMI 1640, 10 % heat inactivated human AB serum, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco-BRL).

Ex vivo INF-γ ELISpot análisis

PBMC were thawed, washed and suspended at a concentration of 2 x 10^6 cells/ml in complete cell culture medium. The cells were then stimulated with the different antigens at the final concentrations indicated above and incubated for 24 h at 37°C, 5 % CO_2 humidified atmosphere. A 96-well nitrocellulose-bottomed plate (Millipore) was coated overnight at 4°C with 10μg/ml anti-human-INFγ primary antibody (clone1-D1K; Mabtech, Sweden). The plates were then washed five times with PBS and blocked with cell culture medium for 1 h at room temperature. The medium was decanted and pre-incubated cells (2 x 10^5 or 1 x 10^5) were added to each well in triplicate and incubated at 37 °C for another 20 h. Assays were terminated by washing plates three times with PBS-Tween 80 (0.05 %) followed by PBS another three times. Secondary antibody (biotin-labeled anti-human-INF-γ; clone 7-B6-1) was added to each well at 1μg/ml and the plate incubated for 2 h at RT. Plates were washed again six times with PBS before application of streptavidine-alkaline phosphatase (1 : 1000 dilution in PBS, 0,5 % FCS, 100 µl/well) for 1 h at RT. After washing wells seven times with PBS, distinct spots were developed by the incubation of plates at RT for 4 to 10 min following the addition of the developing buffer (BCIP/NBT, diluted 1 : 100, Bio-Rad). Spot development was stopped by washing the plates extensively with water and left to dry. Plates were later evaluated using the ELISpot Reader system (AID, Germany) to determine the number of spot forming units (SFU).

ELISA quantification of IL-12 in cell culture supernatants

 Levels of total IL-12 in culture supernatant of PBMC incubated with PHA (10 µg/ml) and PPD (10 µg/ml) for 96 h were determined by ELISA employing a commercial kit (Mabtech, Sweden). Samples were analyzed in triplicates and results expressed as the average of the three readings in an ELISA reader at 450 nm with reference to curves generated using serially diluted recombinant human IL-12. The sensitivity of the assay was 30 pg/ml for total IL-12.

Statistical analysis

Data were analyzed using the STATA program (Stata Corporation). Comparisons among the paired samples were performed using the Wilcoxon signed-rank test whilst the wilcoxon rank-sum test was used to analyze the significance of observed difference in IL-12 secretion between patients and un-exposed controls. For comparing the frequencies of antigen specific IFN-γ secreting cells, the data was transformed to normality using Box-Cox transformation before analyzed by linear regression. Data were considered statistically significant when $P < 0.05$.

Results

Frequency reduction of INF-γ secreting spot forming units in PBMC from BU patients

 Thirteen patients with laboratory-reconfirmed *M. ulcerans* infection, nineteen clinically healthy household contacts and eighteen individuals from a neighboring BU non-endemic area were enrolled for this study (Table I and Table II). The frequency of immediate INF-γ secreting cells in PBMC upon stimulation with IPP, IRIV and PPD was analyzed using an *ex vivo* ELISpot assay. Fig. 1 shows that the mean of SFU upon stimulation with PPD, IPP and IRIV was significantly lower (p=0.0086, p=0.001 and p=0.0002, respectively) in BU patients compared to non-exposed controls. In household contacts, the mean of SFU after IPP and IRIV stimulation was significantly higher compared to BU patients (p=0.005 and p=0.001, respectively)   while in PPD stimulated cultures no significant difference was observed (p=0.82).

Recovery from systemic immuno-suppression after surgical treatment

All patients enrolled for the study were treated by wide surgical excision of the BU lesions. Blood samples were collected several months after the first sampling (Table 2) and PBMC from both time points were analyzed in parallel using the same INF-γ ELISpot assay as above. A 3.9, 3.7 and 3.6 fold median increase in cellular responses against IPP, IRIV and PPD stimulation, respectively, was observed when PBMC taken at the two time points were compared (Fig. 2A). Statistical analysis confirmed this increase of responses as highly significant with p-values of 0.021 and 0.003, respectively, after IPP and IRIV stimulation. PHA stimulated control wells showed only a slight (1.7 fold) median increase between the two different time points analyzed (p=0.17) and in PPD stimulated cultures the boost was not significant (p=0.09). One representative example of an ELISpot analysis is shown in Fig. 2B with the numbers of SFU detectable after IPP, IRIV and PPD stimulation rising between 1st and 2nd sampling. In contrast, medium and PHA control wells remained at a comparable level between the two analyses.

IL-12 production in PPD stimulated PBMC is not affected in BU patients

Next we wanted to determine whether the systemic suppression of INF-γ responses in BU patients is related to a diminished capacity to secrete IL-12. PBMC of nine patients and ten persons from BU non-endemic areas were stimulated with PHA or PPD and the total IL-12 concentration in cell culture supernatants was measured by ELISA (Fig. 3). Interestingly, the IL-12 concentrations in PPD stimulated PBMC of individuals living in *M. ulcerans* non-endemic regions were statistically lower compared to BU patients (p=0.011) while in PHA stimulated cultures no significant difference was observed (p=0.57) (Fig. 3). The mean IL-12 concentrations in supernatants of PPD and PHA stimulated cultures of PBMC obtained before or after surgical treatment showed no difference (Fig. 3).

Discussion

An *ex vivo* ELISpot assay for detection of INF-γ secretion permitting the direct detection of individual antigen-specific T cells at low frequencies was employed in the current study [25]. The 48 h antigen challenge of PBMC suffices to engage cytokine production in the memory/effector T lymphocyte but not in naïve T cells and allows determination of frequencies and cytokine signatures of re-circulating antigen-specific T cells [25]. The frequencies of systemic INF-γ producing SFU after stimulation with IPP, IRIV or PPD was significantly reduced in BU patients compared to individuals living in a neighboring BU non-endemic area. This result is consistent with other reports demonstrating that PBMC from subjects with past or current *M. ulcerans* disease had reduced INF-γ production in response to PPD of *M. tuberculosis* and *M. bovis* or whole killed *M. bovis* BCG or *M. ulcerans* [14-18]. However, our data strongly indicate that reduced INF-γ production is not confined to immune responses specific for mycobacterial antigens or whole mycobacteria but extends to CD4 T cell responses specific for influenza virus [20] and Vγ2Vδ2 T cells [19].

Reduced INF-γ production in BU patients could be the consequence of a genetic abnormality in T cell function predisposing for mycobacterial infections [11]. Therefore, we analyzed PBMC of BU patients sampled at two consecutive time points. When the paired PBMC samples were analyzed in parallel by ELISpot analysis, the numbers of INF-γ secreting cells after IPP, IRIV and PPD stimulation increased significantly after surgical treatment. Comparable numbers of SFU in PHA stimulated PBMC in paired samples excluded a general suppression of cellular immune responses in BU and variations in quality of PBMC sample cryo-preservation. To our knowledge, this is the first report demonstrating that antigen-specific INF-γ production in BU patients is coming back to normal levels after surgical treatment. Hence, confounding genetic defects do not seem to be responsible for the observed immuno-suppression. The question if INF-γ production in patients undergoing spontaneous healing of BU will also improve over time is not addressed here. IL-12 induces T and NK cells to produce several cytokines, including INF-γ [26]. Total IL-12 concentrations in culture supernatants of PPD stimulated PBMC were statistically

higher in BU patients (treated and untreated) than in the controls. This may reflect a compensatory mechanism of the immune system to restore INF-γ production and indicates that the observed reduction in systemic INF-γ responses is not caused by diminished IL-12 production. Different cytokine expression profiles in both PBMC and skin lesions were described in patients suffering from the nodular and ulcerative forms of BU. Nodules were associated with higher INF-γ and lower IL-10 production and BU with lower INF-γ and higher IL-10 production [17]. Within the limited number of BU patients enrolled in our study, a relationship between different stages of BU disease and reduction of INF-γ secretion was not observed.

Vγ2Vδ2 T cells compose the majority of human γδ T cells in circulation and among their defined ligands is IPP, a metabolite found in prokaryotic and eukaryotic cells including mycobacteria [27]. Studies in rhesus monkeys provided evidence that Vγ2Vδ2 T cells contribute to adaptive immune responses in mycobacterial infections [28]. A correlation between the absence or loss of Vγ2Vδ2 T cells and the extent of *M. tuberculosis* disease has been described [29]. An impaired ability of Vγ2Vδ2 T cells to produce cytokines or proliferate in response to phosphorylated microbial metabolites was observed in active *M. tuberculosis* pulmonary disease [30-32]. Interestingly, in 10 out of 13 BU patients analyzed here, the *ex vivo* INF-γ secretion of IPP-reactive Vγ2Vδ2 T cells increased significantly after surgical treatment.

The diffusible macrolide toxins produced by *M. ulcerans* are considered as virulence factors responsible for pathogenicity of *M. ulcerans* and it has been hypothesized that lack of inflammatory responses in BU lesions are related to local immuno-suppressive activities of the mycolactones [6;33;34]. The treatment-associated reversal of immune suppression may indicate that mycolactones exert apart from local also systemic effects. Alternatively, additional other immune-suppressive mechanisms may be operative in chronic *M. ulcerans* disease. PBMC from healthy contacts of TB patients and TB patients with limited disease produce large quantities of   INF-γ in response to whole mycobacteria, PPD of *M. tuberculosis*, ESAT-6, 16-kDa and 38 kDa proteins [35-39]. In contrast, PBMC of active TB patients with advanced disease produce low quantities of

INF-γ after similar stimulation and following effective drug treatment the INF-γ secretion improved [39;40]. Possible candidate structures mediating immune suppression in *M. tuberculosis* isolates like phenolic glycolipids have been described [41;42]. In leprosy induction of regulatory or suppressor T cells mediating immune suppression in affected hosts has been proposed [43;44]. Our findings, within their limits due to the small number of BU patients analyzed and the timing of the blood samples drawn, may indicate that mycolactone-independent immunosuppressive mechanisms common to chronic mycobacterial infections contribute to the reduction of systemic INF-γ responses in BU patients.

Acknowledgment

Characteristic	Buruli ulcer patient (n = 13)	Household contacts (n =19)	Controls from non-endemic area (n = 18)
Sex			
Male	5 (39%)	6 (32%)	8 (44%)
Female	8 (61%)	13 (68%)	10 (56%)
Median age (years)	15	15	29.5
Age range (years)	6 - 45	6 - 56	24 - 65
BCG scar present (%) [a]	69	52	55

Table I: Characteristics of BU patients, household contacts and controls from a BU non-endemic area enrolled in the study.

[a] % values calculated for 13 patients, 14 household contacts and 14 controls, respectively. Five household contacts and four non-exposed controls remained with uncertain scar status.

patient	age	gender	clinical form[a]	BCG status[b]	AFB[c]	culture[d]	PCR[e]	1st blood sample [f]	2nd blood sample [f]
P03	45	f	ulcerative	-	+	+	+	3 mo	11 mo
P04	9	f	plaque	+	-	+	+	0	8 mo
P05	10	m	nodule	+	+	+	+	0	8 mo
P08	16	m	ulcerative	-	+	+	+	4 mo	14 mo
P09	5	f	ulcerative	+	+	-	+	3 mo	12 mo
P11	20	f	ulcerative	+	+	+	+	7 d	7 mo
P12	16	m	ulcerative	+	-	-	+	3 mo	12 mo
P13	11	f	ulcerative	-	+	+	+	7 d	7 mo
P14	15	m	plaque	-	-	-	+	7 d	8 mo
P15	7	f	ulcerative	+	+	+	+	0	9 mo
P19	6	m	nodule	+	+	+	+	0	6 mo
P21	17	f	nodule	+	+	-	+	0	5 mo
P22	17	f	nodule	+	+	+	-	0	5 mo

Table II:

Clinical data of patients presenting with lesions due to confirmed *M. ulcerans* infection

[a] clinical forms of BU disease were graded according to the WHO case definition [1].
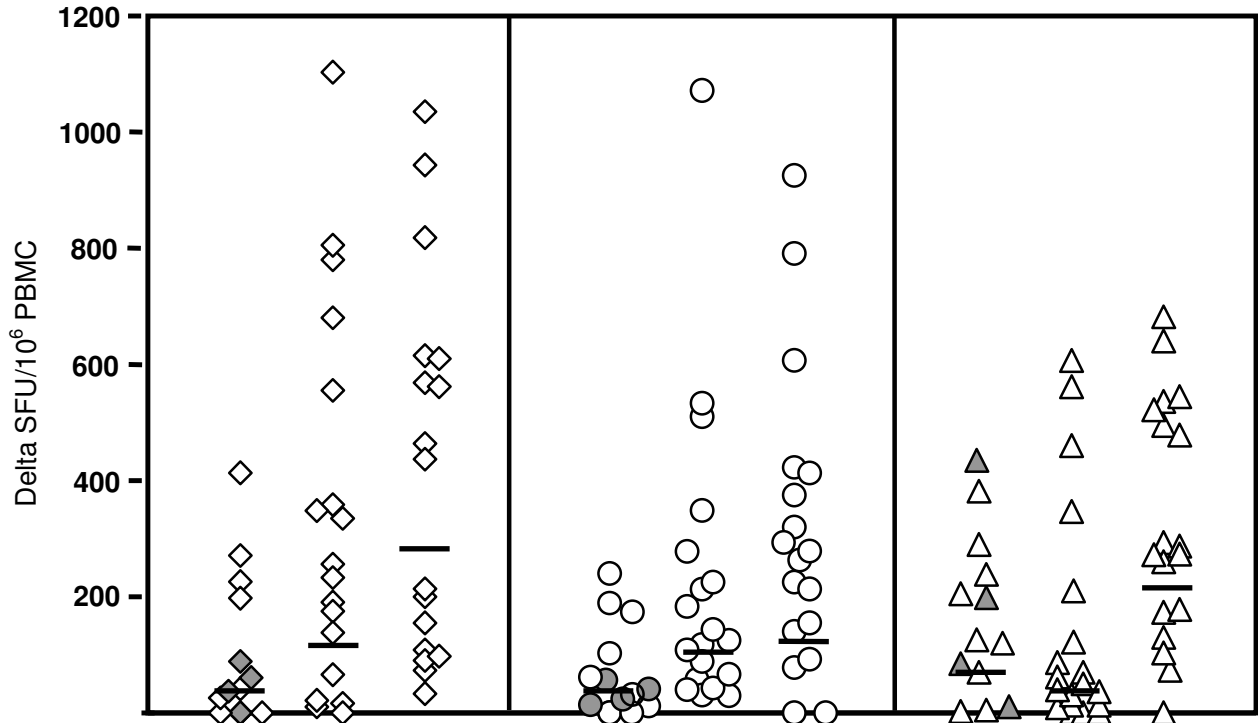
[b] BCG status was determined by confirmation of the presence of a BCG scar by two persons.

[c] Acid fast bacilli detection was performed by direct smearing of tissue exudates followed by Ziehl-Neelsen staining [1].

[d] Culture of *M. ulcerans* according to [22].

[e] IS2404 PCR analysis according to [23].

[f] Time of blood sample collection. Time 0 is time of surgical treatment.

Fig. 1.

Quantification of e*x vivo* INF-γ secreting cells by ELISpot analysis after stimulation of PBMC with IPP (◊), IRIV (○) and PPD (Δ).

PBMC derived from BU patients (BU), household contacts (HC) and controls from a BU non-endemic area (NC) were thawed into cell culture medium and kept overnight in the presence or absence of the different stimuli. For the detection of IFN-γ producing cells, 2 $\times 10^5$ PBMC/well were plated in triplicate wells and spots were developed after 24 h. Delta SFU = mean of SFU of stimulated triplicate cultures – mean SFU of un-stimulated triplicate cultures. Data from each individual analyzed are shown separately. Mean SFU/10^6 PBMC were 26 (6 – 102), 137 (57 – 327) and 262 (157 – 436) after stimulation with IPP in BU patients, HC and NC, respectively. In IRIV-stimulated cells, the mean SFU/10^6 cells were 22 (7 – 70), 135 (83 – 221), 149 (56 – 394) in BU patients, HC and NC, respectively. After PPD stimulation, 88 (35 – 223), 39 (14 – 106) and 212 (100 – 445) SFU/10^6 PBMC were recorded in BU patients, HC and NC, respectively. The values given in brackets are the 95 % confidence intervals of the geometric means of SFU. Data of patients P03, P08, P09 and P12 are shaded in grey.
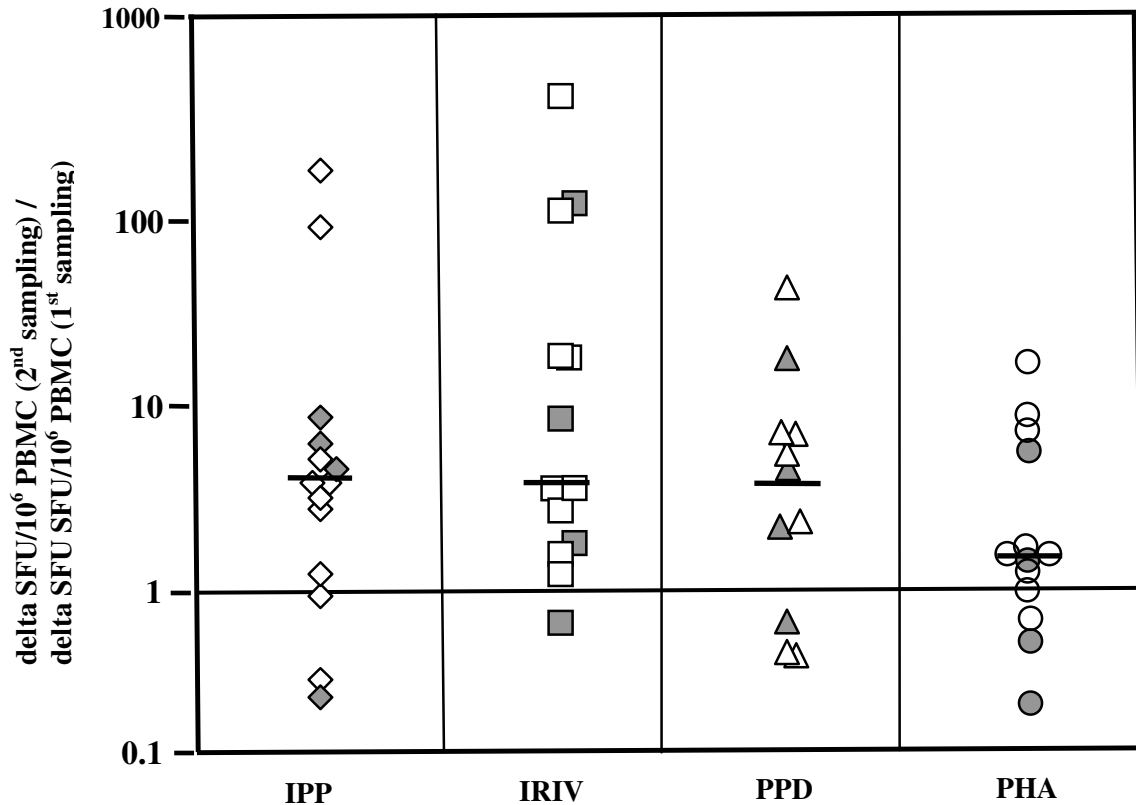
Fig. 2A.

Frequency increase of antigen-specific *ex vivo* INF-γ secreting cells in BU patients.

PBMC of thirteen BU patients sampled at two time points (Table II) were thawed into cell culture medium and kept overnight in the presence or absence of IPP (◊), IRIV (□), PPD (Δ) and PHA (○). For the detection of INF-γ producing cells, 2×10^5 PBMC/well were plated in triplicate wells and spots were developed after 24 h. Delta SFU were calculated as described in Fig. 1. Given is the ratio of SFU obtained after 2nd sampling / 1st sampling. Data of patients P03, P08, P09 and P12 are shaded in grey.
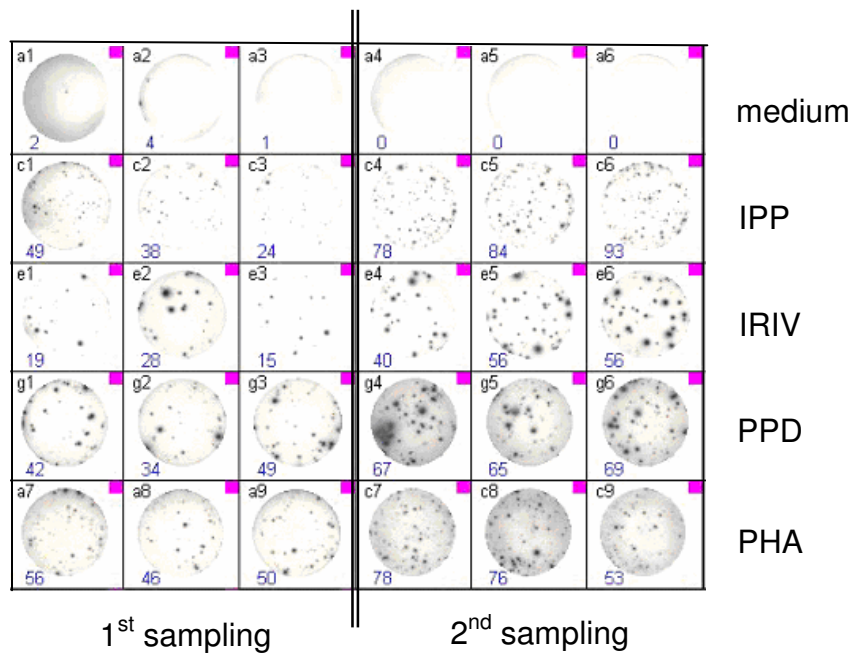
Fig. 2B.

Representative INF-γ ELISpot patterns of BU patient P21.

PBMCs of BU patient P21 were obtained immediately before surgical excision of the BU lesion (1st sampling) and 5 months later (2nd sampling). Parallel analysis of both samples was conducted in triplicates arranged horizontally with 2×10^{5} cells/well plated. The different stimulators used are given on the right and the numbers of SFU/well are shown on the lower left corner.
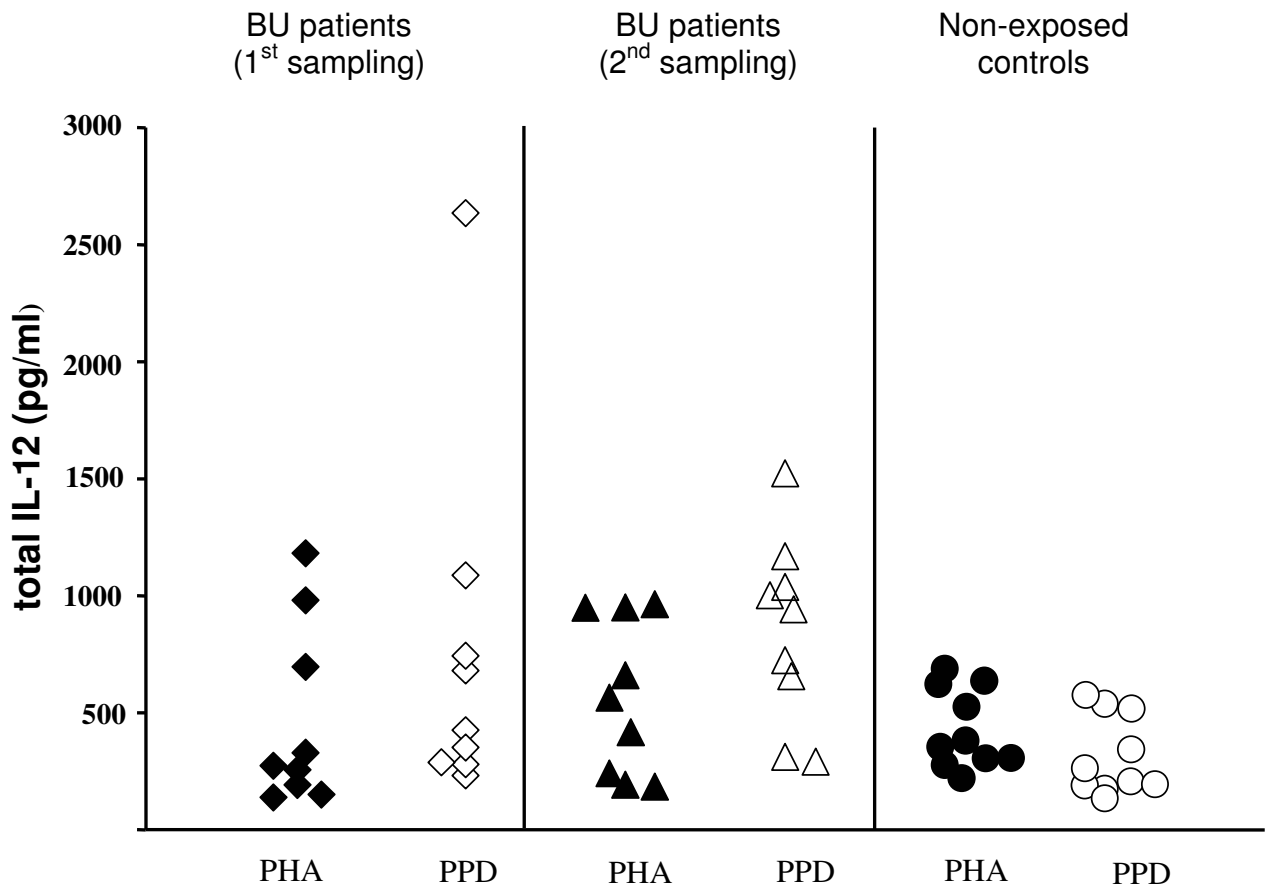
Fig. 3.

Total IL-12 concentrations in cell culture supernatants of PHA and PPD stimulated PBMC of nine BU patients obtained at the two different sampling time points (Table II). Mean of total IL-12 concentration (given in pg/ml) in PPD stimulated cultures was 543 and 750 after 1st and 2nd sampling, respectively, and in PHA stimulated cultures 348 and 472, respectively. PBMC of ten individuals living in a neighbouring *M. ulcerans* non-endemic region were treated similarly and the mean total IL-12 concentrations after PPD and PHA stimulation were 276 and 402, respectively.

Reference List

1. Portaels,F., Johnson,P.D., Meyers,W.M. (2001) Buruli ulcer: Diagnosis of *Mycobacterium ulcerans* disease. World Health Organization.

2. Asiedu,K., Scherpbier,R., Raviglione,M. (2000). Buruli ulcer: *Mycobacterium ulcerans* infection. World Health Organisation.

3. Johnson,P.D., Stinear,T., Small,P.L., Pluschke,G., Merrit,R.W., Portaels,F., Huygen,K., Hayman,J.A., Asiedu,K. (2005) Buruli ulcer (*Mycobacterium ulcerans* infection): new insights, new hope for disease control. *PLOSMedicine* **2**, 0282-0286.

4. Buntine,J., Crofts,K. (2001) Buruli ulcer: Management of *Mycobacterium ulcerans* disease. World Health Organization.

5. Hayman,J., McQueen,A. (1985) The pathology of Mycobacterium ulcerans infection. *Pathology* **17**, 594-600.

6. George,K.M., Chatterjee,D., Gunawardana,G., Welty,D., Hayman,J., Lee,R., Small,P.L. (1999) Mycolactone: a polyketide toxin from Mycobacterium ulcerans required for virulence. *Science* **283**, 854-857.

7. George,K.M., Pascopella,L., Welty,D.M., Small,P.L. (2000) A *Mycobacterium ulcerans* toxin, mycolactone, causes apoptosis in guinea pig ulcers and tissue culture cells. *Infect. Immun*. **68**, 877-883.

8. Dobos,K.M., Small,P.L., Deslauriers,M., Quinn,F.D., King,C.H. (2001) *Mycobacterium ulcerans* cytotoxicity in an adipose cell model. Infect. Immun. **69**, 7182-7186.

9. Dodge,O.G. (1964) Mycobacterial skin ulcers in uganda: histopathological and experimental aspects. *J. Pathol. Bacteriol.* **88**, 169-174.

10. Hayman,J. (1993) Out of Africa: observations on the histopathology of *Mycobacterium ulcerans* infection. *J. Clin.Pathol.* **46**, 5-9.

11. van de Vosse,E., Hoeve,M.A., Ottenhoff,T.H. (2004) Human genetics of intracellular infectious diseases: molecular and cellular immunity against mycobacteria and salmonellae. *Lancet Infect. Dis.* **4**, 739-749.

12. Carmen,I. (1999) Th1/Th2 balance in infection. *Springer Seminars in Immunopathology* **21**, 317-338.

13. Boehm,U., Klamp,T., Groot,M., Howard,J.C. (1997) Cellular responses to interferon-gamma. Annu.Rev.Immunol. **15**, 749-795.

14. Gooding,T.M., Johnson,P.D., Smith,M., Kemp,A.S., Robins-Browne,R.M. (2002) Cytokine profiles of patients infected with *Mycobacterium ulcerans* and unaffected household contacts. *Infect. Immun.* **70**, 5562-5567.

15. Gooding,T.M., Johnson,P.D., Campbell,D.E., Hayman,J.A., Hartland,E.L., Kemp,A.S., Robins-Browne,R.M. (2001) Immune response to infection with *Mycobacterium ulcerans*. *Infect. Immun.* **69**, 1704-1707.

16. Gooding,T.M., Kemp,A.S., Robins-Browne,R.M., Smith,M., Johnson,P.D. (2003) Acquired T-helper 1 lymphocyte anergy following infection with *Mycobacterium ulcerans*. *Clin.Infect. Dis.* **36**, 1076-1077.

17. Prevot,G., Bourreau,E., Pascalis,H., Pradinaud,R., Tanghe,A., Huygen,K., Launois,P. (2004) Differential production of systemic and intra-lesional gamma interferon and interleukin-10 in nodular and ulcerative forms of Buruli disease. *Infect. Immun.* **72**, 958-965.

18. Westenbrink,B.D., Stienstra,Y., Huitema,M.G., Thompson,W.A., Klutse,E.O., Ampadu,E.O., Boezen,H.M., Limburg,P.C., Van Der Werf,T.S. (2005) Cytokine responses to stimulation of whole blood from patients with buruli ulcer disease in Ghana. *Clin.Diagn.Lab. Immunol.* **12**, 125-129.

19. Wesch,D., Marx,S., Kabelitz,D. (1997) Comparative analysis of alpha beta and gamma delta T cell activation by *Mycobacterium tuberculosis* and isopentenyl pyrophosphate. *Eur.J. Immunol.* **27**, 952-956.

20. Schumacher,R., Adamina,M., Zurbriggen,R., Bolli,M., Padovan,E., Zajac,P., Heberer,M., Spagnoli,G.C. (2004) Influenza virosomes enhance class I restricted CTL induction through CD4+ T cell activation. *Vaccine* **22**, 714-723.

21. Green,A.E., Lissina,A., Hutchinson,S.L., Hewitt,R.E., Temple,B., James,D., Boulter,J.M., Price,D.A., Sewell,A.K. (2004) Recognition of non-peptide antigens by human V gamma 9V delta 2 T cells requires contact with cells of human origin. *Clin.Exp.Immunol.* **136**, 472-482.

22. Yeboah-Manu,D., Bodmer,T., Mensah-Quainoo,E., Owusu,S., Ofori-Adjei,D., Pluschke,G. (2004) Evaluation of decontamination methods and growth media for

primary isolation of *Mycobacterium ulcerans* from surgical specimens. *J. Clin.Microbiol.* **42**, 5875-5876.

23. Noeske,J., Kuaban,C., Rondini,S., Sorlin,P., Ciaffi,L., Mbuagbaw,J., Portaels,F., Pluschke,G. (2004) Buruli ulcer disease in Cameroon rediscovered. *Am.J Trop.Med.Hyg.* **70**, 520-526.

24. Zurbriggen,R., (2003) Immunostimulating reconstituted influenza virosomes. *Vaccine* **21**, 921-924.

25. Letsch,A., Scheibenbogen,C. (2003) Quantification and characterization of specific T-cells by antigen-specific cytokine production using ELISPOT assay or intracellular cytokine staining. *Methods* **31**, 143-149.

26. Trinchieri,G. (2003) Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat.Rev.Immunol.* **3**, 133-146.

27. Bonneville,M., Fournie,J.J. (2005) Sensing cell stress and transformation through Vgamma9/Vdelta2 T cell-mediated recognition of the isoprenoid pathway metabolites. *Microbes.Infect.* **7**, 503-509.

28. Shen,Y., Zhou,D., Qiu,L., Lai,X., Simon,M., Shen,L., Kou,Z., Wang,Q., Jiang,L., Estep,J., Hunt,R., Clagett,M., Sehgal,P.K., Li,Y., Zeng,X., Morita,C.T., Brenner,M.B., Letvin,N.L., Chen,Z.W. (2002) Adaptive immune response of Vgamma2Vdelta2+ T cells during mycobacterial infections. *Science* **295**, 2255-2258.

29. Li,B., Rossman,M.D., Imir,T., Oner-Eyuboglu,A.F., Lee,C.W., Biancaniello,R., Carding,S.R. (1996). Disease-specific changes in gamma/delta T cell repertoire and function in patients with pulmonary tuberculosis. *J. Immunol.* **157**, 4222-4229.

30. Gioia,C., Agrati,C., Casetti,R., Cairo,C., Borsellino,G., Battistini,L., Mancino,G., Goletti,D., Colizzi,V., Pucillo,L.P., Poccia,F. (2002) Lack of CD27-CD45RA-V gamma 9V delta 2+ T cell effectors in immuno-compromised hosts and during active pulmonary tuberculosis. *J. Immunol.* **168**, 1484-1489.

31. Boom,W.H., Canaday,D.H., Fulton,S.A., Gehring,A.J., Rojas,R.E., Torres,M. (2003) Human immunity to *Mycobacterium tuberculosis*: T cell subsets and antigen processing. *Tuberculosis* **83**, 98-106.

32. Dieli,F., Sireci,G., Caccamo,N., Di Sano,C., Titone,L., Romano,A., Di Carlo,P., Barera,A., Accardo-Palumbo,A., Krensky,A.M., Salerno,A. (2002) Selective depression of interferon-gamma and granulysin production with increase of proliferative response by Vgamma9/Vdelta2 T cells in children with tuberculosis. *J. Infect. Dis.* **186**, 1835-1839.

33. Pahlevan,A.A., Wright,D.J., Andrews,C., George,K.M., Small,P.L., Foxwell,B.M.(1999) The inhibitory action of *Mycobacterium ulcerans* soluble factor on monocyte/T cell cytokine production and NF-kappa B function. *J. Immunol.* **163**, 3928-3935.

34. Pimsler,M., Sponsler,T.A., Meyers,W.M. (1988) Immunosuppressive properties of the soluble toxin from *Mycobacterium ulcerans*. *J. Infect. Dis.* **157**, 577-580.

35. Al Attiyah,R., Mustafa,A.S., Abal,A.T., Madi,N.M., Andersen,P. (2003) Restoration of mycobacterial antigen-induced proliferation and interferon-gamma responses in peripheral blood mononuclear cells of tuberculosis patients upon effective chemotherapy. *FEMS Immunol.Med.Microbiol.* **38**, 249-256.

36. Dlugovitzky,D., Bay,M.L., Rateni,L., Urizar,L., Rondelli,C.F., Largacha,C., Farroni,M.A., Molteni,O., Bottasso,O.A. (1999) *In vitro* synthesis of interferon-gamma, interleukin-4, transforming growth factor-beta and interleukin-1 beta by peripheral blood mononuclear cells from tuberculosis patients: relationship with the severity of pulmonary involvement. *Scand.J. Immunol.* **49**, 210-217.

37. Dlugovitzky,D., Bay,M.L., Rateni,L., Fiorenza,G., Vietti,L., Farroni,M.A., Bottasso,O.A. (2000) Influence of disease severity on nitrite and cytokine production by peripheral blood mononuclear cells (PBMC) from patients with pulmonary tuberculosis (TB). *Clin.Exp.Immunol.* **122**, 343-349.

38. Lee,J.S., Song,C.H., Kim,C.H., Kong,S.J., Shon,M.H., Kim,H.J., Park,J.K., Paik,T.H., Jo,E.K. (2002) Profiles of IFN-gamma and its regulatory cytokines (IL-12, IL-18 and IL-10) in peripheral blood mononuclear cells from patients with multidrug-resistant tuberculosis. *Clin.Exp.Immunol.* **128**, 516-524.

39. Jo,E.K., Park,J.K., Dockrell,H.M. (2003) Dynamics of cytokine generation in patients with active pulmonary tuberculosis. *Curr.Opin.Infect Dis.* **16**, 205-210.

40. Dieli,F., Friscia,G., Di Sano,C., Ivanyi,J., Singh,M., Spallek,R., Sireci,G., Titone,L., Salerno,A. (1999) Sequestration of T lymphocytes to body fluids in tuberculosis: reversal of anergy following chemotherapy. *J. Infect. Dis.* **180**, 225-228.

41. Reed,M.B., Domenech,P., Manca,C., Su,H., Barczak,A.K., Kreiswirth,B.N., Kaplan,G., Barry,C.E., III, (2004) A glycolipid of hyper-virulent tuberculosis strains that inhibits the innate immune response. *Nature* **431**, 84-87.

42. Manca,C., Reed,M.B., Freeman,S., Mathema,B., Kreiswirth,B., Barry,C.E., III, Kaplan,G. (2004) Differential monocyte activation underlies strain-specific Mycobacterium tuberculosis pathogenesis. *Infect. Immun.* **72**, 5511-5514.

43. Mutis,T., Cornelisse,Y.E., Datema,G., van den Elsen,P.J., Ottenhoff,T.H., de Vries,R.R. (1994) Definition of a human suppressor T-cell epitope. *Proc.Natl.Acad.Sci.U.S.A* **91**, 9456-9460.

44. Belkaid,Y., Rouse,B.T. (2005) Natural regulatory T cells in infectious disease. *Nat.Immunol.* **6**, 353-360.

Chapter 8: Enhanced interferon-gamma and tumor necrosis alpha levels in sera of household contacts of buruli ulcer patients

Dorothy Yeboah-Manu[1], Ernestina Mensah-Quainoo[2], Adwoa Asante-Poku[1], David Ofori-Adjei[1], Gerd Pluschke[3], Claudia Daubenberger[3]

[1] *Noguchi Memorial Institute for Medical Research, University of Ghana, Legon,*

[2] *Ghana Health Service, Amasaman, Ga District, Ghana*

[3] *Swiss Tropical Institute, Molecular Immunology, CH 4002 Basel, Switzerland*

Abstract

The serum concentration of INF-γ, TNF-α, IL-4 and IL-6 was assessed in Buruli ulcer patients (BU), household contacts (HC) and non-exposed controls (NC). Our results demonstrate that about 75 % of BU and HC have enhanced INF-γ and TNF-α serum levels compared to NC indicative for immune activation by *M. ulcerans* exposure.

Buruli ulcer (BU), is a chronic skin disease caused by *Mycobacterium ulcerans* (8). Clinical presentations of BU are variable, but usually the disease starts as painless nodules or papules in the skin and if left untreated gradually causes massive skin ulcerations (5). *M. ulcerans* infection occurs commonly in areas related to rivers, swampy terrain or lacustrine systems and is particularly prevalent among poor populations in remote rural areas in West Africa where modern medical amenities are lacking (1). The mode of transmission of BU remains elusive and it has been suggested that trauma to the skin by a contaminated environment (e.g., soil, water, vegetation, insect vector) constitutes one possibility of spread (11). Alternatively, *M. ulcerans* could be inhaled or ingested and then reactivated in low temperature areas of the body at the sites of trauma (6). Without a serological or cellular diagnostic test system at hand for *M. ulcerans* exposure of humans it is unclear which proportions of people living in an BU endemic focus are actually exposed to *M. ulcerans* and who is prone to progress to clinical disease.

Cytokines regulate cellular immune interactions and are produced by a broad range of cells including lymphocytes, monocytes, macrophages, fibroblasts, neutrophils, endothelial cells or mast cells (2). The presence and concentration of human cytokines can be assessed conveniently in serum or plasma samples using commercially available enzyme immunoassays. Although serum cytokine levels do not necessarily reflect the peripheral blood mononuclear cellular cytokine production they can serve as indicator of the activation of cellular immune system (7). In this study we examined the serum cytokine levels in Buruli ulcer patients (BU) and compared it to contacts of these patients (HC) and individuals residing in neighbouring BU-non-endemic areas (NC). The aim was to establish whether sharing the environment with BU results in immune activation events easily assessable in serum or plasma samples.

This study was approved by the Institutional Review Board of the Noguchi Memorial Institute for Medical Research. 35 BU, 18 HC and 10 NC were involved and informed consent was obtained from all the subjects and or their parents. (Table 1). BU were confirmed microbiologically by culture, polymerase chain reaction and Ziehl-Neelsen staining (16). All patients were treated according to the guidelines of the national BU

control program by surgical excision. The first blood samples were collected from all BU before treatment and from seven BU second and third blood samples were collected one and two months after surgical treatment, respectively. Sera were collected after centrifugation and stored at -70° C until analysis. The serum samples were analyzed for IL-4, IL-6, IFN-γ and TNF-α concentration by using pairs of cytokine-specific monoclonal antibodies according to the manufacturers' instructions (Mabtech, Sweden for IL-14, IL-6 and TNF-α and Endogen for IFN-γ). Each plate included a cytokine standard of recombinant human cytokine and negative controls. All specimens were measured in duplicate and the means of the two values were used in all analyses. Detection limits were 1 pg/ml for IL-4, 8 pg/ml for TNF-α, 8 pg/ml for IFN-γ and 7 pg/ml for IL-6. Data were analyzed using the STATA program (Stata Corporation). Comparisons among the paired samples of the BU patients were performed using the Wilcoxon signed-rank test, while the Wilcoxon rank-sum test was used to analyze the significance of differences among BU, HC and NC. Data were considered statistically significant when $P < 0.05$.

The mean INF-γ serum level in BU, HC and NC was 34.6, 24.7 and 0 pg/ml, respectively, which was significantly higher in BU and HC compared with NC ($p = 0.0002$ and $p = 0.0003$), respectively (Figure 1A). There was no significant difference between serum INF-γ levels in BU patients with pre-ulcerative and ulcerative stages (Figure 1B).

The mean TNF-α concentration in BU, HC and NC was 823.6, 613.3 and 325.3 pg/ml, respectively. The TNF-α levels differed significantly between BU and NC ($p = 0.01$) and HC and NC ($p = 0.02$) (Figure 1A). BU patients with ulcers had significantly lower levels of TNF-α (mean = 586 pg/ml) compared to patients with pre-ulcerative stages (mean = 1341.9 pg/ml) ($p = 0.006$) (Figure 1B).

The mean IL-6 serum concentrations in BU, HC and NC were 38.7, 51.5 and 4.5 pg/ml, respectively. The IL-6 values between BU and NC but not HC and NC differed significantly ($p = 0.04$) (Figure 1A). In BU, HC and NC, the mean IL-4 levels assessed were 306.9, 291.8 and 132.4 pg/ml, respectively and no significant differences between the groups analyzed was detectable (Figure 1A). For all cytokines assessed, no significant changes were observed before and after surgical treatment within a follow up period of

60 days (Figure 1C). In summary, 26 out of the 35 BU (74 %) and 14 out of the 18 HC (78 %) had detectable levels of IFN-γ in their serum. 28 BU (80 %) and 15 HC (83 %) had TNF-α levels higher than 325 pg/ml representing the mean TNF-α level detected in NC. 24 BU (68 %) and 13 HC (72 %) had both enhanced concentrations of IFN-γ and TNF-α compared to NC. 15 BU (45 %) and 4 HC (22 %) showed IL-6 levels above the mean of the NC.

In the present study statistically significant differences in the INF-γ and TNF-α serum concentrations between BU and NC and interestingly between HC and NC were found. Diaz et al., have shown that antibodies specific for an 18 kd heat shock protein of *M. ulcerans* were frequently found in serum of BU and HC, but rarely in African NC. The authors concluded that a considerable fraction of HC is exposed to *M. ulcerans* antigens resulting in specific humoral immune responses {unpublished}. These data prompted us to analyse serum cytokine levels as an indirect measure of immune activation and to shed more light on the potential involvement of distinct cytokines in BU. The approach certainly has limitations since serum cytokine levels can be affected by receptor binding, temperature degradation, urinary excretion and cytokine breakdown within receptor bearing cells.

INF-γ plays a fundamental role in cellular immune responses directed against mycobacteria since patients with defective receptors for INF-γ are highly susceptible to severe mycobacterial diseases (10). Similar to our results, serum INF-γ levels in active *M. tuberculosis* patients were higher compared to healthy controls with positive tuberculin skin tests (13, 14). A number of studies have shown that INF-γ production of PBMC after antigen-stimulation were reduced in BU compared to NC (3, 4, 12, 15, 16). The discordance between enhanced serum INF-γ levels and INF-γ secretion by PBMC of BU suggests that INF-γ might not be mainly derived from PBMC but rather derived from tissue leakage into circulation. Serum IFN-γ levels in BU before and up to 2 months after surgical treatment did not change significantly in contrast to antigen-stimulated PBMC (16).

Side effects of anti-TNF-α therapies have demonstrated that it is crucial for immune control of mycobacterial infections in humans (9). Interestingly, the TNF-α level was

significantly higher in pre-ulcerative lesions of BU (i.e. more localised lesions) compared to later, ulcerative lesions supporting the involvement of this cytokine in immune responses against *M. ulcerans*. Similar to tuberculosis, the serum concentration of IL-6 was significantly higher in BU compared to NC (14) and was highest in pre-ulcerative lesions. Hence, IL-6 might be involved in the immune responses against active *M. ulcerans* infections, particularly during early stages of the disease. In summary, we provided first evidence that about 75 % of HC showed enhanced levels of INF-γ and TNF-α indicative for immune activation. Possibly, exposure to *M. ulcerans* contributes to this immune activation supporting the hypothesis that so far unknown factors contribute to development of clinical disease in BU. In future it would be interesting to monitor persons with elevated levels of INF-γ and TNF-α regularly for eventual development of or protection from BU.

	BU (Pre-ulcer)	BU (ulcer)	HC	NC
	(n = 11)	(n = 24)	(n = 18)	(n = 10)
Age	18 (4-54)	13.2 (3-45)	15.6 [a] (6 – 40)	29.2 (9.5 – 58)
Gender	female 6	female 16	female 8	female 5
	male 5	male 8	male 10	male 5
	Cytokine levels assessed			
IFN-γ	45.3 [b] (0-285.6)	29.7 (0-142.2)	24.7 (0-106)	0
IL-4	427.2 (0-1403)	251.7 (0-1262)	291.8 (0-1115)	132.4 (0-400)
IL-6	59.8 (0-159.4)	29 (0-257)	51.5 (0-372)	4.5 (0-45)
TNF-α	1341.9 (0-3758)	586.0 (0-1610)	613.3 (0-1235)	325.3 (0-957)

Table I:

Overview of persons enrolled in the current study and serum cytokine levels measured by ELISA.

[a] mean age of group, range is given in brackets

[b] calculated arithmetic mean of individual measurements, ranges are given in brackets
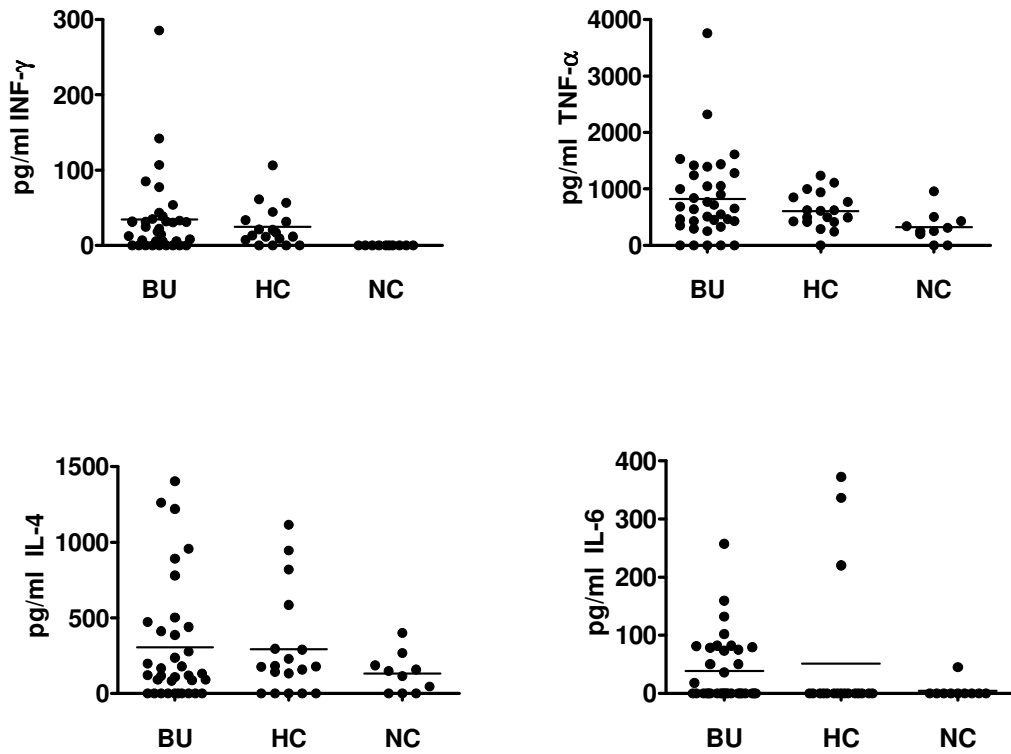
Figure 1A: Circulating IFN- γ, IL-6, IL-4, and TNF-α level in BU, HC and NC. The dots represent individual measurements and the horizontal is the mean value for the different groups.
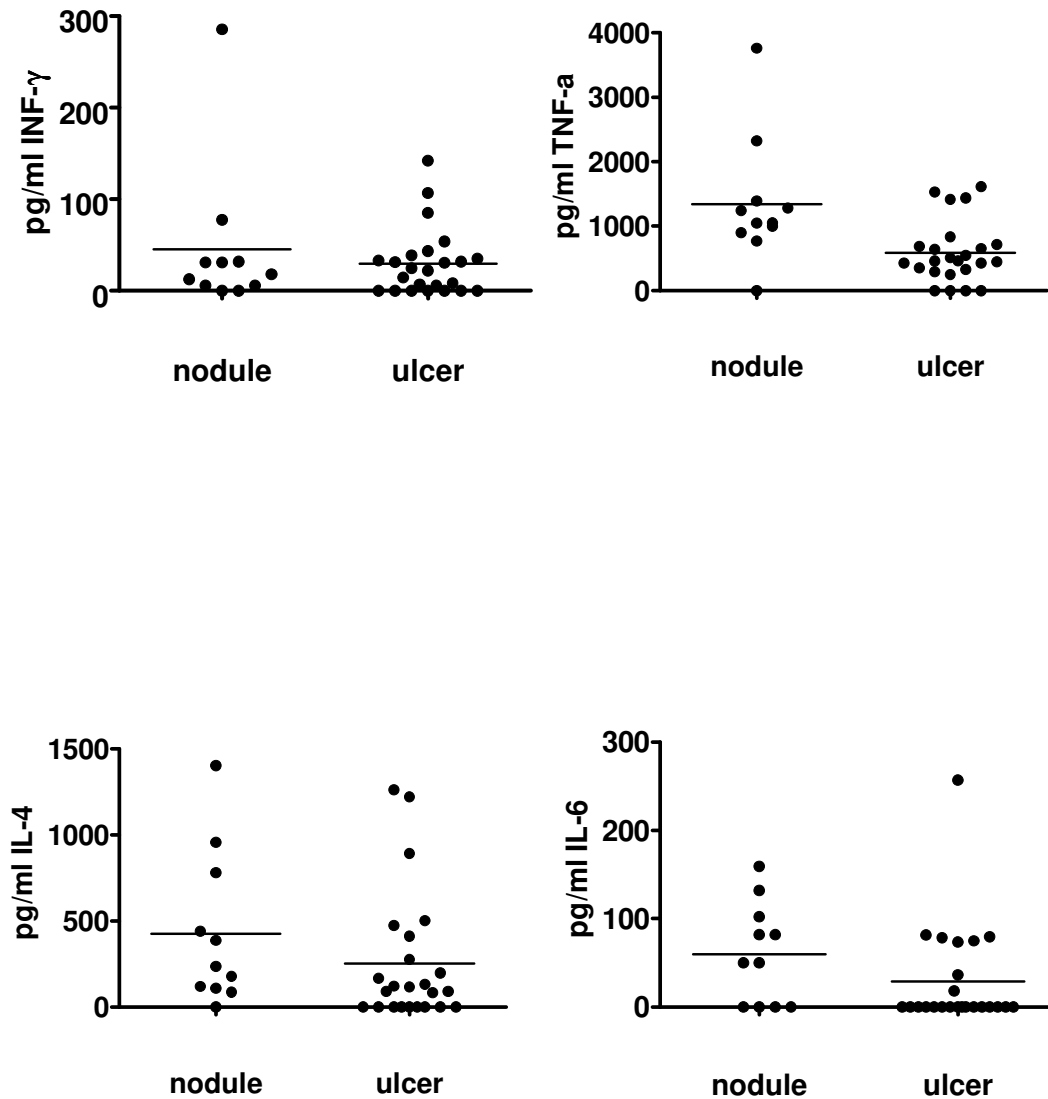
Figure 1B: Comparison of serum levels of IFN-γ, IL-6, IL-4, and TNF-α in BU with pre-ulcerative lesions and ulcerative lesions. The black dots represent individual measurements and the horizontal line is the groups mean values.
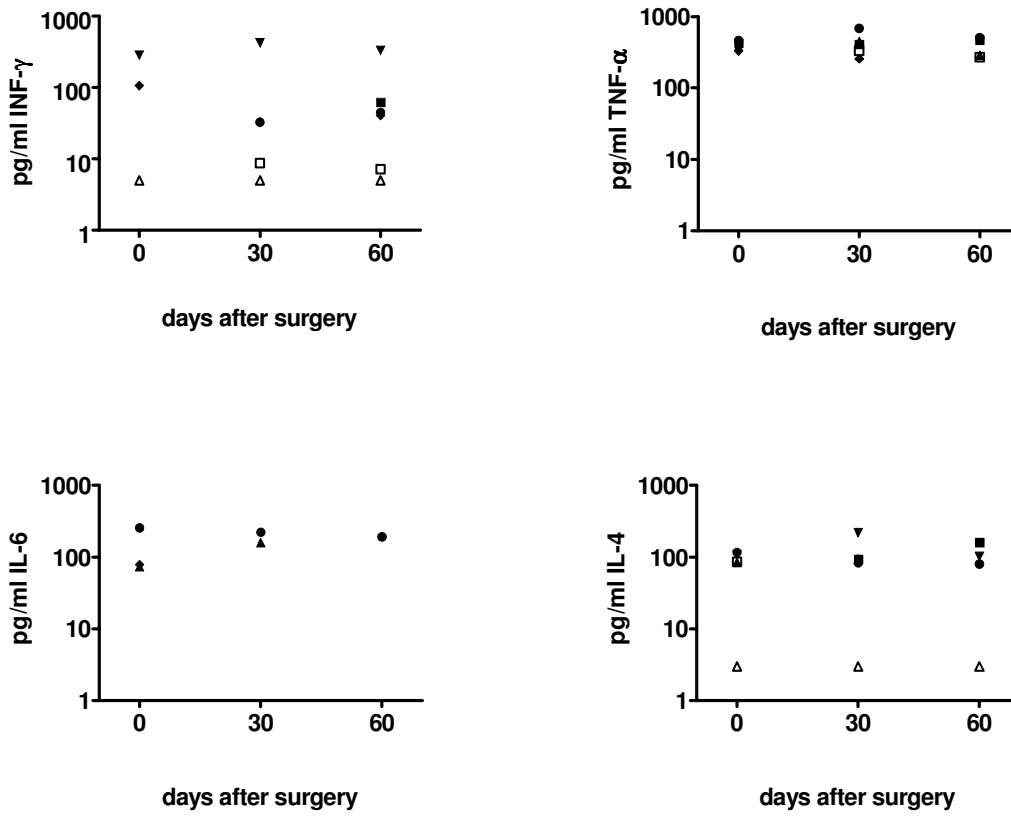
Figure 1C: Serum samples were collected from BU before surgery, 30 and 60 days after surgical treatment. Cytokine levels were analysed by ELISA and expressed in pg/ml. Each individual analyzed is depicted individually by a distinct symbol.

Reference List

1. **Duker, A. A., F. Portaels, and M. Hale**. 2006. Pathways of *Mycobacterium ulcerans* infection: A review. Environ.Int.

2. **Feghali, C. A. and T. M. Wright**. 1997. Cytokines in acute and chronic inflammation. Front Biosci. **2**:d12-d26.

3. **Gooding, T. M., P. D. Johnson, M. Smith, A. S. Kemp, and R. M. Robins-Browne**. 2002. Cytokine profiles of patients infected with *Mycobacterium ulcerans* and unaffected household contacts. Infect Immun **70**:5562-5567.

4. **Gooding, T. M., A. S. Kemp, R. M. Robins-Browne, M. Smith, and P. D. Johnson**. 2003. Acquired T-helper 1 lymphocyte anergy following infection with *Mycobacterium ulcerans*. Clin.Infect Dis. **36**:1076-1077.

5. **Hayman, J.** 1985. Clinical features of *Mycobacterium ulcerans* infection. Australas.J Dermatol. **26**:67-73.

6. **Hayman, J.** 1991. Postulated epidemiology of *Mycobacterium ulcerans* infection. Int.J Epidemiol. **20**:1093-1098.

7. **Jason, J., L. K. Archibald, O. C. Nwanyanwu, M. G. Byrd, P. N. Kazembe, H. Dobbie, and W. R. Jarvis**. 2001. Comparison of serum and cell-specific cytokines in humans. Clin.Diagn.Lab Immunol. **8**:1097-1103.

8. **Johnson, P. D., T. Stinear, P. L. Small, G. Pluschke, R. W. Merrit, F. Portaels, K. Huygen, J. A. Hayman, and K. Asiedu**. 2005. Buruli ulcer (*M. ulcerans* infection): new insights, new hope for disease control. PLOSMedicine **2**:0282-0286.

9. **Mufti, A. H., B. W. Toye, R. R. Mckendry, and J. B. Angel**. 2005. *Mycobacterium abscessus* infection after use of tumor necrosis factor alpha inhibitor therapy: case report and review of infectious complications associated with tumor necrosis factor alpha inhibitor use. Diagn.Microbiol.Infect Dis. **53**:233-238.

10. **Ottenhoff, T. H., F. A. Verreck, M. A. Hoeve, and d. van, V**. 2005. Control of human host immunity to mycobacteria. Tuberculosis.(Edinb.) **85**:53-64.

11. **Portaels, F., K. Chemlal, P. Elsen, P. D. Johnson, J. A. Hayman, J. Hibble, R. Kirkwood, and W. M. Meyers**. 2001. *Mycobacterium ulcerans* in wild animals. Rev.Sci.Tech. **20**:252-264.

12.	**Prevot, G., E. Bourreau, H. Pascalis, R. Pradinaud, A. Tanghe, K. Huygen, and P. Launois**. 2004. Differential production of systemic and intra-lesional gamma interferon and interleukin-10 in nodular and ulcerative forms of Buruli disease. Infect Immun **72**:958-965.

13.	**Vankayalapati, R., B. Wizel, S. E. Weis, P. Klucar, H. Shams, B. Samten, and P. F. Barnes**. 2003. Serum cytokine concentrations do not parallel *Mycobacterium tuberculosis*-induced cytokine production in patients with tuberculosis. Clin.Infect Dis. **36**:24-28.

14.	**Verbon, A., N. Juffermans, S. J. van Deventer, P. Speelman, H. van Deutekom, and P. T. van der**. 1999. Serum concentrations of cytokines in patients with active tuberculosis (TB) and after treatment. Clin.Exp.Immunol. **115**:110-113.

15.	**Westenbrink, B. D., Y. Stienstra, M. G. Huitema, W. A. Thompson, E. O. Klutse, E. O. Ampadu, H. M. Boezen, P. C. Limburg, and T. S. Van Der Werf**. 2005. Cytokine responses to stimulation of whole blood from patients with buruli ulcer disease in Ghana. Clin.Diagn.Lab Immunol. **12**:125-129.

16.	**Yeboah-Manu, D., E. Peduzzi, E. Mensah-Quainoo, A. Asante-Poku, D. Ofori-Adjei, G. Pluschke, and C. A. Daubenberger**. 2006. Systemic suppression of interferon-{gamma} responses in Buruli ulcer patients resolves after surgical excision of the lesions caused by the extra-cellular pathogen *Mycobacterium ulcerans*. J Leukoc.Biol **79**:1150-1156.

Chapter 9: General Discussion and Conclusion

9.1 General discussion

Even though Buruli ulcer (BU) was discovered in the 19th century it was largely neglected until a century later, in 1997, that the World Health Organisation (WHO) recognised it as a disease that creates a considerable public health burden, particularly in West-Africa. At a meeting in Yamoussoukro in 1998 the WHO and the countries affected declared the need to fight this disease. It was realised that to be able to control BU, there is the need to improve the scarce understanding of the various characteristics of the disease. This led to the formation of the BU technical advisory group (1). The 57th WHO Assembly in 2004 not only reiterated the importance of BU but pinpointed a number of areas where research should be intensified: 1) development of simplified diagnostic tools, 2) development of new drugs to improve therapy, 3) host immune response, and 4) development of tools to understand the transmission of the disease in order to enhance control and prevention of the disease.

The present PhD work contributed in the areas of laboratory confirmation of clinical diagnosis and cultivation of *M. ulcerans* from clinical samples, the significance of antibiotics in the treatment of BU, the usefulness of molecular methods in studying transmission and the understanding of immune response in *M. ulcerans* infection.

9.1.1 Cultivation of M. ulcerans and laboratory confirmation of clinical diagnosis

In endemic countries of Africa, cases of BU are diagnosed mainly on clinical grounds, due to limited access to laboratory facilities (2). The presentation of typical indolent, painless, undermined edges and necrotic slough lesion allows a straightforward diagnosis of ulcerative lesions. It is more difficult, however, for the nodule, plaque and oedema forms and even more so for atypical forms, which can be confused with other skin diseases (3). Thus reconfirmation of clinical diagnosis of cases is very important to prevent unnecessary surgical excision and/or antimycobacterial drug treatment of patients with non-BU lesions and for epidemiological surveillance. The available tests for confirmation of cases include: 1) direct smear examination with Ziehl-Neelsen staining to detect the presence of acid-fast bacilli in patholological sample 2) cultivation of *M. ulcerans* on solid medium at 32°C 3) detection of *M. ulcerans* DNA by PCR and 4)

histopathology (3). Of all the above methods the most definite confirmatory is the isolation of *M. ulcerans* by culture from samples obtained from lesions. However, this method is expensive and difficult and the recovery rate can be as low as 20% (4).

In previously reported studies, clinical specimen were collected in remote endemic areas of Africa and shipped to research or diagnostic laboratories in Europe or North America (5,6). A major contribution of the present PhD work was the establishment of a very successful culturing system in an endemic country (Ghana). It was demonstrated that a recovery rate of more than 70% can be achieved within the framework of an efficient partnership between a health delivery institution and a bio-medical institute in an endemic country (chapter 3). The success in primary isolation of *M. ulcerans* depended on the emphasis both partners put on the optimisation of sampling procedures and specimen handling prior to cultivation. We showed that aseptic transfer of the biopsy specimen directly into a special transport medium containing antibiotics, maintenance of the cold chain between health facility and the laboratory, and cultivation within 48 hours of sample collection were critical to obtain an adequate yield of *M. ulcerans*.

 In addition to the sampling process, the removal of contaminating fast growing bacteria by a decontaminating agent before culture was very important. There are two interrelated factors involved in the recovery of *M. ulcerans* from clinical specimens. One is the ability of the decontaminating agent to destroy non acid-fast contaminants, whose presence could cover the detection of *M. ulcerans* growth. The other factor is the decontaminating agent's direct effect on the viability of *M. ulcerans* (7). In a culture optimisation study we demonstrated that oxalic acid decontamination (8) was the best for the recovery of viable *M. ulcerans* from clinical samples and is also the best in the removal of contaminating organisms.

Finally, the medium for cultivating bacteria is important. Mycobacteria differ in their preference for carbon source utilisation, with variation across the genus. This is particularly true with regard to glycerol utilisation. Under laboratory conditions glycerol is the preferred carbon source for a number of mycobacterial species, including *M.*

tuberculosis. However, *M. bovis, M. africanum* and *M. microti* are all unable to use glycerol as a sole carbon source, and pyruvate is therefore routinely added to glycerinated media to enable growth (9). The preferred carbon source for *M. ulcerans* had not been established. Our study showed for the first time that *M. ulcerans* prefers glycerol to pyruvate as source of carbon and so a medium for *M. ulcerans* should contain glycerol.

Even under optimal conditions, culture is a time consuming process and only yields results after several weeks of cultivation. Because of this, and the low recovery rate generally reported, more efforts are being put in the improvement of PCR procedures for detection of *M. ulcerans* DNA in clinical specimen. Most research groups now use the high copy number insertion sequence, *IS2404* in the detection of *M. ulcerans*. Therefore we later included *IS2404* specific PCR and direct acid-fast bacilli staining in a laboratory diagnosis system. The combined methods were used in a large series of patients to assess clinical diagnosis of BU cases seen at the Amasaman Health Centre. This helped the clinician in her case definition and also validated clinical diagnosis (chapter 4). This is essential as sensitivity of clinical diagnosis in experienced hands was only about 50% in one study (10). To the best of our knowledge, this is the first study that involved a large case series assessing the accuracy of clinical diagnosis.

The WHO recommends that case reconfirmation should be done on the basis of positive results for two diagnostic tests (3). In our study, more than 90% of the clinically defined cases were confirmed in the laboratory. Based on this result, we suggest that in an endemic country like Ghana, where clinical features of BU are well known to clinicians, one positive test is regarded sufficient in combination with clinical diagnosis carried out by a clinician who has extensive experience with BU diagnosis. In addition to individual case confirmation, we formally confirmed that the Ga district is endemic for BU as more than 90% of the lesions presented at the Amasaman Health Centre were confirmed microbiologically as BU.

Our findings indicate that there is a need for education of the rural population, as more than 50% of the cases were reported late. Such an education should stress the need for

early detection of nodular lesions, which can reduce the length of stay in hospitals and the need for reconstructive plastic surgery. Also the education should make the rural people know that BU is the result of an infection and not caused by witchcraft.

In spite of the successful establishment of an efficient cultivation system in Ghana, it is not feasible to provide this service nationwide and simple diagnostic assays that are rapid, inexpensive, and do not require highly trained personnel or a complex technological infrastructure are required, for differential diagnosis of very early nodular cases to limi since with the introduction of 8 weeks of drug treatment before surgery (11), it is necessary to conduct pre-surgery confirmation to avoid unnecessary drug treatment as a result of false clinical diagnosis. A very attractive option will be immunodiagnostic procedures, which will not require the use of an invasive procedure for sample collection, as the collection of even needle biopsies may enhance the spread of the pathogen. In a report of a case-control study conducted in Ghana, the authors achieved a specificity of serological testing; 85% BU patients were IgM positive to *M. ulcerans* culture filtrate, 4.5% healthy contacts were positive and none of the TB control patients were positive (12). This promising result may need to be investigated further by identifying specific immunodominant antigens and testing in a larger patient population with appropriate controls. In addition, the possibility of using serodiagnosis for assessing exposure has been evaluated in a pilot study using an immunodominant 18kDa protein (13) and this can be used to identify newly infected people and provide them with preventive therapy, as is being done in the case of tuberculosis for risk individuals (14).

In addition to culture being the most definite method for microbiological confirmation of clinical diagnosis, it also allows the raising of a collection of isolates that is helpful in the understanding of the epidemiology of the microbe being studied. This is very crucial in the case of *M. ulcerans* for which current knowledge is very scanty. Some of the questions that need to be addressed using strain collections are the mode of transmission, strain variability with regard to virulence phenotypes and searching for efficacious drugs for chemotherapy. Within the frame-work of this thesis, we were able to raise a large collection of *M. ulcerans* isolates not only from the Ga district but also from other

endemic districts in Ghana (chapters 3 & 4). These isolates were later used in the analysis of variability in drug sensitivity and for the identification of variable genetic markers within the clonal population of African *M. ulcerans* isolates (chapters 5& 6).

9.1.2 *Antibiotic Treatment*

Prior to 2004 the general impression was that antibiotic therapy is of no benefit in BU treatment and therefore the recommended standard treatment was wide surgical excision followed by skin grafting. Many antimycobacterial drugs were effective in *vitro* but their efficacy was not reflected *in vivo*, at least in humans (15,16). This inconsistency has been explained as being due to the inability of drugs to penetrate tissues and/or to the persistent action of the toxins secreted by the bacillus. In mouse model studies, however a combination of rifampicin and aminoglycosides showed inhibitory activity (17,18). This led to a series of further studies in Ghana (19), Benin (20) and the Ivory-Coast (21). A randomized, placebo-controlled pilot study conducted in Ivory-Coast tested the efficacy of a combination of dapsone and rifampicin, in the management of BU. Their findings indicated a marginal benefit with antibiotic treatment; however the groups were dissimilar at the start of the study. In Ghana, a small clinical trial revealed that a course of at least four weeks of rifampicin and streptomycin inhibits the growth of *M. ulcerans* in pre-ulcerative lesions. In an observational study conducted in Benin all the 99 patients who received a combination of rifampicin and streptomycin were healed after 8 weeks treatment (22).

Based on the results of these studies, the WHO now recommends 8 weeks treatment with a combination of rifampicin and streptomycin before surgery. With this treatment, experts hope to reduce the indications for surgery or the extent of surgery, and also the relapse rates (23). A number of arguments have been put forward against this recommended combinatory treatment (24). The first argument was that there were flaws in the study designs; for example the sample size for the randomised control trial at Agroyesum in Ghana was small and also the study design did not analyse the efficacy in preventing relapse or disseminated infection. Another draw back was, none of the drug trial studies isolated *M. ulcerans* from the patients for in-vitro susceptibility studies. A

second argument is that the use of the combinational therapy for short time without proper microbiological investigation could increase the risk of developing resistant tuberculosis because rates of TB co-infection with BU have not been looked at in-depth in any of the endemic countries. The risk that BU patients are co-infected with TB in the BU endemic regions of Africa is very high, as all the worst affected countries are endemic for tuberculosis and the above antibiotics are very essential for their TB control programs. Consequently, the judicious use of these antibiotics is very essential to prevent an increase in resistant strains of tuberculosis. It is known that in developing countries individuals with TB and other mycobacterial infection at certain time point carry high bacterial loads which predisposes the selection of resistant cases (25). If the rate of BU continues and hence the use of this therapy increases the problem will become serious.

Although there are no reports in literature indicating clinical *M. ulcerans* isolate resistant to rifampicin, there have been previous observations of the ineffectiveness of these drugs in treating BU (26,27). In a mouse model study, Bentoucha et al (28) showed that non-judicious use of rifampicin, as in monotherapy, can cause the *rpo B* gene to mutate, leading to conversion of sensitive strains to resistant ones. There is the danger that *M. ulcerans* will also develop resistant to these drugs. It is therefore imperative to conduct periodic surveys to monitor the susceptibility pattern of MU to these drugs. The inexpensive conventional proportion method in use at present is technically demanding and very slow, requiring at least 8 weeks to complete an experiment (16), while the more rapid BACTEC method requires the use and disposal of radioactive material which is beyond the means of poor endemic countries (29). Within the framework of this PhD a simpler and technically not demanding procedure, Microplate Alamar Blue Assay (MABA) (30,31) was adapted to test the sensitivity of *M. ulcerans* to antibiotics. The adopted MABA, which is a more rapid, easier and cheaper procedure with high throughput, will make it easier to conduct susceptibility assays, which can be used in the screening of new compounds as well as of drugs currently in-use. In developed countries, this assay is usually read with a spectrophotometer (30,31). In our study the end result was read visually and the agreement with the gold standard proportion method was high; more than 90% (chapter 6).

The MABA method we established allowed us to determine the susceptibility pattern of *M. ulcerans* isolates from Ghana and identified both rifampicin and streptomycin resistant strains among our strain collection. This result was reconfirmed using the proportion method (chapter 6). These findings raise major concern and more extensive studies are required to clarify whether resistance to rifampicin and streptomycin is commonly found in African *M. ulcerans* strains. In addition analysis of strains from patients not responding to rifampicin and streptomycin is very important for planning of an alternate regimen for re-treatment.

From the above discussions, it is clear that the development of an effective drug for oral treatment suitable also for the treatment of small children is a research priority in the control of BU. The MABA procedure can be used to screen new compounds to determine *in vitro* activity against *M. ulcerans* for onward *in-vivo* studies. These screenings should take into consideration not only susceptibility to *M. ulcerans* but *M tuberculosis* as well.

9.1.3 *Genetic fingerprinting and transmission*

The mode of transmission of BU is still an enigma. Endemic foci are normally close to areas with slow flowing or stagnant waters, often caused by human environmental disturbance activities. In Uganda, increased numbers of BU cases occurred among refugees when they were living close to the Nile River, but the numbers dropped when the refuges were moved to a different location (32). Also the first documented cases of BU were described among persons living in a swampy area in Bainsdale in Australia (33). In the current work we were able to confirm this with microbiologically-confirmed BU, as more than 90% of all the cases were from both Amasaman and the surrounding villages, or from towns close to the Densu River in the Akwapim south district (chapter 3 & 4). Both Amasaman and its surrounding villages are all dotted with a number of stagnant pools which are the result of major sand winning activities for construction in the near-by cities of Accra and Tema. The Densu River is dammed for the provision of potable water for the above cities and has led to increase in vegetation and flows very slowly along these villages.

An important aspect in the control of infectious disease is the identification of risk factors that increase the chance 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. Genetic fingerprinting of pathogenic bacteria has been extremely useful for monitoring patterns of transmission in human populations. It has helped in tracking the transmission links between individuals, and also demonstrating instances in which related people were infected with unrelated strains. With the inception of active DNA fingerprinting of *M. tuberculosis* isolates, a number of open epidemiological questions with regard to TB control have been answered. An active DNA fingerprinting of *M. tuberculosis* in New-York city identified risk factors (e. g. HIV infection, living in crowded homes) that enhance the risk of acquiring recent tuberculosis and activation of latent infection. High risk individuals who are latently infected are given prophylaxis (14,34-36).

M. ulcerans is said to have a 'very limited polymorphic' genome so standard molecular typing methods have turned out not to be able to discriminate strains of *M. ulcerans* from a specific area. Especially none of the published studies has identified genetic heterogeneity in African isolates (37-39). Within the frame work of this PhD, we applied Variable Number of Tandemly Repeated (VNTR) sequence typing to the large collection of isolates raised from five different districts in Ghana and found for the first time genetic polymorphism in isolates from an African country. More importantly, there were variations in distribution of the different genotypes between the two main endemic areas; while genotype 3 strains were found only in the Ga district, genotype 2 strains were identified only in the Amansie West district. The common allelic combination found in other African isolates (genotype 1) was rarely found in Ghana. Two of the three genotypes which were found in recent Ghanaian isolates were not found in other countries. This showed the emergence and spreading of new variants within Ghana (chapter 5).

Further investigation of the temporal and spatial patterns of emergence and spreading of genotypes may contribute in future to our understanding of the transmission and

epidemiology of Buruli ulcer. This study also confirmed the discriminating power of VNTR-based typing for genotyping of *M. ulcerans* isolates. However, the resolution obtained by the VNTR typing is not enough for more detailed micro-epidemiological studies. Future studies should search for more polymorphic VNTR loci. The possibility of this will be enhanced by the availability of the genome sequence. Future micro-epidemiological studies based on highly discriminating genetic fingerprinting method could answer a number of questions such as: whether most of the recurrences are caused by exogenous re-infection, incomplete surgery or haematogenous spread to other sites; whether in patients with multiple lesions, all lesions are caused by the same strain or by multiple infections; whether person to person transmission is rare or frequent.

In addition we found one dominant strain which seems to be more successful over the rest and phenotypic characterisation will help to understand the basis of some virulence of *M. ulcerans*.

Three major hypotheses exist for the acquisition of *M. ulcerans* infection (40). The first hypothesis is that *M. ulcerans* infection is acquired by contact with a contaminated environment, as reservoirs including biofilm on vegetation in water (41). The environmental bacteria may gain access through wounds or minor abrasions of the skin and sub-cutaneous inoculation may result in multiplication of the pathogen in the sub-cutaneous fat (42). Supporting this hypothesis, Stinear *et al* used a magnetic bead sequence capture-PCR to detect two *M. ulcerans* sequences in water and plant material samples collected from two endemic foci. Both were positive for *M. ulcerans* but, nearby sites and sites unrelated to the endemic areas were negative (43). Case control studies conducted in endemic foci have identified either swimming or fishing as a risk for acquiring infection (44). Distribution of lesions over body surface area in a case series study carried out in Ghana indicated that infection was acquired by activities near the ground such as farming or playing (45). The second hypothesis suggests that infection is acquired by aerosol spray of contaminated water. This is backed by the observation of an outbreak of *M. ulcerans* in area near a golf course irrigation system in Australia (46).

A third hypothesis is that infection may be through intermediate hosts. The third hypothesis is supported by a number of studies: using PCR, *M. ulcerans* has been detected in biting insects (47), mosquitoes from Australia and the salivary glands of the carnivorous insect, *Naucoris spp*. For most of the environmental studies, the *IS2404* PCR which was used as the detection marker is now known not to be absolutely specific for *M. ulcerans* as other environmental mycobacteria have been found to contain this insertion sequence (48,49). Hence the final proof method is the isolation of *M. ulcerans* from these supposed intermediates. In the vast majority of cases it was not possible to culture *M. ulcerans* from the supposed intermediates, as most of the samples were heavily contaminated and had been transported for long distances out of the endemic countries before culturing. Only two pure cultures have been identified from environmental sources (50). The inability to cultivate this organism from environmental sources may be due to inadequate sampling, conditions of transport, decontamination and culture conditions. The establishment of the improved cultivation method in Ghana will now enhance cultivation of *M. ulcerans* from the supposed hosts.

In addition to elucidating transmission among human populations, genetic fingerprinting could also be useful in providing evidence based data to confirm or disprove the hypothesis that an intermediary host is involved by identifying animal reservoirs. The principle behind this is similar to what is used in tracking the source of infections in human populations. That is by comparing the relatedness of genetic fingerprints between human clinical isolates and that of isolates obtained from suspected animal intermediary host/s. Such phylogenetic analysis can help in identifying the pattern/sequence of transmission. Marsollier *et al* carried out genetic fingerprinting on isolates obtained from plants, aquatic insects and clinical isolates from the same region in Ivory-Coast. Findings from this study showed that plant-associated *M. ulcerans* had the same genetic profile as isolates from aquatic insects and the clinical isolates obtained in the same region, as well as clinical isolates from other areas of West-Africa and different from isolates from some other regions (41). These observations implicate aquatic plants and insects as a reservoir of *M. ulcerans*. Nevertheless, the study could not differentiate between the African isolates, and the sample size was small. In our VNTR study we increased the sample size

and were able to find variants in isolates not only within Ghana, but within individual endemic districts. The identification of differences in genotypes in the two main endemic districts of Ghana will enable studies to be made comparing these clinical isolates with environmental isolates from different areas.

9.1.4 *Immune response against M. ulcerans and the potential for vaccine development*
The treatment of BU with antibiotics or surgery is associated with a number of problems. The development of other preventive and control strategies, is therefore an important research priority. Institution of concrete preventive measures for acquiring infection is difficult without a clear understanding of the mode of transmission of *M. ulcerans* (51). The only risk factor identified so far, working or playing close to rivers in endemic areas, (40,44,45) is difficult to eliminate since rivers serve as the main source of water for domestic activities, including cooking, drinking and washing in rural endemic communities. Keeping away from the streams will be difficult for women and children who must daily fetch water from streams. In addition, most of the endemic rural dwellers are farmers who depend on the riverine environments for crop cultivation. Thus an effective vaccine that prevents infection and/or disease seems to represent one of the preferred options for controlling the upsurge in BU cases.

Currently, no effective vaccine for BU is available but there are evidences of some degree of cross-reactive protection conferred by *M. bovis* BCG (BCG) against BU disease. Two different case-controlled studies conducted in endemic regions of Uganda demonstrated that vaccination with BCG confers protection against BU ranging between 18-74% with an overall protection rate of 47% (52,53). Intra-dermal inoculation of unprotected individuals with BCG in an endemic focus offered an overall protection of 47% and this protective efficacy wanes after a year. Furthermore, the vaccinated group and individuals who mounted a delayed-type hypersensitivity response to the *M. tuberculosis* protein lysate, tuberculin, of 4 mm or more in the skin had smaller BU lesions than those in unvaccinated group (52). Portaels *et al* reported that effective BCG vaccination at birth may protect children against the development of severe disseminated disease like osteomyelitis (53). Analysis of the immune mechanisms potentially mediating this cross-protection indicated that the immuno-dominant protein antigen

Ag85A of BCG is recognised by the cellular immune system of *M. ulcerans*-infected mice. Furthermore, vaccination of mice with plasmid DNA encoding Ag85A from BCG was effective in reducing the bacterial load of *M. ulcerans* in the footpad (54). The *M. ulcerans* homolog of Ag85A shared 84.1% amino acid sequence identity and 91% conserved residues with *M. bovis* BCG. These findings indicate that immune responses against common mycobacterial antigens present in the BCG vaccine may offer some protection against BU and *M. tuberculosis*. However, this protection is not long-lasting and complete and therefore BCG has probably failed to control the increasing numbers of BU cases in West and Central Africa. In Ghana, a retrospective study conducted in Amansie-West district showed that there was no difference in the prevalence of BU between BCG vaccinated and non-vaccinated persons (55).

Similar to infections with *M. tuberculosis*, it seems only a proportion of individuals infected with *M. ulcerans* develop active BU disease (12). This idea was corroborated by our analysis of serum concentrations of type 1 and type 2 cytokines in BU patients, household contacts and non-exposed controls, which indicated immune activation in both BU patients and their household contacts but not in the non-exposed controls by *M. ulcerans* (chapter 9). Some BU patients also show spontaneous healing associated with the formation of granulomatous lesions and the development of delayed type hypersensitivity responses in burulin skin tests. These observations show that the induction of immunity to BU through vaccination is possible.

Understanding the potentially protective or harmful immune mechanisms induced or suppressed during natural infection with *M. ulcerans* will support the rational design of vaccine against BU. Current knowledge of the mechanisms involved in protective immune response to *M. ulcerans* is limited. It appears that adaptive immune responses associated with IFN-γ secretion may be protective as individuals with defective receptors for INF-γ are highly susceptible to severe mycobacterial diseases including BU. Persons with past or current *M. ulcerans* disease had reduced INF-γ production in response to PPD of *M. tuberculosis* and *M. bovis* or whole killed *M. bovis* BCG or *M. ulcerans* compared to tuberculin positive individuals in endemic areas (56-58). We showed that

consistently with these reports, an *ex vivo* ELISpot analysis of PBMC revealed that the frequencies of systemic INF-γ producing cells after stimulation with IPP or PPD was significantly reduced in BU patients compared to healthy controls. This depressed INF-γ production was not restricted to immune responses specific for mycobacterial antigens but extended to CD4 T cell responses specific for influenza virus (chapter 7).

In the study of Gooding *et al*, suppression of INF-γ production was not limited to subjects with active disease, but occurred in individuals with healed BU lesions (56). The authors suggested that individuals with BU may have genetic defects in the IFN-γ pathway predisposing for development of clinical disease. We analysed PBMC from the same patients before surgery and after healing in parallel by ELISpot analysis, and showed that the numbers of INF-γ secreting cells after antigen stimulation increased significantly after surgical treatment. By this finding we demonstrated for the first time that antigen-specific INF-γ production in BU patients is restored after healing. Therefore, individuals with BU do not seem to have genetic defects resulting in impaired INF-γ production since the observed suppression in T cell function was closely associated with infection with *M. ulcerans*. It would be of interest to investigate whether the recommended eight weeks of drug treatment in BU also leads to an improvement of the disease associated IFN-γ suppression as has been described in *M. tuberculosis* infections (59).

IL-12 induces T and NK cells to produce several cytokines, including INF-γ (60). We observed that total IL-12 concentrations in culture supernatants of PPD stimulated PBMC were statistically higher in BU patients (treated and untreated) than in the controls (chapter 7). Therefore observed reduction in systemic INF-γ responses does not seem to be a consequence of diminished IL-12 production of the innate immune system. Studies of Prevot and Gooding suggested that Th2 mediated down-regulation of Th1 response may be involved in the reduced IFN-γ production observed in BU. In the study of Prevot, less extensive lesions (nodules) were associated with higher INF-γ and lower IL-10 production and ulcerative lesions with lower INF-γ and higher IL-10 production (58). However, it is currently completely unknown what causes this unfavourable balance between IL-10 and INF-γ in mycobacterial diseases. It is a general belief that

mycolactone produced by *M. ulcerans* is the main virulence factor responsible for the observed immunosuppression in BU. Interestingly, *M. tuberculosis* and *M. ulcerans* infections seem to share some impact on the host immune responses with BU. In both cases, infection leads to suppression in host systemic Th1 response (56,61) which resolves after treatment (59,62). In addition, immune protection by the BCG vaccine is also short lived, and seems to be more effective in controlling systemic infections than infection at tissue sites (63-65). At the time of diagnosis, our analysis of systemic production of IFN-γ after antigen stimulation was suppressed in both disease states but reverses after treatment. Restoration of T cell reactivity was slow such that an optimum response was not yet achieved by two months in both populations (chapter 8).

The present PhD study could not analyse the variations in the composition of different cell subpopulations in the peripheral blood. It is recommended that future studies should look into this as it can be useful in explaining the underlying mechanisms involved in the observed reduced IFN-γ responses.

From the above discussions, it appears that an effective vaccine for BU should prime an individual to mount a strong cellular Th1 response so as to enhance his ability to control initial inoculation. On the other hand, the induction of a neutralising antibody against the polyketide toxin, mycolactone, an essential virulence factor, should in essence disarm the pathogen and prevent it from causing disease. This strategy has been effectively used in the design of successful vaccines for the control of diseases including tetanus and diphtheria (66). Such a strategy will only be effective if the toxin will be highly immunogenic and can be neutralised by specific antibody responses. Mycolactone which has a polyketide structure may be on its own not an ideal structure and future research needs to look at the possibility of coupling neutralizing determinants of the toxin to suitable delivery systems for elucidating high titres of neutralizing antibody responses.

9.2 *Conclusions*

This PhD work was conducted partly in Ghana where closeness to a BU endemic region was a major advantage. Several research stays at the Swiss Tropical Institute in Basel made certain types of laboratory analysis possible and facilitated technology transfer to the laboratories of the Noguchi Memorial Research Institute in Ghana. The most important scientific achievements are:

1. A highly sensitive culturing system was established in an endemic country which enabled us to establish a large collection of *M. ulcerans* isolates from different endemic districts in Ghana, which were later used in analysing the diversity of *M. ulcerans* isolates.

2. A simple in-expensive but rapid method for analysing the antimycobacterial sensitivity pattern was adapted for use in *M. ulcerans*. Screening of Ghanaian *M. ulcerans* isolates with this method for sensitivity to some drugs identified streptomycin and rifampicin resistant strains. This raises major concerns within the context of WHO's recommendations for drug treatment of BU

3. PCR, ZN staining and the established cultivation method were used to validate clinical diagnosis of patients reporting to the Amasaman Health Centre. Results demonstrated that clinical diagnosis performed by an experienced clinician can be very accurate, for both pre-ulcerative and ulcerative lesions.

4. DNA fingerprinting of *M. ulcerans* isolates from Ghana by VNTR typing identified for the first time different genotypes within an endemic country of Africa. The most dominant African genotype was rarely found in Ghana and there seems to be an ongoing microevolution of *M. ulcerans* and spreading of new variants within Ghana

5. The measurement of immediate IFN-γ secretion by ELISpot assay demonstrated that immuno-suppression in BU is not limited to mycobacterial antigens and that susceptibility to BU may not necessarily be due to genetic defects in IFN-γ as immuno-suppression was not permanent but reversed after removal of mycobacterial burden by surgical excision of the BU lesion.

6. Analysis of samples collected at different time points revealed that the reversal of immune suppressions in BU is slow and that mycolactone independent activities may be involved.

7. The serum concentration of INF-γ, TNF-α, IL-4 and IL-6 was assessed in Buruli ulcer patients (BU), household contacts (HC) and non-exposed controls (NC). Our results demonstrate that about 75 % of BU and HC have enhanced INF-γ and TNF-α serum levels compared to NC indicative for immune activation by *M. ulcerans* exposure.

Reference List

1. **van der Graaf, W. T., R. W. Scherpbier, and T. S. van der Werf**. 1999. [Buruli ulcer (Mycobacterium ulcerans infection); report from the International Congress in Yamoussoukro, Ivory Coast]. Ned.Tijdschr.Geneeskd. **143**:312-316.

2. **van der Werf, T. S., W. T. van der Graaf, J. W. Tappero, and K. Asiedu**. 1999. Mycobacterium ulcerans infection. Lancet **354**:1013-1018.

3. WHO. Buruli ulcer: Diagnosis of Mycobacterium ulcerans disease. Portaels, F., Johnson, P., and Meyers, W. M. 2001.
Ref Type: Serial (Book,Monograph)

4. **Sizaire, V., F. Nackers, E. Comte, and F. Portaels**. 2006. Mycobacterium ulcerans infection: control, diagnosis, and treatment. Lancet Infect.Dis. **6**:288-296.

5. **Stienstra, Y., T. S. van der Werf, J. Guarner, P. L. Raghunathan, E. A. Spotts Whitney, W. T. van der Graaf, K. Asamoa, J. W. Tappero, D. A. Ashford, and C. H. King**. 2003. Analysis of an IS2404-based nested PCR for diagnosis of Buruli ulcer disease in regions of Ghana where the disease is endemic. J.Clin.Microbiol. **41**:794-797.

6. **Portaels, F., J. Agular, K. Fissette, P. A. Fonteyne, H. De Beenhouwer, P. de Rijk, A. Guedenon, R. Lemans, C. Steunou, C. Zinsou, J. M. Dumonceau, and W. M. Meyers**. 1997. Direct detection and identification of Mycobacterium ulcerans in clinical specimens by PCR and oligonucleotide-specific capture plate hybridization. J.Clin.Microbiol. **35**:1097-1100.

7. **Allen, B. W.** 1991. Comparison of three methods for decontamination of faeces for isolation of Mycobacterium tuberculosis. Tubercle. **72**:214-217.

8. **Livanainen, E.** 1995. Isolation of mycobacteria from acidic forest soil samples: comparison of culture methods. J.Appl.Bacteriol. **78**:663-668.

9. **Keating, L. A., P. R. Wheeler, H. Mansoor, J. K. Inwald, J. Dale, R. G. Hewinson, and S. V. Gordon**. 2005. The pyruvate requirement of some members of the Mycobacterium tuberculosis complex is due to an inactive pyruvate kinase: implications for in vivo growth. Molecular Microbiology **56**:163-174.

10. **Evans, M. R., R. Phillips, S. N. Etuaful, G. Amofah, J. Adomako, O. Adjei, J. Dennis-Antwi, S. B. Lucas, and M. H. Wansbrough-Jones**. 2003. An outreach education and treatment project in Ghana for the early stage of Mycobacterium ulcerans disease. Trans.R.Soc.Trop.Med.Hyg. **97**:159-160.

11. **van der Werf, T. S., Y. Stienstra, R. C. Johnson, R. Phillips, O. Adjei, B. Fleischer, M. H. Wansbrough-Jones, P. D. Johnson, F. Portaels, W. T. van der Graaf, and K. Asiedu**. 2005. Mycobacterium ulcerans disease. Bull.World Health Organ **83**:785-791.

12. **Okenu, D. M., L. O. Ofielu, K. A. Easley, J. Guarner, E. A. Spotts Whitney, P. L. Raghunathan, Y. Stienstra, K. Asamoa, T. S. van der Werf, W. T. van der Graaf, J. W. Tappero, D. A. Ashford, and C. H. King**. 2004. Immunoglobulin M antibody responses to Mycobacterium ulcerans allow discrimination between cases of active Buruli ulcer disease and matched family controls in areas where the disease is endemic. Clin.Diagn.Lab Immunol. **11**:387-391.

13. **Diaz, D., H Döbeli, D.Yeboah-Manu, .Mensah-Quainoo, A.Friedlein, N.Soder, S.Rondini, T. B. , and G.Pluschke**. 2006. Use of the immunodominant 18kDa small heat shock protein as serological marker for exposure to *M. ulcerans*. Clinical and vaccine immunology **submitted**.

14. **Lavigne, M., I. Rocher, C. Steensma, and P. Brassard**. 2006. The impact of smoking on adherence to treatment for latent tuberculosis infection. BMC.Public Health **6**:66.

15. **Portaels, F., H. Traore, K. De Ridder, and W. M. Meyers**. 1998. In vitro susceptibility of Mycobacterium ulcerans to clarithromycin. Antimicrob.Agents Chemother. **42**:2070-2073.

16. **Thangaraj, H. S., O. Adjei, B. W. Allen, F. Portaels, M. R. Evans, D. K. Banerjee, and M. H. Wansbrough-Jones**. 2000. In vitro activity of ciprofloxacin, sparfloxacin, ofloxacin, amikacin and rifampicin against Ghanaian isolates of Mycobacterium ulcerans. J.Antimicrob.Chemother. **45**:231-233.

17. **Nakanaga, K., H. Saito, N. Ishii, and M. Goto**. 2004. [Comparison of inhibitory effect of rifalazil and rifampicin against Mycobacterium ulcerans infection induced in mice]. Kekkaku **79**:333-339.

18. **Dega, H., A. Bentoucha, J. Robert, V. Jarlier, and J. Grosset**. 2002. Bactericidal activity of rifampin-amikacin against Mycobacterium ulcerans in mice. Antimicrob.Agents Chemother. **46**:3193-3196.

19. **Etuaful, S., B. Carbonnelle, J. Grosset, S. Lucas, C. Horsfield, R. Phillips, M. Evans, D. Ofori-Adjei, E. Klustse, J. Owusu-Boateng, G. K. Amedofu, P. Awuah, E. Ampadu, G. Amofah, K. Asiedu, and M. Wansbrough-Jones**. 2005. Efficacy of the combination rifampin-streptomycin in preventing growth of Mycobacterium ulcerans in early lesions of Buruli ulcer in humans. Antimicrob.Agents Chemother. **49**:3182-3186.

20. **van der Werf, T. S., Y. Stienstra, R. C. Johnson, R. Phillips, O. Adjei, B. Fleischer, M. H. Wansbrough-Jones, P. D. Johnson, F. Portaels, W. T. van der Graaf, and K. Asiedu**. 2005. Mycobacterium ulcerans disease. Bull.World Health Organ **83**:785-791.

21. **Espey, D. K., G. Djomand, I. Diomande, M. Dosso, M. Z. Saki, J. M. Kanga, R. A. Spiegel, B. J. Marston, L. Gorelkin, W. M. Meyers, F. Portaels, M. S.**

Deming, and C. R. Horsburgh, Jr. 2002. A pilot study of treatment of Buruli ulcer with rifampin and dapsone. Int.J.Infect.Dis. **6**:60-65.

22. **van der Werf, T. S., Y. Stienstra, R. C. Johnson, R. Phillips, O. Adjei, B. Fleischer, M. H. Wansbrough-Jones, P. D. Johnson, F. Portaels, W. T. van der Graaf, and K. Asiedu**. 2005. Mycobacterium ulcerans disease. Bull.World Health Organ **83**:785-791.

23. **van der Werf, T. S., Y. Stienstra, R. C. Johnson, R. Phillips, O. Adjei, B. Fleischer, M. H. Wansbrough-Jones, P. D. Johnson, F. Portaels, W. T. van der Graaf, and K. Asiedu**. 2005. Mycobacterium ulcerans disease. Bull.World Health Organ **83**:785-791.

24. **Sizaire, V., F. Nackers, E. Comte, and F. Portaels**. 2006. Mycobacterium ulcerans infection: control, diagnosis, and treatment. Lancet Infect.Dis. **6**:288-296.

25. **Lawn, S. D., E. H. Frimpong, H. Al Ghusein, J. W. Acheampong, A. H. Uttley, P. D. Butcher, and G. E. Griffin**. 2001. Pulmonary tuberculosis in Kumasi, Ghana: presentation, drug resistance, molecular epidemiology and outcome of treatment. West Afr.J.Med. **20**:92-97.

26. **Semret, M., G. Koromihis, J. D. MacLean, M. Libman, and B. J. Ward**. 1999. Mycobacterium ulcerans infection (Buruli ulcer): first reported case in a traveler. Am.J.Trop.Med.Hyg. **61**:689-693.

27. **Darie, H., S. Djakeaux, and A. Cautoclaud**. 1994. [Therapeutic approach in Mycobacterium ulcerans infections]. Bull.Soc.Pathol Exot. **87**:19-21.

28. **Bentoucha, A., J. Robert, H. Dega, N. Lounis, V. Jarlier, and J. Grosset**. 2001. Activities of new macrolides and fluoroquinolones against Mycobacterium ulcerans infection in mice. Antimicrob.Agents Chemother. **45**:3109-3112.

29. **Dhople, A. M.** 2001. Antimicrobial activities of dihydrofolate reductase inhibitors, used singly or in combination with dapsone, against Mycobacterium ulcerans. J.Antimicrob.Chemother. **47**:93-96.

30. **Collins, L. and S. G. Franzblau**. 1997. Microplate alamar blue assay versus BACTEC 460 system for high-throughput screening of compounds against Mycobacterium tuberculosis and Mycobacterium avium. Antimicrob.Agents Chemother. **41**:1004-1009.

31. **Pettit, R. K., C. A. Weber, M. J. Kean, H. Hoffmann, G. R. Pettit, R. Tan, K. S. Franks, and M. L. Horton**. 2005. Microplate Alamar blue assay for Staphylococcus epidermidis biofilm susceptibility testing. Antimicrob.Agents Chemother. **49**:2612-2617.

32. **Barker, D. J.** 1971. Buruli disease in a district of Uganda. J.Trop.Med.Hyg. **74**:260-264.

33. **MacCallum JC, T. P, Buckle G, and Sissons HA**. 1948. A new mycobacterial infection in man. J.Pathol Bacteriol 93-122.

34. **Geng, E., B. Kreiswirth, C. Driver, J. Li, J. Burzynski, P. DellaLatta, A. LaPaz, and N. W. Schluger**. 2002. Changes in the transmission of tuberculosis in New York City from 1990 to 1999. N.Engl.J.Med. **346**:1453-1458.

35. **Moss, A. R., J. A. Hahn, J. P. Tulsky, C. L. Daley, P. M. Small, and P. C. Hopewell**. 2000. Tuberculosis in the homeless. A prospective study. Am.J.Respir.Crit Care Med. **162**:460-464.

36. **Alland, D., G. E. Kalkut, A. R. Moss, R. A. McAdam, J. A. Hahn, W. Bosworth, E. Drucker, and B. R. Bloom**. 1994. Transmission of tuberculosis in New York City. An analysis by DNA fingerprinting and conventional epidemiologic methods. N.Engl.J.Med. **330**:1710-1716.

37. **Ablordey, A., J. Swings, C. Hubans, K. Chemlal, C. Locht, F. Portaels, and P. Supply**. 2005. Multilocus variable-number tandem repeat typing of Mycobacterium ulcerans. J.Clin.Microbiol. **43**:1546-1551.

38. **Jackson, K., R. Edwards, D. E. Leslie, and J. Hayman**. 1995. Molecular method for typing Mycobacterium ulcerans. J.Clin.Microbiol. **33**:2250-2253.

39. **Portaels, F., P. A. Fonteyene, H. De Beenhouwer, P. de Rijk, A. Guedenon, J. Hayman, and M. W. Meyers**. 1996. Variability in 3' end of 16S rRNA sequence of Mycobacterium ulcerans is related to geographic origin of isolates. J.Clin.Microbiol. **34**:962-965.

40. **Duker, A. A., F. Portaels, and M. Hale**. 2006. Pathways of Mycobacterium ulcerans infection: A review. Environ.Int. **32**:567-573.

41. **Marsollier, L., T. Stinear, J. Aubry, J. P. Saint Andre, R. Robert, P. Legras, A. L. Manceau, C. Audrain, S. Bourdon, H. Kouakou, and B. Carbonnelle**. 2004. Aquatic plants stimulate the growth of and biofilm formation by Mycobacterium ulcerans in axenic culture and harbor these bacteria in the environment. Appl.Environ.Microbiol. **70**:1097-1103.

42. **Meyers, W. M., W. M. Shelly, D. H. Connor, and E. K. Meyers**. 1974. Human Mycobacterium ulcerans infections developing at sites of trauma to skin. Am.J.Trop.Med.Hyg. **23**:919-923.

43. **Stinear, T., J. K. Davies, G. A. Jenkin, J. A. Hayman, F. Oppedisano, and P. D. Johnson**. 2000. Identification of Mycobacterium ulcerans in the environment from regions in Southeast Australia in which it is endemic with sequence capture-PCR. Appl.Environ.Microbiol. **66**:3206-3213.

44. **Aiga, H., T. Amano, S. Cairncross, J. Adomako, O. K. Nanas, and S. Coleman**. 2004. Assessing water-related risk factors for Buruli ulcer: a case-control study in Ghana. Am.J.Trop.Med.Hyg. **71**:387-392.

45. **Hospers, I. C., I. C. Wiersma, P. U. Dijkstra, Y. Stienstra, S. Etuaful, E. O. Ampadu, W. T. van der Graaf, and T. S. van der Werf**. 2005. Distribution of Buruli ulcer lesions over body surface area in a large case series in Ghana: uncovering clues for mode of transmission. Trans.R.Soc.Trop.Med.Hyg. **99**:196-201.

46. **Veitch, M. G., P. D. Johnson, P. E. Flood, D. E. Leslie, A. C. Street, and J. A. Hayman**. 1997. A large localized outbreak of Mycobacterium ulcerans infection on a temperate southern Australian island. Epidemiol.Infect. **119**:313-318.

47. **Marsollier, L., R. Robert, J. Aubry, J. P. Saint Andre, H. Kouakou, P. Legras, A. L. Manceau, C. Mahaza, and B. Carbonnelle**. 2002. Aquatic insects as a vector for Mycobacterium ulcerans. Appl.Environ.Microbiol. **68**:4623-4628.

48. **Ross, B. C., P. D. Johnson, F. Oppedisano, L. Marino, A. Sievers, T. Stinear, J. A. Hayman, M. G. Veitch, and R. M. Robins-Browne**. 1997. Detection of Mycobacterium ulcerans in environmental samples during an outbreak of ulcerative disease. Appl.Environ.Microbiol. **63**:4135-4138.

49. **Mve-Obiang, A., R. E. Lee, E. S. Umstot, K. A. Trott, T. C. Grammer, J. M. Parker, B. S. Ranger, R. Grainger, E. A. Mahrous, and P. L. Small**. 2005. A newly discovered mycobacterial pathogen isolated from laboratory colonies of Xenopus species with lethal infections produces a novel form of mycolactone, the Mycobacterium ulcerans macrolide toxin. Infect.Immun. **73**:3307-3312.

50. **Sizaire, V., F. Nackers, E. Comte, and F. Portaels**. 2006. Mycobacterium ulcerans infection: control, diagnosis, and treatment. Lancet Infect.Dis. **6**:288-296.

51. **van der Werf, T. S., Y. Stienstra, R. C. Johnson, R. Phillips, O. Adjei, B. Fleischer, M. H. Wansbrough-Jones, P. D. Johnson, F. Portaels, W. T. van der Graaf, and K. Asiedu**. 2005. Mycobacterium ulcerans disease. Bull.World Health Organ **83**:785-791.

52. **Smith, P. G., W. D. Revill, E. Lukwago, and Y. P. Rykushin**. 1977. The protective effect of BCG against Mycobacterium ulcerans disease: a controlled trial in an endemic area of Uganda. Trans.R.Soc.Trop.Med.Hyg. **70**:449-457.

53. **Portaels, F., J. Aguiar, M. Debacker, C. Steunou, C. Zinsou, A. Guedenon, and W. M. Meyers**. 2002. Prophylactic effect of mycobacterium bovis BCG vaccination against osteomyelitis in children with Mycobacterium ulcerans disease (Buruli Ulcer). Clin.Diagn.Lab Immunol. **9**:1389-1391.

54. **Tanghe, A., S. D'Souza, V. Rosseels, O. Denis, T. H. Ottenhoff, W. Dalemans, C. Wheeler, and K. Huygen**. 2001. Improved immunogenicity and protective

efficacy of a tuberculosis DNA vaccine encoding Ag85 by protein boosting. Infect.Immun. **69**:3041-3047.

55. **Amofah, G. K., C. Sagoe-Moses, C. Adjei-Acquah, and E. H. Frimpong**. 1993. Epidemiology of Buruli ulcer in Amansie West district, Ghana. Trans.R.Soc.Trop.Med.Hyg. **87**:644-645.

56. **Gooding, T. M., P. D. Johnson, M. Smith, A. S. Kemp, and R. M. Robins-Browne**. 2002. Cytokine profiles of patients infected with Mycobacterium ulcerans and unaffected household contacts. Infect.Immun. **70**:5562-5567.

57. **Westenbrink, B. D., Y. Stienstra, M. G. Huitema, W. A. Thompson, E. O. Klutse, E. O. Ampadu, H. M. Boezen, P. C. Limburg, and T. S. van der Werf**. 2005. Cytokine responses to stimulation of whole blood from patients with Buruli ulcer disease in Ghana. Clin.Diagn.Lab Immunol. **12**:125-129.

58. **Prevot, G., E. Bourreau, H. Pascalis, R. Pradinaud, A. Tanghe, K. Huygen, and P. Launois**. 2004. Differential production of systemic and intralesional gamma interferon and interleukin-10 in nodular and ulcerative forms of Buruli disease. Infect.Immun. **72**:958-965.

59. **Torres, M., T. Herrera, H. Villareal, E. A. Rich, and E. Sada**. 1998. Cytokine profiles for peripheral blood lymphocytes from patients with active pulmonary tuberculosis and healthy household contacts in response to the 30-kilodalton antigen of Mycobacterium tuberculosis. Infect.Immun. **66**:176-180.

60. **Trinchieri, G.** 2003. Interleukin-12 and the regulation of innate resistance and adaptive immunity
1. Nat.Rev.Immunol. **3**:133-146.

61. **Raja, A.** 2004. Immunology of tuberculosis. Indian J.Med.Res **120**:213-232.

62. **Yeboah-Manu, D., E. Peduzzi, E. Mensah-Quainoo, A. Asante-Poku, D. Ofori-Adjei, G. Pluschke, and C. A. Daubenberger**. 2006. Systemic suppression of interferon-{gamma} responses in Buruli ulcer patients resolves after surgical excision of the lesions caused by the extracellular pathogen Mycobacterium ulcerans. J.Leukoc.Biol.

63. **Jason, J., L. K. Archibald, O. C. Nwanyanwu, P. N. Kazembe, J. A. Chatt, E. Norton, H. Dobbie, and W. R. Jarvis**. 2002. Clinical and immune impact of Mycobacterium bovis BCG vaccination scarring. Infect.Immun. **70**:6188-6195.

64. **Portaels, F., J. Aguiar, M. Debacker, A. Guedenon, C. Steunou, C. Zinsou, and W. M. Meyers**. 2004. Mycobacterium bovis BCG vaccination as prophylaxis against Mycobacterium ulcerans osteomyelitis in Buruli ulcer disease. Infect.Immun. **72**:62-65.

65. **Surekha, R. H., L. Vijaya, V, G. Sumanlatha, and K. J. Murthy**. 2005. Cell-mediated immune responses in children towards secreted proteins of Mycobacterium bovis BCG. Tuberculosis.(Edinb.) **85**:89-93.

66. **Isomura, S.** 1988. Clinical studies on efficacy and safety of acellular pertussis vaccine. Tokai J.Exp.Clin.Med. **13 Suppl**:39-43.

Appendix: Recovery of immediate effector function of Vγ2Vδ2 T cells in Buruli ulcer and tuberculosis patients during treatment.

Dorothy Yeboah-Manu[1], Ernestina Mensah-Quainoo[2], Gerd Pluschke[3] and Claudia Daubenberger[3]

[1] *Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, Ghana*

[2] *Ghana Health Service, Amasaman, Ga District, Ghana*

[3] *Swiss Tropical Institute, Molecular Immunology, CH 4002 Basel, Switzerland*

Abstract

Interferon-gamma (IFN-γ) release after stimulation of peripheral blood mononuclear cells with specific antigens was analyzed in Buruli ulcer and tuberculosis patients undergoing treatment. Recovery of antigen-specific INF-γ production was quantified up to 2 months during treatment. Our result indicates that *Mycobacterium ulcerans and M. tuberculosis* infections cause long-lasting functional impairment of IFN-γ secretions.

Buruli ulcer (BU), caused by *Mycobacterium ulcerans* (*M. ulcerans*) infections, is the third most common mycobacterial disease in immuno-competent people, after tuberculosis and leprosy (14). Clinically, BU presents in the skin as chronic disease with either non-ulcerative (papules, nodules, plaques and edematous forms) or ulcerative lesions (7). Histo-pathological findings suggest that cell-mediated immunity plays a role in healing of BU. This is supported by the finding that delayed type hypersensitivity response to crude preparations of *M. ulcerans* (Burulin) was observed primarily in BU patients with healed or active disease (9). However, the involvement of the different components of the cellular immune system in protection is currently unclear. While the vast majority of mature T lymphocytes carry a T cell receptor (TCR) molecule composed of α and β chains, 1 – 5 % of circulating T cells express the γδ TCR hetero-dimer (3). T cells expressing the Vγ2Vδ2 TCR can account for up to 95 % of the γδ T cells in peripheral blood (3). After activation they secrete interferon-γ (INF-γ) and show cytotoxic T lymphocyte and natural killer cell-like effector functions (12,16). IPP represents a small phospho-antigen recognized exclusively by Vγ2Vδ2T cells and is produced during the largely prokaryotic non-mevalonate pathway of isoprenoid synthesis. It is assumed that Vγ2Vδ2T T cells sense proliferating pathogens that secrete very low amounts of intermediates of the non-mevalonate isoprenoid biosynthesis pathway providing a link between innate and adaptive immunity to a wide range of pathogens (2,8).

From studies in *M. tuberculosis* it is known that chronically active pulmonary tuberculosis appears to down-regulate Vγ2Vδ2T T cell responses resulting in impaired cytokine production or proliferation to *in vitro* stimulation with phospho-antigens (10). We and others have shown that BU patients show impaired INF-γ production after stimulation of PBMC with live or dead mycobacteria, PPD, isopentenyl pyrophosphate and also to non-mycobacterial antigens (6,17,19). Several months after surgical excision of BU lesions, INF-γ responses against all antigens used for stimulation recovered significantly, indicating that the measured systemic immuno-suppression was not the consequence of a genetic defect predisposing for BU but is rather related to the presence of *M. ulcerans* bacteria (19). In the current study we extended and refined our previous

results in a group of BU patients by analysing the rate and pace of changes in INF-γ production after surgical removal of the BU lesions and compared to TB patients undergoing drug treatment.

Seven TB and seven BU patients were involved in this study. The age range and clinical presentations for the BU and TB patients are indicated in tables 1 and 2 respectively. The BU patients were confirmed microbiologically by one of the following methods: *IS2404* PCR, culture and direct smear microscopy (18). The diagnosis of clinical TB was made on the medical history, physical examination and two acid-fast bacilli smear positive sputum samples according to the guidelines of the Ghana national tuberculosis program. The TB patients are new and had not received any therapy at the onset of the study. The first blood samples were collected from all patients before treatment. BU patients were all treated by surgical excision and TB patients by chemotherapy. For BU patients second and third blood samples were collected later after one and two months respectively. A second sample was collected two months later from the TB patients after being treated by DOTS with rifampicin/isoniazid/streptomycin and have converted to sputum smear negative. Peripheral blood mononuclear cells (PBMC) were separated within four hours after drawing peripheral venous blood, cryo preserved in Ghana and transported on liquid nitrogen to Basel for further analysis. This study was approved by the Institutional Review Board of the Noguchi Memorial Institute for Medical Research, and informed consent was obtained from the all subjects or their parents. The *ex vivo* ELISpot analysis was conducted essentially as described (19). The antigens used for stimulation of PBMC included, isopentenyl pyrophosphate (IPP) described as main stimulatory for Vγ2Vδ2 lymphocytes , PPD of *M. tuberculosis* that is stimulatory for CD4+ and Vγ2Vδ2 T cell subset and mitogen PHA. Spots were counted by the ELISpot Reader system (AID, Germany). Antigen-specific cells per 10^6 PBMC were calculated by subtracting spot numbers in media control wells from spot numbers in antigen-containing wells. Data were analyzed using the STATA program (Stata Corporation). Data was considered significant when P< 0.05.

In Figure 1, the SFU for each individual patient during the longitudinal study are given. After stimulation with PHA, all BU patients showed considerable INF-γ secretion. Hence, BU patients were able to respond to mitogen stimulation with INF-γ secretion with responses remaining stable over the time points analyzed. Therefore, a general immuno-suppression of INF-γ production and variations in quality of PBMC sample cryo-preservation could be excluded (Figure 1). In contrast, in four BU patients, the IPP induced INF-γ secretion increased significantly two months after surgery. Three patients remained at the same level during the course of investigation (Figure 1). Two out of these four patients showed also improvement of INF-γ secretion in PPD stimulated PBMC (Figure 1). As shown in figure 2, only two of the seven TB patients had a significant increase in IFN- γ secretion in response to IPP stimulation, and one had a marginal increase within two months of treatment. Two of these three patients together with three other patients improved significantly in IFN- γ secretion upon PPD stimulation of their PBMC. Contrary to the BU patients, there was a difference in response to PHA between before and during treatment (p=0.01). This finding agrees with previous findings, that T cell anergy in advanced TB patients extends to mitogens (4,15). As indicated in table 2 most of the TB patients were advanced smear positive cases.

In light of previous studies suggesting Vγ2Vδ2 T cell dysfunction in *M. tuberculosis* and HIV infections, which improves upon effective drug treatment (1,11), we investigated changes in immediate effector function in a group of BU and TB patients undergoing surgical and drug treatment. Our result indicates that, even though antigen induced cytokine secretion improved in some of the patients (in both groups) two months within treatment, the mean response was still low compared to healthy Ghanaian controls (19), especially after IRIV stimulation (result not shown). We thus confirmed that in *M. tuberculosis* and *M. ulcerans* infections, after appropriate treatment regimens, cellular immune responses improved significantly which might be indicative for shared immuno-pathogenic mechanisms of mycobacteria. Given the slow recovery of INF-γ secretion after surgery, our results question the idea that the toxin mycolactone produced by *M. ulcerans* presents the only virulence factor of *M. ulcerans* (5).

Currently, the exact mode of transmission and the reservoirs involved in BU remains to be determined (13). Additionally, it is presently unclear what proportions of people that have contact with the pathogen develop actually the clinical disease. In future, population based studies would help to define the stage of *M. ulcerans* exposure and/or infection that results in suppression of immediate effector function of Vγ2Vδ2T cells. Additionally, it would be of interest to follow BU patients without improvement of INF-γ production after surgical treatment in terms of recurrence and/or super-infection with *M. ulcerans*. In summary, previous observations of with systemic immuno-suppression of Vγ2Vδ2 T cell effector function that resolves gradually after surgical removal of bacteria are confirmed and refined.

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patient	age	gender	clinical form [a]	BCG status [b]	AFB [c]	Culture [d]	PCR [e]	1st sample [f]	2nd sample [f]	3rd sample [f]
P23	10	f	ulcerative	+	+	+	+	0	1 mo	2 mo
P26	16	f	ulcerative	ND	+	+	+	0	1 mo	2 mo
P27	16	m	nodule	ND	-	+	_	0	1 mo	2 mo
P28	4.5	m	ulcerative	+	+	+	+	0	1 mo	2 mo
P29	3.5	f	ulcerative	+	+	+	+	0	1 mo	2 mo
P30	4	m	nodule	+	+	+	+	0	1 mo	2 mo
P31	5	f	ulcerative	+	-	-	+	0	1 mo	2 mo
P32	3	m	ulcerative	+	+	+	+	0	1 mo	2 mo
P34	40	f	ulcerative	+	+	+	+	0	1 mo	2 mo

Table I: Clinical data of patients presenting with lesions due to confirmed *M. ulcerans* infection

[a] clinical forms of BU disease were graded according to the WHO case definition .

[b] BCG status was determined by confirmation of the presence of a BCG scar by two persons.

[c] Acid fast bacilli detection was performed by direct smearing of tissue exudates followed by Ziehl Neelson staining . [d] Culture of *M. ulcerans* according to . [e] IS2404 PCR analysis according to . [f] Time of blood sample collection. Time 0 is time of surgical treatment

patient	age	gender	clinical form[a]	BCG status[b]	AFB[c]	1st blood sample [f]	2nd blood sample [f]
TB01	21	M	Pulmonary	+	+	0	2 mo
TB02	38	M	Pulmonary	nd	+	0	2 mo
TB05	30	F	Pulmonary	nd	++	0	2 mo
TB06	21	M	Pulmonary	+	+	0	2 mo
TB09	35	M	Pulmonary	-	+++	0	2 mo
TB10	24	F	Pulmonary	+	++	0	2 mo
TB12	36	M	Pulmonary	-	+++	0	2 mo

Table II: Clinical data of patients presenting with lesions due to confirmed tuberculosis infection

[b] BCG status was determined by confirmation of the presence of a BCG scar by two persons.

[c] Acid fast bacilli detection was performed by direct smearing of tissue exudates followed by Ziehl Neelson staining .

[f] Time of blood sample collection. Time 0 is time of diagnosis before treatment
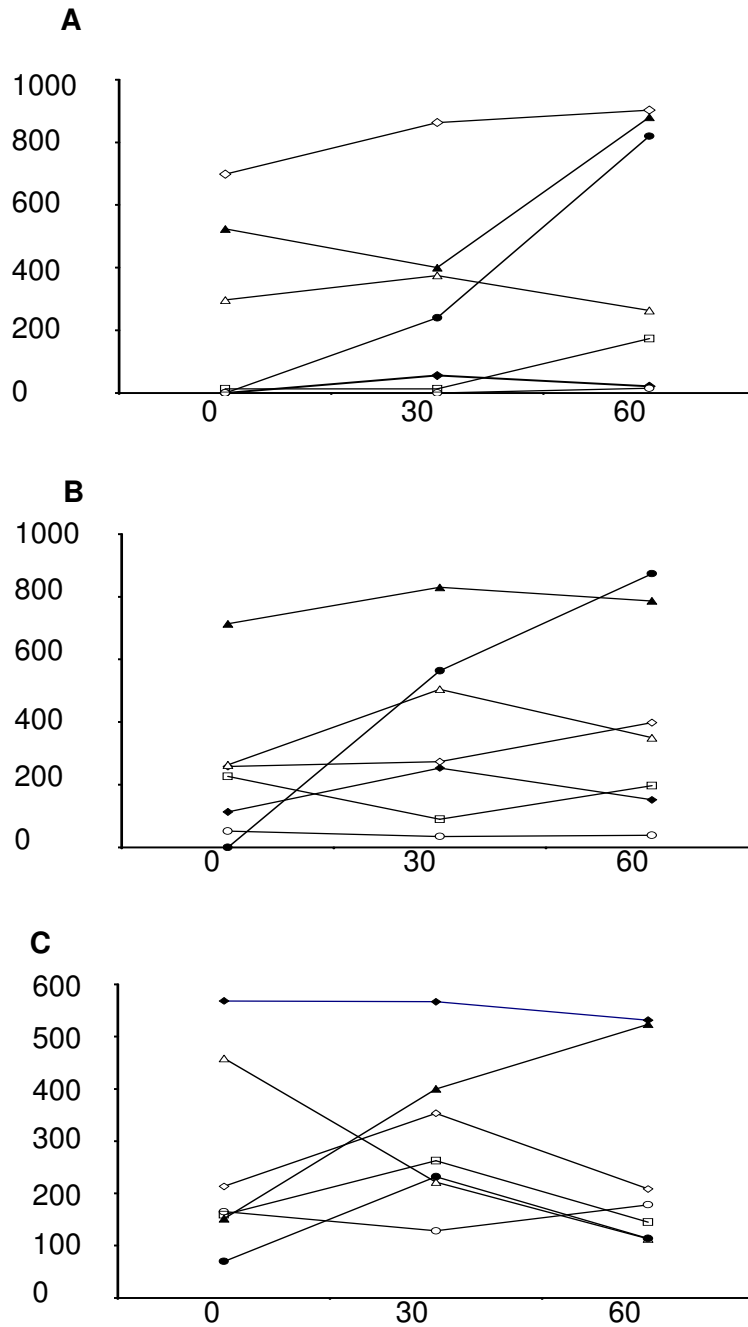
Figure 1: Increase in antigen-specific *ex vivo* INF-γ secreting cells in BU patients after surgical treatment. PBMC of seven BU patients sampled at day 0, 30 and 60 post surgery (p.s.) (Table I) were thawed into cell culture medium and kept overnight in the presence or absence of IPP (a), PPD (b) and PHA (c).
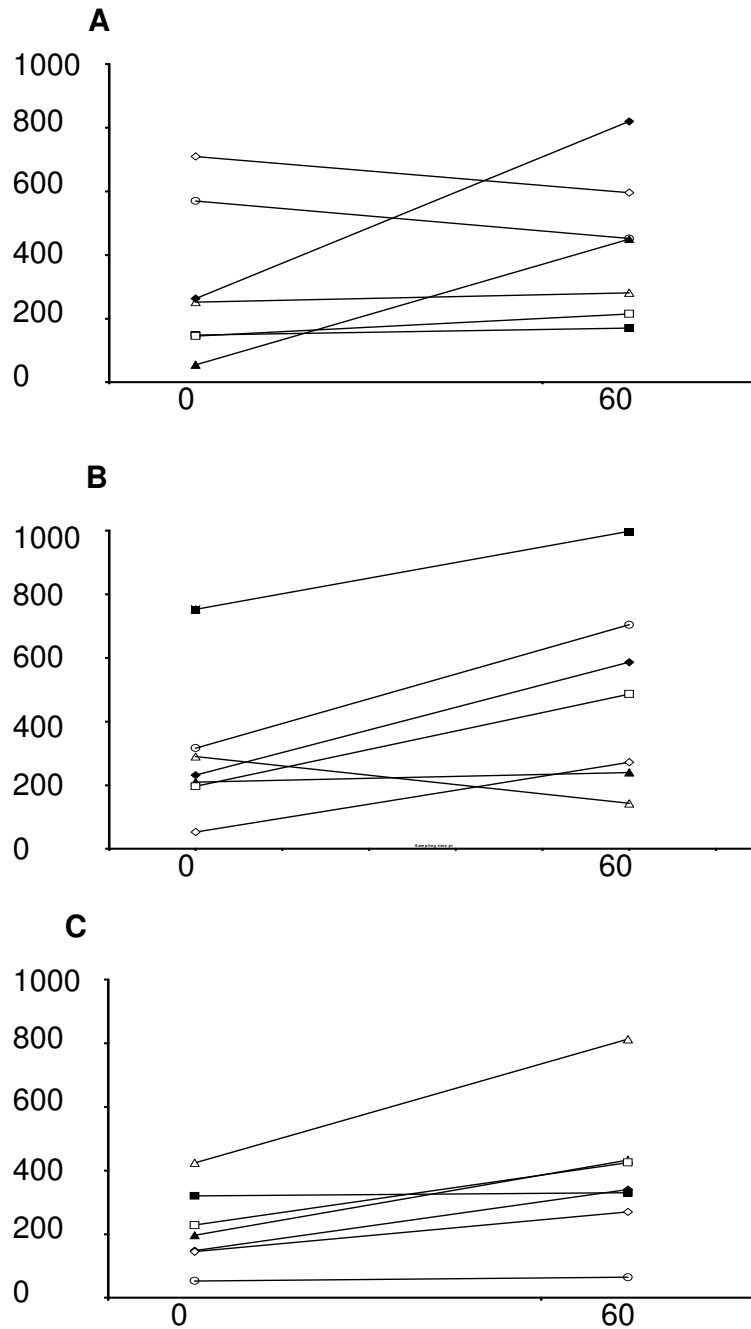
Figure 2: Increase of antigen-specific *ex vivo* INF-γ secretion in TB patients during treatment. PBMC of seven TB patients sampled at day 0, and 60 of treatment (Table 2) were thawed into cell culture medium and kept overnight in the presence or absence of IPP (a), PPD (b) and PHA (c).

Reference List

1. **Al Attiyah, R., A. S. Mustafa, A. T. Abal, N. M. Madi, and P. Andersen**. 2003. Restoration of mycobacterial antigen-induced proliferation and interferon-gamma responses in peripheral blood mononuclear cells of tuberculosis patients upon effective chemotherapy. FEMS Immunol.Med.Microbiol. **38**:249-256.

2. **Belmant, C., E. Espinosa, R. Poupot, M. A. Peyrat, M. Guiraud, Y. Poquet, M. Bonneville, and J. J. Fournie**. 1999. 3-Formyl-1-butyl pyrophosphate A novel mycobacterial metabolite-activating human gammadelta T cells. J.Biol.Chem. **274**:32079-32084.

3. **Chen, Z. W.** 2005. Immune regulation of gammadelta T cell responses in mycobacterial infections. Clin.Immunol. **116**:202-207.

4. **Dlugovitzky, D., M. L. Bay, L. Rateni, L. Urizar, C. F. Rondelli, C. Largacha, M. A. Farroni, O. Molteni, and O. A. Bottasso**. 1999. In vitro synthesis of interferon-gamma, interleukin-4, transforming growth factor-beta and interleukin-1 beta by peripheral blood mononuclear cells from tuberculosis patients: relationship with the severity of pulmonary involvement. Scand.J.Immunol. **49**:210-217.

5. **George, K. M., D. Chatterjee, G. Gunawardana, D. Welty, J. Hayman, R. Lee, and P. L. Small**. 1999. Mycolactone: a polyketide toxin from Mycobacterium ulcerans required for virulence. Science **283**:854-857.

6. **Gooding, T. M., P. D. Johnson, M. Smith, A. S. Kemp, and R. M. Robins-Browne**. 2002. Cytokine profiles of patients infected with Mycobacterium ulcerans and unaffected household contacts. Infect.Immun. **70**:5562-5567.

7. **Guarner, J., J. Bartlett, E. A. Whitney, P. L. Raghunathan, Y. Stienstra, K. Asamoa, S. Etuaful, E. Klutse, E. Quarshie, T. S. van der Werf, W. T. van der Graaf, C. H. King, and D. A. Ashford**. 2003. Histopathologic features of Mycobacterium ulcerans infection. Emerg.Infect.Dis. **9**:651-656.

8. **Jomaa, H., J. Feurle, K. Luhs, V. Kunzmann, H. P. Tony, M. Herderich, and M. Wilhelm**. 1999. Vgamma9/Vdelta2 T cell activation induced by bacterial low molecular mass compounds depends on the 1-deoxy-D-xylulose 5-phosphate pathway of isoprenoid biosynthesis. FEMS Immunol.Med.Microbiol. **25**:371-378.

9. **Kiszewski, A. E., E. Becerril, L. D. Aguilar, I. T. Kader, W. Myers, F. Portaels, and P. R. Hernandez**. 2006. The local immune response in ulcerative lesions of Buruli disease. Clin.Exp.Immunol. **143**:445-451.

10. **Li, B., M. D. Rossman, T. Imir, A. F. Oner-Eyuboglu, C. W. Lee, R. Biancaniello, and S. R. Carding**. 1996. Disease-specific changes in gammadelta T cell repertoire and function in patients with pulmonary tuberculosis. J.Immunol. **157**:4222-4229.

11. **Martini, F., R. Urso, C. Gioia, A. De Felici, P. Narciso, A. Amendola, M. G. Paglia, V. Colizzi, and F. Poccia**. 2000. gammadelta T-cell anergy in human immunodeficiency virus-infected persons with opportunistic infections and recovery after highly active antiretroviral therapy. Immunology **100**:481-486.

12. **Olin, M. R., C. K. Hwa, J. Lee, and T. W. Molitor**. 2005. Gammadelta T-lymphocyte cytotoxic activity against Mycobacterium bovis analyzed by flow cytometry. J.Immunol.Methods **297**:1-11.

13. **van der Werf, T. S., Y. Stienstra, R. C. Johnson, R. Phillips, O. Adjei, B. Fleischer, M. H. Wansbrough-Jones, P. D. Johnson, F. Portaels, W. T. van der Graaf, and K. Asiedu**. 2005. Mycobacterium ulcerans disease. Bull.World Health Organ **83**:785-791.

14. **van der Werf, T. S., W. T. van der Graaf, J. W. Tappero, and K. Asiedu**. 1999. Mycobacterium ulcerans infection. Lancet **354**:1013-1018.

15. **Vilcek, J., A. Klion, D. Henriksen-DeStefano, A. Zemtsov, D. M. Davidson, M. Davidson, A. E. Friedman-Kien, and J. Le**. 1986. Defective gamma-interferon production in peripheral blood leukocytes of patients with acute tuberculosis. J.Clin.Immunol. **6**:146-151.

16. **Wen, L., D. F. Barber, W. Pao, F. S. Wong, M. J. Owen, and A. Hayday**. 1998. Primary gamma delta cell clones can be defined phenotypically and functionally as Th1/Th2 cells and illustrate the association of CD4 with Th2 differentiation. J.Immunol. **160**:1965-1974.

17. **Westenbrink, B. D., Y. Stienstra, M. G. Huitema, W. A. Thompson, E. O. Klutse, E. O. Ampadu, H. M. Boezen, P. C. Limburg, and T. S. van der Werf**. 2005. Cytokine responses to stimulation of whole blood from patients with Buruli ulcer disease in Ghana. Clin.Diagn.Lab Immunol. **12**:125-129.

18. **Yeboah-Manu, D., T. Bodmer, E. Mensah-Quainoo, S. Owusu, D. Ofori-Adjei, and G. Pluschke**. 2004. Evaluation of decontamination methods and growth media for primary isolation of Mycobacterium ulcerans from surgical specimens. J.Clin.Microbiol. **42**:5875-5876.

19. **Yeboah-Manu, D., E. Peduzzi, E. Mensah-Quainoo, A. Asante-Poku, D. Ofori-Adjei, G. Pluschke, and C. A. Daubenberger**. 2006. Systemic suppression of interferon-{gamma} responses in Buruli ulcer patients resolves after surgical excision of the lesions caused by the extracellular pathogen Mycobacterium ulcerans. J.Leukoc.Biol.

Curriculum Vitae
PERSONAL DETAILS

- Surname: YEBOAH-MANU
- Maiden Name: AMOAH
- Other Names: Dorothy Kyerewah
- Date of Birth: 27th November 1966
- Nationality: Ghanaian
- Marital Status: Married

EDUCATION

- **Ofori Panin Secondary School:** 1979-1986. 8 GCE Ordinary & 4 Advanced Levels.

- **Kwame Nkrumah University of Science and Technology:** 1988-1992 BSc, (Hons) First Class in Biochemistry. I carried out a project on the microbiological quality of street foods sold in Kumasi, Ghana.

- **London School of Hygiene and Tropical Medicine:** 1999-2000, MSc. in Applied Molecular Biology of Infectious Diseases. The title of my thesis was 'A rapid and easy method for the differentiation of members of the *Mycobacterium tuberculosis* complex'. I was sponsored by Ghana Government Scholarship.

- **Swiss Tropical Institute, University of Basel:** 2003-2006, Ph.D in Microbiology. The title of my thesis is 'Bacteriological and immunological studies toward the effective management and control of *Mycobacterium ulcerans* disease.

. WORK EXPERIENCE

- June- September 1991:   Vacation training, Cadbury Ghana
- April 1993 to April1998:   Senior Research Assistant Noguchi Memorial Institute for Medical Research (NMIMR)
- May 1998 to Nov. 2001:   Principal Research Assistant (NMIMR)
- Dec. 2001 to date: Chief Research Assistant (NMIMR)

RESEARCH INTEREST

- Microbial quality of foods in Ghana

- Diarrhoea diseases in children

- Mycobacterial diseases of public health importance in Ghana.

COURSES ATTENDED

- Mar-96 to Dec-96: I attended a nine-month course in current molecular biology techniques and their application in bacteriology at the Institute of Bacterial Disease Research, Osaka University, Japan. I was sponsored by JICA

- Aug-02 to Sep-02: Surveillance of Transmissible diseases. Advanced module of the Master in International Health, held at the Swiss Tropical Institute and organised by WHO/STI.

CONFERENCES AND WORKSHOPS ATTENDED

- FAO inter-country workshop on street foods in Africa held at the FAO office in Accra. 27April – 1 May 1992

- SGS Ghana workshop on Occupational Health and Safety held in Accra. 23–25 February 1993.

- WHO centenary celebration of ORT in Accra. I was the rappauter for the occasion. I was also a member of the report writing committee. 9 December 1994

- Japan Association of Microbiology centenary celebration conference in Tokyo, Japan.17 –19 April 1996

- African Health Sciences Congress, held in Accra, Ghana. I presented a paper titled "Street food from Accra Ghana: How safe? 19 - 23 April 1999

- International Mini-symposium on Buruli ulcer. Evaluation of decontamination methods and growth media for the primary isolation of *Mycobacterium ulcerans* from surgical specimens.Basel, Switzerland. March 12-13, 2004.

- WHO Afro workshop on Buruli ulcer in Accra, Ghana. Jan 2004

- Medicine and Health in the Tropics. Systemic suppression of interferon-gamma responses in Buruli ulcer patients resolves after surgical excision of the lesions

caused by the extracellular pathogen Mycobacterium ulcerans (poster). Marseille, France. September 11-15, 2005

- Annual Congress of the Swiss Society of Tropical Medicine and Parasitology. Systemic suppression of interferon-gamma responses in Buruli ulcer patients resolves after surgical excision of the lesions caused by the extracellular pathogen Mycobacterium ulcerans (poster). Ascona, Switzerland, November 2-3, 2005.

PUBLICATIONS

1. Patience Mensah**,** M Armar-Klemesu, **D Yeboah-Manu**, A Ablordey, K Rikimaru, K Taniguchi, F. Nkrumah, H. Kamiya. Microbial quality of infant foods from peri-urban Ghana. *African Journal of Health Sciences 1995;2:282-286.*

2. Patience Mensah, Kwaku Owusu-Darko, **Dorothy Yeboah-Manu**, Anthony Ablordey, H Kamiya[1] & FK Nkrumah. The role of street food vendors in the transmission of enteric pathogens in Accra. *Ghana Medical Journal 1999; 33:1,19-29*

3. **Dorothy Yeboah-Manu,** Malcolm D. Yates and Stuart Mark Wilson. Application of a simple multiplex polymerase chain reaction to aid in the routine work of the mycobacterium reference laboratory. *J. Clin Microbiology 2001;39:4166-4168*

4. Patience Mensah, **Dorothy Yeboah-Manu**, Kwaku Owusu-Darko & Anthony Ablordey. Street foods in Accra, Ghana: How safe are they? *Bulletin of the World health Organisation 2002; 80:546-554*

5. Patience Mensah, **Dorothy Yeboah-Manu**, Owusu-Darko Kwaku, Ablordey Anthony, M Armar-Klemesu, G. Armah. Food Handling and Household Factors have predisposing roles in Persistent Diarrhoea in Ghana. *Bulletin of the World Health Organisation (Submitted).*

6. **Dorothy Yeboah-Manu**, Thomas Bodmer, Ernestina Mensah-Quainoo, Samuel Owusu, David Ofori-Adjei and Gerd Pluschke. Evaluation of decontamination methods and growth media for the primary isolation of *Mycobacterium ulcerans* from surgical specimens. *J. Clin Microbiol 2004;* 42(12):5875-5876

7. Agbodaze D, Nmai PNA, Robertson FC, **Yeboah-Manu D**, Owusu-Darko K, Addo KK Microbial quality of "khebab" consumed in the Accra Metropolis *Ghana Medical Journal*2005;39(2):46-49

8. Hilty M*, **Yeboah-Manu D***, Boakye D, Mensah-Quainoo E, Rondini S, Schelling E, Ofori-Adjei D, Portaels F, Zinsstag J, Pluschke G. Genetic Diversity in Mycobacterium ulcerans isolates from Ghana revealed by a newly identified locus containing a Variable Number of Tandem Repeats. *J. Bacteriol* 2006; 188(4):1462-5. * Contributed equally

9. **Dorothy Yeboah-Manu** , Elisabetta Peduzzi , Ernestina Mensah-Quainoo , Adwoa Asante-Poku , David Ofori-Adjei , Gerd Pluschke, Claudia Dauberbenger. Systemic suppression of interferon-gamma responses in Buruli ulcer patients resolves after surgical excision of the lesions caused by the extracellular pathogen Mycobacterium ulcerans. *J Leukoc Biol* 2006;79(6):1150-6.

10. D. Diaz, H Döbeli, **D. Yeboah-Manu**, E. Mensah-Quainoo, A. Friedlein, N. Soder, S. Rondini, T Bodmer, and G. Pluschke. Use of the immunodominant 18kDa small heat shock protein as serological marker for exposure to *M. ulcerans*. *Clinical vaccine development(submitted)*

11. **Dorothy Yeboah-Manu**, Adwoa Asante-Poku, Kwaku Owusu-Darko, Ernestina Mensah-Quainoo, Markus Hilty, David Ofori-Adjei, Gerd Pluschke. Identification of streptomycin and rifampicin resistance M. ulcerans isolates in Ghana. To be submitted to *Antimicrobial chemotherapy*.

12. Ernestina Mensah-Quainoo, **Dorothy Yeboah-Manu**, Caroline Asebi, Francis Patafuor David Ofori-Adjei, Thomas Junghanss, Gerd Pluschke. Diagnosis of *Mycobacterium ulcerans* disease: an assessment of the accuracy of clinical judgement at a treatment centre in Ghana. To be submitted to *Tropical medicine and international health*

13. **Dorothy Yeboah-Manu** , Ernestina Mensah-Quainoo , Adwoa Asante-Poku , David Ofori-Adjei , Gerd Pluschke, Claudia Dauberbenger. Enhanced serum levels of interferon-gamma and tumor necrosis alpha in household contacts of patients with *Mycobacterium ulcerans* disease**.** Ready for submission to *Clinical Vaccine Immunology*

14. **Dorothy Yeboah-Manu**, Ernestina Mensah-Quainoo, Gerd Pluschke and Claudia Dauberbenger. Recovery of immediate effector function of Vγ2Vδ2 T cells in Buruli ulcer and tuberculosis patient during treatment. To be submitted to *Infection and Immunity*

ABSTRACTS

1. Patience Mensah, M Armar-Klemesu, **D Yeboah-Manu**, A Ablordey, K Rikimaru, K Taniguchi, F. Nkrumah, H. Kamiya. Microbial quality of infant foods from peri-urban Ghana. 16th African Health Sciences Congress, Nairobi, Kenya, 6-10 February 1995.

2. Patience Akpedonu, **Dorothy Yeboah-Manu**, A. Ablordey and Kwaku Owusu-Darko. Street Food From Accra: How Safe. 20th African Health Sciences Congress Accra, Ghana, April 19-23 1999: Abstract 0-25.

3. Patience Akpedonu, Kwaku Owusu-Darko, **Dorothy Yeboah-Manu**, Anthony Ablordey. The Role of Street Food Vendors in the Transmission of Enteric Pathogens in Accra. 20th African Health Sciences Congress Accra, Ghana, April 19-23 1999:Abstract 0-26.

4. Patience Mensah, M Armar-Klemesu, K. Owusu-Darko, **D Yeboah-Manu**, A Ablordey. Risk Factors for Persistent diarrhoea in Accra, Ghana. First World Congress on Paediatric Gastroenterology and Nutrition, Boston, Massachusetts, USA, 5-9 August 2000. Abstract 906, pp S232.

5. Addo K. K., **Yeboah-Manu D. K.**, Dan-Dzide M. N., Owusu-Darko K., Caulley P., Minamikawa M., Akpedonu P., Bonsu F. The dilemma of incorporating a TB microscopy quality assurance framework into a regional health system in Ghana. 14th Conference of the African region IUATLD, Durban South Africa, 11-14 June 2002. Abstract 023.

6. Addo K. K., **Yeboah-Manu D. K.**, Owusu-Darko K., Dan-Dzide M. N., Caulley P., Minamikawa M., Ak[edonu P., Bonsu F., Leinhardt C. Situational analysis of TB microscopy centres in Ghana. 14th Conference of the African region IUATLD, Durban South Africa, 11-14th June 2002. Abstract 043,

7. K. Addo, **D. K. Yeboah-Manu**, M. N. Dan-Dzide K. Owusu-Darko, P. Caulley, M. Minamikawa, P. Akpedonu, F. Bonsu and C. Lienhardt. Improving laboratory diagnosis of TB in Ghana: the impact of a quality assurance system. 33rd world conference of the International Union against TB and Lung disease, Montreal, Canada, 6-10 October 2002. Abstract 206-PD ppS111

DOCUMENTS

1. Kennedy K. Addo, Phillomena Caulley, **Dorothy K. Yeboah-Manu,** Kwaku Owusu-Darko, Marilyn Dan-Dzide, Mariko Minamikawa and Patience Akpedonu. *Tuberculosis Microscopy: A laboratory manual for Ghana*. 2001
2. Kennedy K. Addo, **Dorothy K. Yeboah-Manu,** Kwaku Owusu-Darko, Marilyn Dan-Dzide, Mariko Minamikawa and Frank Bonsu. *Tuberculosis Microscopy: Establishment of quality assurance system for the TB national control program in Ghana (in press).*