Development of new molecular genetic tools to study *Mycobacterium ulcerans* infection (Buruli ulcer)

INAUGURALDISSERTATION

zur

Erlangung der Würde einer Doktorin der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

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aus

Viterbo (Italien)

Basel, 2005

AKNOWLEDGMENTS

The present PhD project was undertaken within a network of collaboration in Switzerland and abroad, and it saw the contribution of numerous people who became involved in many different ways. Thanks to their invaluable participation, the realization of this work was made possible. To all of them go my most grateful acknowledgments.

My first thanks go to my supervisor at the Swiss Tropical Institute, Prof. Gerd Pluschke, who provided me with the best scientific and moral support I could ever have desired. I am especially grateful to him for his trust and guidance experienced during the whole period of the PhD, which, combined with his continuous and stimulating participation, kept me highly motivated and enthusiastic.

I would like to thank Dr. Ernestina Mensah-Quainoo, Amasaman District Health Director, for her competence and energy, which created an excellent base for our successful collaboration. Together with her, I also would like to thank the whole Amasaman team of nurses, anaesthetists and health managers, who welcomed me warmly and invaluably committed themselves to the project.

Sincere thanks goes to Dr. Thomas Junghanss, for his support in the field and for his medical and inter-personal skills. His was irreplaceable in solving unexpected problems in the field, while always maintaining the best collaborative atmosphere.

The accessibility to the other field station in Cameroon was guaranteed by Dr. Jürgen Noeske and by the Aide aux Lepreux Emaus Suisse (A.L.E.S.). I thank them for the organization of the Buruli ulcer survey and for the collection and shipment of the samples.

Particular thanks are addressed to Mrs. Dorothy Yeboha for her culturing several strains of *M. ulcerans* at the Noguchi Memorial Institute for Medical Research.

Without the participation of the Buruli ulcer patients and the help of the local health workers, this study would have not taken place, so my deep gratitude goes to all of them for their willingness to take part in the project and for their trust in its relevance.

During the entire PhD, I was able to take advantage of a strong partnership with the Unit of Medical Genomics at Hoffman-LaRoche, represented by Prof. Ulrich Certa. His generous scientific and technical contribution was essential to developing the microchip-array used to perform molecular epidemiology. I would also like to acknowledge the members of his team, with whom I created a very enjoyable and efficient collaboration: Adriana Ille, Michel Tessier, Cyrill Mangold, Gregor Dernick and Oliver Gutmann. All of them offered their skills and commitment, which resulted in a remarkable exchange of expertise.

Part of the work was undertaken at the Institute Pasteur in Paris, in the Unité de Génétique Moléculaire Bactérienne department. I am very grateful to Dr. Timothy Stinear, who organized my stay there and who constantly supported me with his most recent findings in the functional genomics of *M. ulcerans*. He provided me with the plasmid library used to construct the microarray and with extremely valuable comments and suggestions.

The Real-Time PCR measurements were performed at Solvias, Basel, thanks to collaboration with Dr. Heike Troll. I am grateful to her for instructing me on how to use the apparatus, and for her constant assistance in the analysis of the data.

The origin of all the *M. ulcerans* strains used derived either from the Institute for Infectious Diseases in Bern, where we collaborated with Dr. Thomas Bodmer, or from the Institute of Tropical Medicine in Antwerp, kindly provided by Prof. François Portaels. I am extremely grateful to both of them, who ensured the starting material for any study I undertook.

The histopathology results, needed to study *M. ulcerans* spreading, were provided by Prof. Sebastian B. Lucas and Catherine Horsfield at the Department of Histopathology, St. Thomas's Hospital in London. I would like to thank them for their excellent collaboration.

I am very grateful to Michael Käser for sharing the work load on *M. ulcerans* deletions identification, and to Martin Nägeli for his commitment in performing the numerous PCRs and sequencing reactions required.

A special thank goes to Laura Gosoniu, who patiently helped me in the statistical analysis of the data and to Uwe Niesner for his help in German translations.

Very warm thanks are reserved to my friends and colleagues from the MPI, who created an ideal collaborative working atmosphere, so important for me during the last four years: Elisabetta Peduzzi, Diana Diaz, Marija Curcic, Jean Pierre Dangy, Julia Leimkugel, Markus Müller, Denise Vogel, Sybille Siegrist, Valentine Pflüger, Lucy Ochola, Shinji Okitsu, Marco Tamborrini, Christine Banholzer, Therese Ruf, Max Bastian, Toby Jäggi, Rolf Spirig, Tatjana Zalac, Daniela Schütte, Franziska Schwager, Niels Pfeiffer, Selina Bopp, Christian Flueck, Mirjam Kaestli, Sebastian Rusch, Jutta Marfurt, Christian Scheurer, Sonja Bernhard, Michael Oberle, Naomi Maina, Kerstin Gillinwater and Nina Schild.

I extend many thanks to the senior scientists who helped me in one way or another:

Prof. Niklaus Weiss, PD. Dr. Claudia Daubenberger, Prof. Tom Smith, Dr. Penelope Vainatsu, PD. Dr. Ingrid Felger, Prof. Hans-Peter Beck, Prof. Jacob Zinsstag and Prof. Reto Brun.

I would also like to thank Christine Walliser, Isabelle Bolliger, Eliane Ghilardi and Agnès Doré, Madlene Buholzer, Beatrice Waeckerlin for their administrative support.

Particular thanks are devoted to Bernadette Peterhans, Axel Hoffmann, Erna Schaefer and Caroline Meyer for their help and enthusiasm during the coordination of the Health Management in Tropical Countries (HCMTC) course, which I attended before starting the PhD.

My sincere thank goes to Prof. Marcel Tanner for having welcomed me at the STI, by giving me the opportunity to attend the course of Health Care Management in Tropical Countries. I also would like to thank him for his support and sharing of a common vision of "science as service" for health improvement.

Many other people of the STI contributed to making the period of my PhD a wonderful experience, among them: Monica Daigl, Flavia Pizzagalli, Yvette Endriss, Werner Rudin, Abdallah Abouihia, Angelika Barry, Martin Baumann, Markus Hilty, Bandula Kurukulasooriya, Heidi Immler, Hanspeter Marti, Bianca Pluess, Giovanna Raso, Martin Raab, Wilson Sama and Christian Lengeler.

Finally, I would like to thank my parents Rolando and Gabriela, my sister Cristiana, my love Steve and my best friends Elisabetta and Ursula for their never-ending support, patience and affection.

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Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät

Der Universität Basel auf Antrag der

Herren Prof. Dr. N. Weiss, Prof. Dr. P. Erb und Prof. Dr. G. Pluschke.

Basel, 12 Juli 2005

Prof. Dr. Hans-Jakob Wirz

Dekan

dedicated to my family

with gratitude and love

SUMMARY

Buruli ulcer is an infectious disease caused by an environmental pathogen, *Mycobacterium ulcerans,* which is the third major mycobacterial pathogen of man, after *M. tuberculosis* and *M. leprae.* Since 1980, dramatic increases in the incidence of Buruli ulcer have been reported from West African countries, sometimes associated with man-made environmental changes. After the first international conference on Buruli ulcer in 1998 (Yamoussoukro meeting), attention has been drawn to the severity of this neglected disease and to its many poorly understood features. Since then new initiatives have been undertaken to promote control and research efforts. Within the framework of WHO identified research priorities, the present PhD project focused on the development of new molecular genetic tools to investigate *M. ulcerans* epidemiology and pathology.

Apart from the association of Buruli ulcer with swampy environments, little is known about risk factors, environmental reservoirs and pathways of transmission. One factor that impairs research on these issues is the lack of suitable fine typing methods to track different *M. ulcerans* subclones and their spreading within a community. The comprehension of the population structure itself and of the mechanisms leading to genetic variability also suffers from this lack of tools. For this reason, we developed a new plasmid-based microarray approach, which was used to perform a comparative genomic analysis of 30 *M. ulcerans* strains, from different geographical origins. Fifteen large sequence polymorphisms were identified affecting genes of all major functional categories. Results obtained with this prototype microarray demonstrated that insertional/deletional events, often associated with insertion sequences are the most important mechanisms of genetic diversification in *M. ulcerans.* Analysis of strain diversity with a larger microarray should represent a suitable tool for micro-epidemiological studies.

Within the framework of a Buruli ulcer survey in Cameroon, an optimized diagnostic PCR was developed. The method, operating on genetic material extracted directly from swab samples, demonstrated the usefulness of such highly sensitive technique for epidemiological studies. Neglected Buruli ulcer foci have been rediscovered and an association between Buruli ulcer cases and slow flowing water basins have been reconfirmed.

A quantitative PCR specific for *M. ulcerans* DNA, the *IS2404* real-time PCR, was developed with the aim to gain insights into the pathology of the disease. The very high sensitivity and specificity of the method allowed the quantitative assessment of the dissemination of the mycobacteria in Buruli ulcer lesions, and its comparison with histopathological changes.

Although the heaviest mycobacterial burden was detected in the central foci of the lesions, we could measure significant amounts of mycobacterial DNA and microcolonies in samples from peripheral regions and occasionally in healthy appearing excised tissue margins. Additional peaks of mycobacterial DNA clearly marked sites where satellite lesions were developing. Even when granulomas provided evidence for the development of cell-mediated immunity, development of satellite lesions by contiguous spreading was not completely prevented. The technique offers also the potential to predict recurrences: in one case we could demonstrate that a relatively small number of mycobacteria that have spread into healthy appearing tissue can lead to the development of a recrudescence. These data altogether support the concept that wider surgical excision improves the chance of healing of Buruli ulcer. The application of our approach for assessing the mycobacterial burden in excision margins, combined with long term follow-up of patients, should help to improve current guidelines for surgical treatment of Buruli ulcer.

It is becoming more and more evident that mycobacterial spreading can occur even at distant sites from the original primary ulcer, producing so called “metastatic lesions”. The contribution of re-activation versus re-infection is not clear, neither is the mode of spreading into the body known. In the case of a HIV+ patient, we could report the insurgence of multifocal aggressive lesions leading to osteomyelitis. The time span interposing between the primary Buruli manifestations and the recurrence at the new sites, together with the physical distance of the patient from the endemic area, is such to argue about eventual persistence of *M. ulcerans* in an immunocompromised individual.

ZUSAMMENFASSUNG

Buruli Ulkus, eine Infektionskrankheit verursacht durch *Mycobacterium ulcerans*, ist nach Tuberkulose und Lepra die dritt häufigste durch Mykobakterien verursachte Erkrankung. Seit 1980 ist die Inzidenz von Buruli Ulkus in ostafrikanischen Ländern dramatisch angestiegen. In einigen Fällen ist dies mit durch den Menschen hervorgerufenen Veränderungen der Umwelt assoziiert. Die erste internationale Konferenz über Buruli Ulkus im Jahre 1998 (Yamoussoukro Konferenz) richtete erstmals die Aufmerksamkeit auf die Schwere und die wenig verstandenen Mechanismen dieser bis dato weitgehend unbeachteten Erkrankung. Innerhalb der von der WHO ausgerufenen Forschungsschwerpunkte versucht diese Doktorarbeit durch Entwicklung molekular genetischer Methoden dazu beizutragen, die Epidemiologie und Pathologie der *M. ulcerans* Infektionen zu klären.

Bis auf die Verbindung des Auftretens von Buruli Ulkus mit Feuchtgebieten ist über Risikofaktoren, Umweltreservoir und Übertragungswege wenig bekannt. Ein wichtiger Faktor, der die Klärung dieser Fragen erschwert, ist das Fehlen geeigneter Typisierungsmethoden um *M. ulcerans* Subklone zu unterscheiden und so die Ausbreitung der Erreger innerhalb eines endemischen Gebietes zu verfolgen. Das Verständnis der Populationsstruktur der Mycobakterien und der Mechanismen die zu genetischer Variabilität des Erregers führen, ist hiervon ebenso betroffen. Um diese Fragen zu klären, haben wir einen DNA Mikroarray entwickelt, auf dem Plasmide immobilisiert wurden, die genomische Sequenzen des Erregers enthalten. Dieser Prototyp wurde benutzt, um eine vergleichende genomische Analyse von 30 *M. ulcerans* Stämmen unterschiedlicher geographischer Herkunft durchzuführen. Es wurden 15 große Sequenz- Polymorphismen identifiziert, die Gene aller wichtigen funktionellen Kategorien umfassten. Diese Ergebnisse zeigten, dass das Auftreten von Deletionen, häufig assoziiert mit der Insertion von Sequenzen, der wichtigste Mechanismus der genetischen Diversifikation von *M. ulcerans* darstellt. Ein das ganze Genom von *M. Ulcerans* umfassender Mikroarray wird daher voraussichtlich detailliertere Analysen der genomischen Unterschiede zwischen Erregerstämmen und damit mikroepidemiologische Studien zur Ausbreitung genetischer Varianten des Erregers ermöglichen.

Für eine Studie über die Verbreitung des Buruli Ulkus in Kamerun, wurde eine diagnostische Polymerase Kettenreaktion (PCR) optimiert. Die erfolgreiche Extraktion genetischen Materials aus Wundabstrichen gefolgt von der sensitiven PCR-basierten Detektion des Erregers bewies die Eignung dieser Technik für epidemiologische Studien.

ABBREVIATIONS

AFLP	Amplified Fragment Lenght Polymorphism
ALES	Aide aux Lepreux Emaus Suisse
BCG	Bacillus Calmette-Guèrin
BU	Buruli Ulcer
CDS	Coding Sequences
dNTP	Deoxyribonucleosidetriphosphate
DRC	Democratic Republic of Congo
ELISA	Enzyme-linked Immunoabsorbent Assay
H&N	Hematoxylin-Eosin
InDels	Insertions and Deletions
IgG, M	Immunoglobulin G, M
IL-4, 10….	Interleukin 4, 10…
INFγ	Interferon Gamma
IS	Insertion Sequence
LSP	Large Sequence Polymorphism
MIRU	Microsatellites Interspersed Repetitive Units
MLST	Multilocus Sequence Typing
mRNA	Messenger Ribonucleic Acid
PCR	Polymerase Chain Reaction
PFGE	Pulsed-field Gel Electrophoresis
PRPA	PCR-restriction Profile Analysis
RD	Region of Diversity
RFLP	Restriction Fragment Length Polymorphism
rRNA	Ribosomal Ribonucleic Acid
SNP	Single Nucleotide Polymorphism
STI	Swiss Tropical Institute
TH	T cell Helper
VNTR	Variable Number Tandem Repeats
WHO	World Health Organization
ZN	Ziehl-Neelsen staining

Chapter 1

INTRODUCTION

1.1 History and epidemiology of Buruli ulcer

Buruli ulcer is a disease of skin and soft tissue caused by an environmental pathogen, *Mycobacterium ulcerans,* which has the capacity to produce a damaging toxin. The main burden of disease falls on children living in sub-saharan Africa but healthy people of all ages, races and socioeconomic classes are susceptible.

M. ulcerans is the third most important mycobacterial pathogen of man, after *M. tuberculosis* and *M. leprae*[1]. The definitive description of *M. ulcerans* was published in 1948, when MacCallum and others reported 6 cases of an unusual skin infection in Australia, caused by a mycobacterium that could only be cultured in Löwenstein-Jensen medium when the incubation temperature was set lower than for *M. tuberculosis*[2]. In Africa, large ulcers almost certainly caused by *M. ulcerans* had been described by Sir Robert Cook in 1897 and by Kleinschmidt in northeast Congo during the 1920s[3].

Prior to the 1980s, foci of *M. ulcerans* infection were reported in several countries in sub-Saharan Africa including Congo, Uganda, Gabon, Nigeria, Cameroon and Ghana[4]. The Uganda Buruli Group coined the name "Buruli ulcer" because the cases they described were first detected in a Buruli county, near lake Kyoga[5].

Since 1980, dramatic increases in the incidence of Buruli ulcer have been reported from the West African countries of Benin, Côte d'Ivoire and Ghana. New foci were also discovered recently in Togo and Angola[4] and older ones have been re-discovered in Cameroon[6]. Estimates vary, but there have probably been more than 30,000 cases in West Africa in the last 20 years. In southern Benin, a recent study has reported detection rates of 21.5/100,000 per year, higher than for either tuberculosis or leprosy[7]. The same holds true for a national case search performed in Ghana in 1999, where the prevalence was estimated to be 20.7/100,000, exceeding that of leprosy[8]. These figures do not show the complete picture as there is considerable under-reporting. Due to the focal distribution of the disease, Buruli ulcer represents the major health problem in some of the highly endemic areas of West Africa, for example in Côte d'Ivoire local prevalence of Buruli ulcer can be as high as 16.3%[9], and in Ghana, in areas of high incidence, up to 22% of the population is affected [10]. Several other countries outside Africa are also endemic including rural areas of Papua New Guinea, Malaysia, French Guyana and Mexico. In Australia, the disease remains uncommon but there have been increases in both the incidence and endemic areas in the last 10 years[11,12].

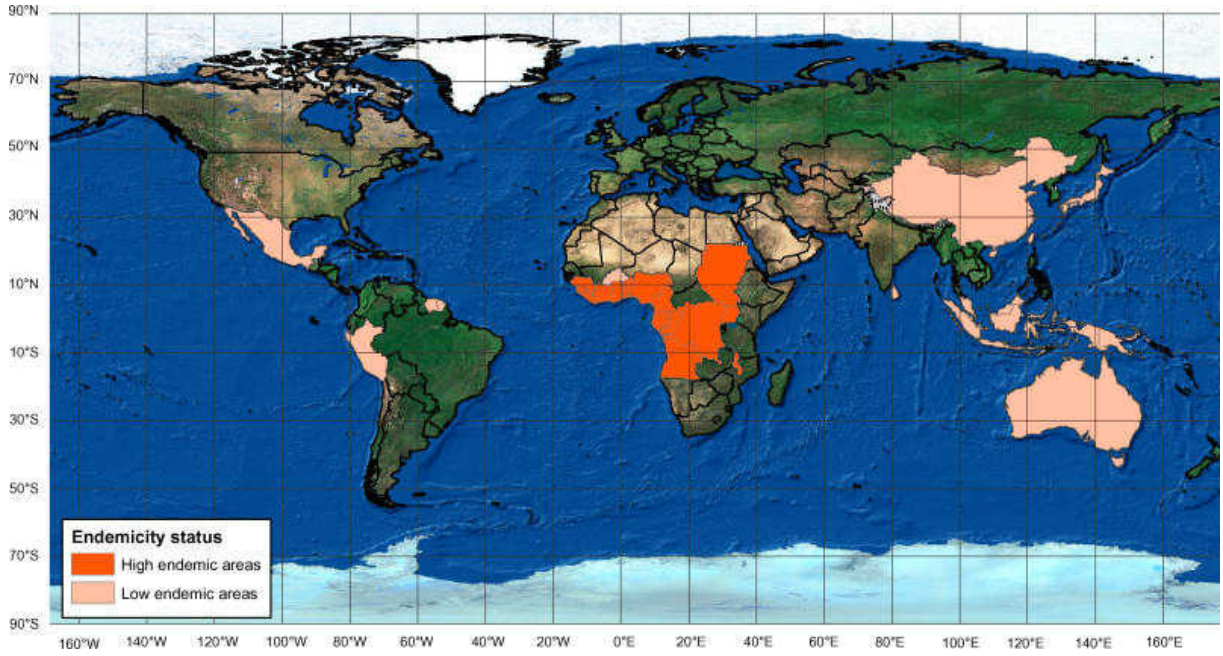

Figure 1. Countries reporting Buruli ulcer (Source: Johnson et al, *PLoS Med*. 2005)

1.2 Causative organism and toxin secretion

M. ulcerans belongs to a group of mycobacteria that are potentially pathogenic for humans and animals. These are sometimes called "opportunistic mycobacteria" or "occasional pathogens". Most species belonging to this group are found widespread in the environment and may become pathogenic under special circumstances[13].

M. ulcerans is a slow growing mycobacterium. Its generation time is about 20 hours and primary cultures may take between 6 and 8 weeks to be positive[3]. Incubation at about 32ºC is essential for its isolation in primary culture[14]. Another important factor is the oxygen concentration. Reduced oxygen concentration enhances the growth of *M. ulcerans*, suggesting a preference of this organism for microaerophilic environments[15].

M. ulcerans is unique among mycobacteria in that much of the pathology appears to be mediated by production of toxic macrolides, the mycolactones that are required for virulence[16]. These soluble toxins have immunosuppressive and cytotoxic properties *in-vitro* and can be isolated from the culture filtrate of the mycobacterium. When injected into healthy guinea pigs, histopathological changes compatible to Buruli ulcer lesions, were induced[17]. Mycolactones induce cell death by apoptosis, which may explain the absence of an inflammatory immune response despite the extensive tissue damage[18].

In contrast to the wild type *M. ulcerans,* mycolactone negative mutants fail to colonize the salivary glands of water insects, suggesting that these molecules may play a role in the ability of *M. ulcerans* to colonize reservoir species[16,19].

Until now no cell receptor has been found to explain the cascade of effects induced by mycolactones[20].

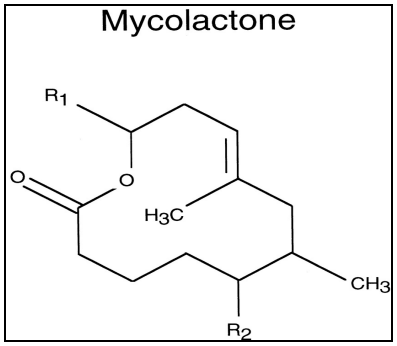

Figure 2. Mycolactone core structure: 12-membered ring to which two polyketide-derived side chains (R_1 and R_2) are attached. (Source: George et al, *Science* 1999)

1.3 Pathogenesis

1.3.1 Clinical forms

Clinically *M. ulcerans* disease manifests as papules, nodules, plaques, oedematous forms and ulcers. The disease may be active (ongoing infection) or inactive (previous infection with characteristic depressed stellate scars with or without sequelae). Any part of the body can be affected but it mostly occurs on limbs.

Early Buruli lesions may initially appear as a mobile subcutaneous nodule, a papule or a raised plaque[14]. A subgroup of patients presents with rapidly progressive oedema of a whole limb, abdominal wall or side of face without an obvious focal lesion. Part or all of the affected area will subsequently ulcerate. Recent anecdotal reports suggest that timely antibiotic therapy may greatly reduce the resulting necrosis.

Ulceration can be extensive and disfiguring, often affecting 50% or more of a limb. Patients usually present to hospital when large ulcers are established, or when secondary infections cause new symptoms of pain and fever. Sometimes, in advanced lesions, the infection spreads beyond the deep fascia, affecting the bone[21]. In Benin it has estimated that up to 10% of the patients have severe osteomyelitis[22]. Some lesions may arise from haematogenous spread[23, 24]. Contractures are common and up to 10% of cases admitted to a hospital require amputation of a limb. Lesions close to the eyes may lead to blindness.

1.3.2 Histopathology

Progressive *M. ulcerans* infection causes characteristic tissue changes. Initially there is coagulative necrosis of the lower dermis and subcutaneous fat. The organisms are present in clumps or in smaller microcolonies in the centre of the lesion. In these early lesions there is little or no evidence of an inflammatory response or the development of granulomas. As the disease progresses all elements of the skin are affected including nerves and blood vessels. Healing is associated with the formation of granulation tissue at the edges of the ulcer. In this stage the number of bacilli decreases and granulomas containing epithelioid cells and Langherans giant cells may be seen[25].

Histological specimens typically show large clumps of extracellular acid fast organisms surrounded by areas of necrosis and a poor or absent inflammatory response[26]. Subcutaneous fat is particularly affected, but underlying bone may also become involved in advanced cases[3]. Later in the natural history of the disease the immunosuppressive effect of the toxin is somehow overcome by the host: cell mediated immunity develops and healing commences[1].

It is possible that macrophages may initially engulf the organism after entry trough the skin, an accumulation of toxin may sequentially cause lysis of the macrophages and paralysis of the functions of infiltrating lymphocytes or macrophages. In turn, this localized immunosuppression may contribute to a delay in an early systemic immune response to mycobacterial antigens. This may account for the observation that patients with active lesions are often unresponsive to *M. ulcerans*-derived antigen (burulin) on skin testing[27]. Later, during the healing phase, characterized by the appearance of granulomas, there is conversion to a positive burulin test indicating that a specific cellular response develops.

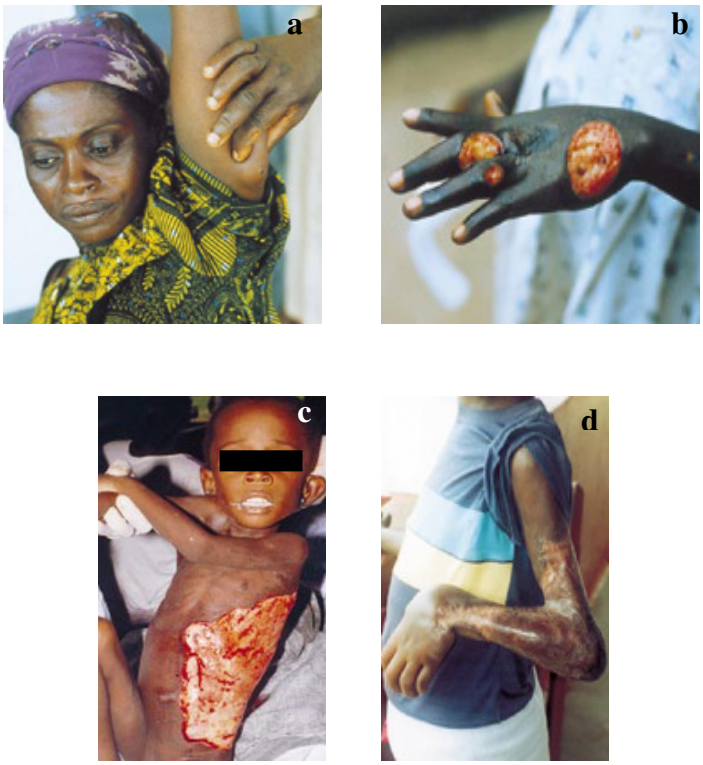

Figure 3. Clinical forms of Buruli ulcer
a: nodule; b: early ulcer; c: late ulcer; d: crippling deformity after self-healing
(Source: Asiedu et al, WHO 2000)

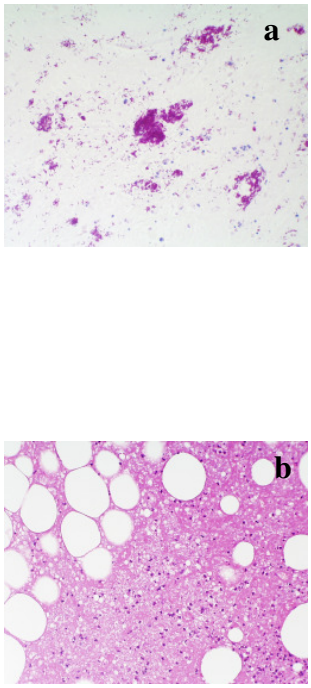

Figure 4. BU Histology
a: ZN staining
b: Buruli type fat necrosis

1.4 Diagnosis

Early treatment of *M. ulcerans* disease provides a better outcome than treatment of the ulcerative forms, but it is often impaired by the difficulties of diagnosis. In a known endemic area, an experienced person can diagnose an advanced *M. ulcerans* infection on clinical grounds. The commonly used diagnostic laboratory tests are: i) detection of mycobacteria by Zihel-Neelsen (ZN) staining, a technique that lacks sensitivity and specificity, ii) culture of *M. ulcerans*, which may take several months, iii) detection of characteristic histopathological changes in excised tissue and iv) detection of *M. ulcerans* DNA by PCR, representing a rapid, sensitive and specific diagnostic method[14].

1.4.1 PCR approaches

The application of PCR approaches as diagnostic test constitutes a big improvement: the repetitive element 2404 (IS2404), an insertion sequence present in multiple copies in the *M. ulcerans* genome, is used as DNA target and it guarantees the superior sensitivity and specificity of the method.

The PCR diagnosis permits the confirmation of the clinical diagnosis in a short time, as long as high laboratory standards are ensured to avoid the risk of contamination and consequent false positive results. Because of the inherent fragility of this technique, improvements and optimization of PCRs protocols have been made to allow the use of starting material obtained through non-invasive sampling approaches (like swab samples).

PCR applications to environmental samples demonstrated for the first time the presence of *M. ulcerans* in the environment[28], when it became clear its nature as ubiquitous mycobacterium[29,30,31,32]. After refinements of culture methods, in very few cases, it was also possible to isolate it and grow it[33,19]. Recent evidence indicates, however, that some other mycobacteria also harbour IS2404[34].

In addition to this conventional diagnostic PCR, the recently developed real-time PCR method uses the TaqMan system (IS2404 TaqMan) to quantify *M. ulcerans* DNA by monitoring the real-time amplification of IS2404[35]. This method offers the possibility to measure the starting amount of target DNA in clinical specimens and other samples, thus providing a measure of mycobacterial burden. Real-time PCR has several advantages over the conventional end-point PCR which include the reduction of risk of contamination, by eliminating the post-PCR processing and a diminished sensitivity to PCR inhibitors.

1.5 Treatment

1.5.1 Surgical excision

The mainstay of treatment is surgical excision of early lesions, which is often curative. Unfortunately many patients do not present until there is extensive and disfiguring ulceration, when there is no alternative but wide excision followed by skin-grafting, and sometimes even amputation[21]. Currently it is not clear how extensive surgeries should be performed and it is largely left to the individual judgment of the surgeon to find the right balance between an oversized excision and an incomplete removal of the pathogen, thus increasing the risk of recurrence.

Observations at St. Martin's Hospital, Agroyesum, in the Amansie west District of Ghana suggest that further surgery is needed for recurrent disease in more than 15% of cases and another study by Teelken et al. showed that relapse after surgery may occur in between 15-47% of cases[36].

Untreated Buruli ulcer will eventually subside with the gradual development of host immunity. However, by this time, tissue damage may be very extensive and scarring can lead to permanent functional and cosmetic deformity. Successful treatment will shorten the course of the disease and minimise deformity. Skilled surgery, expert post-operative nursing care and restorative physiotherapy are required to achieve this. The cost of those interventions may be beyond the means of local rural health services. It has been estimated that the average cost to treat Buruli ulcer in Ghana is over 780 US dollars per person[14,10]. Even in Australia with access to universal health care, the cost and complexity of treating *M. ulcerans* infections can be considerable.

1.5.2 Susceptibility to antibiotics

M. ulcerans is susceptible to several antimycobacterial drugs *in vitro* but the only promising combination in the mouse footpad model was the combination of rifampicin and amikacin[17]. A human trial has recently shown that early nodular lesions may be rendered culture negative after a minimum of 4 weeks therapy with rifampicin plus streptomycin[37].

Further research to identify cheap, safe and effective oral drug combinations that can be used as an adjuvant to surgery or which could even replace surgery is urgently required.

1.6 Transmission

The disease often occurs in people who live or work close to rivers and stagnant bodies of water. Changes in the environment, such as the construction of irrigation systems and dams, seem to have played a role in the resurgence of the disease. For instance, in the Busoga district in Uganda, Barker postulated that the outbreak was related to unprecedented flooding as a result of heavy rainfall[38]. In Nigeria, infections have emerged when a small stream was dammed to make an artificial lake[39].

In Phillip Island, Australia, a recent outbreak of the disease was temporally associated with the formation of a small swamp that, after its improved drainage, was followed by a cessation of cases[12].

Although the transmission of Buruli ulcer is not clearly explained, there is some mounting evidence for an association of focal outbreaks with flooding, human migration[38] and man-made topographical modifications such as dams and resorts. Deforestation and increased basic agricultural activities may have also significantly contributed, especially in West Africa, where the disease is rapidly emerging.

Trauma is probably the most frequent means by which *M. ulcerans* is introduced into the skin from environmental sources. The initial trauma can be as slight as a hypodermic needle puncture or as severe as a gunshot[40]. Epidemiological data has not spoken for frequent person-to person transmission[41].

In a study from Cote d'Ivoire[9], the wearing of long trousers was a protective factor against Buruli ulcer in communities with a high prevalence of the disease. In another study in Ghana, of 96 patients with *M. ulcerans* infection there was a significant association between lesion and occupational exposure[42].

M. ulcerans was first detected in the environment in the 1990s by Australian researchers using polymerase chain reaction (PCR)[43,44]. Subsequently, PCR was used by others to detect *M. ulcerans* in aquatic insects obtained from endemic areas in Africa, leading to the hypothesis that *M. ulcerans* may be transmitted by biting water bugs of the insect order Hemiptera. In support of this proposal, *M. ulcerans* has been detected in the salivary glands of *Naucoris* spp. (Naucoridae), and has been transmitted to laboratory mice via these aquatic insects[19]. There is additional evidence that *M. ulcerans* DNA can be detected by PCR in other aquatic insect predators (e.g., Odonata, Coleoptera), as well as in aquatic snails, small fish, and the biofilm of aquatic plants[45,30]. Despite this, only two pure cultures of *M. ulcerans* have been obtained from environmental sources[46,19]. In Australia, it has been postulated that aerosols arising from

contaminated water may disseminate *M. ulcerans* and infect humans via the respiratory tract, or through contamination of skin lesions and minor abrasions, but this has yet to be proven[47]. Recent progress has been rapid, but the exact mode of transmission and the key environmental reservoirs remain to be elucidated.

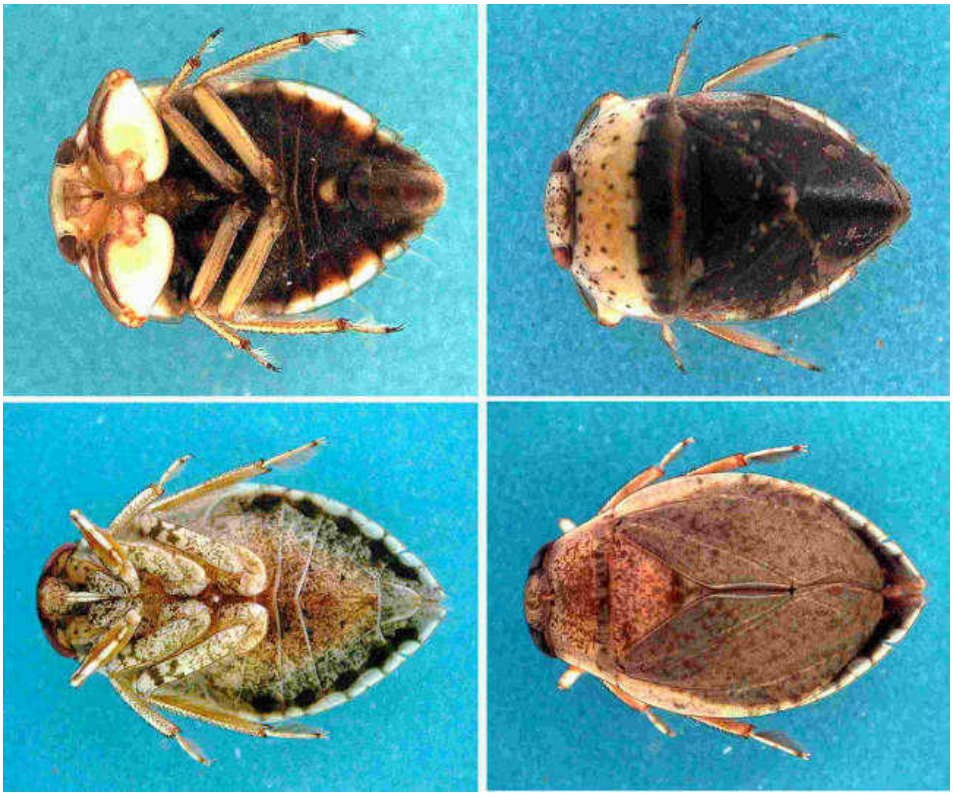

Figure 4. Semi-Aquatic Hemiptera positive for *M. ulcerans*
(Source: Johnson et al, *PLoS Med*. 2005)

1.7 Immune response

M. ulcerans is unique among mycobateria, not just for its toxin production, but also because the pathogen multiplies extracellularly in the host and there is little inflammatory response.
It is likely that soon after infection, mycolactone production prevents a local immune response[48]. Nevertheless, human lesions do heal spontaneously. The histological appearance of late lesions is similar to other mycobacterial disease, with small numbers of organisms and granuloma formation (contrasting with the large clumps of bacilli in early lesions). In a late stage of infection, T cells become reactive as evidenced by the human delayed hypersensitivity response to burulin[27]. Other evidence exists for the protective role of acquired

cellular immunity: bacillus Calmette-Guèrin vaccination is protective in mice against low-dose inoculation[49] and to some extent in man[50]. Finally, data from Benin suggest that there is a second peak of incidence in the elderly, which may correspond with declining immunity[51]. Interestingly, patients with a past history of *M. ulcerans* infection typically have a strong Th-2 cytokine response when their lymphocytes are exposed *in vitro* to *M. ulcerans*. In one fascinating case study, the development of ulcerative *M. ulcerans* disease was associated with a switch from the Th-1 to Th-2 phenotype[52].

Antibody responses may also play a protective role, but this is difficult to study because of the strong overlap between different species of mycobacteria.

Another observation is noteworthy: if the progress of the disease is determined by the ability of the host to mount a cell mediated immunoresponse, it would be expected that HIV co-infection would influence its course, but this does not appear to be the case. Anecdotal evidence from observations on families in endemic areas raises the possibility that host factors influence susceptibility to *M. ulcerans* infection[53,54].

There is no specific vaccine against *M. ulcerans* available, but *M. bovis* - BCG offers some protection, albeit short lived[54,50]. BCG may also provide more enduring protection against the most severe forms of Buruli[55].

Current prospects for better vaccines include improved or repeated BCG vaccination, rational attenuation of a *M. ulcerans* isolate and subunit vaccines, aimed at immunodominant protein antigens or the toxin itself[56].

1.8 Genome and bacterial population structure

Mycobacteria are known to have a very limited genomic diversity, which makes it particularly difficult to develop finger printing methods for microepidemiological studies. *M. tuberculosis* complex is constituted by 5 different members (*M. tuberculosis*, *M. africanum*, *M. microti*, *M. bovis*, *M. canetti*) which share a high genomic similarity, even though specialized in infecting different hosts. *M. ulcerans* DNA is closely related (99.6% identity) to another mycobacterium species, *M. marinum*, from which it could have recently diverged by the acquisition and concomitant loss of DNA, in a manner analogous to the emergence of *M. tuberculosis*, where species diversity is being driven mainly by the activity of mobile DNA element[57]. Hallmark is the acquisition of the virulence plasmid[58]. Several conventional and newly developed typing techniques have attempted to describe the population structure of *M. ulcerans* and to investigate its evolution.

Multilocus Sequence Typing (MLST). This technique compares the sequences of housekeeping genes derived from different isolates. The application of this method on 18 different *M. ulcerans* strains resulted in the identification of 6 genotypes related to the 6 geographical areas of Suriname, Mexico, China/Japan, Africa and Victoria (Australia). Comparative analysis between *M. marinum* and *M. ulcerans* confirmed their relatedness, suggesting a recent divergence of *M. ulcerans,* by the acquisition and concomitant loss of DNA, like the specific insertions sequences IS2404 and IS2606[57].

16S rRNA sequencing. This method employs the sequencing of 16S rRNA genes, which have changed little over millions of years as organisms evolved. The slight changes that have occurred provide clues as to how closely or distantly various organisms are related. This technique revealed identical signature sequences in *M. marinum* and *M. ulcerans*[59,60]: the only sequence differences within this locus are two nucleotides at the 3' end of the gene, which varies only in certain *M. ulcerans* strains[61,62].

Amplified Fragment Length Polymorphism (AFLP). This technique is based on digestion of whole-genomic DNA with two endonucleases, ligation of double stranded oligonucleotide adaptors to the restriction halfsites and selective amplification of the modified restriction fragments with adapter specific primers. Starting from 12 *M. ulcerans* strains from Australia, Malaysia and Africa, it could discriminate just two groups of isolates: the African group and the Australian (and Malaysian) group[63].

PCR-restriction Profile Analysis (PRPA). This method involves an amplification step of the 3' 16S rRNA and the subsequent cutting with three restriction enzymes. The *M. ulcerans* strains tested were split into three categories: African, Australian and Mexican[33].

Restriction Fragment Length Polymorphism (RFLP). The basis of this technique is to identify the number of a particular repeated element (like IS2404) and its own distinctive loci, by cutting the whole genome with different restriction enzymes and then probing it with the chemically labelled element. The attempt to differentiate 14 *M. ulcerans* isolated by using IS2404 as probe, led to the identification of 6 groups belonging to Africa, Australia, South Asia, Asia, South America and Mexico, respectively[46].

When a polymorphic GC-rich repeat sequence was used as probe, it was possible to characterize 11 distinct RFLP types, distinguishing even strains from Benin and Zaire[64].

2426 PCR. This technique is based on the amplification of DNA between the insertion sequences (IS2404 and IS2406), using outward-directed primers specific for such ISs. It allowed the identification of 9 distinctive profiles correlating with the geographical areas. Within each group there was lack of genotype variation[44].

Combination of IS2404 PCR and GC-rich repeated sequence. This method uses the outward-directed IS2404 specific primers in combination with an oligonucleotide targeting the GC-rich repeated motif. Comparison of banding profiles revealed 10 different patterns corresponding to the geographical origin of the isolates. The technique described above confirmed the clonal population structure of *M. ulcerans* within a given area and an overall remarkable genomic architecture homology[65].

Pulsed Field Gel electrophoresis. This technique, based on the migration of large DNA fragments in an electric field, employs the principle that large DNA fragments require more time to reverse than do small DNA fragments. By alternating the direction of the current during gel electrophoresis, it is possible to resolve DNA fragments of 100 -1,000 kb. This approach was used to compare genome sizes of *M. marinum* versus *M. ulcerans* and it showed shrinkage of the *M. ulcerans* genome by 200 kb[57].

When undigested *M. ulcerans* DNA was used, a band of about 174 kb was detected, corresponding to a circular plasmid (pMUM001) which comprises 81 protein-coding sequences. The primary function of such plasmid is mycolactone toxin production[66].

The occurrence of inter-strain variability was also discovered at the plasmid level, both related to the mycolactone structure that can be produced by different isolates[16] and related to frequent genetic rearrangements that render the virulence plasmid particularly unstable[67].

MIRU-VNTR. Mycobacterial interspersed repetitive units are 46 to 100 bp long sequences that are interspersed in different copy numbers throughout the genome. The method is routinely used for the genotyping of *M. tuberculosis*: the amplification of such loci with primer specific oligos produce amplicons whose size can be used to estimate the number of repeats at each locus. A combination of 4 MIRU loci on 39 *M. ulcerans* strains identified seven different profiles largely grouped according to their geographical origins. Once again, all the African isolates produced a single profile[68, 65].

All the above mentioned typing methods proved a limited discriminatory power, particularly insufficient to differentiate among isolates from the same area. This makes them unsuitable to perform micro-epidemiological studies, where the fingerprinting of the strains is aimed at revealing transmission pathways and environmental reservoirs. Whole genome analysis, allowing genome-wide comparisons, might represent the best approach to deal with this issue. There is urgent need for innovative techniques able to attempt this type of investigation. New opportunities will come from the publication of the whole *M. ulcerans* genome sequence in

2005. Microarray analysis of genomic DNA is one of such methods that could prove extremely helpful to discriminate *M. ulcerans* variants.

Microarray analysis

The ability to immobilize thousands of DNA fragments on a surface, such as a coated glass slide or a membrane, has led to the development of DNA microarray technology. The two common applications of DNA microarray technology in microbiology are the exploration of genome-wide transcriptional profiles and the measurement of the similarities or differences in genetic contents among different microbes. In this context microarray could probably be suitable to distinguish the highly homogeneous *M. ulcerans* population, to perform micro-epidemiological studies.

In the case of *M. tuberculosis* complex this approach has led to the identification of large sequence polymorphisms (LSP) that allowed the distinction between *M. bovis* BCG and *M. tuberculosis*, through the identification of at least 18 regions of diversity (RD1 to RD18)[69, 70].

The construction of a microarray allowed detection of small-scale genomic deletions among 19 clinically and epidemiologically well-characterized isolates of *M. tuberculosis*[71], showing that the deletions are likely to contain ancestral genes whose functions are no longer essential for the organism's survival, whereas genes that are never deleted constitute the minimal mycobacterial genome. These examples demonstrated that array-based comparative genomics constitutes a promising approach to exploring molecular epidemiology, microbial evolution, and pathogenesis of those species where deletion/insertion events account for most of the genomic variation, while single nucleotide polymorphism (SNP) rates are low.

A major drawback to the implementation of microarray is the need to have a fully sequenced genome, before proceeding either with the oligos synthesis (in the case of oligos-based chips), or with the whole-genome primers design (in the case of PCR fragment-based chips). Shotgun DNA microarrays may overcome this problem, by offering the possibility for genome-scale experiments, even in the absence of a complete genomic sequence[72]. However, they do not overcome the laborious and time consuming preparation of PCR fragments suitable for spotting, which often constitutes the bottleneck of the technique.

The development of a plasmid-based microarray can circumvent both the need for a completely sequenced genome and for the generation of PCR fragments.

1.9 Research framework on Buruli ucer

Buruli ulcer is mainly a disease of poor people, living in remote rural communities with little economic or political influence. This has hampered recognition of Buruli ulcer as major problem: national surveillance systems did not pick up the appearance of new outbreaks and affected populations often believe that there is no medical treatment, which discourages them from seeking help[73,74].

On the other hand, the absence of a potential first-world market has meant that there has been little private investment in drugs, diagnostics and vaccine development. Also research on Buruli ulcer was not very intense for a long time.

A first step towards the recognition of the importance of BU was undertaken in December 1997, when Dr Hiroshi Nakajima, then Director-General of the World Health Organization (WHO), announced that WHO would take the lead to mobilize the world's expertise and resources to fight the emergence of Buruli ulcer as a serious public health problem. In 1998, WHO launched the Global Buruli Ulcer Initiative to coordinate control and research efforts, and organized the first International Conference on Buruli ulcer control and research in Yamoussoukro, Côte d'Ivoire (WHO Press Office, 1998, Fact Sheet No. 199). The resulting Yamoussoukro Declaration on Buruli ulcer drew attention to the severity of the disease and expressed concern about its many poorly understood features. Five key aspects have been identified as most likely to provide immediate direct benefit to Buruli ulcer patients in the medium term:

1. identification of the mode of transmission
2. development of methods for early diagnosis
3. improvement of treatment
4. BCG trials and development of new vaccines
5. cultural and socio-economic studies

It is within such a framework of priorities that the present PhD project was developed, and it mainly focused on investigating the first three points, through the use of molecular and genetic tools.

The field work and the accessibility to Buruli ulcer endemic areas constituted an important aspect of the work and the collaboration with the Amasaman Health Centre in the Ga district

of Ghana and with Aide aux Lepreux Emaus Suisse (A.L.E.S.) active in the Nyong river basin in Cameroon proved providential for the successful outcome of the project.

In addition, the requirement for highly sophisticated DNA microarray technologies was met through collaboration with Prof. U. Certa (Hoffman La Roche, Basel), where part of the work to develop and implement new microepidemiological tools was undertaken.

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Chapter 2

GOAL AND OBJECTIVES

2.1 Goal

To contribute to the improvement of the health status of patients affected by Buruli ulcer, by expanding basic knowledge on the disease.

2.2. Objectives

1. To develop a microarray to study the basis of the genetic variation of *M. ulcerans,* with the aim to obtain a new fine typing tool suitable for micro-epidemiological studies.
2. To study the epidemiology of *M. ulcerans* in a rediscovered endemic area in Cameroon, thorough the implementation of a diagnostic PCR approach.
3. To develop a new real-time PCR to quantify *M. ulcerans* burden in tissues and environmental samples.
4. To study mechanisms of pathogenesis and spreading of *M. ulcerans* in Buruli ulcer lesions to improve guidelines for the surgical procedure.
5. To study the risk of recurrence in Buruli ulcer patients
6. To evaluate the possibility of *M. ulcerans* reactivation versus reinfection.

Chapter 3

Detection of insertional/deletional genomic diversity in *Mycobacterium ulcerans* using a plasmid-based DNA microarray

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This article will be submitted in:

Molecular Microbiology

Abstract

The analysis of genetic variability within natural populations of pathogens contributes to the understanding of genome function and evolution. In the case of *Mycobacterium ulcerans*, standard molecular typing methods such as multi-locus sequence typing, restriction fragment length polymorphism, and fingerprinting using variable number of tandem repeats have revealed a remarkable lack of genetic diversity and a clonal population structure within given geographical regions. Using a prototype plasmid-based microarray, with theoretical 15% genome coverage, we have initiated comparative genomic analysis of *M. ulcerans*. Fifteen large deletions comprising 1.8 kb to 53.1 kb were detected when 30 clinical isolates of diverse geographical origin were analysed. In many cases deletions were associated with the insertion of DNA sequences, thus representing InDel events. A total of 241 coding sequences that covered all major functional categories were found to be deleted in comparison to a reference isolate from Africa. Genes predicted to encode metabolic and information pathways as well as cell wall proteins were underrepresented, while pseudogenes, insertion sequence elements (ISE), unique hypothetical proteins and genes involved in detoxification-adaptation were overrepresented. Isolates coming from the same continent usually shared distinct large sequence polymorphisms. In some instances more than one type of deletion was observed for a given sequence region, suggesting recombination hot spots or selective advantage for loss of a particular region. Analysis of the sequences flanking deletions suggested that two highly abundant ISE (IS*2404* and IS*2406*) are driving InDel variation. Given the significant strain variation uncovered by this small prototype microarray a larger genome-wide array may be a useful tool for micro-epidemiological studies.

Introduction

The study of the genetic diversity within bacterial species has provided important information on aspects such as virulence[1,2], antibiotic resistance[3], epidemiology and microbial evolution[4,5,6,7]. In the case of mycobacterial species, such as *M. tuberculosis* and *M. ulcerans*, very low intra-species diversity limits the use of genetic fingerprinting techniques that are based on sequence diversity in selected genetic elements. In other mycobacteria such as *M. tuberculosis* and BCG [8-11] genome-wide microarray analyses have led to the identification of large sequence polymorphisms. However, the complete genome sequence of an organism is required for the design of synthetic oligonucleotide or PCR product-based microarrays. When this information is not available, an alternative is a PCR product-based shotgun DNA microarray[12]. To avoid the laborious and time-consuming production of a large set of PCR products, we have developed a plasmid-based microarray. We have used this method for the differential genomic analysis of *M. ulcerans,* a human pathogen for which the fully assembled and annotated genome sequence is not yet available.

M. ulcerans is the causative agent of Buruli ulcer (BU), an infectious disease characterized by chronic necrotizing skin ulcers[13]. BU is an emerging infectious disease particularly in West-African countries, but is also found in tropical and sub-tropical regions of Asia, the Western Pacific and Latin America[14]. Genetic analyses suggest the recent divergence of *M. ulcerans* from *M. marinum*, a well known fish pathogen, which can cause limited granulomatous skin infections in humans[15]. 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 polyketide toxin mycolactone. This toxin appears largely responsible for the massive tissue destruction seen in BU[16]. The epidemiology and mode of transmission of *M. ulcerans* disease is not fully understood, partly because no molecular typing method is available that has sufficient high resolution for micro-epidemiological analyses. The apparent

lack of genetic diversity of *M. ulcerans* within individual geographical regions is indicative of a clonal population structure. The genotyping technique that has shown the highest discriminatory power so far is based on the use of outward-directed primers specific for the insertion sequence IS*2404*, in combination with an oligonucleotide targeting a repeated GC-rich motif[17]. The application of this method allowed the resolution of 10 different *M. ulcerans* genotypes, corresponding to the geographic origin of the isolates. However, this level of resolution is not sufficient for micro-epidemiological analyses. We hypothesized that, as for *M. tuberculosis*[18], deletional and insertional events mediated by repetitive sequence elements are the major mechanism for genomic variation in *M. ulcerans*. To test this hypothesis we developed a plasmid-based microarray and analysed genomic DNA from 30 *M. ulcerans* isolates of diverse origins.

Materials and Methods

Plasmid-based DNA microarray

Three hundred and fifty two *E. coli* plasmids (pCDNA2.1 Invitrogen) were randomly selected from shotgun clone library, that was generated for the *M. ulcerans* genome project (http://genopole.pasteur.fr/Mulc/BuruList.html). Each plasmid contained a *M. ulcerans* DNA fragment from strain Agy-99 within the size range 2.3-2.7 kbp. Given a genome size of 5800 kbp (T. Stinear, unpublished observations), this collection of 352 plasmids represents a theoretical coverage of approximately 15% of the Agy98 genome. Plasmid DNA was prepared using a Biomek 2000 Workstation. DNA yield was determined by measurement of optical density at 260nm using a GeneQuant spectrophotometer. Plasmids were dissolved at a concentration of 150ng/µl in 3xSSC buffer (20xSSC stock solution is 3M NaCl, 0.2M NaCitrate, pH 7.0)

Using a Topspot spotter (Genescan), DNA was spotted onto glass slides (Superfrost) coated with poly-L-lysine and cleaned with nanopure water. The DNA samples, loaded on a piezo-dispensing head containing 24 channels, were deposited by the robot device at identical locations on each of the slides. The resulting spots had an average diameter of 270µm, corresponding to a spotted volume of approximately 1nl and were 500µm apart when measured centre to centre. After spotting, the slides were incubated at 4ºC overnight, rehydrated under a controlled atmosphere with 50-60% humidity at room temperature (RT) for 1 hour and stored in the dark at RT until use.

The layout of the spotted microarrays consisted of 2 replicates per field, each of them containing 32 controls and 352 plasmids. Two identical fields were printed on each glass slide, enabling the hybridization of two different probes on a single slide. Human Cot-1 DNA and plasmid DNA without insert served as negative controls. To assess DNA deposition on the microarrays, a 500bp DNA fragment comprising part of the Beta-lactamase gene present

in all plasmids was labelled with Cy3 and spotted. A biotinylated preparation of this fragment was used as another positive control.

Preparation of biotinylated fragments of *M. ulcerans* genomic DNA for hybridization

M. ulcerans strains used in this study are listed in Table 1. Bacterial pellets of about 60mg (wet weight) were heated for 1 hour at 95°C in 500µl of extraction buffer (50mM Tris-HCl, 25mM EDTA, 5% monosodium glutamate). One hundred microliters of a 100mg/ml lysozyme solution were added. After 2 hours of incubation at 37°C, 70µl Proteinase K-10X buffer (100mM Tris-HCl, 50mM EDTA, 5% SDS, pH 7.8) and 10µl of a 20mg/ml proteinase K solution were added. After incubation at 45°C overnight, the samples were subjected to bead beater (Mikro-Dismembrator, Braun Biotech International) treatment with 300µl of 0.1mm zirconia's beads (BioSpec Products) at 3000 rpm for 7 minutes. Beads and undigested tissue fragments were removed by brief centrifugation and the supernatants were transferred to fresh tubes. An equal amount of phenol-chloroform (Fluka) was added and the DNA contained in the upper phase was precipitated with ethanol and resuspended in 150µl of water. The DNA yield was measured using a spectrophotometer (GeneQuant) and DNA quality (i.e. high molecular size and purity from RNA) was checked on a 1% agarose gel.

Seven µg of *M. ulcerans* genomic DNA were digested with 3U of *Sau*3A1 (New England Biolabs) at 37ºC for 2 hours in a 60µl reaction volume. Efficiency of the digestion was controlled by analysing the size of the obtained DNA smear, which was between 100 and 2000bp on a 1% agarose gel. The digested genomic DNA was purified by ethanol precipitation and biotinylated according to Pollack et al[19] using a BioPrime kit (Gibco/BRL). Briefly, 2µg of DNA were mixed with 20µl of 2.5x random primer solution, brought to a final volume of 44µl with H_2O and denatured for 5 min at 95ºC. Five µl of 10x dNTP mix (2mM dATP, dGTP and dTTP, 1mM dCTP and 1mM biotin-14-dCTP) were added on ice. One µl of Klenow fragment (40U/µl) was added, the total reaction volume being 50µl. After 2 hours of

incubation at 37ºC, the reaction was stopped by addition of 5µl stop buffer. The biotinylated DNA was purified using a Microcone YM-30 filter (Amicon/Millipore) and its concentration was measured by optical density at 260nm.

Hybridization

Five micrograms of biotinylated DNA were mixed with 30µg human Cot-1 DNA (1µg/µl, Roche Applied Science) and 100µg yeast tRNA (Gibco/BRL). The hybridization mix was concentrated to a final volume of 12µl using a Speed Vac Concentrator System. Prior to hybridization, 2.5µl of 20XSSC and 0.5µl of 10% SDS were added, the mix was denatured at 95ºC for 3 min and subsequently incubated at 37ºC for 30min.

Before use, the microarrays were cleaned with a N_2 flow and exposed to UV in a Stratalinker 2400 at 650X100µJ to fix the DNA on the chip. Immediately before application of the hybridization mix, the microarray was heated at 95ºC for 5 min. Subsequently it was placed in a hybridization chamber and 30µl of water were added at each end in the chamber to prevent dehydration of the hybridization mix. For each field of the array, 13µl of the hybridization mix was applied and a coverslip was placed on top. After hybridization at 65ºC for 16-20 hours, the microarray was washed once with 2xSSC, 0.03%SDS for 5 min at 65ºC, twice with 1xSSC for 5 min at RT and finally with 0.2xSSC for 5 min at room temperature. The coloration step was performed with 2ml staining solution containing 50% caseine, 1x maleic acid buffer (Roche Applied Science) and 2µg Streptavidin Cy3 Fluorolink (1µg/µl, Amersham) at RT for 30 min. Thereafter, the slide was washed twice for 5 min each with 1X TBS (0.15M NaCl, 0.02M Tris, pH 7.5) and 0.1 TBS. After drying with N_2, the microarray was kept in the dark until scanning.

Scanning of microarrays and data analysis

Images of the microarrays were acquired using a dual laser scanning microscope (GenePix 4100A scanner; Axon Instruments Inc.) with an excitation wave length of 532nm and an emission wavelength of 570nm. Gain of the photomultiplier as well as parameters of contrast and brightness at which the image was viewed were kept constant for all measurements. The resulting acquired image was analyzed by the software GenePix Pro 4.1 (Axon Instrument Inc.), allowing the assignments of an average fluorescence intensity for each spot of the array. Both the images and the mean intensity values were used for data interpretation.

Three types of hybridizations were carried out as quality control on the microarrays. A first hybridization with a Cy3-labeled random oligonucleotide (Microsynth) was performed to estimate the amount of spotted DNA. The 9mer oligonucleotides were diluted in a hybridization buffer containing 25% formamide, 5xSSC, 0.1% SDS, to a concentration of 15pmol/µl. After 2 min of denaturation at 90ºC, 20µl of this solution were hybridized to a microarray for 5 min at RT. The microarray was then washed for 1 min with 2xSSC, 0.1% SDS and for 1 min with 0.05x SSC, dried with N_2 and scanned. A second hybridization with the biotinylated β-lactamase gene fragment was carried out to specifically quantify the spotted plasmids. Four ng of biotinylated DNA were mixed with 30µg of human Cot-1 DNA (1µg/µl, Roche Applied Science) and 100µg yeast tRNA (Gibco/BRL) and the microarray was processed as already described above. A third hybridization served as a negative control and in this case no DNA was added to the hybridization mix.

To select the spots of the microarray that yielded evaluable results, five hybridizations were performed using *M. ulcerans* Agy-99 genomic DNA. The 10 replicate values obtained for each spot served to select the spots to be included in the analysis of genomic diversity of *M. ulcerans.* All spots which yielded a signal lower than 2x the one given by the plasmid without insert (negative control) and all spots whose coefficient of variation was >30% were rejected.

232 spots with an average signal above the threshold and good signal stability were included in the subsequent comparative genomic hybridization analysis.

Comparative genomic hybridization

The DNA of 30 *M. ulcerans* strains was processed and hybridized under identical conditions. Hybridization were performed at least twice in order to obtain four sets of data. Spots associated with anomalies like dust particles or satellite spots due to the deposition of a second droplet of DNA material near the principal spot were rejected. For each plasmid the average signal value, standard deviation and coefficient of variation were calculated and a signal ratio in comparison to the reference strain was assessed. Outlier spots with a ratio higher than U2 (U2=Upper quartile+3x InterQuartile) were identified through a box plot analysis.

Characterization of large sequence polymorphisms

Microarray data indicating the presence of a deletion were verified by PCR analysis using primer pairs spanning the insert sequences of the respective plasmids and/or flanking regions. 5' and 3' limits of reconfirmed genomic deletions with respect to the genome of strain Agy-99 were determined by PCR analysis using multiple sets of primers complementary to flanking genomic regions. PCRs bridging the genomic breakpoints were performed using a long range PCR polymerase mix (Fermentas) according to the manufacturer's description. PCR products were cloned into pGEM-T (Promega, Catalys AG) and sequenced using an ABI PRISM 310 genetic sequence analyzer (Perkin-Elmer).

Results

Comparative genomic hybridization of *M. ulcerans* isolates

A microarray was constructed based on a random selection of 352 *E. coli* plasmids obtained from the shotgun library of the *M. ulcerans* genome project. Each plasmid contained a DNA fragment from the *M. ulcerans* strain Agy-99, a recent clinical isolate from Ghana. DNA hybridization signal intensities from 30 *M. ulcerans* clinical isolates were compared to those obtained with strain Agy-99. Box-plot analysis (Fig. 1) identified plasmids yielding outlier signals with respect to strain Agy-99. PCR analysis reconfirmed for 19 out of 20 of these plasmids an association of the outlier signal with a genomic deletion. Only one low hybridization signal represented a false positive result (refer p188 from strain 940511 Côte d'Ivoire, Fig. 1). The number of confirmed outlier plasmids per isolate ranged from zero in the case of most African isolates to nine for the isolates from Suriname and from French Guyana (Tab. 1).

Three of the 19 plasmid inserts (p111, p299 and p341) that yielded reconfirmed outlier signals contained sequences from the virulence plasmid pMUM001 of *M. ulcerans*. Among the other 16 plasmids derived from the *M. ulcerans* chromosome some contained fragments overlapping the same region (Fig. 2). Hybridizing regions were almost identical for p60 and p61. Both plasmids yielded outlier values with the isolates from Suriname and French Guyana. In the case of p88, p153 and p360, inserts were overlapping. A cluster of overlapping inserts were observed for p88, p153 and p360 and these produced outlier values for both of the Mexican isolates. The same pattern was seen with p291 and p124, which have inserts that are located in close proximity to each other in the genome (Fig. 2). These results from related inserts demonstrate the reproducibility of the differential hybridization analysis.

Characterization of genomic regions of difference

The 5' and 3' limits of the genomic deletions with respect to the genome of strain Agy-99 were determined by PCR analysis using multiple sets of primers complementary to plasmid inserts and to flanking genomic regions. The size of the deletions ranged from 1.8 kb to 53.1 kb (Tab. 2). Some of the inserts were part of the same deletion (p124-p291, p60-p61 and p88-p153-p360, respectively), so altogether 12 chromosomal regions of difference (RDs) were identified.

In three of the 12 RDs (RD3, 9 and 12) two distinct types of overlapping but independent deletions (designated A and B) were observed. These deletions shared neither the 5' nor the 3' end sequences. The RD3 strains from Australia had a 3.5 kb deletion, while strains from Suriname and French Guyana had a slightly larger (3.8 kb) deletion. The RD9 isolates from Suriname and French Guyana had a larger (25.4 kb) deletion than the RD9 isolates from Japan and China (17.7 kb). The largest deletion (53.1 kb) was designated RD12 and was observed in strains from Japan and China. Isolates from Suriname and French Guyana had a significantly smaller RD12 deletion (35.2 kb). In RDs1, 2, 4, 7, 8, 10 and 11, deletions of identical size were observed in two isolates from the same geographical region. A 19.7 kb deletion in RD6 was found in isolates from two different geographical regions (Mexico and Japan/China, respectively).

In order to assess whether polymorphisms undetected by the microarray analysis would frequently occur in the identified RDs, a detailed sequence analysis was performed in all *M. ulcerans* strains included in this study for two randomly selected RDs (RD5 and 12). Four distinct primer pairs were used to span the insert sequence plus 5' and 3' flanking sequence stretches. In the case of RD12, the PCR analysis reconfirmed the presence of a deletion in the four strains presenting outlier signals in the microarray analysis, but no evidence for polymorphisms in the other strains was obtained. In the case of RD5, PCR analysis confirmed the presence of a deletion in the two Mexican strains with outlier signals (Fig. 3, lanes 28 and

29). Unexpectedly the analysis identified the presence of an insertion in strains from Japan, China, Suriname and French Guyana (Fig 3, tracks 2 -5). The sequence of this 765bp DNA insert was identical for all three strains, it had a G+C content of 64% and showed no significant homology with known genomic sequences.

Association of deletions with insertions reveals InDel events

One of the 15 identified genome rearrangement events (deletion 3A retrieved in two Australian isolates) was found to be a mere deletion with the genomic sequences flanking the 5' and 3' borders of the 3451 bp deletion being directly joined (Fig. 4). Analysis of the other 14 deletions revealed that the loss of DNA in a given strain with respect to the genome of Agy-99 was associated with the insertion of substituting sequences of varying sizes and unrelated to the deleted regions. The occurrence of insertions in combination with deletions reflects InDel events that have resulted in the substitution of original DNA. As an example, the larger (3784 bp) deletion 3B found in the isolates from Suriname and French Guyana was associated with the insertion of an unrelated DNA fragment (Fig. 4).

In this case, the substituting DNA comprises the transposable element IS*2404* plus an additional stretch of 163 bp. Interestingly, several of the analysed deletion borders seem to be in direct proximity to either IS*2404* or IS*2606*. These ISE can be situated either in the genomic sequences flanking the deletion, or in the deleted parts, or in the substituting sequence stretches as in the case of deletion 3B.

Analysis of genes encoded by the deleted DNA sequences

The identified 15 distinct deletions identified 241 protein-coding sequences (CDS), representing 4.4% of the 5482 CDS annotated thus far for strain Agy-99 (T. Stinear, unpublished results). The number of deleted CDS ranged from 2 (RD4) to 54 (RD8), with an average of 18.6 CDS per deletion (Tab. 2). Twenty-two percent of all affected CDS were

predicted pseudogenes, mostly due to the presence of point mutations introducing frame-shift mutations or premature stop codons. The CDS were classified into 11 functional categories plus pseudogenes[20]. Predicted proteins involved in detoxification, unique hypothetical proteins, insertion sequences and pseudogenes were significantly overrepresented among these 241 deleted CDS. Genes involved in information pathways, metabolism and cell wall processes were underrepresented (Fig. 5). Eighty-nine of the deleted functional CDS had orthologues with >50% amino acid sequence identity to proteins from *M. tuberculosis* H37Rv genome. Only one of these orthologues (the transcriptional repressor *Mce3R*) is considered to be an essential gene required for growth of *M. tuberculosis* (Sassetti 2003).

Discussion

In this report we describe the use of a plasmid-based DNA microarray for identifying DNA deletion polymorphisms in a collection of 30 *M. ulcerans* strains of geographically diverse origin. A set of plasmids randomly selected from an *E. coli* shotgun library of genomic DNA of *M. ulcerans* strain Agy-99 genomic plasmid library was spotted onto glass slides. This is a newly developed technology and it is highly suitable for situations where the complete genome sequence of a microorganism is not available. The prototype array used comprised 232 useable plasmids, selected for giving a reproducible signal, which contained *M. ulcerans* genomic DNA fragments of approximately 2.5 kb, thus reaching a genome coverage of 15%. In spite of this incomplete coverage, 12 distinct genomic and 3 distinct virulence plasmid-based RDs were identified. Fifteen distinct deletions with sizes between 1.8 kb to 53.1 kb were found and characterized in detail by sequence analysis within the 12 genomic RDs. The deletions identified were in most cases found in more than one *M. ulcerans* isolate, demonstrating that they are not events occurring during *in vitro* cultivation. Recombination events between adjacent copies of IS*6110* in *M. tuberculosis* and IS*100* in *Y pestis* have been shown to promote the deletion of intervening DNA segments[21-25]. Close association of RDs with the high copy number insertion sequence elements of *M. ulcerans* indicates that these are involved in InDel events in this mycobacterium.

Although genome coverage with the prototype plasmid-based microarray used here was low, several geographical types of *M. ulcerans* could be differentiated. These were the African (including isolates from Ghana, Benin, Côte d'Ivoire, DRC, Angola and Togo), Papua New Guinean, Australian, the Asian (Japan and China), the South American (Suriname and French Guyana), the Mexican groups and an Australian subgroup differing from the other Australian isolates in RD3. Use of a microarray that covers the whole genome may lead to the

development of a genomic fingerprinting method urgently needed for micro-epidemiological studies aiming to characterise transmission pathways and environmental reservoirs.

The 15 distinct genomic deletions we have identified affected 6.2% of the *M. ulcerans* Agy-99 genome, or 4.4% of the genes. When a whole-genome microarray[26] was used to compare genomic DNA of 100 *M. tuberculosis* isolates, 5.5% of the genes were found to be affected. Considering the limited genome coverage of the *M. ulcerans* prototype array used here, findings demonstrate a remarkably high degree of InDel diversity in *M. ulcerans.* In contrast, single nucleotide polymorphisms are rare[27]. The genome of *M. ulcerans* is about 500 kb smaller than that of the closely related *M. marinum* (http://www.sanger.ac.uk/Projects/M_marinum/). Our comparative analysis of the genomes of *M. ulcerans* isolates indicates that genome contraction is an ongoing process in this emerging pathogen. Studies in other groups of microorganisms indicate that genome reduction is usually associated with adaptation to a more stable environment. It remains to be investigated, to which ecological niche(s) in the environment or in host organisms *M. ulcerans* is adapting.

Among the affected CDS, insertion sequences and pseudogenes were overrepresented, whereas genes associated with cell wall-processes, metabolic and information pathways were underrepresented. Genes involved in detoxification-adaptation, including the mammalian cell entry (*mce) 3* operon were over-represented among the deletions. In *M. tuberculosis* the *mce* operons have been shown to code for genes important for entry and survival of the pathogen in mammalian cells [28, 29]. The four m*ce* operons of *M. tuberculosis* have homologues among other mycobacteria. In particular, the *mce3* operon has been found in *M. avium* and *M. smegmatis*, while its deletion in *M. bovis* has been also documented[30]. The 12.7 kb region coding for the *mce3* operon is located near the 3' end of the RD2 element[31] present in *M. bovis*, but absent in some strains of *M. bovis* BCG, which suggests the potential instability of

this region. Using a mouse model of intradermal infection, it has recently been shown, that *M. ulcerans* is initially captured by phagocytes [32]. *In vitro* studies suggest that the *M. ulcerans* intracellular stage is transient, since phagocytic cells enter apoptosis-mediated cell death within one day. It would be interesting to investigate whether the *Mce3* operon plays a role during the transient invasion of host cells by *M. ulcerans.*

Among the deleted genes involved in cellular metabolism, 42% are dehydrogenases, central enzymes in anaerobic metabolism[33]. As speculated for *M. tuberculosis*[34], the loss of such genes, important for survival in poorly oxygenated environments like in soil, could reflect the adaptation to a new life style. It remains to be determined whether *M. ulcerans* is primarily adapting to persist in a specialised environmental habitat, in arthropod hosts[35] or in chronic wounds of mammalian hosts.

p	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
8			■	■																										
34			■	■																										
58			■	■	■	■																								
60			■	■																										
61			■	■																										
88																											■	■		
92	■	■																									■	■		
100			■	■																										
111	■					■					■	■															■	■		
124																											■	■		
144	■	■	■	■																										
153																											■	■		
170																											■	■		
188													■																	
291																											■	■		
299	■																													
305	■	■																												
315	■	■	■	■																										
341	■	■	■	■																										
360																											■	■		

Table 1. Distribution of outlier plasmids among isolates
p: plasmid

1. 8756 Japan
2. 980912 China
3. 842 Suriname
4. 7922 French Guyana
5. 5147 Australia
6. 5142 Australia
7. 9540 Australia
8. 9550 Australia
9. 940339 Australia
10. 8849 Australia
11. 5151 DRC
12. 5150 DRC
13. 940511 Côte d'Ivoire
14. 940662 Côte d'Ivoire
15. 94511Côte d'Ivoire
16. 960658 Angola
17. 960657 Angola
18. 970680 Togo
19. 970321 Ghana
20. 970483 Ghana
21. 970359 Ghana
22. 940111 Benin
23. 001441 Benin
24. 940886 Benin
25. 970104 Benin
26. 940512 Benin
27. 5143 Mexico
28. 5114 Mexico
29. 941331 PNG
30. 9357 PNG

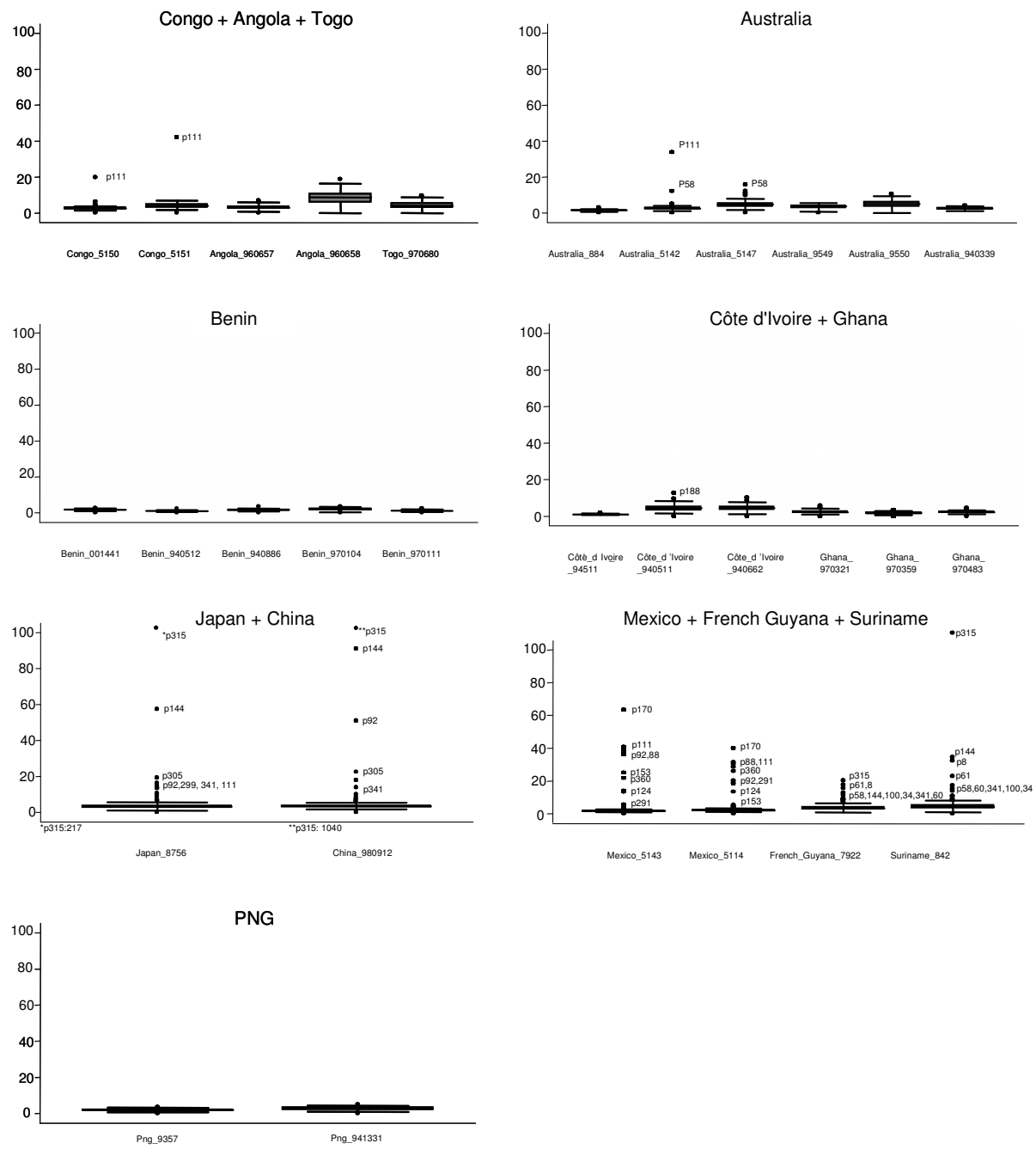

Figure 1. Box plot analysis of the 30 *M. ulcerans* strains for identification of the plasmids yielding outlier signals

The median of the data is represented by the line in the center of the rectangular box. The two ends of the rectangles represent the upper quartile UQ, corresponding to the 75th percentile (the value such that 75% of the data values are below it) and the lower quartile LQ, corresponding to the 25th percentile (the value such that 25% of the data values are below it). The inter quartile IQ is equal to UQ-LQ. The other two values shown are the maximum and

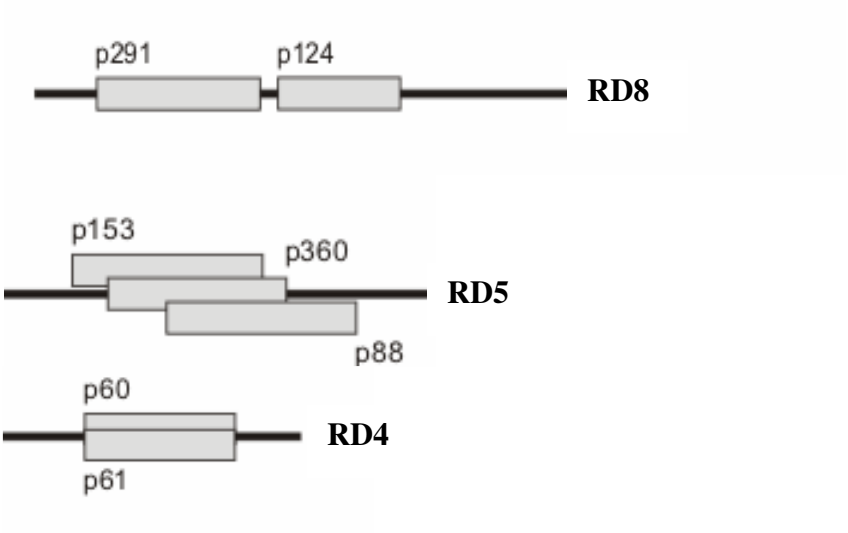

Figure 2. Regions of differences including overlapping or adjacent plasmids

RD	Deletion Number	Plasmid	Strains	Size of deletion (kb)	Number of CDS
1	1	p8	SU/FG	11,1	14
2	2	p34	SU/FG	6,5 – 7,0	4
3	3A	p58	AU1/AU2	3,5	5
	3B	p58	SU/FG	3,8	6
4	4	p60, p61	SU/FG	1,8 – 2,4	2
5	5	p88, p153, p360	ME1/ME2	27,1 – 27,4	23
6	6	p92	JP/CH/ ME1/ME2	19,7	24
7	7	p100	SU/FG	14,9 – 15,5	13
8	8	p124, p291	ME1/ME2	52,7 – 53,1	54
9	9A	p144	JP/CH	18,1	15
	9B	p144	SU/FG	25,4	20
10	10	p170	ME1/ME2	8,2 – 8,7	10
11	11	p305	JP/CH	4,5	7
12	12A	p315	JP/CH	53,1	50
	12B	p315	SU/FG	35,2 – 35,7	32

Table 2. Summary of the features of the 12 different RDs

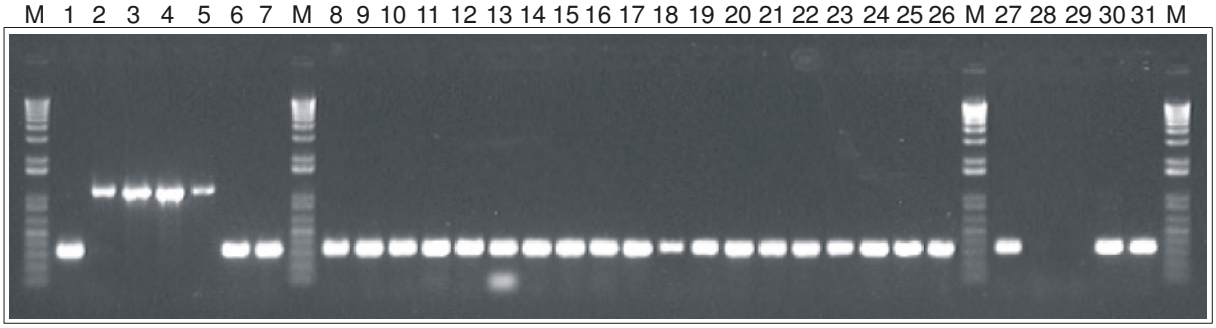

Figure 3. Additional polymorphism in RD5.

Probing of genomic regions by PCR within the deletion obtained in the Mexican strains (lane 28 and 29) as compared to the reference strain Agy-99 (lane 1) reveals an insertion of approx. 750 bp for the *M. ulcerans* isolates from Asia and South America (lanes 2, Japan; 3, China; 4, Suriname; 5, French Guyana). This insertion is located within the 2,8 kb hybridizing region of Plasmid p88 between primers 5'-AATACCACCTCCTGGTGCAG (position 1238 bp) and 5'-GACGACGATGAACTCCCAAT (position 1613bp). M, 1 kb marker; 6-11, Australia; 12-13, Congo; 14-16, Côte d'Ivoire; 17-18, Angola; 19, Togo; 20-22, Ghana; 23-27, Benin; 30-31, Papua New Guinea.

1. Agy-99
2. 8756 Japan
3. 980912 China
4. 842 Suriname
5. 7922 French Guyana
6. 5147 Australia
7. 5142 Australia
8. 9540 Australia
9. 9550 Australia
10. 940339 Australia
11. 8849 Australia
12. 5151 DRC
13. 5150 DRC
14. 940511 Côte d'Ivoire
15. 940662 Côte d'Ivoire
16. 94511Côte d'Ivoire
17. 960658 Angola
18. 960657 Angola
19. 970680 Togo
20. 970321 Ghana
21. 970483 Ghana
22. 970359 Ghana
23. 940111 Benin
24. 001441 Benin
25. 940886 Benin
26. 970104 Benin
27. 940512 Benin
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29. 5114 Mexico
30. 941331 PNG
31. 9357 PNG

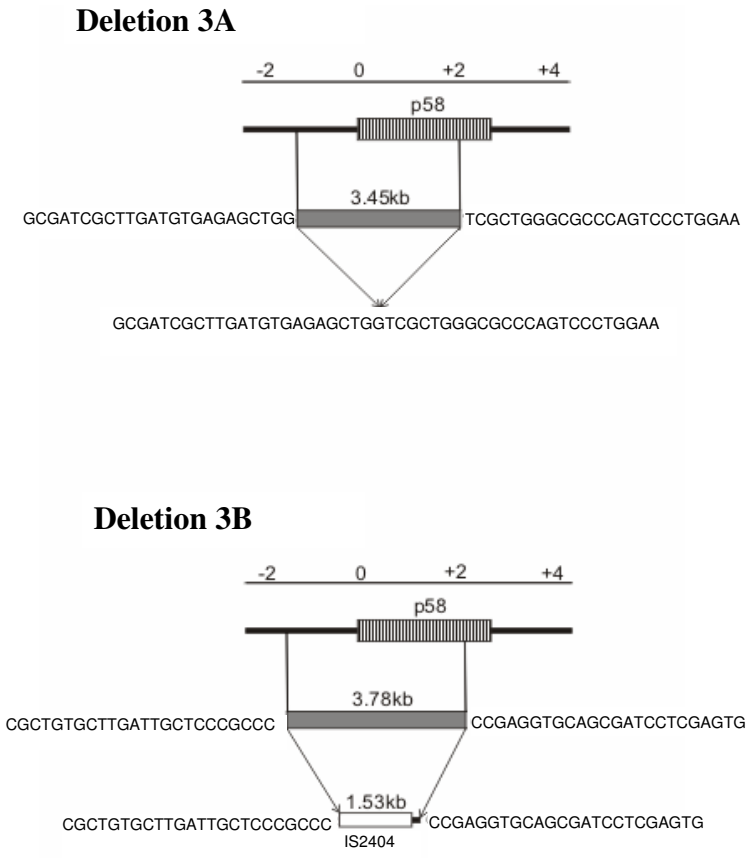

Figure 4. Deletion 3A and 3B, showing two examples of a mere deletion event (3A), and of a deletion (3B) associated with an insertion event (InDel)

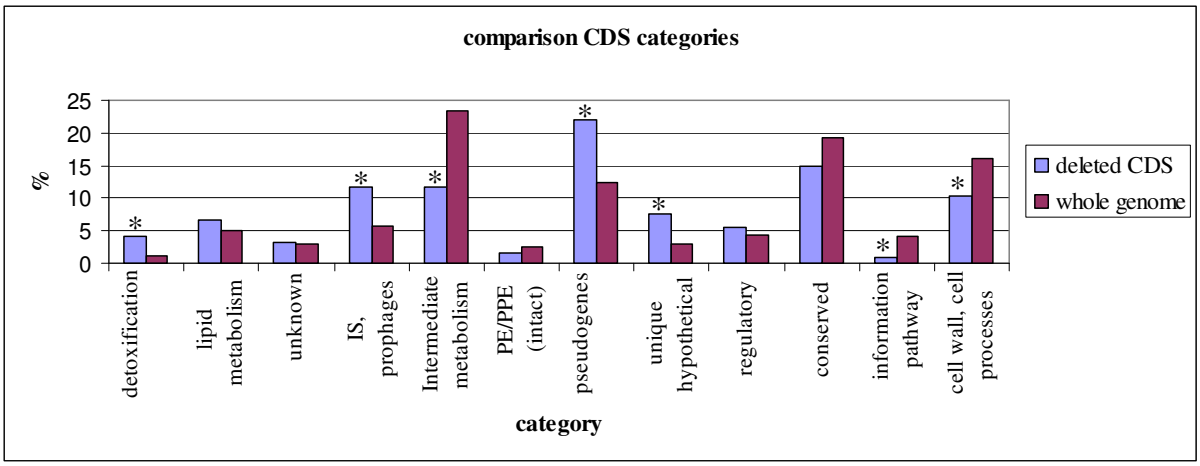

Figure 5. Comparison of the distribution of CDS functional categories between the RDs and the *M. ulcerans* genome

* depicts significant differences ($p<0.05$)

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presented with active lesions while 234 (54%) were inactive cases. Twenty-five of 202 active cases (12.4%) were recurrent cases. While the overall prevalence of active and inactive BU in the surveyed area was 4.4‰, the highest prevalence for active cases found in a particular settlement was 8%. Disease prevalence for active and/or inactive cases was higher in villages closer to the river Nyong (Figure 2). It was principally the rural, impoverished part of the population with limited geographical and economic access to health facilities that was affected. Sixty-six (28.2%) of the 234 cases with healed ulcers had severe chronic functional disabilities as a result of contraction deformities. In one case amputation of a finger was observed.

Figure 3 shows the age and sex distribution of the 202 cases with clinically diagnosed active BU. A total of 115 (56.9%) of these were male and 87 (43.1%) were female patients, resulting in a sex ratio of 1.3 in favor of the male sex. The age of patients with active BU ranged from 2 to 90 years with a median age of 14.5 years. Fifty per cent of the cases were under 15 years of age but no case was observed in children aged less than 2 years. The rate of illness did not differ significantly between the sexes or between different age groups.

A total of 243 active BU lesions were found in the 202 active cases as some patients presented with multiple active lesions. Figure 4 shows the distribution of lesions according to where they were located on the body. Most lesions (93%) were located on the extremities, with the lower limbs being involved twice as frequently as the upper limbs. The distribution of lesions by site of localization on the body did not show any significant differences with respect to age or sex. The right or left distribution of lesions was similar both in the upper and the lower limbs ($p = 0.20$ and $p = 0.12$, respectively).

The mean duration of the lesions was 10.5 months (range: 1 week - 8 years; median 5.5 months). The clinical spectrum of lesions observed was as follows: 187 (92.8 %) of the cases presented with ulcers while 15 (7.4%) cases had non-ulcerative lesions (4 cases with nodules, 7 cases with plaques and 4 cases with edema). The average diameter of ulcerative lesions was 10.3 cm (range

1 – 107 cm). Bone involvement was clinically suspected in 30 (14.9%) of the 202 cases. Involvement of the eyes and genitalia was seen in two cases each. The extent of the lesion or its proximity to a joint (elbow, wrist, knee, ankle) made severe complications after spontaneous healing probable in 58 (28.7%) of the cases.

A BCG vaccination scar was observed in 105 (52.0 %) of the 202 active cases. Multiple lesions defined as lesions present simultaneously on different parts of the body were observed more often in children (≤ 15 years old) without a BCG scar as compared to those with the scar (7/20 vs. 1/30, P <0.01) suggesting a protective effect of this vaccination against more severe forms of BU in this age group. No such association was found for bone involvement in BU even after stratifying patients by age groups.

One hundred and seventeen cases (57.9 %) said that a family member or relative had suffered in the past or was currently suffering from BU. In one family three children and in three other families two children were suffering at the same time from active BU. Relatives of patients with active BU, or members of their households who had had BU in the past, were asked when the illness had occurred. 75% reported that it was within the last three years. In 5% it was reported as having occurred more than 10 years ago. The different types of sources of (drinking) water (water tap, river, well, spring) were not significantly associated with the presence of BU. Probably patients go to the swamps for other domestic activities.

An analysis of the health seeking behavior of the cases revealed that more than two thirds (67.3%) of them were exclusively under treatment given by traditional healers and less than 10% of them were being treated exclusively by trained health personnel.

Samples for laboratory confirmation of the presence of *M. ulcerans* in the lesions were taken from 164 (81.1%) of the 202 clinically diagnosed active cases. Samples could not be taken from the other 38 cases either because they presented with non-ulcerative lesions such as nodules, plaques or edema (n = 15) or because they presented with ulcers in an advanced stage of healing (n = 23). Direct microscopic examination of the 164 samples stained by the ZN technique revealed AFB in 38 (23.2%) cases. A total of 162 samples were analyzed by the *IS2404* PCR technique (two PCR samples were lost). 135 (83.3 %) of these samples were positive for *M. ulcerans* DNA. In 34 cases, the both ZN staining and PCR were positive.

Discussion

During the last decade, the existence of BU disease in Cameroon has occasionally been suspected, but none of the cases notified on the basis of clinical symptoms was confirmed by laboratory diagnosis. In our study we identified on clinical grounds 436 persons with active (n=202) or inactive BU (n=234) in an endemic area situated in the Nyong basin in the Centre Province of Cameroon, where *M. ulcerans* disease was described for the first time more than 30 years ago. *M. ulcerans* DNA was identified by the diagnostic *IS2404* PCR assay in 135 (83.3%) of the 162 analyzed active cases with ulcerative lesions. However, confirmation by two independent laboratory methods as required according to WHO guidelines[8] to positively diagnose BU was achieved in only 34 (17%) of these patients. For logistical reasons we had to renounce on systematic biopsy taking. If that had been possible more extensive analyses of specimens by culture of *M. ulcerans,* staining of AFB, PCR analysis and histopathological examination of excisional biopsies would probably have yielded far more ‘double positive’ laboratory results.

The differential diagnosis of the clinically identified 15 pre-ulcerative lesions (i.e., 4 cases with nodules, 7 with plaques and 4 with edema) was the most problematic, since it had to be done without any laboratory re-confirmation. All cases with inactive (healed) BU had – for evident reasons – to be diagnosed exclusively clinically, too. Diagnostic misclassification may thus have overestimated the overall prevalence of BU. On the other hand, it is generally admitted that the clinical diagnosis of ulcerating BU by an experienced clinician is relatively straightforward in a known endemic area.[17] Other common chronic ulcerative lesions encountered in Cameroon (tropical and venous ulcers, ulcers due to burns) can be differentiated from BU by their clinical aspects and case history. We therefore included all the 202 clinically diagnosed cases with active lesions in the analyses of the clinical presentation and of possible risk and protective factors.

When supposing that clinical diagnosis together with partial confirmation by laboratory procedures was mostly correct, we may ask, alternatively, whether it was likely that we missed a substantial number of cases. The delimitation of the survey area was empirical, depending substantially on the ability of the local population to identify the disease. However, the population and peripheral local health personal appeared to identify BU even in its early, pre-ulcerative forms surprisingly well. On the other hand, we may have missed cases because presentation was voluntary and this could have resulted in a selection bias. Therefore the survey's results may rather have underestimated than overestimated prevalence. On various occasions we even noticed traditional healers forbidding their patients to present themselves to the survey team. People with inactive BU were not included in the analysis of the clinical presentation and possible risk and protective factors, for two reasons. Firstly, we assumed that the presentation of cases on a voluntary basis would induce a selection bias towards active cases. Secondly, case-histories from people with lesions were likely to be less accurate when the disease was acute some years ago.

The endemic area had characteristics similar to those found in foci elsewhere [4,5,6,7] and the clinical characteristics of active cases notified resemble what has been found in similar situations.[14] The endemic area is located in the swampy banks along the large meanders of a slow flowing river Nyong and some of its affluents, and it is isolated. The frequency of identified cases diminished with distance to the river. Similar to findings in Côte d'Ivoire [18], more than half of the cases were children =/< 15 years old with the highest rate of infection in the group of 10-14 years. No preponderance of women in the group of adult (=/> 15 years) patients was found, as was reported in earlier descriptions of this focus and in other studies.[6-13]The ulcerative forms predominated in prevalent active BU cases; our finding of 92.8% compares with 89.5% or 96.4% found in prevalence studies in Côte d'Ivoire and in Benin, respectively.[5,6] However, in a more recent study, performed on 1700 patients from Bénin (from 1997 to 2001), there was no statistical difference between the percentage of ulcerated and non-ulcerated forms. The increasing number of non-ulcerated forms detected from 1997 to 2001 is a result of increasing activity of public health programs that raised awareness of BU clinical forms (Debacker et al, manuscript in preparation).

Our study showed a relatively high frequency (14.9%) of bone involvement, although similar frequencies have been reported in other studies.[19] This high frequency may have been due to a selection bias due to our case-finding method and use of clinical diagnosis. Lesions on the limbs were predominant. In the first - restricted - case series of the Nyong focus upper limbs were predominantly affected, but our findings confirm the general finding of lower limbs being most affected.[13] Even after stratification for sex and age, no unequal right-left distribution of lesions on limbs as reported in some other studies [4,17,18] was found. Similarly in a recent study in Bénin, there were no significant differences between the number of lesions on the right or left limbs (Debacker et al., manuscript in preparation).

In 1969, the Uganda Buruli Group demonstrated in a controlled trial that BCG vaccination confers partial protection against BU during at least six months.[20] This finding was confirmed by Smith and colleagues in 1974.[21] In Benin, BCG vaccination appeared to partially protect children of less than 15 years of age with confirmed BU against the osseous forms of BU.[22] In our study population children not vaccinated with BCG appeared to have a significantly higher risk of multiple lesions, confirming the partial protective effect of BCG vaccination against severe forms of BU in children.

BU tends to affect several members of the same family which was already observed in early studies of the disease in Uganda.[2] Reported figures about the proportion of households with several BU cases are scarce, varying between 0% and 5%.[13, 23] Our figure of almost 58% of a history of cases in the family or among people living in the same compound appears high. But it is not really comparable with figures communicated by other studies as our questionnaire did not allow differentiating between other family members suffering currently from BU and family members known to have suffered in the past from BU. The data suggest that within the endemic area scattered disease foci of different importance subsist during time.

Even with only partial laboratory re-confirmation, we conclude on the basis of clinical and epidemiological evidence that the area surveyed constitutes a focus of emergent endemic BU of considerable magnitude. The accumulation of family cases during the past three years suggests BU to be recently re-emerging on a larger scale in the survey area. However, this focus has probably existed without interruption and - when considering the distribution of active and inactive cases - without major transposition of the different individual foci, for more than thirty years in a well-circumscribed area without triggering off any intervention by health authorities.

Further support was given to the idea that the disease had never really disappeared from the area by the fact that it was evidently well known and described by the local population.

Patients' preference of traditional medicine reflects a situation where the health system has had nothing to offer to assist these patients and where, consequently, the population has lost confidence in the ability of the health system to assist. Currently, surgery is the only proven effective treatment of *M. ulcerans* disease.[16] Limiting factors include inadequate surgical facilities, need for prolonged stay in hospital and high treatment costs. Estimating the mean duration of the disease without medical intervention to be one year, the annual incidence could be estimated to be a 100-150 new cases of BU in an area largely identical with the survey area. With more than 25% of these patients being at high risk to develop severe long-term disabilities, a comprehensive public health intervention by health authorities and the health districts concerned is urgently needed. Control strategies promoted by the Global Buruli Ulcer Initiative include strengthening of the health care capacity in BU endemic areas by upgrading surgical facilities, health education and staff training in the communities to promote early detection and rapid referral, development of motivational strategies and rehabilitation of those already deformed by the disease.[16]

Acknowledgements:

We thank Dr. Ayissi Christopher, Delegate of Public Health of the Centre Province for his assistance. We are also grateful to late Simon Bitoto and Daniel Ze Bekolo, Leprosy District Officers for Ayos and Akonolinga health districts, as well as to Petrus Nkamsse and Arlette Ngassam for the invaluable help they gave us in different aspects of this study. We thank Dr. Thomas A. Smith for helping with the design of a map of the survey area showing prevalence by location of the survey area and Jeniffer Jenkins for editing the manuscript.

Financial support:

This study was funded by 'Aide aux Lépreux Emmaüs-Suisse' and 'Médecins Sans Frontières Switzerland' (MSF-CH). FP was supported by the Fund for Scientific Research, Flanders (Belgium) (FWO-Vlaanderen contract nr. G-0301-01).

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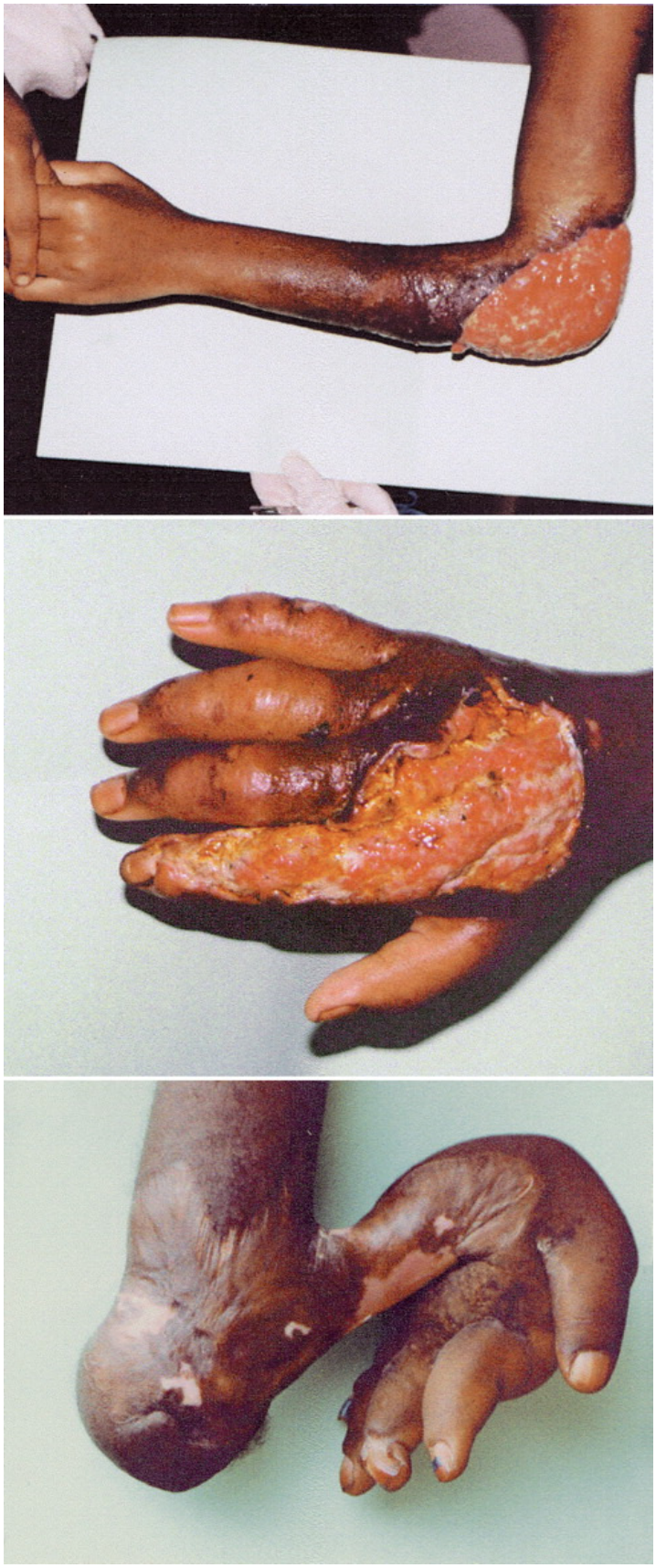

FIGURE 1. Presentation of fully developed Buruli ulcer lesions and of a contracture deformity resulting from scarring.

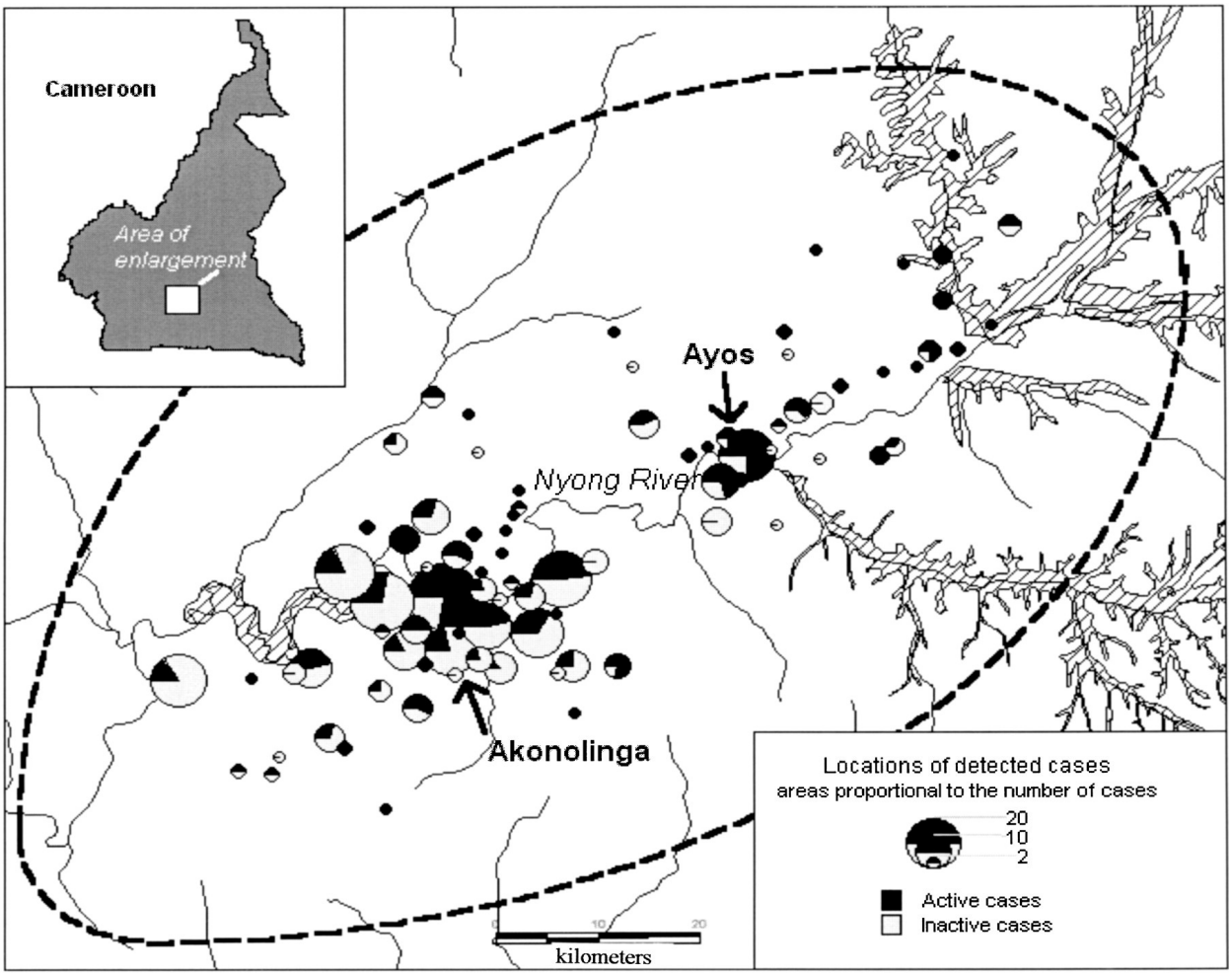

FIGURE 2. Prevalence of active and inactive Buruli ulcer cases in 83 villages and settlements in the study area in Cameroon along the Nyong River and some of its main tributaries. The survey area encircled.

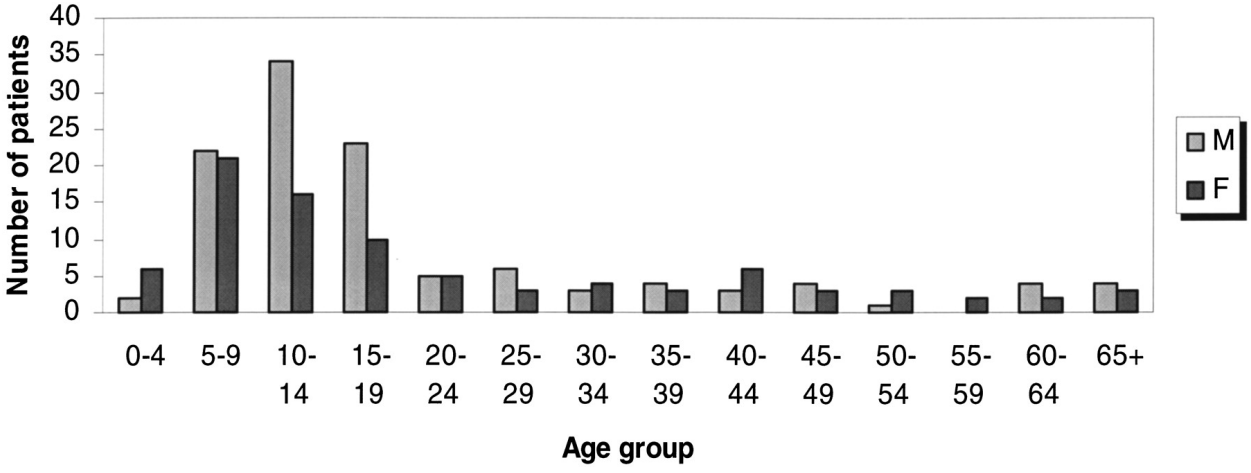

FIGURE 3. Age (in years) and sex distribution in 202 clinically diagnosed patients with active Buruli ulcer.

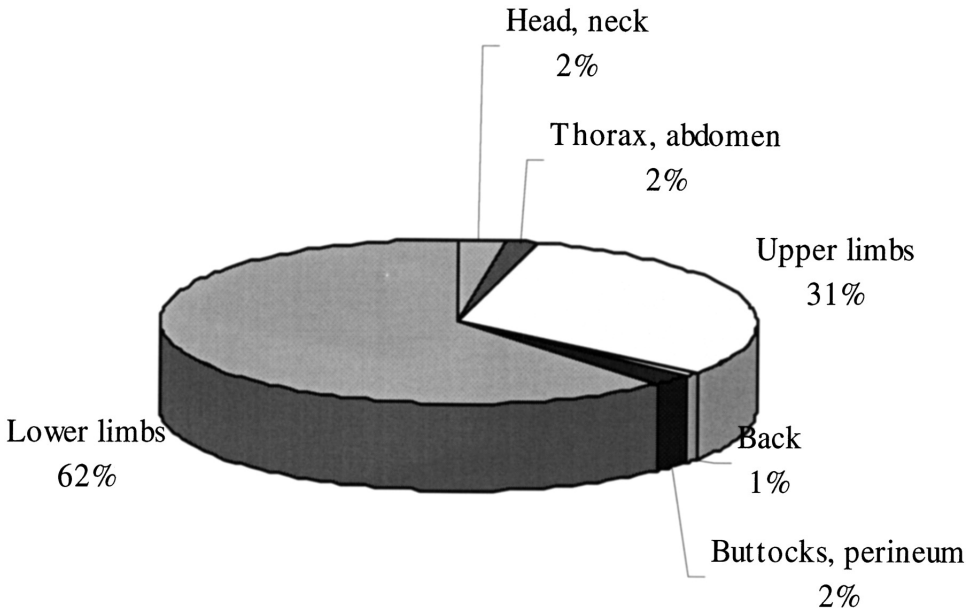

FIGURE 4. Distribution of active lesions in 202 clinically diagnosed patients with Buruli ulcer.

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Chapter 5

Development and application of real-time PCR assay for quantification of *M. ulcerans* DNA assay

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This article has been published in:

Journal of Clinical Microbiology 2003 Sep;41(9):4231-7.

Abstract

Buruli ulcer, an infection caused by *Mycobacterium ulcerans* is after tuberculosis and leprosy the third most common mycobacterial disease. The mode of transmission of *M. ulcerans* is not exactly known, but since Buruli ulcer often occurs in focalized swampy areas, it is assumed that there is a reservoir of the pathogen in stagnant water. Buruli ulcer usually starts as a painless nodule and can lead to massive destruction of skin, subcutaneous tissue and eventually muscle and bone. Currently the only recommended treatment is wide surgical excision. In this report we describe the development of a real-time PCR method for the quantification of *M. ulcerans* DNA (IS2404 TaqMan). The highly specific assay is based on the detection of the *M. ulcerans* specific insertion sequence IS2404. The IS2404 TaqMan assay turned out to be about ten times more sensitive than the available conventional PCR-based diagnostic test. It is demonstrated that the Taqman IS2404 assay is suitable for the quantitative assessment of the dissemination of the mycobacteria in Buruli ulcer lesions. Prototype results obtained with excised tissue from a patient with a late pre-ulcerative Buruli ulcer lesion reconfirmed earlier histopathological findings indicating that tissue damage occurs far beyond the regions in which large numbers of mycobacteria are detectable. The IS2404 TaqMan assay should be a useful tool for both diagnosis and research into the pathology and mode of transmission of this still inadequately investigated mycobacterial disease.

Introduction

Mycobacterium ulcerans is a slow growing environmental mycobacterium, which causes a disfiguring condition known as Buruli ulcer. After tuberculosis and leprosy Buruli ulcer is the third most common mycobacterial infection in humans. It often occurs in people who live close to rivers and stagnant bodies of water. *M. ulcerans* infections have been found in more than thirty tropical and sub-tropical countries of Africa, Asia, Latin America and the Western Pacific, but are most common and severe in West Africa (1).

The mode of transmission of *M. ulcerans* is not clear, but it is assumed that the environmental pathogens enter the body through small lesions in the skin (1,5). Aquatic insects may be involved in some cases (1,17). After entry, the bacteria proliferate in the subcutaneous tissue. In African patients, early lesions are characterized by coagulative necrosis of lower dermis and subcutaneous fatty tissue associated with some calcification. Production of mycolactone (12), a toxin with affinity for fatty cells (11) may be important for the pathogenesis of the disease by causing necrosis and thus providing a favorable milieu for the mycobacteria. It is likely, however, that additional bacterial factors are involved in the pathogenesis (9). Even in the most minimal lesions, necrosis of fatty tissue seems to be a primary event (18). In contrast to other pathogenic mycobacteria, *M. ulcerans* is not a facultative intracellular pathogen, but is found primarily as extracellular microcolonies (13,18)

Depending on its progression, Buruli ulcer has different clinical manifestations. The disease often starts as a painless swelling in the skin and as it progresses, all elements of the skin become affected. The early pre-ulcerative forms (nodules, papules, plaques and edemas) are followed by the ulcerative stage. Necrosis of the subcutaneous fatty tissue with vascular

occlusion results in sloughing and secondary ulceration of the overlying skin. Acid-fast bacilli (AFB) stained by the Ziehl-Neelsen (ZN) technique in tissue sections seem to be largely confined to the necrotic slough and surrounding necrotic fatty tissue. In the late stages massive areas of skin, subcutaneous tissue and sometimes muscle and bone are destroyed, leading to gross deformities. If a healing response takes place, fibrosis, scarring, calcification and contractures with permanent disabilities may result (29). To date the treatment of choice is surgery, since current antimicrobial therapies appear to be ineffective, but recurrence of the disease after surgical treatment is a common problem that may be due to incomplete removal of the mycobacteria and inadequate excision.

Early treatment of *M. ulcerans* disease provides a better outcome than treatment of the ulcerative forms, but is often impaired by the difficulties of diagnosis. The commonly used diagnostic tests are: i) detection of mycobacteria by ZN staining, a technique that lacks sensitivity and specificity, ii) culture of *M. ulcerans*, which may take several months, iii) detection of characteristic histopathological changes in excised tissue and iv) detection of *M. ulcerans* DNA by PCR, representing a rapid, sensitive and specific diagnostic method (20). IS2404, an insertion element present in multiple copies in the *M. ulcerans* genome is commonly used as target sequence for this purpose (25). IS2404 is specific for *M. ulcerans* and encodes a 328 amino acids long transposase (26).

In this report we describe the development of a PCR method for the quantification of *M. ulcerans* DNA by monitoring the real-time amplification of IS2404, using the TaqMan system (IS2404 TaqMan). Beyond the possibility of measuring the starting amount of target DNA in clinical specimens and other samples, real-time PCR has several advantages over

the conventional end-point PCR. These include reduction of risk of contamination, by eliminating the post-PCR processing and a diminished sensitivity to PCR inhibitors. By applying IS2404 TaqMan PCR to samples coming from Buruli ulcer patients, it will be possible to correlate dissemination of *M. ulcerans* with progression of the disease. The method may also help to determine the optimal extent of surgical excision in order to reduce recurrence.

Materials and Methods

Mycobacterial isolates

Mycobacterial isolates that have been employed in this study are listed in Table 1. Clinical isolates were identified to the species level by the partial sequencing of the 16S ribosomal DNA (rDNA) gene and by conventional methods (19).

Clinical specimens

Samples of about 100mg were taken from tissue excised from a Buruli ulcer patient (male, 12 years old) in Amasaman, Ghana with a pre-ulcerative plaque lesion at the ventral side of his left distal forearm. Care was taken to avoid cross-contamination among the different sample areas.

Cotton swabs were used to collect diagnostic samples from the base of undermined margins of lesions of Buruli ulcer patients with ulcerative lesions. The samples were maintained in the cold chain until analyzed.

DNA preparation

Extraction of DNA from cultivated mycobacteria was done as previously described by Telenti *et al*. (28). The tips of the cotton swabs were cut and heated for 30 minutes at 95°C in screw cap tubes containing 600µl of extraction buffer (0.2% SDS, 0.05M NaOH). After a brief centrifugation, 40µl of a 20mg/ml proteinase K solution was added to the supernatants. After 30 minutes of incubation at 60°C the samples were vortexed for 2 minutes with 200µl of 100µm glass beads and the DNA in the supernatants was precipitated and washed with ethanol. After another washing with acetone and air drying, the precipitated DNA was resuspended in 60µl of water and 0.5µl of the template solutions were used for PCR analysis.

Tissue samples of about 100mg were heated for 1 hour at 95°C in 500µl of extraction buffer (50mM Tris-HCl, 25mM EDTA, 5% monosodium glutamate). One hundred microliters of a 100mg/ml lysozyme solution were added. After 2 hours of incubation at 37°C, 70µl Proteinase K-10X buffer (100mM Tris-HCl, 50mM EDTA, 5% SDS, pH 7.8) and 10µl of a 20mg/ml proteinase K solution were added. After incubation at 45°C overnight, the samples were subjected to bead beater (Mikro-Dismembrator, Braun Biotech International) treatment with 300µl of 0.1mm zirconia beads (BioSpec Products) at 3000 rpm for 7 minutes. Beads and undigested tissue fragments were removed by brief centrifugation and the supernatants were transferred to fresh tubes. An equal amount of phenol-chloroform (Fluka) was added and the DNA contained in the upper phase was precipitated with ethanol and resuspended in 150µl of water. The DNA yield was measured using a spectrophotometer (GeneQuant) and 50ng of DNA per each sample were subjected to amplification with conventional and real time PCR.

Conventional PCR

DNA was amplified in a 50µl reaction mixture containing 1µM of each primer (MU1, MU2; Table 2), 2.5U of HotStart DNA Polymerase (Qiagen), 200µM of each deoxyribonucleotide triphosphate, 1.5mM $MgCl_2$ and 1xPCR Buffer (Qiagen). PCRs were performed in a Gen Amp PCR System 2400 (Perkin Elmer) thermal cycler with the following protocol: denaturation at 94°C for 10 minutes, amplification for 35 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min, and a final extension at 72°C for 7 min. Ten microliters of amplified DNA were subjected to electrophoresis in a 1% agarose gel and detected by ethidium bromide staining and UV transillumination. A 1Kb ladder was used as a size marker.

Real-time PCR

Primer and probe sequences (Table 2) were selected from a region of the IS2404 sequence with minimal homology with human DNA. The probe was labeled with the fluorescent dyes 5-carboxyfluorescein (FAM) on the 5' end and N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA) on the 3' end. When the dyes are close to each other, the 5' reporter fluorophore (FAM) transfers laser induced excitation energy to the 3' quencher (TAMRA). When the oligoprobe hybridizes to its template, the fluorophores are released by the 5'-3' exonuclease activity of the TaqPolymerase, leading to the separation of the dyes in solution. Once they are separated the reporter emission is no longer quenched and it is possible to measure the increasing fluorescence which is proportional to the amount of target DNA produced (15). DNA extracted from *M. ulcerans* strain Agy98 was used as standard in all experiments.

Real-time PCR reaction mixtures contained template DNA, 0.3µM of each primer, 0.1µM of the probe and TaqMan Universal PCR Mastermix (Applied Biosystem) in a total volume of 25µl. Amplification and detection was performed with the ABI Prism 7700 Sequence Detection System, using the following profile: 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 10 min and 40 cycles of 95°C for 15 sec and 60°C for 1 min. Analyses were done in triplicates. For quantification, an external standard curve with *M. ulcerans* Agy98 DNA serially diluted over 6 logs was used. Negative controls were included in each amplification experiment. To analyze the potential inhibitory effect of human DNA on the real-time PCR reaction, increasing amounts of human genomic DNA from 10 to 1000ng were added to a fixed amount of 20fg of *M. ulcerans* DNA per PCR reaction mix.

Results

Development of an IS2404 based real-time PCR assay

Using IS2404, an insertion sequence unique for *M. ulcerans* as target, an ABI Prism Sequence Detection System (TaqMan) quantitative real-time PCR method was developed. Primers F1 and R1 comprised residues 406 - 424 and 465 – 449 in the genebank IS2404 sequence AF003002, respectively (Table 2). They were used in combination with the probe P1 comprising residues 447 – 426. The standard curve obtained with a serially diluted *M. ulcerans* genomic DNA preparation was linear over six orders of magnitude with a coefficient of correlation of 0.999 and a slope of 3.51 corresponding to a PCR efficiency of >90% (Figure 1). Assuming that 1 genome copy corresponds to 5fg of DNA (27), the detection limit was 0.2 genome copies per reaction mixture. Since it is expected that the genome of *M. ulcerans* isolates contain approximately 50 – 100 copies of IS2404, the detection limit thus corresponded to 10 - 20 copies of IS2404. With amounts of *M. ulcerans* DNA equivalent to 0.2 to 200000 genome copies, Ct values ranging from 35 to 16 were obtained with a coefficient of variation less than 2 (Table 3).

The potential inhibitory effect of human genomic DNA was evaluated by spiking fixed amounts of *M. ulcerans* DNA with increasing concentration of human genomic DNA (Table 4). Human DNA on its own yielded no signal and amounts of up to 500ng of human DNA per reaction mixture had no inhibitory effect on the amplification of a starting amount of 20fg of *M. ulcerans* DNA. Only addition of 1µg of human genomic DNA resulted in some inhibition (Table. 4). This was compatible with *M. ulcerans* DNA quantification in clinical specimens, since amounts of human DNA in extracts from affected tissue were

much smaller (50ng). Genomic DNA of none of the other 22 mycobacterial species tested (Table 1) produced an amplification product, demonstrating the high specificity of the IS2404 TaqMan assay.

Use of the IS2404 TaqMan assay for the analysis of clinical specimens

Swab samples taken from the undermined edges of lesions from patients with ulcerative forms of Buruli ulcer were subjected to conventional and quantitative PCR. The conventional PCR allowed the detection of the expected 568bp band, with some variation in the intensity of the signal among the different samples (Fig 2). Southern blot hybridization confirmed that IS2404 derived PCR products were obtained (data not shown). The real-time PCR revealed more than 400 fold differences in the amounts of *M. ulcerans* DNA, ranging from 13 to 5267 genome copies/µl of extract (Table 5).

To demonstrate the suitability of the IS2404 TaqMan assay for the quantitative assessment of the dissemination of the mycobacteria in Buruli ulcer lesions, a surgical specimen widely excised from a patient with a non-ulcerative plaque on the ventral side of the left forearm was analyzed. In addition to the firm and slightly elevated lesion (B, B* and C in Fig. 3), the surgeon had excised a clear margin of normal tissue (A and A* in Fig. 3). In the centre of the plaque a more elevated region (C in Figure 3) was detectable, which was presumably the location of a papule or nodule in the earlier stage of the disease. Nine samples of about 100mg extending from the skin into the subcutaneous fatty tissue were taken at different distances from this centre of the lesion in a plane parallel to the skin surface (level 1 in Fig.3). In the center of the lesion, deep subcutaneous tissue and fascia were also severely

affected. The surgeon therefore removed two additional layers into the deeper tissues at this location, with the lowest one (level 3 in Fig. 3) extending deep down to the flexor tendon. Six samples were taken from these tissue specimens (levels 2 and 3 in Fig. 3), which exhibited pathologic changes both in color and texture.

Result of a conventional PCR analysis with the tissue samples demonstrated the presence of *M. ulcerans* DNA in all the specimens taken and already gave some indication that the amounts of mycobacterial DNA template present in the samples varied (Fig. 4). But only the result of the IS2404 TaqMan assay fully demonstrated the dramatic differences in the burden of *M. ulcerans* at different locations of the lesion (Fig. 3). The specimen taken from the middle layer in the very centre of the lesion (C2 in Fig. 3) contained about 20 times more mycobacterial DNA than the 14 other samples altogether. Three neighboring samples from layers 1 and 2 still contained 1-2% of the amount of *M. ulcerans* DNA found in the focus sample. Levels of M. *ulcerans* DNA in the additional samples from the plaque lesion (from B, B* and level 3 in Fig. 3) and in the excised margins of apparently healthy tissue (A and A* in Fig. 3) were comparable. None of these samples contained more than 0.3% of the amount of *M. ulcerans* DNA found in the sample with the highest burden. Values from split tissue samples showed little variation (data not shown), indicating that the yield of *M. ulcerans* DNA obtained with the optimized DNA extraction method used was quite reproducible. Nevertheless the absolute values presented may represent underestimates, since release of mycobacterial DNA may still have been incomplete.

Discussion

In this paper the development of an IS2404 based real-time PCR assay for the quantification of *M. ulcerans* DNA is described. IS2404 is also the target of a conventional PCR assay (24) recommended in a slightly modified form (20) by WHO as test for the confirmation of the clinical diagnosis of *M. ulcerans* infection. While conventional PCR assays basically provide end-point measurements suitable for qualitative "yes/no" results, the real-time IS2404 TaqMan assay quantifies the starting concentration of mycobacterial DNA by monitoring the amount of reaction product during the exponential phase of the amplification cycles. The remarkably broad dynamic range of the IS2404TaqMan assay allowed us to analyze samples with widely varying content of *M. ulcerans* DNA without prior estimation of template concentrations. The IS2404 TaqMan assay exhibited an about ten times higher sensitivity than the conventional IS2404 based PCR, which has been described to have a detection limit of two genome copies. No amplification products were detected when the DNA of 22 other mycobacteria species were tested with the IS2404 TaqMan assay, which reconfirms specificity data described for the conventional IS2404 PCR (24).

As expected, IS2404 TaqMan analyses demonstrated that the amounts of *M. ulcerans* DNA present in extracts from wound swabs as taken routinely for PCR confirmation of clinical diagnosis vary widely. Endpoint determinations by conventional PCR did not fully reflect these differences. Amounts of DNA in the swab extracts were at the lowest end close to the detection limit of the conventional PCR. Already small variations in the reaction conditions may in such cases cause inconsistencies in the results obtained in

parallel analyses performed by the same laboratory or by different laboratories. Introduction of the more sensitive IS2404 TaqMan assay will thus improve PCR confirmation of clinical diagnosis of Buruli ulcer and yield fewer false negative results. Due to their high sensitivity, PCR based assay systems are prone to yield false positive results caused by cross-contamination. Real time PCR reduces this risk considerably, by avoiding the post-PCR handling of the samples containing vast amounts of PCR products. Nevertheless it is advisable, even with the IS2404 TaqMan assay, to strictly adhere to the 'three-room' principle: room one for the preparation of the PCR reaction mix, room two for the processing of samples and the preparation of template DNA in a biosafety cabinet and room 3 for PCR amplification.

Apart from the confirmation of clinical diagnosis, real-time PCR tests have a broad range of potential applications including the quantification of target DNA in clinical specimens and environmental samples. *M. ulcerans* is an environmental mycobacterium and it appears that infection with it is related to swampy environments. *M. ulcerans* has been isolated from aquatic bugs belonging to the genus *Naucoris* (17) and detected by conventional PCR both in water samples (23) and in water insects (21) (22) (25). The IS2404 TaqMan assay may help in future to identify the major environmental reservoir(s) of this pathogen.

Currently surgery is the only proven effective treatment of *M. ulcerans* disease (3). Lesions in advanced stages, i.e. late pre-ulcerative and ulcerated forms may require excisions that include deep fascia and sometimes even muscle. In all forms excisions must include healthy tissue at the lateral and deep margins. The IS2404 TaqMan assay may help to answer the

question of how wide surgical excision has to be to avoid recurrences. Currently the decision on the size of excision is largely left to the experience and judgment of the surgeon. The surgeon has to compromise between the risk of recurrence and an oversized excision, associated with the need for more extensive skin grafting, increased risk of secondary infections and longer hospitalization. IS2404 TaqMan analyses will be more suitable for the analysis of the dissemination of mycobacteria in Buruli ulcer lesions than the more labor-intensive and less specific and sensitive enumeration of AFB in ZN stained tissue sections. Cultivation of *M. ulcerans* is not a suitable quantification method, since decontamination methods have a detrimental effect on the organism and primary cultivation requires between six and eight weeks or longer (20). As a typical example, data obtained with excised tissue from a patient with a pre-ulcerative plaque lesion on the forearm are presented in this report. The heaviest mycobacterial burden was found in a sample of subcutaneous tissue located about 0.5cm below the surface in the centre of the lesion. It is likely that this sample is part of the original focus of the infection, which seemed to be still relatively small in this late pre-ulcerative stage, since three other samples taken from the centre of the plaque (C in Fig. 3) contained less than 2% of this amount of *M. ulcerans* DNA. Levels were still lower in all the other samples taken from more peripheral affected or from macroscopically healthy tissues. In agreement with these findings, immunohistopathological studies of Buruli ulcer lesions have indicated that tissue necrosis extends far beyond the regions in which microcolonies of AFB are detected (4). These results support the hypothesis that diffusible toxins are associated with the pathology of *M. ulcerans* infection (9,12). After introduction of the mycobacteria into the dermis or subcutaneous tissue there is presumably a latent phase during which the slow growing

bacteria proliferate and elaborate sufficient toxin to destroy the surrounding tissue. The subsequent necrosis, especially of fatty tissue, may then provide a favorable milieu for further proliferation (18). Analyses of the spreading of the mycobacteria may contribute to our understanding of the pathogenesis of Buruli ulcer and guide surgical treatment. Lesions appear to progress differently and it is possible that differences in the dynamics of mycobacterial spreading can be distinguished, that require different types of intervention. It is remarkable, that levels of M. *ulcerans* DNA in affected tissue outside the centre of the plaque lesion and in the excised margins of apparently healthy tissue were comparable. During the collection of tissue samples and the preparation of template DNA extreme care was taken to avoid cross-contamination. Nevertheless, it cannot be completely excluded, that at least part of the signals observed with these more peripheral samples are related to contamination from the infection focus during excision. However based on concentration gradients observed with excised samples from more advanced Buruli ulcer lesions (unpublished results) it appears likely that the signals observed reflect spreading of relatively small numbers of mycobacteria. Removal of the complete burden of *M. ulcerans* may not be possible even with very wide excision. After surgery, the primed immune system may, however, be able to control small numbers of residual mycobacteria devoid of a protective cloud of necrotic tissues and bacterial toxin(s).

To date no antibiotic treatment has proven to be consistently effective in the treatment of Buruli ulcer (3). In a mouse footpad model of *Mycobacterium ulcerans* infection, treatment with a combination of rifampin and amikacin has yielded promising results (6) and clinical trials with combinations of antibiotics are currently in progress (10). Monitoring of the

response to chemotherapy may represent another useful application of the IS2404 TaqMan assay. Real time PCR has been used to monitor the efficacy of antimalarial (14), antibacterial (2) and antiviral (16,30) treatment. In the case of an IS6110 TaqMan assay for *M. tuberculosis* it has been found, that the amounts of DNA quantified in sputum corresponded well with the numbers of AFB counted by microscopy (7). Before initiation of antituberculosis therapy, measures of AFB, *M. tuberculosis* DNA, and cultivable bacilli were comparable, suggesting that quantification of DNA is a good method for measuring the initial bacillary load. However, the decline in cultivable bacilli in the specimen did not correlate with the rate of disappearance of both AFB and *M. tuberculosis* DNA. Therefore, these tests were not appropriate for monitoring treatment efficacy (7). In contrast, the rapid disappearance of *M. tuberculosis* mRNA suggested that it is a good indicator of microbial viability and a useful marker for monitoring the efficacy of chemotherapy (8). The same may hold true for *M. ulcerans* disease which may also require the development of a real time RT-PCR system. IS2404 encoding a 328 amino acids long transposase should be a suitable target also for such an assay.

Acknowledgments

We thank Thomas Junghanss for his critical reading of the manuscript and for his help during the sample collection. This work was in part supported by the Stanley Thomas Johnson Foundation

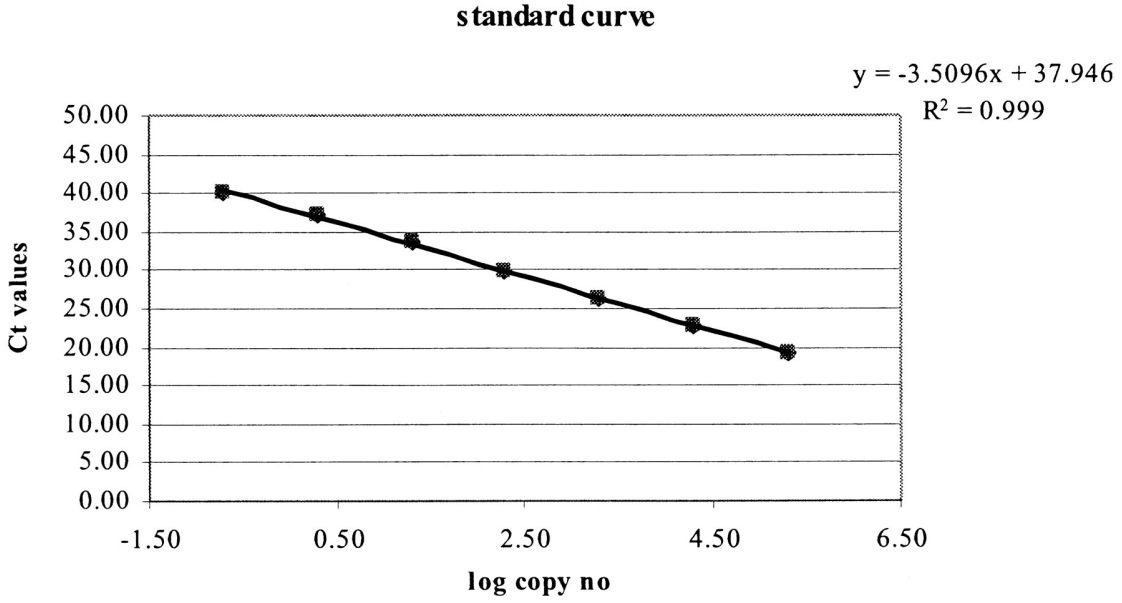

FIG. 1. Standard curve generated by the analysis of known amounts of genomic *M. ulcerans* DNA with the IS2404 TaqMan assay. The regression line calculated for the data points is shown; the coefficient of correlation is more than 0.99.

TABLE 1. List of mycobacterial strains used in this study

Mycobacterial species	Source	Medium	Temperature
M. abscessus	ATCC 19977	mod. LJ*	35°C
M. africanum	Swiss NCM ***	mod. LJ	35°C
M. avium subsp. *avium*	MAC101	mod. LJ	35°C
M. bohemicum	Clinical isolate	mod. LJ	35°C
M. bovis	ATCC 19210	mod. LJ	35°C
M. bovis biovar. BCG	ATCC 35734	mod. LJ	35°C
M. bovis subsp. *caprae*	Clinical isolate	mod. LJ	35°C
M. chelonae	DSM 43804	mod. LJ	35°C
M. fortuitum	ATCC 49403	mod. LJ	35°C
M. gordonae	Pasteur 14021.001	mod. LJ	35°C
M. haemophilum	ATCC 29548	MB 7H10**	28°C
M. intracellulare	Clinical isolate	mod. LJ	35°C
M. kansasii	NCTC 10268	mod. LJ	35°C
M. lentiflavum	Clinical isolate	mod. LJ	35°C
M. malmoense	NCTC 11298	mod. LJ	35°C
M. marinum	ATCC 927	mod. LJ	28°C
M. scrofulaceum	Pasteur 14022.0031	mod. LJ	35°C
M. simiae	Clinical isolate	mod. LJ	35°C
M. smegmatis	Pasteur 14133.0001	mod. LJ	35°C
M. terrae	Clinical isolate	mod. LJ	35°C
M. tuberculosis H37Rv	Pasteur 14001.0001	mod. LJ	35°C
M. xenopi	Clinical isolate	mod. LJ	35°C

[a] mod. LJ, modified Löwenstein-Jensen; MB 7H10, Middlebrook agar with 10% oleic acid-albumin-dextrose-catalase enrichment supplement with 0.4% ferrum-ammonium-citrate. [b] Swiss NCM: Swiss National Centre for Mycobacteria, Zurich, Switzerland.

TABLE 2. Primer and probe sequences used for the IS2404 TaqMan assay and conventional PCR

	Sequence (5'-3')	Reference
Primers		
F1	ATTGGTGCCGATCGAGTTG	This study
R1	TCGCTTTGGCGCGTAAA	This study
MU1	GGCAGGCTGCAGATGGCATA	Ross *et al*.
MU2	GGCAGTTACTTCACTTGCACA	Ross *et al.*
Probe		
P1	FAM-CACCACGCAGCATTCTTGCCGT-TAMRA	This study

TABLE 3. Measurement of replicate standard curves ranging from 0.2 to 200,000 genomes of *M. ulcerans* per reaction mixture[a]

Genome copy number	LOG copy number	Ct for the following reactions					
		1	2	3	Mean	SD	CV
0.2	-0.70	35.0	35.5	34.6	35.05	0.49	1.39
2	-0.30	31.5	32.5	31.8	31.94	0.52	1.62
20	1.30	29.1	29.1	28.7	28.95	0.20	0.70
200	2.30	26.0	25.7	26.0	25.88	0.18	0.68
2000	3.30	22.2	22.2	22.3	22.25	0.02	0.10
20000	4.30	18.8	18.9	18.8	18.85	0.06	0.29
200000	5.30	15.8	15.8	16.0	15.89	0.11	0.68

[a] Abbreviations: Ct, threshold cycle number; SD, standard deviation; CV, coefficient of variation.

TABLE 4. Evaluation of the potential inhibitory effect of human genomic DNA in quantification of *M. ulcerans* by IS2404 TaqMan

Human genomic DNA per PCR reaction (ng)	Ct values
0	28.51
10	28.69
50	28.48
100	28.37
200	28.37
500	28.85
1000	30.20

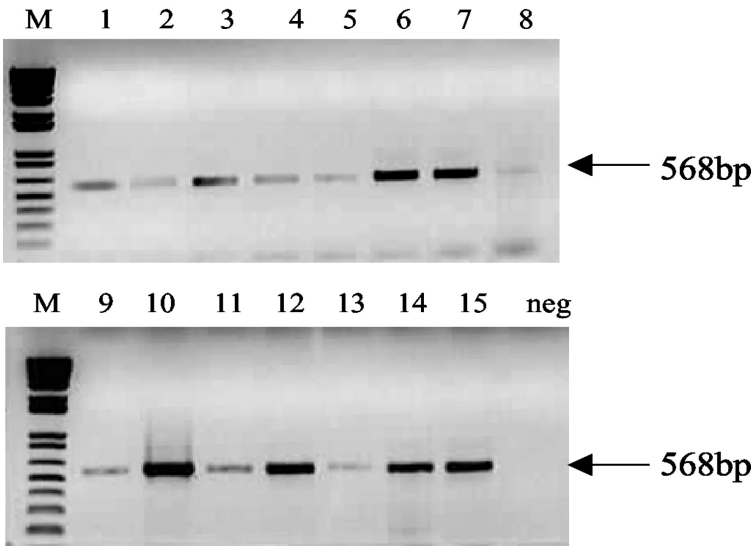

FIG. 2. Amplification products obtained by conventional diagnostic IS2404 PCR with DNA extracted from swabs used for taking samples from undermined edges of Buruli ulcer lesions (lanes 1 to 15). The lane marked "neg" was loaded with an aliquot from a negative control amplification containing no template DNA.

TABLE 5. Numbers of genome copies of *M. ulcerans* in swabs of patients with Buruli ulcer

Sample	copy no/µl*	SD	CV
1	20	1.1	10.3
2	106	4.0	7.5
3	240	10.0	8.3
4	13	0.4	5.9
5	20	0.9	9.4
6	333	11.5	6.9
7	720	28.2	7.9
8	16	2.5	31.4
9	46	26.5	11.5
10	5267	115.5	4.4
11	460	200.0	8.7
12	105	1.5	2.9
13	14	0.6	8.9
14	970	7.1	1.5
15	1090	21.2	3.9

[a] 60 µl of extract was obtained per swab.

[b] SD, standard deviation.

[c] CV, coefficient of variation.

TABLE 6. Measurement of genomic *M. ulcerans* DNA from tissue samples[a]

Reaction 1[e]	Reaction 2[e]	Reaction 3[e]	Mean	SD[b]	CV[c]
286	308	328	307	21	6.75
245	200	270	238	35	14.73
32	31	30	31	0.7	2.28
39	34	36	36	2	6.59
402	391	433	409	22	5.42
8,333	8,392	ND[d]	8,362	42	0.50
7,782	8,340	8,388	8,170	337	4.12
1,273	1,483	1,550	1,435	144	10.04
351	327	325	334	15	4.24
502,378	499,420	493,558	498,452	4,489	0.90
4,585	4,674	4,794	4,684	105	2.25
596	585	489	557	59	10.54
160	161	ND	161	0.4	0.25
153	165	161	160	6	4.08
127	119	123	123	4	3.22

[a] Corresponding to 1 µl of tissue extract (100mg/150µl), the values are ordered referring to tissue samples coming from left to right and from levels 1 to 3 according to the schema of the lesion shown in Fig. 3.

[b] SD, standard deviation.

[c] CV, coefficient of variation.

[d] ND, not detected.

[e] Values are numbers of genome copies.

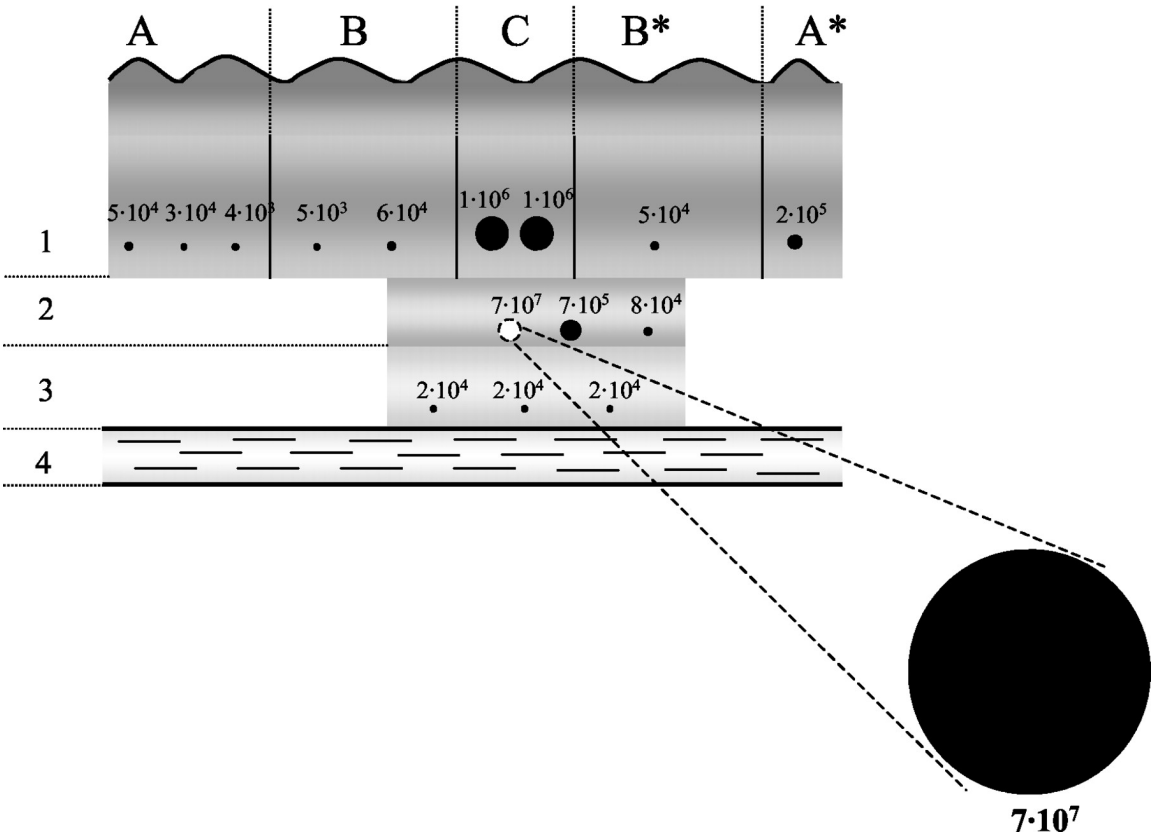

FIG. 3. Schema of the location of tissue samples taken from a surgically removed Buruli ulcer plaque lesion. The dots show the location of the specimens subjected to IS2404 TaqMan assay, and their sizes indicate the amounts of *M. ulcerans* DNA detected. The values correspond to the mycobacterial genome copies found in 100 mg of tissue sample. C, center of the plaque (diameter, about 1.5 cm); B and B*, firm and slightly elevated area of the plaque as identified by the surgeon (about 4 cm); A and A*, excised margins of apparently healthy tissue (3 and 1 cm, respectively) (A* faces towards the distal part of the forearm); 1, upper layer comprising epidermis and subcutaneous tissue; 2 and 3, deeper layers of subcutaneous tissue; 4, flexor tendon.

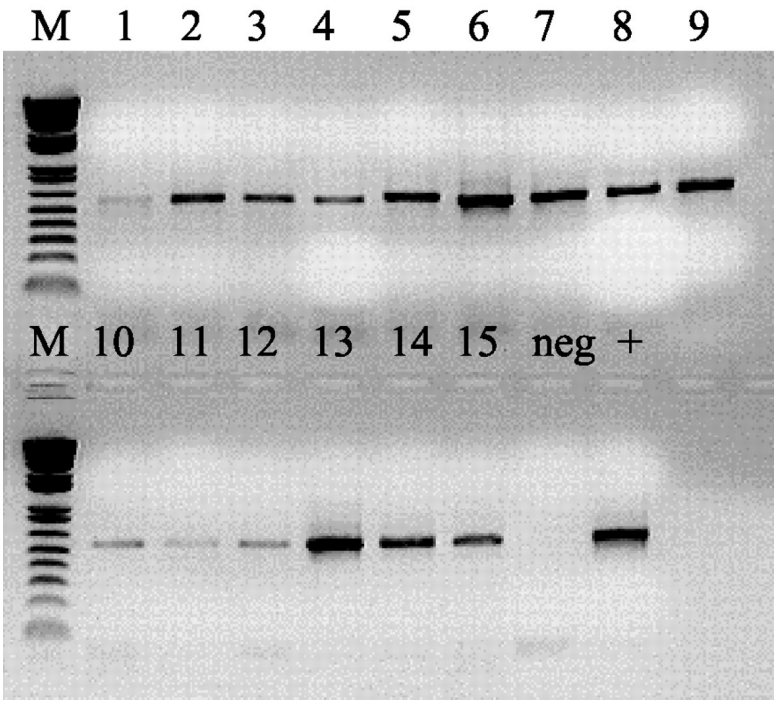

FIG. 4. Amplification products obtained by conventional diagnostic IS*2404* PCR with DNA extracted from tissue samples taken from a surgically removed Buruli ulcer plaque lesion. Lanes 1 to 9, specimens collected from left to right belonging to the layer 1 of Fig. 3; lanes 10 to 12, specimens collected from left to right belonging to the layer 3 of Fig. 3; lanes 13 to 15, specimens collected from left to right belonging to the layer 2 of Fig. 3. The lane marked "neg" was loaded with an aliquot from a negative control amplification containing no template DNA. The lane marked "+" was loaded with an aliquot from a positive control amplification containing *M. ulcerans* genomic DNA.

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Chapter 6

Contiguous spread of *Mycobacterium ulcerans* in Buruli ulcer lesions analysed by real-time PCR quantification of mycobacterial DNA and histopathology

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This article has been submitted in:

Journal of Pathology

Abstract

The distribution of *M. ulcerans* in Buruli ulcer lesions was analysed by IS*2404* real-time PCR quantification of *M. ulcerans* DNA and by semi-quantitative microscopic assessment of the number of Acid Fast Bacilli. Mycobacterial burden was compared with histopathological changes. Focal distribution of tissue destruction extending into areas with high and low mycobacterial burden was a feature in all lesions analysed. Even where most of the mycobacteria were washed out of ulcerative lesions, peaks of mycobacterial DNA and Acid Fast Bacilli in the necrotic base of the ulcers still marked the position of the primary infection focus. Significant amounts of mycobacterial DNA and microcolonies were also present in samples from more peripheral regions and occasionally in excised margins of macroscopically and histologically healthy appearing excised tissue margins. Additional peaks of mycobacterial DNA clearly marked sites where satellite lesions were developing. Even when granulomas provided evidence for the development of cell-mediated immunity, development of satellite lesions by contiguous spreading was not completely prevented. Areas free of mycobacterial DNA were found between primary and secondary infection foci and around scarring tissue of healing lesions. Results demonstrate that IS*2404* real-time PCR analyses represent a better tool than the less sensitive and only semi-quantitative microscopic enumeration of AFB for studying the dynamics of *M. ulcerans* infection *in situ*.

Keywords: *M. ulcerans*, real-time PCR, histopathology, satellite lesions.

Introduction

Mycobacterium ulcerans infection (Buruli ulcer) is a progressive necrotizing infection of the skin and the subcutaneous tissue [1]. The disease has been reported from at least 30 countries worldwide, mostly in tropical areas, where it mainly affects poor people living in rural areas. It can involve all age groups, though children below 15 are predominantly affected. Prevalence rates can be as high as 22% as it has been recorded in an endemic community in Ghana [2]. The mode of transmission of Buruli ulcer is not entirely clear [2], but once *M. ulcerans* is introduced into the dermis or subcutaneous tissue, it proliferates and produces a toxin, designated mycolactone [3]. This polyketide toxin has cytopathic activity [4] and causes necrosis of the dermis, panniculus and fascia, usually leading to a single, painless, subcutaneous nodule, oedema, or plaque [5]. Fat cells enlarge and lose their nuclei and the skin that covers these lesions eventually sloughs off, together with the underlying tissues, forming an ulcer with undermined edges [6]. Inflammatory cells are scarce in active lesions, but after an unspecified time period, a granulomatous response in the dermis and panniculus may be observed, followed by healing and scarring [7]. In many cases calcification occurs [7]. *M. ulcerans* may spread, presumably by lymphatic and haematogenous pathways, to distant foci. Occasionally metastatic bone lesions arise [8].

The currently accepted definitive treatment is the surgical removal of the infected tissue including healthy tissue at the peripheral and deep margins [1]. Due to the non-specific clinical manifestations of the disease and its indolent course, those affected tend to postpone their presentation to the health facilities [9]. This delay can necessitate complicated surgical intervention [10]. While early clinical forms can be easily excised

and closed by primary suturing, extended lesions require general anaesthesia and skin grafting [1]. Currently it is not clear how extensive surgical excision with a curative intent should be. It is largely left to the individual judgement of the surgeon to find the right balance between an oversized excision and an incomplete removal of the pathogen, thus increasing the risk of recurrence. Since recurrence rates between 16% [11] [12] and 47% [13] have been described, it is important to learn more about the distribution of *M. ulcerans* within lesions.

In this report, we have quantified the distribution of mycobacterial DNA in Buruli ulcer lesions using real-time PCR. This method has a detection limit of about 50 copies of the targeted sequence IS2404, high PCR efficiency (>90%) and specificity [14]. Mycobacterial burden was compared with histopathological changes. Results obtained with four selected patients are presented, illustrating contiguous dissemination and development of secondary lesions in the proximity of primary lesions.

Materials and methods

Clinical specimens

Patients presenting with ulcerative lesions at the Amasaman Health Centre in the Ga district of Ghana were enrolled in this study in July 2003. Clinical diagnosis of Buruli ulcer was reconfirmed by IS*2404* PCR, microscopic detection of Acid Fast Bacilli (AFB) and observation of characteristic histopathological changes. All of the patients who enrolled in the study (Table 1) received the standard treatment, comprising wide surgical excision of lesions including margins of macroscopically healthy tissue followed by skin grafting. The study had no influence on treatment modalities. Ethical approval for analysing patient specimens was obtained from the local ethical review board of the Noguchi Memorial Institute for Medical Research. Informed consent was obtained from the patients or their guardians before enrolment.

The excised tissue was divided into two halves across the centre of the lesion and spatially matching samples of each half were taken for histopathological and real-time PCR analysis, respectively (Fig. 1).

For PCR sampling, care was taken to avoid cross contamination, by starting sampling from the macroscopically healthy looking margins and working towards the centre of the lesion, using a separate disposable scalpel to cut each piece. Samples for histopathology were stored in 10% formalin, samples for PCR analyses in liquid nitrogen.

Real-time PCR analysis

Extraction of DNA from tissue specimens was done as previously described [14], Briefly, the samples were heat inactivated and then subjected to enzymatic and mechanical lyses; the DNA was first purified with phenol-chloroform, then precipitated with ethanol and finally resuspended in water. The DNA yield was measured with a spectrophotometer (GeneQuant) and 50ng of DNA per sample were used for real-time PCR. Quantification of *M. ulcerans* DNA by IS*2404* real-time PCR using an ABI Prism 7700 sequence detection system was done as described [14]. The real-time PCR efficiencies were >90% and the coefficients of correlation were from 0.955 to 0.997 (average: 0.985). Samples were analysed in triplicate and negative controls were included in each experiment. All PCR negative samples were positive both in a control PCR specific for the human β-actine gene (forward primer: GGACTTCGAGCAAGAGATGG reverse primer: AGCACTGTGTTGGCGTACAG) and in IS2404 PCR when spiked with 50 pg of *M. ulcerans* DNA, ruling out that negative results were caused by co-purification of PCR inhibitors or DNA template degradation

Histopathologic examination

Formalin-fixed strips of excised tissue were cut and sequentially blocked. Precise positions from where samples had been taken for PCR analysis from the matching strip were marked with ink. Specimens were embedded and processed into paraffin blocks. Four micrometer thick sections were cut from each block and stained with Haematoxylin and Eosin (H&E) and Ziehl-Neelsen (ZN) according to standard protocols. Each of the H&E sections were examined microscopically and semi-quantitatively assessed for the

following parameters: inflammation (acute – neutrophils; chronic – plasma cells; histiocytes; eosinophils; mild/moderate/dense infiltrate) and for the presence or absence of granulomas, dermal and fat necrosis, panniculitis, calcification, neuritis and venulitis (inflammation of the wall of the veins).

Each of the ZN stained sections were double-screened for the presence of AFB, and a semi-quantitative logarithmic score of the numbers of AFB detected was recorded (0 : no AFB; 10^0 : a single AFB within the entire section; 10^1 : up to 10 AFB within the entire section; 10^2 : up to approximately 100 AFB within the section; 10^3 : up to approximately 1000 AFB within a section; 10^4 : up to approximately 10 000 AFBs, characterised by dense clumps of bacilli on ZN stained sections.).

Results

Results obtained with four selected Buruli ulcer patients are presented, illustrating contiguous dissemination and development of secondary lesions in the proximity of primary lesions.

Each patient is presented separately, as they had distinct microbiological-histopathological patterns.

Patient I. Focal distribution of *M. ulcerans* in a primary ulcerative lesion.

Patient I presented with an ulcerated plaque (size of the ulcer: 4cm x 5cm) with necrotic slough at the centre, on the dorsal aspect of the left upper arm (Fig. 2).

a. PCR. The real time PCR analysis showed that the highest mycobacterial DNA burden was present in the samples at the base of the ulcer (Fig. 2, samples F, G and H), and decreased, generally with a steep gradient towards the margins. There were, however, small but significant (i.e. PCR value greater than 10) amounts of mycobacterial DNA present in the samples from more peripheral and clinically normal parts of the skin (Fig. 2, samples C and M).

b. Histopathology. There was typical necrosis and inflammation in the centre of the lesion (Fig. 2, Fig. 6a, 6b), with irregular clusters of AFB detected in the areas corresponding to the highest mycobacterial DNA concentrations (Fig. 6d). The typical Buruli necrosis was also seen in zones where there was DNA but no AFB (I). Occasional single AFB were detected in peripheral sections (D and L), where the tissue was inflamed

with (Fig. 6c), or without necrosis. AFB were not detected in samples C or M where the tissue was histologically normal or nearly so but where PCR identified infection. No granulomas were seen in any zone.

Patient II. Contiguous dissemination and development of a satellite lesion.

Patient II presented with an ulcerating plaque on the left upper arm measuring 7cm x 5cm (Fig. 3). The ulcer, located centrally in the lesion, measured 1.4cm x 1.8cm and had a necrotic sloughing base.

a. PCR. The *M. ulcerans* DNA distribution had one peak at the centre of the tissue adjacent to the ulcer (Fig. 3, sample F), and a second and higher peak of *M. ulcerans* in sample D (plaque, clinically). Significant genome copy numbers were also detected at both surgical resection margins (clinically healthy skin).

b. Histopathology. Typical Buruli-type fat necrosis and panniculitis were confluent in the central part of the specimen, which incorporated both *M. ulcerans* PCR peaks (sections D to F) (Fig. 6e, 6f). Typical dermal necrosis was present within sections C and D, with an additional focus in section G. Large numbers of AFBs were detected in the central sections (D and E) (Fig. 6g) and low numbers of AFBs were present within the more peripheral sections (Fig. 3 samples F, G and H), where fat necrosis and acute inflammation was also detected (Fig. 6i). The surgical resection margins were histologically normal and no AFB were present, despite the presence of significant quantities of DNA. Granulomas were present within the central zones D to G, as well as

neuritis (Fig. 6h), venulitis and calcification. No AFB were seen within the inflamed nerves.

Thus the centre of the lesion was an advanced Buruli ulcer, and there was a secondary infection focus developing (sample D) as indicated by the peak of mycobacterial DNA. Clinically the texture of the tissues was different in this secondary focus.

Patient III. Absence of mycobacteria in tissue separating two neighbouring lesions.

Patient III presented with a small ulcerated lesion and a larger non-ulcerated nodule located about 3cm apart on the dorsal aspect of the right elbow (Fig. 4). Although the ulcer itself was only 1cm in diameter, wide surgical excision was performed to remove both lesions "en masse".

a. PCR. The *M. ulcerans* DNA distribution had two peaks, a smaller peak associated with the ulcerated region and a second, much larger peak, associated with the non-ulcerated nodular lesion. The concentration of *M. ulcerans* DNA decreased in a very steep gradient from the centre of the nodule to the adjacent tissue samples F and D, which both contained 1000 fold less DNA than sample E. In tissue sample G, between the two lesions, no *M. ulcerans* DNA was detected.

b. Histopathology. Typical dermal and fat necrosis was present within sections E (Fig. 6j) and F (Fig. 6l), corresponding to the nodular lesion. AFB were found by microscopy only in sample E (the nodule). PCR identified DNA in zones which were histologically normal and had no AFB (C). Granulomas were present across the tissue from nodule to ulcer (D

to H) (Fig. 6k) and beyond, without detectable AFB or DNA in the lateral part (I to K). In the central zone (G), AFB were absent, as was mycobacterial DNA, showing that the ulcer and the nodule were not part of an active continuous lesion (there were granulomas in this part indicating previous infection and damage). Probably the nodule represented a new infection focus resulting from previous contiguous spread of mycobacteria.

Patient IV. Absence of mycobacterial DNA in a healing lesion in proximity to an active lesion

Patient IV presented with two ulcerative lesions, a large ulcer on the medial and a smaller one on the anterior aspect of the left thigh. The larger ulcer showed signs of previous treatment and was surrounded by scar tissue. The smaller ulcer was located 5cm away from the larger ulcer. The excised tissue comprised both ulcers (Fig. 5).

a. PCR. *M. ulcerans* DNA was detected neither in the healing margin of the larger ulcer (sample A) nor in the tissue separating the two lesions (samples B and C). However, *M. ulcerans* DNA was detected at the base of the small ulcer (D and E).

b. Histopathology. In the healing margin of the larger ulcer (A, B, C), there was non-specific mild dermal inflammation, without dermal necrosis, fat necrosis, AFB, or granulomas. Samples D and E, corresponding to the small ulcer, showed typical appearances of Buruli ulcer, with dermal and fat necrosis, and large numbers of AFBs (Fig. 6m, 6n). The AFB corresponded to the peaks of PCR detected DNA. No granuloma formation was found in the healing margin of the large ulcer at the time of sampling.

Correlation of PCR, AFB and histopathology

Tables 2a to 2e correlate PCR-DNA positivity, AFB presence, typical Buruli necrosis and granuloma formation in 44 samples from the four cases.

Across all the lesions, more samples had positive DNA (66%, 29/44) than had AFB detected (34%, 15/44), and just 1 sample had AFB without DNA present. All but one of the samples with Buruli-type necrosis had DNA detected (94%, 16/17) compared with 76% (13/17) with AFB seen. In samples without necrosis, mycobacterial DNA was identified more often (48%, 13/27) compared with detection of AFB (7%, 2/27). Granulomas, considered to be one feature of the healing response in Buruli ulcer [6] were associated with DNA in 67% (8/12) and AFB in 42% (5/12) of samples. Calcification of necrotic tissue correlated with granuloma formation, consistent with a chronic lesion. Conversely, in the pre-granuloma phase, 66% (21/32) had DNA compared with 31% (10/32) that had histologically visible AFB.

The distribution of AFB with respect to necrosis was irregular, with clumps of abundant AFB (density = 10^3 or 10^4) seen in some necrotic zones, whereas others in the same patient's samples were negative or very low density (Fig 6).

Discussion

This is the first study of Buruli ulcer correlating real-time quantitative PCR with histopathology. The use of these two techniques on the same samples produced complementary information that gave a clearer picture of *M. ulcerans* distribution in lesions. It provided data on the relationship between mycobacterial burden and associated tissue damage, as assessed by histopathological assessment, and contributed to the understanding of the pathological processes involved in progression of the disease, healing and recurrence.

Mycolactone, secreted by *M. ulcerans*, has affinity for fatty cells and, by causing necrosis, it may create a favourable milieu for mycobacterial proliferation. The histological signs of severe tissue damage coincided with high concentrations of mycobacterial DNA, and less consistently with high numbers of detectable AFB. In addition, there were signs of severe tissue damage in areas where few mycobacteria were detected. It is likely that this is due to the diffusion of mycolactone. The preferred localisation of *M. ulcerans* in the subcutaneous fatty tissue [15] was reconfirmed by real-time PCR analysis of superficial and deeper tissue samples from patients III and IV: the deeper portions (mainly fatty tissue) showed up to 1000 fold higher concentrations of mycobacterial DNA than the upper ones (data not shown).

It is generally assumed, from the previous clinico-pathological descriptions of Buruli ulcer [16] [17] that the onset of a granulomatous response marks the beginning of containment and eventual healing of a *M. ulcerans* infection [18] [19]. However, results

obtained with the specimens from patients II and III demonstrated that new satellite lesions can still develop after the onset of a cellular immune response. The relationship of cell-mediated immunity, i.e. granuloma formation, to healing in this infection requires further research. Calcification, associated with granuloma formation is presumably dystrophic in origin. The significance of the neuritis (seen in patients I and II) is not clear, but it is unusual in other dermal inflammation conditions apart from leprosy. No AFB were seen in the nerves.

While IS*2404* real-time PCR quantifies the entire load of mycobacterial DNA in a tissue block, the less sensitive microscopic enumeration of AFB (estimated detection limit: 1000 bacilli per ml) only reflects the mycobacterial burden in the individual sections examined. Due to the clustered distribution of mycobacteria in microcolonies (Fig 6c, 6d) [7] microscopic quantification of AFB was in general less reliable than DNA quantification and AFB staining did not reliably demonstrate contiguous spreading. Taking the limitations of the microscopic enumeration of AFB into account, an overall good correlation between the PCR-based and the microscopic analysis was observed.

The highest quantity of *M. ulcerans* DNA (i.e. log 5 or more) was found focussed in small tissue areas representing new infection foci with necrosis. Even when most of the mycobacterial DNA was washed out from ulcerated foci, peaks of DNA in the necrotic base of the ulcers still marked the position of the original infection focus, as in patient I. Although the vast majority of AFB and mycobacterial DNA remained confined to the central infection foci, some lateral spreading, sometimes extending into macroscopically

and histologically healthy appearing margins, was observed. In a patient not presented in this study, we observed a recurrence at a site, where the healthy appearing tissue contained significantly more mycobacterial DNA than in specimens taken from other sites (Rondini, unpublished observation).

Care was taken to avoid DNA cross-contamination between samples used for PCR analysis. Areas free of mycobacterial DNA were found between primary and secondary infection foci and around scarring tissue in proximity to an active mycobacterial DNA containing focus, demonstrating that it is possible to avoid carry over of significant amounts of DNA.

Surgical treatment of Buruli ulcer entails to the loss of large amounts of tissues. Early detection and surgical removal of small lesions prevents many complications and reduces costs [10]. Therefore it is particularly important to understand the progression of the disease with the goal to minimize the size of the excision, whilst at the same time reducing the risk of recurrences. Follow-up of the four patients enrolled in this study showed that they did not develop any recurrence during a period of 16 months. The wide surgical excision applied at the Amasaman Health Centre appeared to be effective in removing both primary infection foci and satellite lesions. This result is in agreement with a recent retrospective analysis comparing treatment outcome in two hospitals with different surgical treatment procedures for Buruli ulcer [13]. Long term follow-up of patients supported the concept that wider surgical excision improves the chance of healing of Buruli ulcer.

Our results emphasize, that careful macroscopic evaluation of the tissue surrounding primary foci is required prior to and during surgery to identify and excise newly developing satellite lesions. Application of the molecular approach described here for assessing excision margins, combined with long term follow-up of patients, should help to improve current guidelines for surgical treatment of Buruli ulcer.

Table 1 List of patients enrolled in the study

Patient	Age	Sex	Type of lesion	Site	Lenght of examined tissue
I	11	M	plaque with ulcer	left upper arm, dorsal	15cm
II	5	F	plaque with ulcer	left upper arm, ventral	10cm
III	21	F	(1) small nodule with central ulcer (2) large nodule	(1)right elbow, lateral (2) right elbow, dorsal	12cm
IV	45	F	(1) small ulcer (2) large ulcer	(1) left thigh, frontal (2) left thigh, lateral	15cm

Table 2 Correlation of PCR, AFB and histopathology

	DNA+	DNA-	Total
AFB+	14	1	15
AFB-	15	14	29
Total	29	15	44

Table 2a

	Buruli necrosis +	Buruli necrosis -	Total
AFB+	13	2	15
AFB-	4	25	29
Total	17	27	44

Table 2b

	Buruli necrosis +	**Buruli necrosis -**	**Total**
DNA+	16	13	29
DNA-	1	14	15
Total	17	27	44

Table 2c

	Granuloma +	**Granuloma-**	**Total**
DNA+	8	21	29
DNA-	4	11	15
Total	12	32	44

Table2d

	Granuloma +	**Granuloma -**	**Total**
AFB+	5	10	15
AFB-	7	22	29
Total	12	32	44

Table 2e

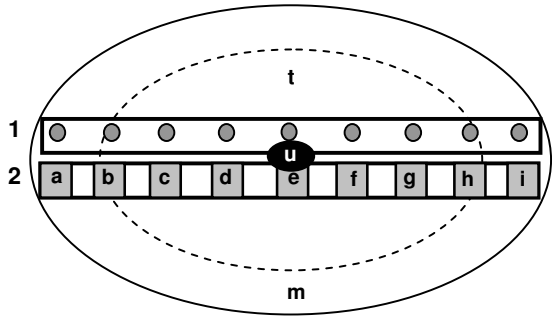


Fig. 1 Schematic view of the sampling method

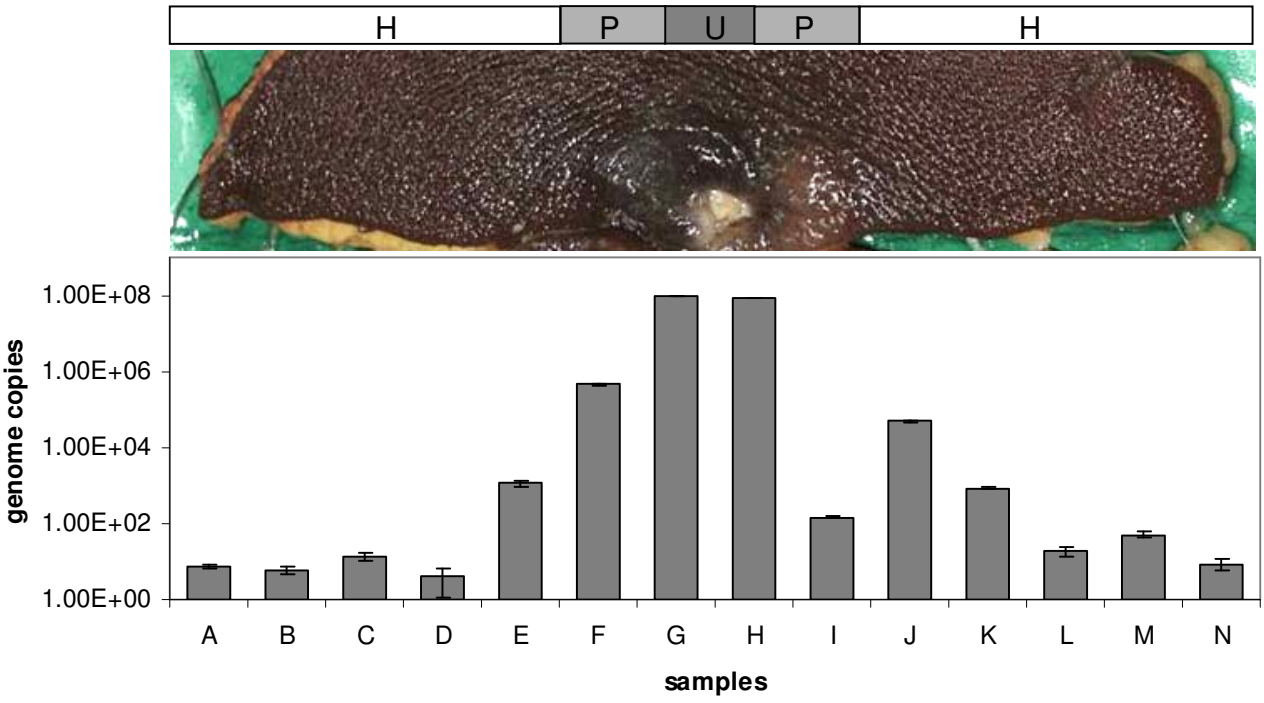
1: first half of excised tissue used for histopathology. Sections were taken in correspondence with the marked dots

2: second half of excised tissue used for real time PCR; a-i: samples matching the marked dots, where DNA quantification was performed

t: tissue macroscopically affected

u: ulcer site

m: margins macroscopically healthy



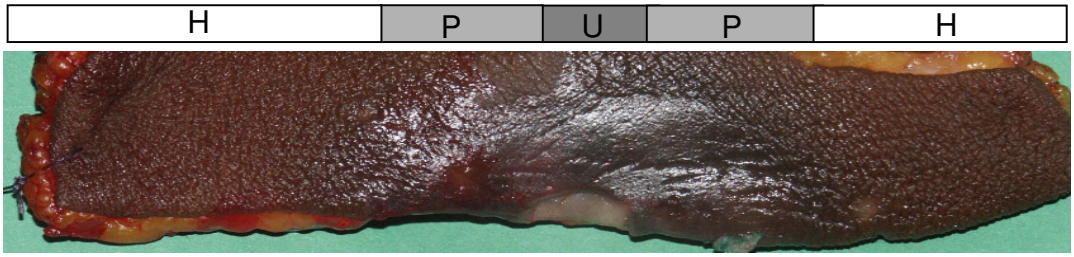
A	B	C	D	E	F	G	H	I	J	K	L	M	N
<10 ±0.8	<10 ±1.6	13.8 ±3.1	<10 ±2.8	$1.1x10^3$ $\pm1.6x10^2$	$4.5x10^5$ $\pm6.5x10^3$	$9.2x10^7$ $\pm1.4x10^4$	$8.3x10^7$ $\pm9.4x10^4$	$1.5x10^2$ ±7.1	$4.8x10^4$ $\pm7.9x10^2$	$8.3x10^2$ $\pm5.5x10^1$	19.5 ±5.5	50.6 ±5.5	<10 ±2.6

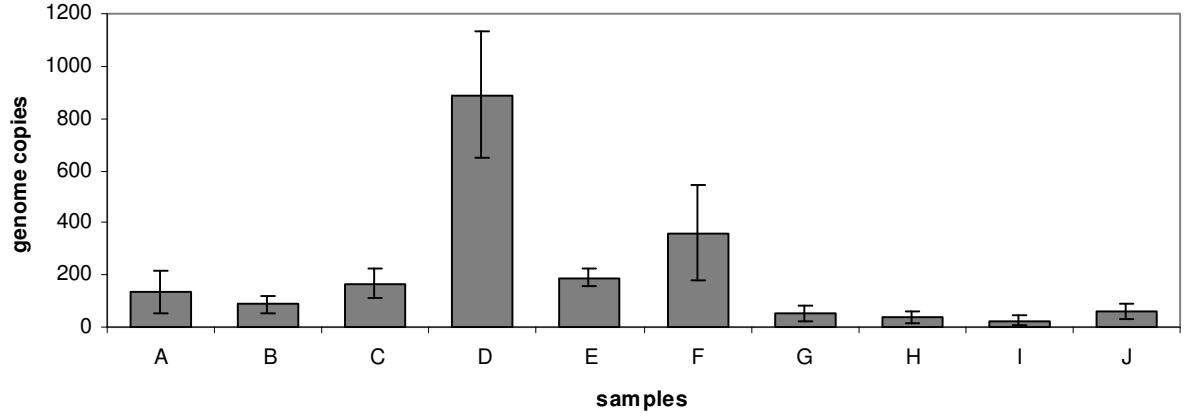
Tissue sample	A	B	C	D	E	F	G	H	I	J	K	L	M	N
AFB	0	0	0	10^1	10^1	10^4	10^1	10^2	0	1	0	1	0	0
panniculitis	no	no	no	no	yes	yes	yes	yes	yes	yes	no	no	no	no
granulomas	no	no	no	no	no	no	no	no	no	no	no	no	no	no
calcification	no	no	no	no	no	no	no	no	no	no	no	no	no	no
venulitis	no	no	no	no	no	no	yes	yes	no	no	no	no	no	no
neuritis	no	no	no	no	no	no	no	yes	no	no	no	no	no	no
histiocyte	no	no	mild	mild	mild	moderate	moderate	mild	mild	mild	mild	no	no	no
plasma cells	no	no	no	no	no	moderate	moderate	mild	mild	mild	no	no	no	no
neutrophils	no	no	no	mild	mild	moderate	severe	mild	mild	no	no	no	no	no
eosinophils	no	no	no	no	no	mild	no	no	no	no	no	no	no	no
dermal necrosis	no	no	no	yes	yes	yes	yes	yes	yes	no	no	no	no	no
fat necrosis	no	no	no	yes	yes	yes	yes	yes	yes	no	no	no	no	no

Fig. 2 Patient I

Genome copies corresponding to 50ng extracted DNA

H, healthy looking tissue P, plaque U, ulcer





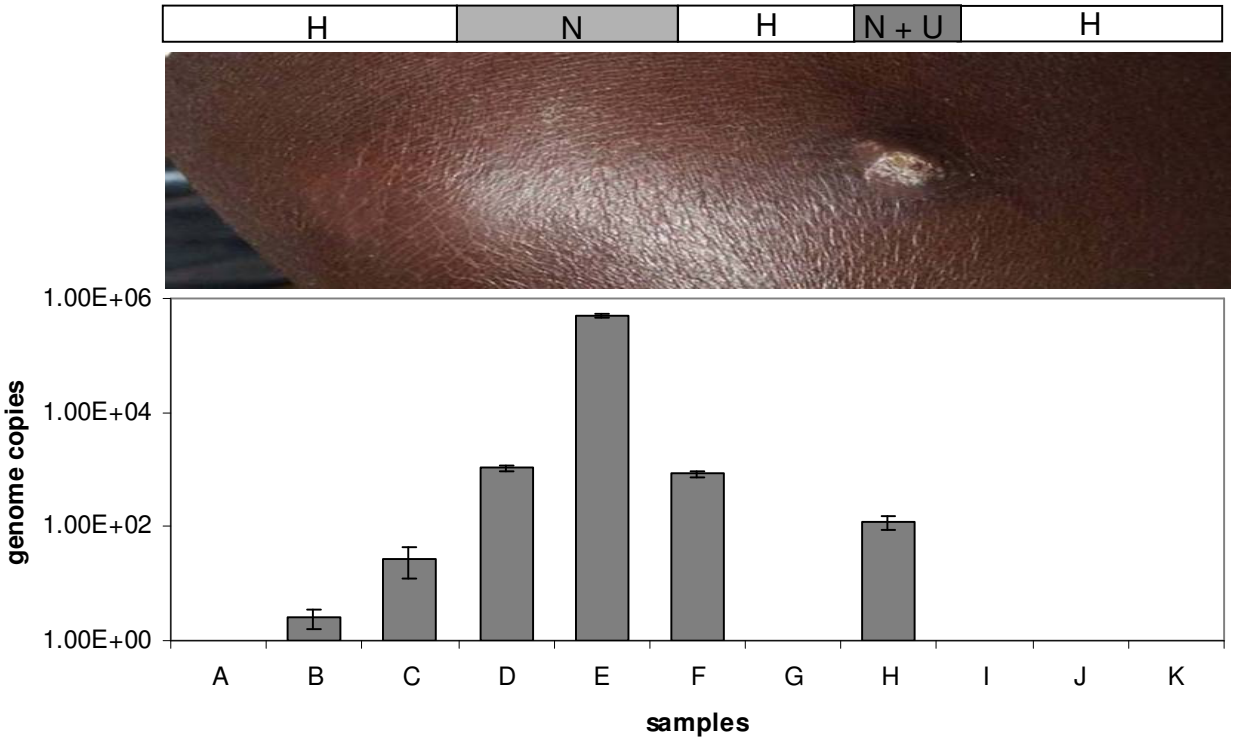
A	B	C	D	E	F	G	H	I	J
134.3 ±79.2	86.8 ±33.0	167.4 ±57.1	890.4 ±241.1	187.7 ±35.5	361.7 ±184.4	50.89 ±29.0	33.7 ±22.1	26.0 ±19.4	59.4 ±31.9

Tissue sample	A	B	C	D	E	F	G	H	I	J
AFB	0	0	0	10^2	10^3	10^1	10^1	10^1	0	0
panniculitis	no	yes	yes	yes	yes	no	yes	yes	yes	no
granulomas	no	no	no	yes	yes	yes	yes	no	no	no
calcification	no	no	no	yes	yes	yes	no	no	no	no
venulitis	no	no	yes	yes	yes	yes	yes	yes	no	no
neuritis	no	no	no	yes	yes	yes	yes	yes	no	no
histiocyte	no	mild	moderate	moderate	mild	mild	mild	mild	mild	mild
plasma cells	no	mild	mild	mild	moderate	mild	mild	mild	no	no
neutrophils	no	no	no	mild	moderate	mild	mild	mild	no	no
eosinophils	no	no	no	no	no	no	no	no	no	no
dermal necrosis	no	no	yes	yes	no	no	yes	no	no	no
fat necrosis	no	no	yes	yes	yes	yes	yes	yes	no	no

Fig. 3 Patient II

Genome copies corresponding to 50ng extracted DNA

H, healthy looking tissue P, plaque U, ulcer



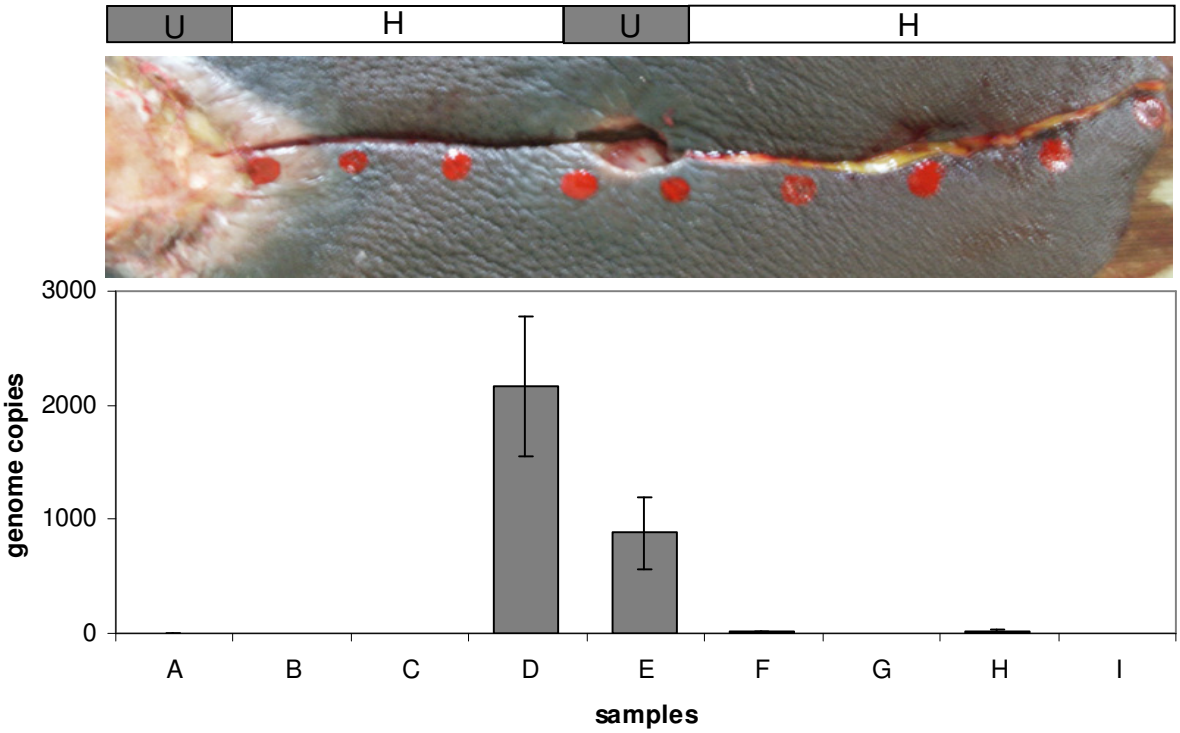
A	B	C	D	E	F	G	H	I	J	K
0 -	<10 ±0.9	$2.8x10^{1}$ $±1.5x10^{1}$	$1.0x10^{3}$ $±9.8x10^{1}$	$4.9x10^{5}$ $±3.6x10^{4}$	$8.3x10^{2}$ $±1.1x10^{2}$	0 -	$1.2x10^{2}$ $±3.2x10^{1}$	0 -	0 -	0 -

Tissue sample	A	B	C	D	E	F	G	H	I	J	K
AFB	0	0	0	0	10^{2}	0	0	0	0	0	0
panniculitis	no	no	no	no	yes	yes	no	no	no	no	no
granulomas	no	no	no	yes	yes	yes	yes	yes	yes	yes	yes
calcification	no	no	no	no	no	no	no	no	no	no	no
venulitis	no	no	no	no	no	yes	no	no	no	no	no
neuritis	no	no	no	no	no	no	no	no	no	no	no
histiocyte	no	no	no	mild	mild	mild	mild	mild	mild	mild	mild
plasma cells	no	no	no	no	no	no	no	mild	no	mild	no
neutrophils	no	no	no	no	mild	mild	no	no	no	no	no
eosinophils	no	no	no	no	no	no	no	mild	no	no	no
dermal necrosis	no	no	no	no	yes	yes	no	no	no	no	no
fat necrosis	no	no	no	no	yes	yes	no	no	no	no	no

Fig. 4 Patient III

Genome copies corresponding to 50ng extracted DNA

H, healthy looking tissue N, nodule U, ulcer



A	B	C	D	E	F	G	H	I
<10 ±3.1	0 -	0 -	$2.1x10^3$ $\pm6.2x10^2$	$8.8x10^2$ $\pm3.2x10^2$	11 ±2.6	0 -	11 $\pm2.0x10^1$	0 -

Tissue sample	A	B	C	D	E	F	G	H	I
AFB	0	0	0	10^4	10^4	0	0	0	0
panniculitis	no	no	no	yes	yes	no	no	no	no
granulomas	no	no	no	no	no	no	no	no	no
calcification	no	no	no	no	no	no	no	no	no
venulitis	no	no	no	yes	no	no	no	no	no
neuritis	no	no	no	no	no	no	no	no	no
histiocyte	mild	mild	mild	mild	mild	mild	no	no	no
plasma cells	mild	mild	no	no	mild	no	no	no	no
neutrophils	no	no	no	mild	mild	no	no	no	no
eosinophils	no	no	mild	mild	mild	no	no	no	no
dermal necrosis	no	no	no	yes	yes	yes	no	no	no
fat necrosis	no	no	no	yes	yes	yes	no	no	no

Fig. 4 Patient III

Genome copies corresponding to 50ng extracted DNA

H, healthy looking tissue U, ulcer

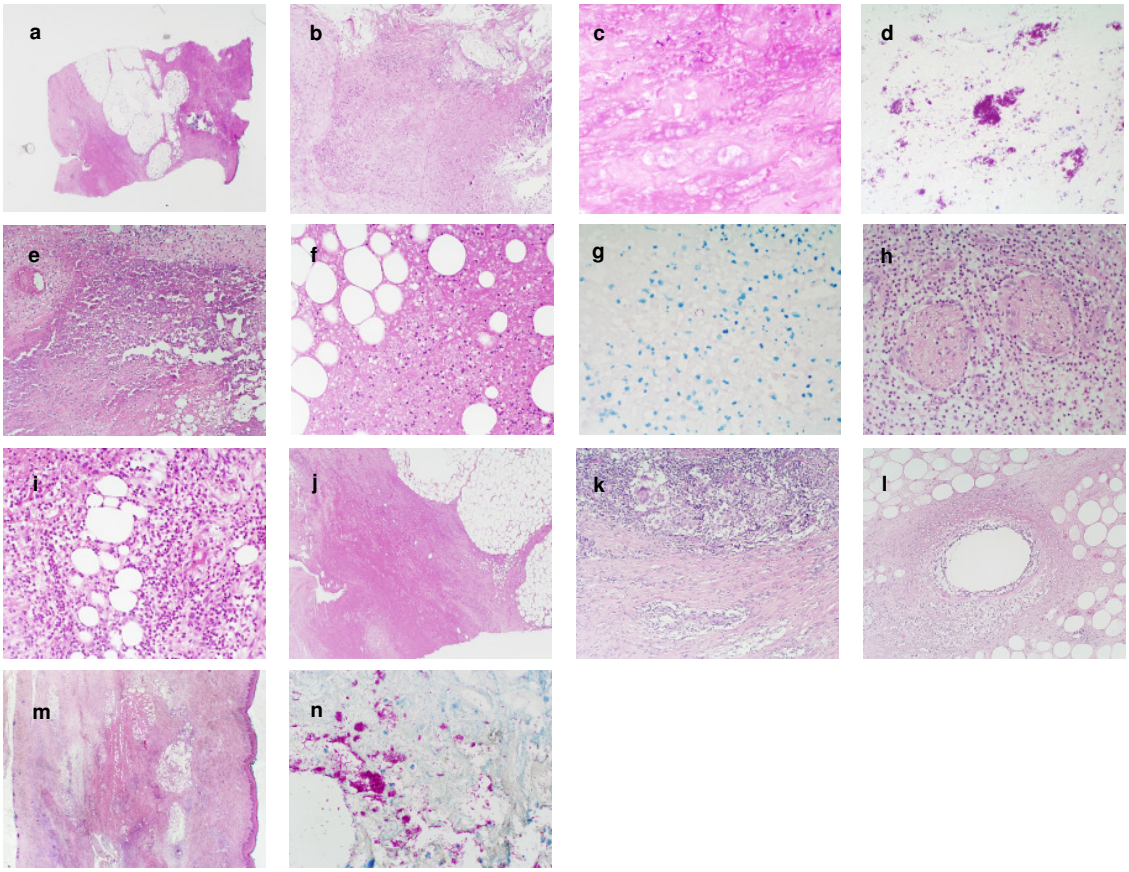


Fig. 6

a-d Patient I. **a** sample H: low power image of Buruli ulcer showing extensive Buruli-type (coagulative) necrosis throughout the mid and deep dermis, with extensive fat necrosis of subcuticular adipose tissue. There is a characteristic absence of inflammatory cells. **b** sample G: high power ulceration, with calcification. **c** sample D: dense clumps of AFB visible on H&E stain. **d** sample H: ZN stain showing dense clumps (10x4) of AFB.

e-i Patient II. **e** sample E: Buruli-type necrosis, with calcification and acute inflammation. **f** sample E: high power image of typical Buruli-type necrosis, fat necrosis and a moderate neutrophilic infiltration. **g** sample D: ZN stain showing aggregates of AFBs, lining dead fat spaces, and scattered single bacilli. **h** sample E: neuritis - neutrophilic infiltration of nerve associated with oedema and degenerative appearances to the nerve. **i** sample H: Non-specific fat necrosis and acute inflammation

j-l Patient III. **j** sample E: high power image of typical Buruli-type necrosis with fat necrosis, lacking inflammation. **k** sample H: very high power of granulomas, with giant cells adjacent to dermal scarring (fibrosis). **l** sample F: venulitis - inflammation of the vein wall with oedema and focal fibrinoid necrosis

m-n Patient IV. **m** sample E: low power, including fat necrosis, dermal 'Buruli-type' necrosis. **n** sample D:ZN stain showing clumps of AFBs.

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Chapter 7

Detection of *Mycobacterium ulcerans* DNA in the margin of an excised Buruli ulcer lesion predicts the site of recrudescence

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This article will be submitted in:

Clinical Infectious Diseases

Abstract

A real-time PCR assay was used to measure the amount of *Mycobacterium ulcerans* DNA in the excised lesion of a Buruli ulcer patient. While the heaviest mycobacterial burden was found in the centre of the lesion, macroscopically healthy appearing tissue was also partially infiltrated. A recrudescence that developed at the edge of the excision was located in an area, where the excised healthy margin contained a significant amount of *M. ulcerans* DNA. These results demonstrate that a relatively small number of mycobacteria that have spread into healthy appearing tissue can lead to the development of a recrudescence

The newly developed real-time PCR assay (IS2404 Taqman) has enabled the quantitative detection of *Mycobacterium ulcerans* DNA in lesions of patients affected by Buruli ulcer (BU) (Rondini et al) The application of the method to examine the margins of an excised lesion could provide valuable information in order to forecast possible recrudescence. This is the first report correlating the development of a recrudescence in a BU patient who underwent surgical treatment, with its original mycobacterial burden. Although the lesion had a focal *M. ulcerans* distribution, small numbers of more widespread mycobacteria were sufficient to establish a new infection focus at the edge of the previously excised lesion

Case report

A 23-year-old man from the Ga district of Ghana presented in November 2002 with an ulcerated plaque (23 x 15 cm) on the right forearm (Fig 1) at the Amasaman Health Centre. Within the plaque both an ulcer (3 x 2 cm) and - about 4 cm laterally of it - a non-ulcerated nodule was observed. After clinical diagnosis of Buruli ulcer, the plaque lesion was surgically removed *en block* with a margin of 2-3 cm (Fig. 2).

For the analysis of the spreading of *M. ulcerans* in the lesion, *s*amples were collected along the long axis of the excised tissue (Fig. 2) and the concentration of *M. ulcerans* DNA was determined by real-time PCR as described [1]. The risk of cross contamination was minimized by taking the samples starting from the macroscopically healthy looking margins and working towards the centre of the lesion, using a separate disposable scalpel to cut each piece. Twenty-two specimens adjacent to each other were obtained from this strip of tissue. Two extra samples (23 and 24 in Fig. 2) were taken from the margin in a region where, based on the macroscopic appearance of the tissue, the surgeon had decided to extend the excision beyond the originally planned margins (Fig. 2). Laboratory analyses (PCR, culture and histopathology) confirmed the clinical diagnosis of BU. Real-time PCR analysis revealed a focal distribution of *M. ulcerans*, with the highest mycobacterial burden at the site of the

nodule (sample 14 in Fig. 3). While the *M. ulcerans* content was >100 fold higher in this sample than in any other sample, smaller peaks were also observed at the margins of the ulcer (samples 8 and 11). No significant amounts of *M. ulcerans* DNA were found in some of the samples from the healthy appearing margins (samples 1, 2 and 24) while mycobacterial spreading had extend to others (samples 22 and 23).

The patient was subjected to skin grafting after 30 days of hospitalization and discharged in good condition 60 days after admission. Seven months after surgery, he presented to the Health Centre with a BU recrudescence located at the site where the excised margin contained a significant amount of *M. ulcerans* DNA (sample 23). After excision of the satellite lesion and successful skin grafting (Fig. 2), no further recurrence has developed up to now.

Discussion

M. ulcerans disease, commonly called Buruli ulcer, is a progressive necrotizing infection of the skin and the subcutaneous tissue [2]. The mode of transmission of Buruli ulcer is not entirely clear, but once *M. ulcerans* is introduced into the dermis or subcutaneous tissue, it proliferates and produces a toxin, known as mycolactone [3]. This polyketide toxin has cytopathic activity [4] and causes necrosis of the dermis, panniculus, and fascia, usually leading to painless manifestations like subcutaneous nodules, ulcers, oedema, or plaques. Focal distribution of mycobacteria with tissue destruction extending into areas with low mycobacterial burden is a common feature of Buruli ulcer lesions. Additional peaks of mycobacterial DNA mark sites where satellite lesions in the vicinity of the primary focus are developing (Rondini et al., submitted). *M. ulcerans* may also spread, presumably by lymphatic and haematogenous pathways, to distant locations, where metastatic skin and occasionally bone lesions arise [5]. The currently accepted definitive treatment of Buruli ulcer is the surgical removal of the infected tissue [6]. Due to the frequent delay between the first appearance of the disease and the presentation to the health facilities, patients often

necessitate extended surgical interventions and long periods of hospitalization. Recrudescence after surgery has been observed in up to 47% of cases [7].

In the Buruli ulcer lesion described in this report, the highest mycobacterial load was detected at the site of a nodule, which probably represented a satellite infection focus. Most of the mycobacteria were obviously washed out from the primary focus, the neighbouring ulcer. Small, but significant amounts of mycobacterial DNA in the excised margins of healthy appearing tissue demonstrate that even an excision with wide margins does not always ensure that the entire mycobacterial burden is removed. Development of the recrudescence in the vicinity of the infected margin indicates that even small numbers of mycobacteria missed by surgical treatment can cause a relapse. Even when granulomas provide evidence for the development of cell-mediated immunity, development of satellite lesions by contiguous spreading is not completely prevented (Rondini et al., submitted). The immune status of a patient may, however, determine the mycobacterial load that can still be successfully contained. As soon as a microcolony containing a critical number of *M. ulcerans* cells has developed by focal bacterial multiplication, a cloud of mycolactone may cause a local impairment to the cellular immune system.

Traditionally drug therapy on its own has been considered ineffective for treatment of Buruli ulcer. Based on recent data, WHO treatment guidelines now recommend to combine surgery with a combination of anti-mycobacterial drugs [8]. Combined with conservative surgery adjunct antibiotic treatment may impair development of satellite lesions from small numbers of *M. ulcerans* bacteria. It remains to be demonstrated in formal clinical trials, to what extent the adjunct antibiotic treatment improves the outcome for patients. Quantification of *M. ulcerans* DNA by real-time PCR can provide insight into the dynamics of *M. ulcerans* infection and is a valuable tool to monitor effects of adjunct antibiotic treatment on mycobacterial spreading.

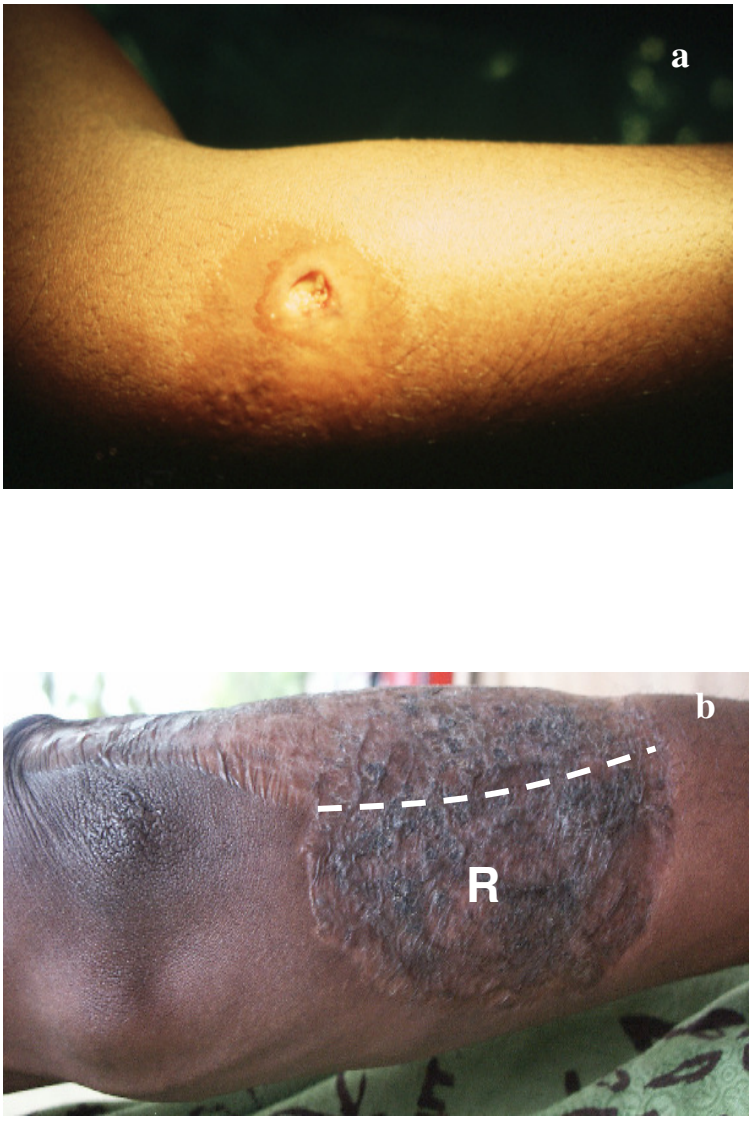

Fig.1

(a) BU patient presenting an ulcerated plaque on the right forearm (before surgery – Nov. 2002)

(b) Extension of the skin-graft due to recurrence (R) (July, 2003)

Fig.2 Surgically excised affected tissue

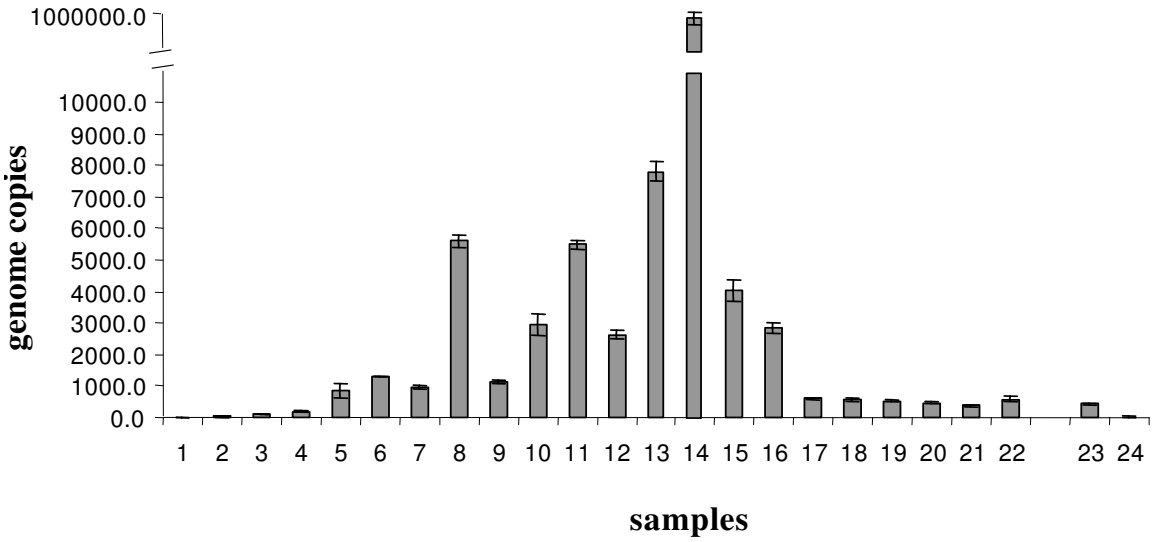

Fig. 3 Real-time PCR quantification of mycobacterial genome copies corresponding to 50ng extracted DNA

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Chapter 8

Aggressive multifocal Buruli ulcer with associated osteomyelitis in a HIV positive patient

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This article has been accepted in:

Journal of Clinical and Experimental Dermatology

Summary:

Mycobacterium ulcerans infection causes a skin disease known as Buruli ulcer (BU), a disorder manifested usually as a solitary and painless nodule or papule that progresses to massive necrotizing destruction and cutaneous ulceration. When healing occurs, it often results in disabling deformities. Buruli ulcer is considered the third most common mycobacterial disease in immunocompetent people, after tuberculosis and leprosy. Although the emergence of Buruli ulcer in Western African countries over the past decade has been dramatic, it has been scarcely reported in industrialised countries.

We report an HIV-positive Equatorial Guinean patient presenting aggressive and multifocal BU associated with an underlying destructive osteomyelitis, in which only an aggressive surgical approach yielded to a resolution of the disease. In a global world, with increasing migratory population fluxes, an increased awareness to dermatologists regarding the clinical, histopathological and microbiological features of BU is important in order to avoid significant delays in diagnosis and treatment.

Case report

A 27-year-old woman was referred to our Department for assessment of chronic ulcers on her legs. Five years earlier, in 1998, while living in a rural area on the border between Equatorial Guinea and Cameroon, she had noticed a painless nodule on her right shoulder that became ulcerated. The lesion was surgically excised in 2000 in Malabo. In 1999, while living in Malabo, a non endemic BU area, new ulcerated lesions had appeared on her left leg. In April 2003, she noticed new painless ulcerations on the inner aspect of her right leg and she travelled to Spain seeking a diagnosis and treatment. Past medical history revealed only several intestinal parasitism episodes.

Physical examination revealed destructive scarring changes on the left leg, severe ankylosis of the knee and several large ulcerations with granulation-like tissue changes (fig. 1a). On the right leg, a cutaneous ulcer, 3cm in diameter, was observed and a non-tender subcutaneous nodule was also palpated (fig. 1a).

A radiological survey disclosed severe bone destruction of the left foot and bone erosions on the left tibia, which were confirmed by a computed tomography (fig.1b). A chest x-ray film and an abdominal ultrasonography were normal.

Laboratory studies only disclosed a normocytic anaemia (haemoglobin levels: 9.4 g/dl). She had positive anti-HIV antibodies detected by Microparticle Enzyme Immunoarray (MEIA; Abbott Laboratories) and confirmed by an Inno LiPA assay (Innogenetics). She had a viral load of 48.500 copies, a CD4 count of 322/mm^3 and a CD4/CD8 ratio: 0.38 (normal 0.6-2.8).

Several skin biopsies were taken from ulcers on both legs. A dermal and subcutaneous granulomatous inflammatory infiltrate, extensive fat necrosis, and calcification foci were observed (fig. 2a). Specific stains for microorganisms (PAS, Giemsa, Gram and Ziehl-Neelsen) were negative. A biopsy from the right leg nodule disclosed in addition to the aforementioned changes, several clusters of acid-fast resistant bacilli (positive Ziehl-Neelsen stain)(fig. 2b). The specific *M. ulcerans* IS2404 PCR amplification was performed after DNA extraction from all the paraffin-embedded biopsies. In all the samples the *M. ulcerans* DNA target was detected.

Wide-spectrum antibiotics and antiretrovirals including lopinavir/ritonavir, didanosine and tenofovir were prescribed. Amputation of the left leg above the knee was performed. Both lesions on the right leg were also excised, and full-thickness skin grafts were placed. No further relapses have been detected after a 6-monthly follow-up.

Buruli ulcer (BU), caused by *M. ulcerans*, is becoming an emerging infectious disease with an impact soon to surpass that of leprosy. Hundreds of cases in well-circumscribed foci have been described in Western Africa, establishing a broad endemic area in equatorial regions.

Although its mode of transmission is unknown, it is considered that *M. ulcerans* is acquired from the natural environment, after penetrating injuries in the exposed parts of the body. Recently, some authors have suggested that some aquatic insects can also transmit the disease.[1]

BU frequently involves legs and arms of young people living in rural areas with limited access to health care. The incubation period varies from 2 weeks to 3 years, although it

is usually shorter than three months. A firm, non-tender nodule is usually the first manifestation of the disease in African patients, which evolves to a painless ulcer with undermined edges. Ulceration can be extensive and disfiguring, often affecting 50% or more of a limb. Although necrosis rarely penetrates beyond the fascia to underlying muscle, an increase in the incidence of osteomyelitis has recently been reported.[2] The lesions tend to heal after months or years, leading to scarring, ankylosis, and contractures. The absence of a host-derived inflammatory response is a characteristic feature.

BU has occasionally been observed in volunteers or journalists[3] who have travelled to Africa, as well as in immigrants who contracted the disease while living in endemic areas.[4] Most patients who were treated in non-endemic areas were seeking a diagnosis.[5,6]

From 4% to 15% of patients may develop multifocal BU. In such instances, *M. ulcerans* may spread by lymphatic, and haematogenous pathways to distant foci, usually the skin and bone.[7] Reinfection or autoinoculation mechanisms have also been postulated.[8] Reinfection seems improbable in our patient as new lesions appeared while living in a non-endemic area.

Unlike tuberculosis, the emergence of BU has not been linked with co-infection by HIV. Although HIV is not considered a risk factor, some authors have suggested that an immunological impairment such as HIV infection may facilitate multifocal aggressive forms of the disease.[8,9]

The diagnosis can easily be achieved in endemic areas when characteristic clinical and epidemiological features are met. Cultures may be negative due to critical decontamination treatments. Similarly, as the Ziehl Neelsen staining has a low sensitivity, a negative result does not rule out the diagnosis of BU. PCR techniques may be especially useful to confirm the diagnosis.[10]The differential diagnosis should include several disorders clinically characterized by persistent nodules or chronic destructive cutaneous ulcers such as pyogenic cellulitis, tropical ulcer, leishmaniasis, mycetoma and tuberculosis. An early, aggressive surgical approach is the only effective treatment at present.

The diagnosis of BU should be considered when dealing with a chronic skin ulcer in a patient who has recently lived in or travelled to an endemic region. Surgical treatment should not be delayed, as left untreated this infection can lead to extensive sequelae.[1] Considering increasing immigration and international travelling, a future rise in the number of cases of BU observed in industrialized countries seems to be predictable.

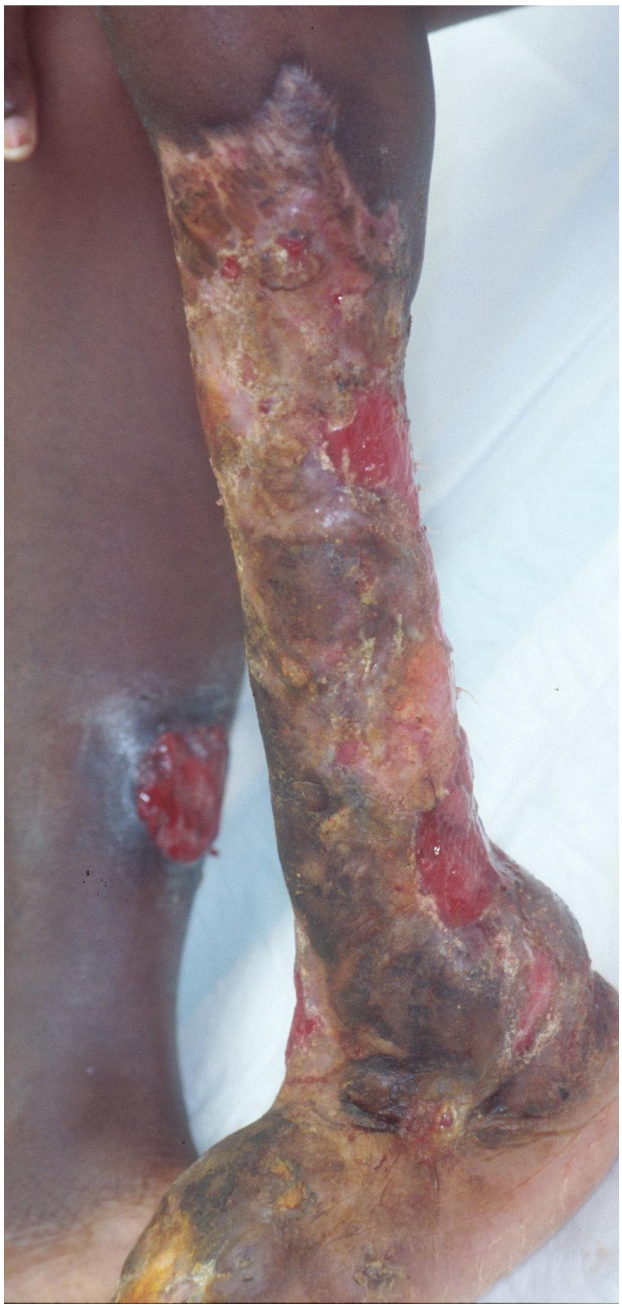

Figure 1a

Destructive scarring changes and several large ulcerations with granulation-like tissue changes on the left leg. On the right leg, a cutaneous ulcer, 3cm in diameter, with slightly undermined edges is observed

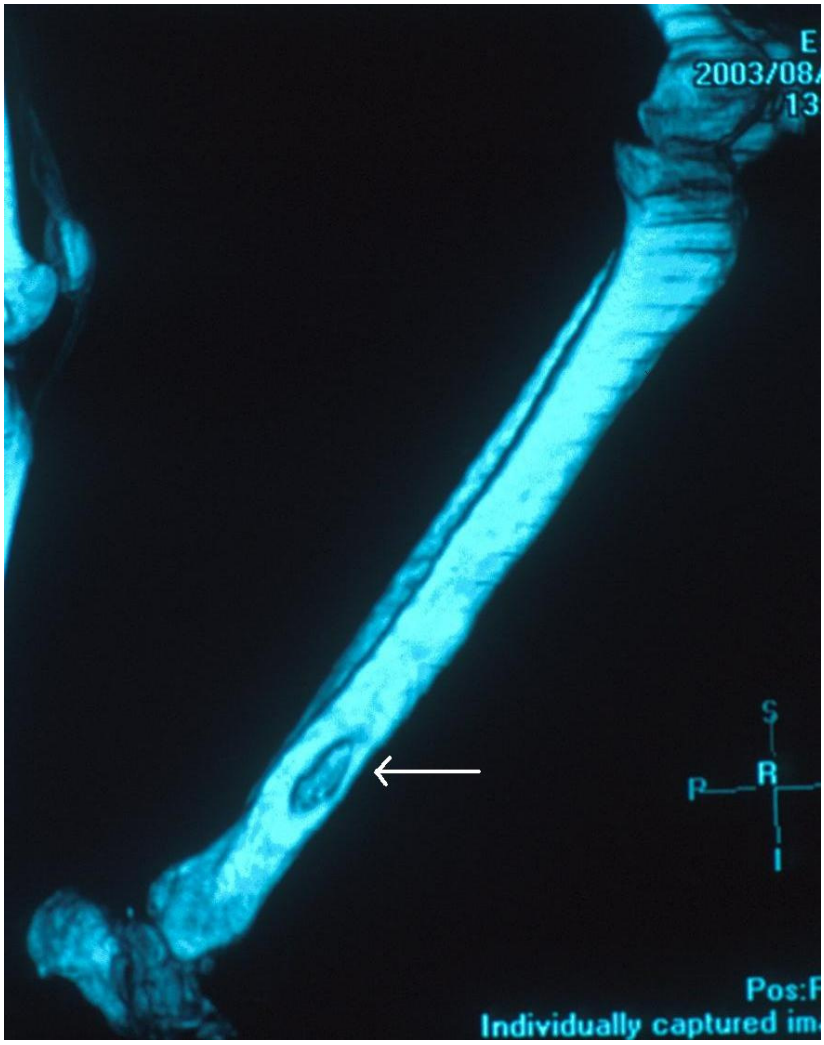

Fig. 1b

Computed tomography showing one erosion on the left tibia.

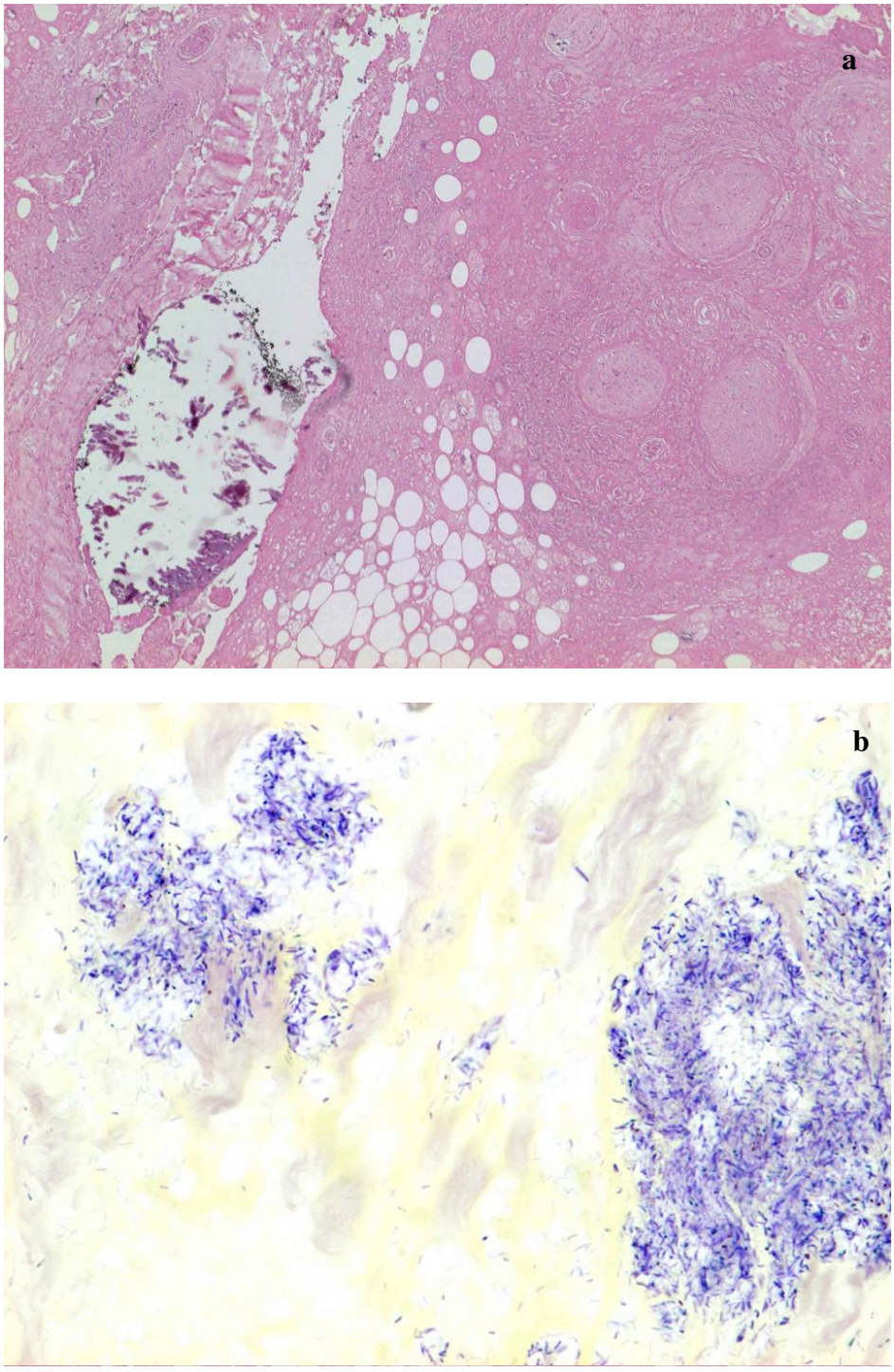

Figure 2 (a) Granulomatous inflammatory infiltrate, fat necrosis, and calcification foci (hematoxylin and eosin, x60). (b) Multiple bacilli are demonstrated by a positive Ziehl Neelsen stain (x60).

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Chapter 9

GENERAL DISCUSSION AND CONCLUSION

9.1 Implications of the main findings and suggestions for further research

After the first International Conference on Buruli ulcer Control and Research in 1998, research on this neglected disease has been intensified and new important features have been discovered. However, there are still many unsolved questions that need to be clarified, mainly in the fields of epidemiology and transmission, treatment and prevention, pathology and diagnosis.

Within the framework of the present thesis, the development of new molecular tools to study *M. ulcerans* allowed investigating several aspects of the disease. The most important findings contributed to our knowledge on *M. ulcerans* genetic variation, epidemiology & transmission and disease progression.

9.1.1. Genetic variation in M. ulcerans

The studies on *M. ulcerans* diversity performed with different typing techniques have all revealed a clonal population structure within specific geographical regions[1-6] and a low level of genetic variation, typical of mycobacterial species, as observed also for *M. tuberculosis*. More in detail, MIRU and MLST analysis evidenced an extremely homogeneous genomic architecture among isolates from the same region, and an overall low rate of single nucleotide polymorphism[7,8]. Although several studies have been undertaken, the basis of *M. ulcerans* genetic diversification has not been elucidated yet.

The major contribution of the present work in this field is represented by the description of insertional/deletional polymorphism and its implications for functional genomics (Chapter 3).

M. ulcerans belongs to the category of non tuberculous mycobacteria, where many other environmental species are grouped as well. This is a very heterogeneous group including non pathogenic species (saprophytes like *M. smegmatis* or *M. vaccae*) and opportunistic bacteria (facultative pathogens like *M. marinum*, *M. chelonae* and *M. fortuitum*). A phylogenetic analysis on 16s rRNA has suggested a very close genetic relationship between *M. ulcerans* and *M. marinum*, indicating a divergence of the species dated less than 100 000 years ago. *M. ulcerans* is thus of relatively recent evolutionary origin[9,10]. The discovery of the virulence plasmid pMUM001, absent in *M. marinum*, further enlightened the basis of *M. ulcerans* specific pathology[11]. The source of this 174 kb plasmid is not clear, but *M. ulcerans* might have acquired it by lateral gene transfer from other microbial communities (probably among the actinomycetes), conferring the capability of mycolactone secretion. Whether *M. ulcerans* existed in the environment in a non pathogenic form before the acquisition of the virulence

plasmid it is not known, although it is clear that the production of a functional toxin is needed for growth in insects' salivary glands and in human tissues. Recent data have also shown that the inherent instability of the plasmid can generate various mycolactone forms and that insertion-deletion events can occur among the highly homologous modules of the plasmid[12]. After this first report on *M. ulcerans,* showing the importance of lateral gene transfer to acquire virulence, a more dynamic scenario, although limited at the plasmid level, was proposed.

The results we obtained through comparative genomic hybridizations of different *M. ulcerans* isolates, using a new plasmid-based microarray technology, demonstrated that large sequence polymorphisms, mediated by insertion-deletion events, may represent the dominating driving force of genetic variation. This is a common feature within the mycobacterium genus, and more specifically within the *M. tuberculosis* complex, where the progressive deletions of 14 regions of difference has mediated the sub-branching, from a common *M. tuberculosis* ancestor, of all the other members till the highly attenuated BCG Pasteur[13].

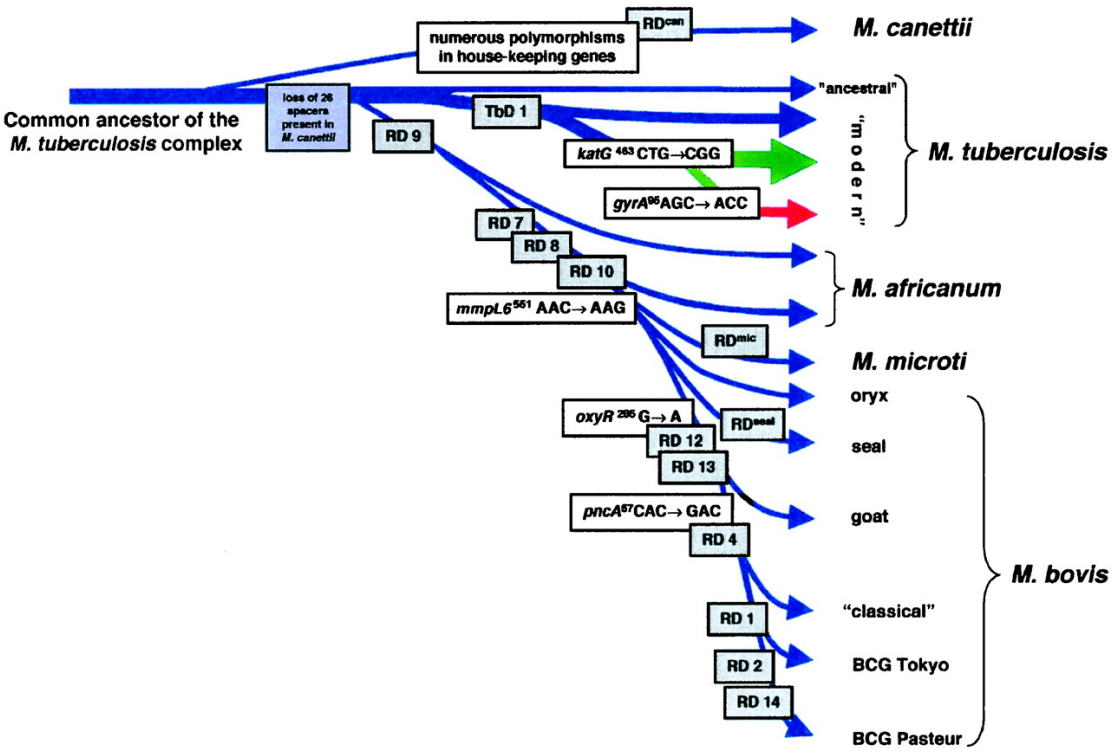

Figure 1. Scheme of the proposed evolutionary pathway of the tubercle bacilli illustrating successive loss of DNA (Source: Brosch et al, *PNAS* 2002)

The *M. ulcerans* genome is substantially smaller than the *M. marinum* genome; on top of that we could map deletions of considerable size (up to 50 kb, which already represents the genetic variation occurring between *M. bovis* and *M. tuberculosis*). Preliminary results from the Buruli ulcer genome project, has confirmed a certain level of genomic decay, with the presence of insertion sequences covering 5% of the whole genome and many pseudogenes[14]. Not surprisingly, among the deleted CDS that we were able to identify, insertion sequences and pseudogenes were deleted in excess, with the last ones alone accounting for the 22% of the all deleted CDS. Together with these disposable elements, also genes involved in detoxification were overrepresented among the deletions, showing that their importance is limited for the extracellular *M. ulcerans* bacilli. According to the pattern of deletions observed, we could postulate that *M. ulcerans* might be facing an adaptation phase from an environmental mycobacterium to an organism which is getting accustomed to a more stable environment. The well documented genomic downsizing that already occurred in *M. leprae,* with a genome comprising only 3.27 Mb and 1600 genes, is an example of reductive evolution to a minimal gene-set required for a pathogenic mycobacterium[15]. At the moment it is not clear whether *M. ulcerans* is adapting to restrict its multiplication in a mammalian host, or in another unidentified ecological niche.

The publication of the fully annotated *M. ulcerans* genome will open new possibilities to perform direct comparisons among different mycobacteria, in order to identify genetic fingerprinting methods for epidemiological investigations, and new antigens for both diagnostic and vaccination studies.

9.1.2 Epidemiology and transmission

The epidemiology of *M. ulcerans* has not been fully clarified yet, and due to the patchy distribution of the cases and the considerable underreporting, it is hard to draw an overall picture of its prevalence. It is in sub-Saharan Africa that the main burden of the disease falls, where, after the first reports of the Uganda Buruli Group in the '60s[16], new endemic foci are being discovered[17-23]. One of the contributions of the present work lies in the rediscovery of a hidden pocket of Buruli transmission in the Nyong River basin in Cameroon (Chapter 4). This endemic area, after its first description in 1969, has been ignored for about 30 years, till when our cross-sectional survey identified more than 430 Buruli ulcer cases urging health care interventions. The case of Cameroon can be regarded as a successful example of the impact that such studies may have on the establishment of beneficial interventions for the affected populations: the obtained results led to the beginning of a surgical treatment program

conducted by Aide aux Lepreux Emaus Suisse (ALES) and by Médicins Sans Frontières who have treated several hundreds patients so far. Beyond the usefulness of these emergency initiatives, it is important to draw attention also on the need to implement long-term strategies like control programs, upgrading surgery facilities, promoting health education, training the local staff to detect early cases, and providing rehabilitation for those already deformed by the disease.

Understanding the mode of transmission would constitute the best way to prevent (if possible), or to control the infection. But, beyond the empirical observation that the proximity to swamps and wetlands is often associated with Buruli ulcer insurgence, the exact mode of transmission remains unknown.

Hayman, studying *M. ulcerans* epidemiology in Australia, postulated such association[24], which was further supported by several independent lines of evidence, like the detection of bacilli in water samples during an outbreak[25], their isolation from aquatic insects collected in an endemic region of Côte d'Ivoire[26], and the finding that aquatic snails might act as passive hosts[27]. These results could implicate that the source of infection is water-related and its proximity might increase the risk of getting infected. The results obtained in Cameroon confirmed this hypothesis: the villages closer to the slow flowing Nyong river presented a higher prevalence than the others (Chapter 4). To further investigate how exactly the mycobacteria come into contact with the human host, several possibilities have been postulated: (1) by aerosol spray of contaminated water sources, (2) by water-related activities (like fishing, bathing, washing clothes, or fetching water), or (3) by an animal reservoir present in the endemic areas. There are convincing data for all the above cited possibilities. On the contrary, person to person transmission does not seem to play an important role.

(1) The first possibility is sustained by the results obtained from the Buruli ulcer outbreak that occurred on Phillip Island between 1992 and 1995[25]. This sudden increase of Buruli cases could be associated with an irrigation system of a golf-course, which had the capacity to aerosolize the bacteria over a wide area. Interestingly, when the swamp where the water used for irrigation came from, was reclaimed, also the cases ceased to appear.

(2) To support the second hypothesis, a recent case-control study performed in Ghana[28] reported a significant greater risk of developing Buruli ulcer for people who swim in the river on a regular base. Although it was not possible to exclude whether the transmission would occur in the river itself, it seemed plausible that the contact with riverbanks vegetation, which

was found to stimulate the growth and biofilm formation of *M. ulcerans*[29] and where insects live[26], could constitute a major risk factor.

(3) The third possible pathway of transmission is sustained by several data on the detection of *M. ulcerans* in various animals, and by the fact that it was possible to induce Buruli ulcer lesions in mice, after the bites of water bugs of the Naucoridae genus. Up to now, however, the contribution of this other potential transmission mode has not been defined.

It would be interesting to investigate, among the affected people documented in Cameroon, if an association between behaviour and disease can be found, and which are the most likely sources of Buruli ulcer for the local population. A more ecological type of approach should be undertaken to monitor different water insects that might behave as vectors of *M. ulcerans,* in order to understand whether their biting behaviour could be directly associated with infection, or if they are part of a more complex transmission chain.

Another suggestion for future studies could be the application of our newly developed real-time PCR assay (Chapter 5) to monitor environmental samples from the Buruli ulcer endemic area, with the goal to discover its ecological niche. Conventional PCR has shown *M. ulcerans* presence in many different organisms and environmental samples, but the type of results obtained so far were only qualitative, but not quantitative, and thus did not provide information about preferential accumulation of bacteria in particular type of samples. By applying real-time PCR on water bugs, snails, fish, or riverbank vegetation, it might be possible, on one hand to follow the chain that brings it into contact with humans, on the other hand to identify environmental reservoirs and risk factors. In the literature there are already examples of such studies, like the detection of *Vibrio vulnificus* in seawater and oyster samples[30], or the monitoring of the fate of *Escherichia coli* O157:H7 in irrigation water on soils and plants[31].

These types of studies may be rendered more complicated by the recent findings that IS2404, used as target to perform conventional and real-time PCR, is not unique in *M. ulcerans*, as once believed, but it has been found in other mycobacterial isolates[32-34]. The real risk of misinterpretation of PCR data provoked by these newly discovered mycobacteria still needs to be assessed.

9.1.3 Disease progression

Much of the *M. ulcerans* pathology has been attributed to the secretion of the mycolactone toxin, whose injection in guinea pigs' limbs mimicked typical Buruli ulcer lesions[35]. In human lesions it is possible to detect scattered large clumps of AFB mycobacteria (most likely

secreting mycolactone) in the centres of the ulcers, together with severe histopathological changes like fat necrosis, infarction of blood vessels, neural destruction and calcification. Interestingly, lesions especially in their late stages present little or no inflammatory response, with virtually no host cellular response[36,37], while, during the healing phase, the lesions account for an increased number of immune cells and far less AFB[38,39]. The subcutaneous fatty tissue, with its affinity for *M. ulcerans* (which has a very hydrophobic cell surface) and mycolactone, seems to constitute the primary site of bacterial multiplication, from where the mycobacteria are able to spread further. Spreading to distant sites seems to be relatively rare, since recurrences usually occur at the sites of excision, most probably due to incomplete mycobacterial removal. It seems likely that the initial accumulation of the mycolactone guarantees a favourable milieu for more rapid mycobacterial proliferation[40]. In some patients, however, development of multiple "metastatic" lesions, speaks for haematogenous spreading.

Since the only quantitative detection tool available so far has been the AFB enumeration –a technique lacking sensitivity and specificity- it was not yet possible to evaluate the spreading of the bacteria within a lesion. For this reason, one of the most important contributions of this PhD work was the development of a much more sensitive real-time PCR assay that allowed quantifying the mycobacterial burden within excised tissue samples (Chapter 5). The most interesting findings, resulting from the application of such method combined with histopathology, enabled a deepening of the current knowledge on *M. ulcerans* distribution and spreading, and on the relationship between tissue damage and mycobacterial presence (Chapter 6). Furthermore, we could use the newly developed technique to evaluate the effectiveness of the actual surgical procedure, and the risk of getting a recrudescence at the excision site (Chapter 6-7).

From the results obtained it was possible to confirm that the initial infection focus locates within the subcutaneous fatty tissue. At the moment it is not clear what the dominating infection route is and whether the primary inoculation can reach the subcutaneous fatty tissue directly (i.e. via wound or insect bite). Probably supported by the local accumulation of mycolactone, the mycobacteria proliferate and invade surrounding areas: the fact that we noticed signs of severe tissue necrosis, in areas with very low mycobacteria levels, gives an indication for the "preparatory" role that the toxin may have before the establishment of new mycobacterial foci. Another interesting feature is the development of the so called "satellite lesions" in the neighbouring regions of a primary ulceration. The lack of mycobacteria and of histopathological changes in intervening samples between the primary and the satellite lesion, showed that these contiguous dissemination events are not merely the result of a

homogeneous horizontal propagation, rather they represent newly established foci of "migrating" mycobacteria. The data obtained indicate that even a granulomatuous response mounted in the primary ulcerated focus of infection, as an attempt to contain the bacteria, is not sufficient to prevent the escape and the development of a new focus that constitutes a newly formed nodule. The most probable way of spreading of such escaped mycobacteria could be through the lymphatics, considering both the temperature barrier of 37 degrees and the observed necrosis of the blood vessels.

A better understanding of the dynamics of *M. ulcerans* spreading will be useful to promote guidelines for the surgeons performing excision operations, which represent, at the moment, the gold standard of Buruli ulcer treatment. The experience and, more recently, additional comparative studies[41] have shown that the wider the excision, the least the risk of getting a recrudescence, although it might mean a more complicated intervention, more skin to graft and higher risk of secondary bacterial infection. However, finding a balance is not easy, considering also that there is probably a certain mycobacterial threshold the immune system can cope with. The quantification of such threshold is not straightforward and is probably dependent on host-related factors.

In one case we could demonstrate that the presence of small but significant levels of mycobacteria at one side of the healthy margin of the excised tissue was enough to provoke a recurrence at the same site, reinforcing the concept that in each surgery wide healthy appearing margins should be included (Chapter 7).

The use of real-time PCR to predict recurrences is already used in tumour therapy, where the detection of melanoma micrometastases in sentinel lymph nodes is critical for the prognosis of the patients[42]. The application of the *M. ulcerans* real-time PCR to a larger number of cases thoroughly documented and scrupulously followed up, could enlighten possible associations between clinical appearance and mycobacterial presence according to the particular stage of the disease, in order to predict what type of intervention would be best suited for the patient to minimize the risk of relapses.

Real-time PCR approaches have been also used to detect and quantify *M. tuberculosis*[43] and *M. leprae*[44] in tissue samples, and it was proposed to use them to monitor treatment efficacy. Treatment failures with *M. tuberculosis*, due to the spreading of drug-resistant strains, make mandatory an early evaluation of the efficacy of the drug regimen used, considering also the long duration of the complete therapy and the overall costs on the health system. A major draw back of real-time PCR assays is represented by the measurement of "total mycobacterial

DNA content", meaning "viable counts" plus dead bacilli, which could hamper an effective monitoring of the decline of bacilli after drug treatment[45]. This type of problem may also arise if treatment efficacy is monitored in Buruli ulcer. To overcome such difficulty it would be advantageous to develop a real-time reverse transcription-PCR system for *M. ulcerans*, considering that mRNA disappearance would be a better indicator to monitor chemotherapy efficacy[46].

An important issue in *M. tuberculosis* infection is the fact that even a prolonged drug regimen does not guarantee sterilization. The mycobacteria may persist in the host according to two different models: the "static equilibrium", in which bacterial replication is slow or absent, or the "dynamic equilibrium", in which continued replication is balanced by an equally rapid rate of killing by the host immune response[47]. In this second situation, there would be an accumulation of dead bacteria, whose number would be continually growing compared with the number of live ones. Recent results supported the first model, with the existence of a static equilibrium between host and pathogen[48]. The existence of persistent forms of *M. ulcerans* has not been proven yet, but anecdotal cases indicate that some patients develop new Buruli lesions at new sites after prolonged time intervals after excision interventions. This phenomenon may be related to an inherently higher host susceptibility to reinfection, but it could also be caused by metastatic spreading of the same infecting bacterial strain, which might be able to persist in the patient for long periods of time and provoke the formation of new lesions at distant sites.

The post-operation re-insurgence of Buruli ulcer lesions, which we could monitor in an immunocompromised patient after his migration to a non endemic region (Chapter 8), led us to speculate whether *M. ulcerans* reactivation instead of reinfection has occurred. The physical distance of the patient from the original endemic region and the time span interposing between the primary Buruli manifestations and the recurrent forms was such to postulate eventual persistence of *M. ulcerans* in an immunocompromised individual. This would be a completely new field to explore, but at the moment, the lack of animal models resembling this condition hampers those studies aimed to the discovery of eventual *M. ulcerans* persistent forms, and to the identification of their preferential location.

As initial approach to study *M. ulcerans* persistence, it would be interesting to start investigating the contribution of reactivation versus reinfection in patients presenting Buruli ulcer recurrent forms. To allow such differentiation, fine typing tools able to discriminate among isolates from the same endemic district would be needed. For example, when such

tools became available for *M. tuberculosis* genotyping, it became clearer that the ratio of reinfection versus reactivation is proportional to the transmission rates of the particular region: in high incidence areas the contribution of reinfection is more consistent that reactivation and vice versa[49,50]. As already described, the microarray technique, permitting whole genome analysis, would have the potential to constitute such tool (Chapter 3) and an upscaled version of the already developed prototype microarray could be employed to characterize strains isolated from relapsing patients.

9.2 Conclusions

In this work the development of new molecular genetic tools to investigate *M. ulcerans* infection is described. The application of such methods contributed to our understanding of the genetic diversity and pathology of *M. ulcerans*. Based on the findings generated during these studies, the following conclusions can be drawn.

1. A microarray-based comparative analysis of the genomes of *M. ulcerans* isolates confirmed a remarkable lack of genetic diversity and a clonal population structure within given geographical regions. As for *M. tuberculosis*, large sequence polymorphism appears to constitute the most important source of genetic variation for *M. ulcerans.* The deletions discovered among the tested 30 *M. ulcerans* strains revealed a total of 241 genes missing, including all major functional categories. Genes predicted to encode metabolic, information pathways and cell wall proteins were underrepresented, while pseudogenes, (accounting for the 22% of all the deletions) insertion sequences, unique hypothetical proteins and genes involved in detoxification and adaptation were overrepresented. The potential of the microarray approach to detect deletions may represent a suitable basis for the development of a genotyping system required for micro-epidemiological studies.

2. A survey performed in Cameroon led to the rediscovery of a Buruli ulcer focus after more than 30 years of no reports, urging control program implementation. This study also reconfirmed the relationship between insurgence of Buruli ulcer and presence of water: the villages closer to the slow flowing Nyong river presented a higher prevalence than those located further away. *IS2404* PCR represented the most sensitive approach to reconfirm clinical diagnosis.

3. The development of a real-time PCR method for the quantification of *M. ulcerans* DNA (*IS2404* TaqMan) represented a new highly specific research tool, with a ten times higher sensitivity than the conventional PCR-based diagnostic test. We could demonstrate that its application to samples derived from Buruli ulcer lesions allowed a quantitative assessment of the dissemination of the mycobacteria. The IS2404 TaqMan assay therefore represents a useful tool for both diagnosis and research into the pathology and mode of transmission of Buruli ulcer.

4. The combination of histopathology and *IS2404* real-time PCR on Buruli ulcer tissue samples allowed a direct comparison between mycobacterial burden and damage provoked by *M. ulcerans* infection. Significant amounts of mycobacterial DNA and microcolonies were also present in samples from more peripheral regions and occasionally in excised margins of macroscopically and histologically healthy appearing tissue. Additional peaks of mycobacterial DNA clearly marked sites where satellite lesions were developing. Even when granulomas provided evidence for the development of cell-mediated immunity, development of satellite lesions by contiguous spreading was not completely prevented. *IS2404* real-time PCR analyses represented a better tool than the less sensitive and only semi-quantitative microscopic enumeration of AFB for studying the dynamics of *M. ulcerans* infection *in situ*.

5. The real-time PCR assay allowed the identification of a site in a Buruli ulcer patient prone to develop a recurrence after surgery. The recrudescence developed in fact at the edge of the excision, in an area where the excised healthy margin contained a significant amount of *M. ulcerans* DNA. This study demonstrated that even a relatively small number of mycobacteria that have spread into healthy appearing tissue can lead to the development of a recrudescence.

6. The development of a new Buruli ulcer lesion was documented in a HIV+ patient, who moved to a non Buruli endemic region. This case represented an example of *M. ulcerans* reactivation instead of reinfection, due to the physical distance of the patient from the endemic region and the time span interposing between the primary Buruli manifestations and the recurrent forms.

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EDUCATION

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  Advisor: Prof. Gerd Pluschke
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RESEARCH EXPERIENCE

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  Post graduate research assistant, Department of Pharmacy
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- **University of Tuscia, Viterbo-Italy** 1998-1999
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- **Interdepartmental Centre of Electron Microscopy** 1998
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  Graduate research assistant
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HONORS / AWARDS

- **Dissertation fellowship** 2001-present
- **1-year scholarship for research activity abroad** 2000
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- **National "Ulrich Prize"** for the best diploma work in 1999
  Phytotherapy awarded by the Italian Phytotherapy
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FIELDWORK AND COURSES

- **Samples collection and cases documentation** 2001-present
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PUBLICATIONS

1. **Rondini S., Kaeser M., Stinear T., Tessier M., Naegeli M., Portaels F., Certa U. and Pluschke G.**
   Detection of insertional/deletional genomic diversity in *Mycobacterium ulcerans* using a plasmid-based DNA microarray
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2. **Rondini S., Mensah-Quainoo E., Junghanss T. and Pluschke G**.
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9. **Rondini S., Ovidi E., Taddei A. R., Ceci M., Rambelli A., Tiezzi A**.
   Extracts of *Aloe arborescens* leaves present cytotoxic activity on murine myeloma cells.
   Atti Accademia dei Fisiocritici, Siena, 1999. Serie XV, Tomo XVIII, pg 27-32