# Development of Tools for the Control of *Mycobacterium ulcerans* Disease (Buruli Ulcer)

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**Dedicated to my late grandma, H.I. Edet, and to the rest of my wonderful family.**

*Chance favours the prepared mind.*
*Louis Pasteur (1822 – 1895)*

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## Table of contents

## Summary

Buruli ulcer (BU) – or *Mycobacterium ulcerans* disease – is a neglected tropical disease endemic in over 30 countries worldwide in tropical and subtropical regions. BU manifests as chronic ulcerated or non-ulcerated lesions in skin and soft tissues, with permanent disabilities and disfigurements being typical outcomes of the disease in low-resource endemic areas. The current BU control strategy is contingent upon the diagnosis and treatment of every case. No reliable preventive measures are currently available. However, BU control is hampered by the difficulties inherent in the real-world application of available diagnostics, and by the lengthy antibiotic and surgical interventions necessary especially for severe disease, which limit patient adherence and increase the risk of relapses.

In this thesis, we have developed a variety of tools that could potentially ameliorate some of the challenges affecting BU control efforts. The immunoassays described are highly sensitive, being able to detect 1 – 2 ng of mycolactone, and could potentially become BU-specific diagnostics, since mycolactone is unique to *M. ulcerans.* The mycolactone competitive ELISA could detect the toxin in laboratory samples such as extracts from *M. ulcerans* cultures and tissues from *M. ulcerans*-infected mice. It has also proven capable of detecting mycolactone in clinical samples, in limited pilot studies. This assay is currently being converted into a lateral flow format, which could then be amenable to point-of-care diagnosis. The mycolactone capture ELISA is the first report of such an assay for the hapten-like mycolactone molecule. Similar to the competitive assay, the mycolactone capture assay is highly specific and sensitive, and could detect the toxin in a variety of samples, including clinical samples. Further optimisation of this assay, and ultimately conversion into a lateral flow format, is envisaged. Additionally, our PMA-qPCR method represents a culture-independent means of differentiating live and dead *M. ulcerans* cells. As an extension of the diagnostic gold standard IS*2404* qPCR, this PMA-qPCR method could be directly suitable for applications where live/dead discrimination is necessitated but for which routine microbial culture is infeasible.

New treatment options will help in BU control by easing treatment (thus improving patient adherence), and would reduce the overreliance on rifampicin – the only highly active antibiotic against *M. ulcerans* in clinical use. We took advantage of drug discovery efforts for other diseases by identifying scaffolds that could be repurposed for BU treatment. Two topical agents developed for chronic wound management, and compounds from the tuberculosis drug development pipeline, were assessed for efficacy against *M. ulcerans*. The topical agents – a wound care disinfectant and a

bismuth-based antimicrobial agent – are already marketed for the management of chronic wounds of other aetiologies, and could therefore be applied to BU management in a relatively straightforward manner. Scaffolds with antituberculosis activity – which included arylvinylpiperazine amides, quinazoline amines, pyrrolopyridine diones, and quinolone carboxamides – were active against *M. ulcerans* in nanogram to low microgram concentrations. Further optimisation, including *in vivo* assessment in a BU mouse model, will help guide the repurposing of these scaffolds into novel BU treatment options.

BU prevention by breaking transmission chains is currently not feasible because these transmission routes are still unknown. Vaccination could be one option for BU prevention, and is currently an actively researched field. We assessed the utility of three mycolactone-based vaccine candidates and found that potent mycolactone-neutralising monoclonal antibodies could be reproducibly generated using PG-203, a protein-conjugated non-toxic derivative of mycolactone comprising the invariant parts of the mycolactone molecule. PG-203, therefore, could serve as the basis for the development of a toxoid vaccine against BU.

Altogether, the tools developed in the framework of this thesis could facilitate BU control by simplifying diagnosis and treatment of the disease, and contributing to the development of a vaccine against BU.

# 1. INTRODUCTION

*Mycobacterium ulcerans* disease – also known as Buruli ulcer (BU) or Bairnsdale ulcer – is a chronic necrotising disease of skin and soft tissue found in over 33 countries worldwide. The areas with the highest endemicity are found in parts of West and Central Africa, with Australia being the only sub-tropical region with a considerable proportion of the global BU cases [1]. Despite the many advancements in the understanding of this neglected tropical disease (NTD), considerable knowledge gaps still exist, with transmission routes still largely unknown and no reliable preventive measures being available. BU diagnosis and treatment are interwoven into the control of the disease, with control aimed at reducing the morbidity and economic burden of the disease [2]. Diagnostics with different sensitivities and applicability are used in the different endemic settings, the results of which help guide treatment. BU is treatable with an 8-week antibiotic combination therapy, although several barriers to treatment exist, which adversely affect BU control measures. These various elements are discussed below in greater detail.

## 1.1 Epidemiology

Although the name Buruli ulcer was derived from the Buruli County (now known as Nakasongola District) in Uganda, where large numbers of cases were reported in the 1960s, the disease was first described by Sir Albert Cook in 1897 in Kampala, Uganda [3]. Another report of the disease was recorded in 1948 by the Australian pathologist Peter MacCallum who described the disease in six patients from Bairnsdale and Melbourne, leading to the disease being also known as Bairnsdale ulcer [4].

Historically, BU has been reported in tropical and subtropical countries in Africa (particularly West and Central Africa), Asia (China and Japan), the Americas, and

Australasia. Only 14 countries in these endemic regions report incidences regularly to the World Health Organisation (WHO), and in 2019, there were 2,271 new cases reported from 11 endemic countries, with Nigeria reporting the highest incidence of 943 cases (Fig. 1). The true burden of BU is, therefore, likely not known due to underreporting of cases, a situation exacerbated by lack of awareness of the disease and poor access to healthcare in low-resource endemic areas. Equally worrisome are recent reports of BU cases in areas in which the disease was historically not known to be present, such as in Senegal [5, 6], South Sudan [7], and Jordan [8]. Even in countries that are known endemic regions, BU cases are beginning to be found in parts of the country outside of the typical hotspots [9, 10, 11]. This suggests that BU could be an emergent or re-emerging disease.

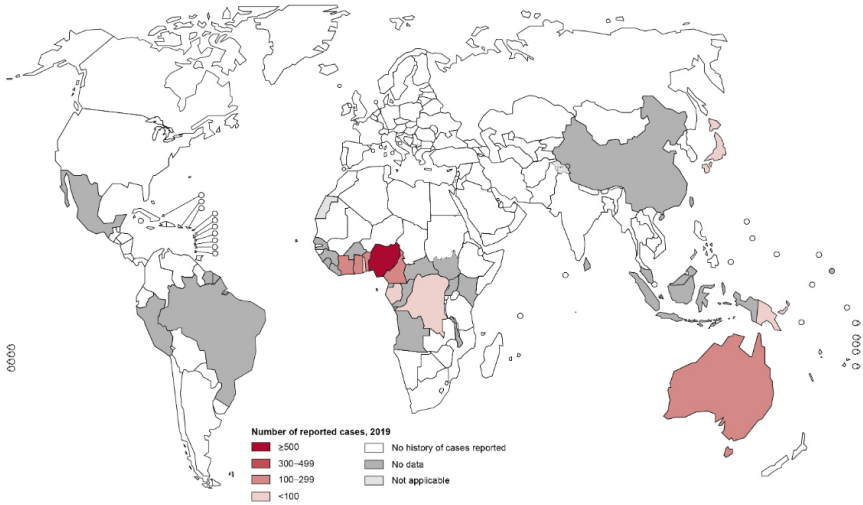


**Figure 1**. BU incidence in 2019 [12].

Susceptibility to BU is age-related, but not significantly associated with sex [1]. In African BU-endemic areas, BU is more commonly seen in children aged 5 – 15 years compared

to young adults, and in the elderly aged >65 years [13]. In the Australian context, BU is more commonly seen in the elderly >65 years of age [14]. In Japan, the majority of cases are in middle-aged adults [15]. It is thought that one's likelihood of developing BU is an interplay between exposure and immune status. In the highly endemic rural areas in Africa, exposure to *M. ulcerans*-contaminated environments would likely begin from around early childhood, as that is the age when children start to venture away from home for recreation (e.g. swimming in streams and rivers) and chores (e.g. fetching water from streams). Occupational exposure (e.g. during fishing and farming) could also begin early in childhood and persist throughout adulthood. The immature nature of the immune system in children could then play a role in their risk of developing BU following contamination. Similarly, the decline in immune function that occurs with age could predispose elderly individuals to BU disease. Further evidence for the role of immune status in the initiation and severity of BU is seen in the higher rate of BU in immunocompromised individuals. Indeed, BU has a higher incidence and greater severity in HIV-positive individuals compared to their HIV-negative counterparts, with the infection more likely to manifest as multiple lesions [16, 17]. The role of differential exposure and immune status in BU development also explains why children <5 years are rarely affected; although, their immune system is less developed than that of older individuals, children in that age group typically stay closer to home, and their exposure to *M. ulcerans*-contaminated environments is thereby minimised. BU in Australia is typically found in coastal recreational areas, where many elderly people have their retirement homes. Exposure is therefore more likely to occur in this age group, who may also have higher susceptibility due to age-related decline in immune status.

## 1.2 Clinical presentation

BU lesions are predominantly on the extremities, particularly the lower extremities, although other body sites can be affected. BU typically starts out innocuously as a

plaque, papule, or nodule, usually several weeks to months after the presumed contamination of the skin [1, 18]. Due to the painlessness of these early forms of the disease, those affected often do not seek medical intervention until later stages of the disease when the lesion has deteriorated into an open ulcer. BU can also present as widespread oedema, which contrary to the other forms of the disease, is more frequently associated with pain [19]. Regardless of the early presentation, all non-ulcerated forms (i.e. plaques, nodules, or oedema) may eventually ulcerate as disease advances.

Plaques, papules, and nodules are typically small and localised, while oedema can affect much larger areas of the body. Ulcers typically start out small, but without prompt treatment, can enlarge enough to cover large swaths of the body. Buruli ulcers at critical body sites, such as on the face or near the genitalia, can be particularly debilitating, resulting in permanent loss of organs such as the eyes (Fig. 2). Both immunocompetent and immunosuppressed individuals can be afflicted by BU, although the progression of disease is faster in the latter group of individuals. Consequently, HIV infection is considered a risk factor for severe BU [16, 17].

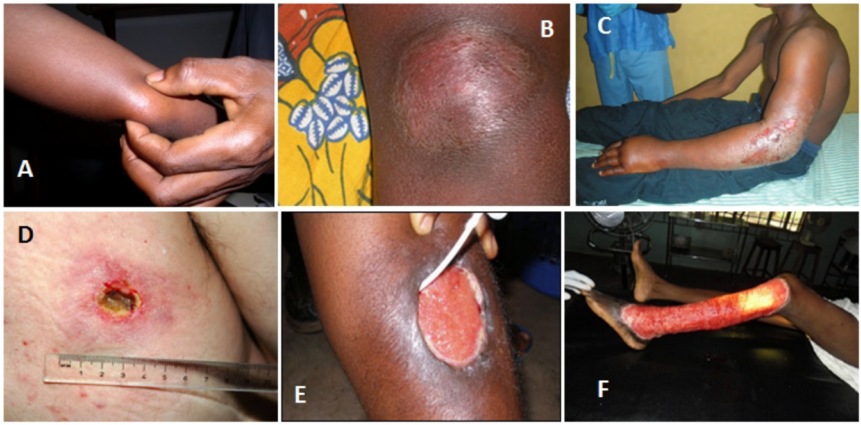

**Figure 2. Clinical presentation of BU**. (A) Nodules are typically painless, firm, subcutaneous lesions, measuring 1 – 2 cm in diameter. (B) Plaques are painless, raised, hypo- or hyperpigmented lesions with well-demarcated borders, typically >2 cm in diameter. (C) BU oedema is non-pitting, often extensive, and more likely to be associated with pain and discomfort than nodules and plaques. (D – F) Ulcers may be small or quite extensive at presentation, characterised by undermined edges (as shown in E) and a cotton wool-like necrotic centre. Ulcers are typically painless, but may be minimally painful, particularly when the lesion is probed, for example, during sampling the undermined edges (pictures taken from [19, 20, 21]).

The WHO categorisation of Buruli lesions based on the dimensions and severity of disease is shown in Table 1. In endemic areas with well-developed health systems and good access to healthcare, most BU cases are category I lesions, which are more amenable to treatment. The reverse is the case in endemic areas with less adequate access to healthcare, where most patients present with category III lesions.

**Table 1. Classification of Buruli lesions** [19].

| WHO Category | Description |
|---|---|
| **I** | Small single lesion (nodule, papule, plaque, or ulcer <5 cm, borders defined by palpitation) |
| **II** | Non-ulcerated and ulcerated plaque and oedematous forms<br>Large single ulcer 5 – 15 cm in diameter (borders defined by palpitation) |
| **III** | Large single ulcer > 15 cm in diameter (borders defined by palpitation)<br>Multiple small or large ulcers<br>Lesions in the head and neck regions, and at other critical body sites (e.g. breasts, genitalia)<br>Osteomyelitis, osteitis, joint involvement |

Category I disease may spontaneously resolve without treatment, but often needs to be treated for complete resolution without relapse. Ulcerated forms of the disease typically require adequate medical intervention for sequelae-free resolution of the disease. Since Buruli ulcers tend to heal with the formation of contractile scars, consistent surgical and

non-surgical interventions (e.g. physiotherapy) are essential during healing to prevent deformity (Fig. 3A – C). This is especially crucial with ulcers that span a joint. With category III disease, there is high risk of losing affected body parts, e.g. loss of eyes with large ulcers on the face, and loss of limbs from amputation following irreversible bone damage due to osteomyelitis (Fig. 3D – E). In rare cases, squamous cell carcinoma, a life-threatening condition, could occur following long-term infection (Fig. 3F). It is therefore imperative that the disease is diagnosed early and treatment is initiated promptly.


**Figure 3. Sequelae of BU**. (A - C) Contractures are a common occurrence when BU lesions heal without adequate physiotherapy during the healing process. (D) Loss of limbs often results in cases of bone involvement. (E) Loss of eyes and other external body organs resulting from BU lesions at such critical body sites. (F) Squamous cell carcinoma is a rare but serious complication of severe BU (pictures taken from [19]).

BU is thought to be rarely fatal, however, with the high prevalence of underreporting, and the higher chances of loss-to-follow-up of patients living far away from health centres, the true mortality rate of the disease cannot be conclusively ascertained.

Superinfection of ulcerated forms of BU is a common occurrence, which carries a high risk of sepsis. Indeed, a range of contaminating bacteria and fungi have been cultivated from superinfected Buruli lesions, including *Staphylococcus aureus, Pseudomonas aeruginosa,* as well as enteric bacteria such as *Escherichia coli, Proteus mirabilis,* and *Klebsiella pneumoniae* [22, 23]. Many of these species are typically associated with antibiotic resistance; therefore, while BU may not be directly fatal, it is conceivable that more serious complications could arise in patients with poorly managed ulcers.

## 1.3 Pathogenesis

### 1.3.1 pMUM and Mycolactone

Mycolactone, the plasmid-borne polyketide cytotoxin produced by *M. ulcerans*, is the main virulence factor of the organism, and is responsible for the main features of the disease. The pMUM plasmids encode the polyketide synthases (PKSs) that catalyse the biosynthesis of mycolactone, as well as accessory genes thought to be necessary for mycolactone production (Fig. 4).

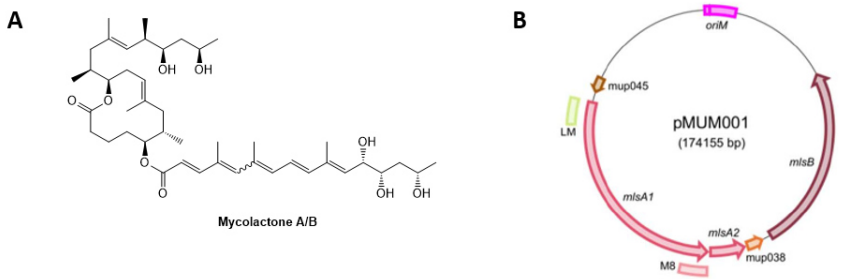


**Figure 4.** (A) Structure of mycolactone A/B. (B) Organisation of the pMUM plasmid (figure taken from [24]).

Like all known modular Type I PKSs, the pMUM plasmid synthesises mycolactone in a modular process reminiscent of an assembly line, where each module receives the product of the preceding module, adds to it, and passes it off to the next module, until the end where the product is released from the PKS. There are three PKS genes encoded on pMUM – *mlsA1* (50,973 bp), *mlsA2* (7,233 bp), and *mlsB* (42,393 bp). The core lactone ring and upper side chain of mycolactone are the products of mlsA1 and mlsA2. The lower acyl side chain is synthesised by mlsB (Fig. 5).

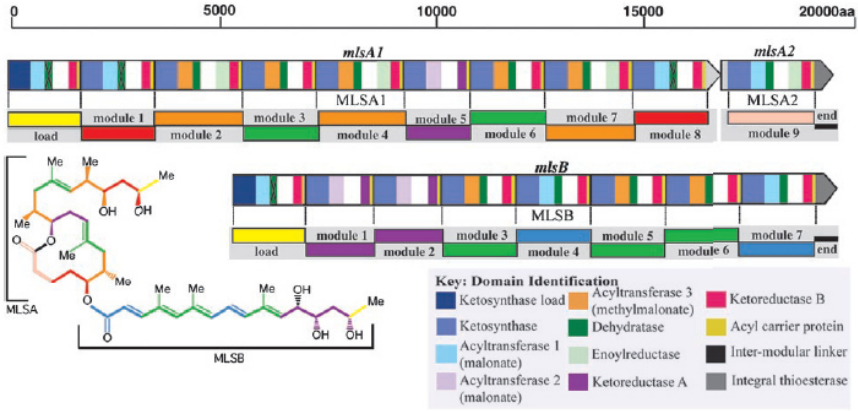


**Figure 5**. Modular arrangement of genes on the *mls* loci (figure taken from [25]).

As is the norm with modular PKSs, each module begins with a **ketosynthase** (**KS**) which is followed by an **acyltransferase** (**AT**), and the module terminates with the **acyl carrier protein** (**ACP**); these three enzymes are the indispensable parts of a PKS module [26]. Between the start and end of a module are other pertinent enzymes such as **dehydratases** (**DHs**), **ketoreductases** (**KRs**), and **enoylreductases** (**ERs**), all of which collectively comprise the reductive loop that impart different modifications to the growing polyketide. Functionally speaking, a KR reduces a carbonyl group to a hydroxyl

(OH) group; a DH removes this OH group with an adjacent hydrogen atom thus dehydrating the molecule and resulting in the formation of a double bond; while an ER reduces this double bond by the addition of extra hydrogen atoms (Fig. 6). An ACP carries the nascent polyketide. An AT selects the appropriate acyl-CoA to be added to the growing polyketide from the available acyl-CoAs in the intracellular milieu; it is always situated near the KS. This acyl-CoA could either be an acetate moiety (supplied as malonyl-CoA) or a propionate moiety (supplied as methylmalonyl-CoA). Thus, the elongation of the polyketide is either by the addition of 2 or 3 carbons, respectively. Lastly, a KS receives the load of the preceding ACP and catalyses the condensation reaction with the acyl-CoA borne on the AT. The product of this condensation passes off sequentially to the remaining enzymes in the module (KR, DH, or ER) until it gets to the ACP which passes it off to the next module. At the end of the assembly line, the product is released by the action of a thioesterase (TE) [27, 28, 29].

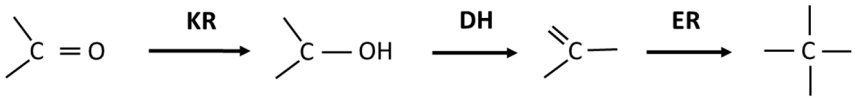


**Figure 6**. Enzymatic conversion of PK substrates.

There are two types of KRs; B-type KRs are part of modules that also contain a DH while A-type KRs are found in modules without a DH. For pMUM, only module 5 of mlsA1 and modules 1 and 2 of mlsB contain A-type KRs. All other modules of pMUM contain B-type KRs [30]. This accounts for the abundance of unsaturation found in the mycolactone molecule, especially for the lower side chain made by mlsB, which has five of its seven modules containing B-type KRs (which means it contains DHs in 5 of 7 modules). There are three types of ATs, which differ based on the acyl-CoA they can carry. In mlsA, modules 2, 3, 4, 6, and 7 contain AT1 or AT2, which use acetate; while modules 1, 5, 8,

and 9 contain AT3, which uses propionate. This accounts for the methyl groups seen on parts of the molecule synthesised by AT3-containing modules [26, 31].

There are three known forms of the pMUM plasmid, designated pMUM001, pMUM002, and pMUM003, each of which encodes PKSs producing different variants of mycolactone. pMUM001 is found in the human *M. ulcerans* strains and encodes Mycolactone A/B produced by the African and Japanese strains, Mycolactone C produced by the Australian strains, and Mycolactone D produced by the Chinese strains. pMUM002 is found in the frog strain *M. ulcerans* subsp. *Liflandii* and encodes Mycolactone E. pMUM003 is found in the fish strain *M. ulcerans* subsp. *marinum* and encodes Mycolactone F [26]. All pMUM-containing mycobacteria are collectively referred to as mycolactone-producing mycobacteria (MPMs) [32].

All mycolactones described so far are identical with respect to the lactone core and upper side chain, which are altogether encoded by *mlsA1* and *mlsA2*. The structural differences of the mycolactone variants all arise from the module structure of *mlsB* (Fig. 7). The pMUM PKSs are highly unusual in the degree of sequence similarity that exists between modules. Typical PKSs show between 40 – 70% similarity between domains of the same module (e.g. between AT and DH in one module) and even less similarity between equivalent domains of different modules (e.g. between AT in module 2 and AT in module 3). In contrast, the pMUM PKSs have extremely high sequence identity, ranging between 98 – 100% (e.g. the AT domains in different modules are nearly completely identical). This unprecedented domain similarity implies that substrate specificity is less stringent in the pMUM PKSs, and points to potential instability in the integrity of the plasmid [25]. It also explains why mycolactones A/B, C, and D are all encoded by the same type of plasmid, pMUM001 (Fig. 7). Nevertheless, although extensive passage in the laboratory can lead to loss of parts of the plasmid with concomitant loss of mycolactone production, it has not been possible to cure the

bacteria of the plasmid, indicating that pMUM carriage plays a crucial role to *M. ulcerans*. It is, however, not clear what the roles of the plasmid and encoded toxin are outside of the context of human infection.

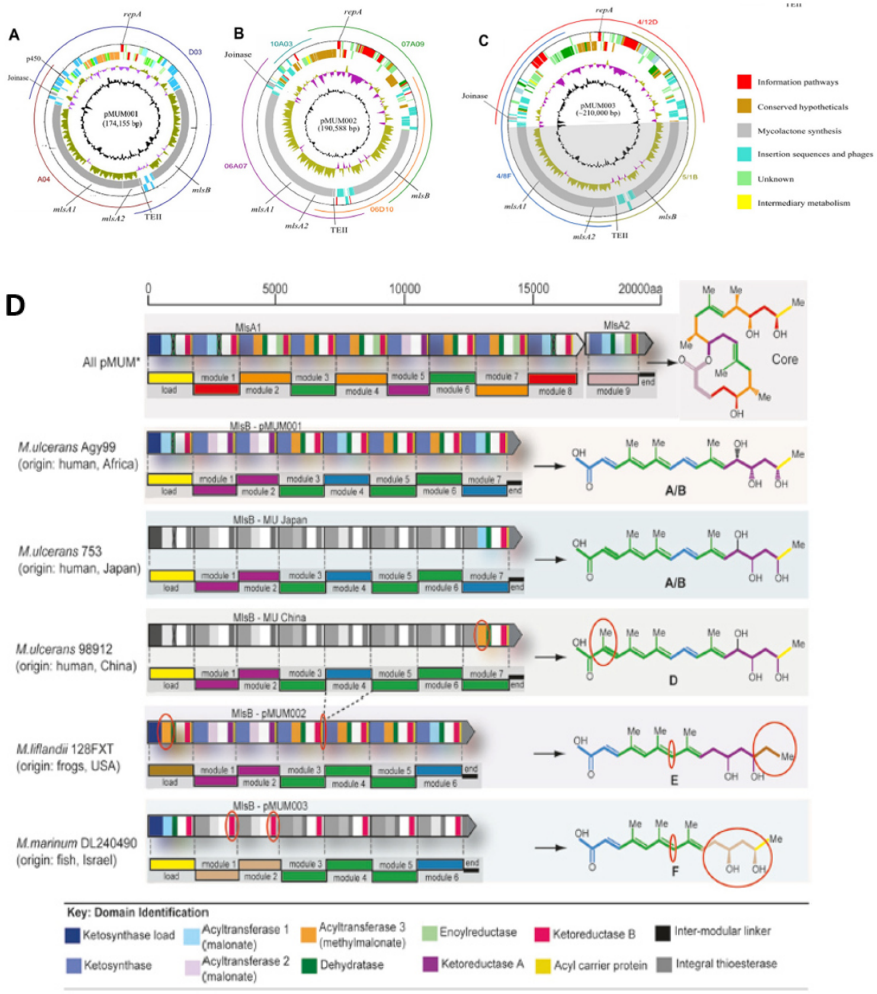

**Figure 7**. (A – C) Structures of the different pMUM plasmids and (D) their encoded mycolactones (figures adapted from [26]).

### 1.3.2 Cellular targets and mechanisms of action of mycolactone

Mycolactone is thought to passively diffuse across the cell membrane and accumulate in the cytoplasm of host cells [33]. Consequently, mycolactone can gain access to a range of cell types, leading to a broad spectrum of outcomes. Indeed, the toxin has been directly implicated in the development of typical BU symptoms, such as local and systemic immune dysregulation, local analgesia, and cell death via apoptosis and necrosis. This broad-spectrum activity indicates that mycolactone might engage a variety of cellular targets. Research over the years has elucidated a number of mechanisms through which mycolactone is thought to act. These are summarised in Fig. 8 below, and briefly outlined in the following section.

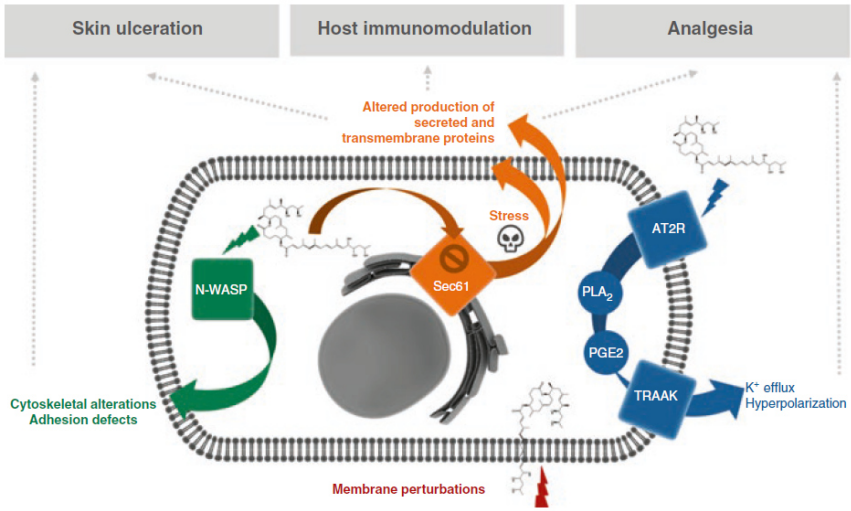

**Figure 8**. **Proposed cellular targets and mechanisms of action of mycolactone** (figure taken from [34]). Skin ulceration can result from at least three different pathways of apoptotic cell death: (i) Mycolactone inhibits mTOR signalling, resulting in Bim-mediated induction of apoptosis; (ii) Mycolactone induces the integrated stress response as a consequence of Sec61 blockade, resulting in the induction of apoptosis; and (iii) Mycolactone destabilises cell cytoskeleton and adhesion via N-WASP hyperactivation, resulting in apoptosis via anoikis. Host immunomodulation is predominantly via mycolactone-mediated Sec61 blockage, leading to downregulation of an array of immunological factors. Analgesia results directly from mycolactone-mediated downregulation of neuronal signalling, and indirectly from mycolactone-mediated reduction of Sec61-dependent substrates important for pain sensation. Mycolactone has also been shown to perturb membranes.

### i. Immunomodulation: Sec61 blockade

The Sec61 translocon is a heterotrimeric protein that facilitates entry of nascent proteins into the secretory pathway of cells. Almost all secreted proteins – as well as proteins destined for endosomes and lysosomes, glycosylphosphatidylinositol (GPI)-anchored proteins, and integral transmembrane proteins (TMPs) – require entry into the endoplasmic reticulum (ER), and on to the Golgi, via Sec61 [35]. Proteins that fail to access the ER are degraded in the cytoplasm by the proteasome in a ubiquitin-dependent manner. Therefore, by blockading Sec61, mycolactone directly downregulates the expression of a wide range of proteins, including cytokines and other immunological factors (e.g. TNF-α, IFN-γ, IL-2), and membrane proteins (e.g. IFN-γR, VCAM-1, L-selectin) [36, 37, 38].

Sec61 blockade also induces a stress response in cells (the integrated stress response) that contributes to the induction of apoptosis in affected cells [35]. Thus, mycolactone can, not only reduce the activity of immune cells, but also cause cell death.

### ii. Bim-mediated apoptosis: Inhibition of mTOR signalling

The mechanistic target of rapamycin (mTOR) is a 298-kDa serine/threonine kinase involved in the regulation of cell growth and proliferation, and is conserved throughout evolution. It forms two distinct complexes – mTOR complex 1 (mTORC1) and mTOR

complex 2 (mTORC2) – which together sense extracellular signals to positively regulate cell growth by promoting a variety of anabolic processes and inhibiting catabolic processes [39].

Mycolactone has been shown to inhibit mTORC2 signalling by preventing assembly of the complex. mTORC2 is required for the activation of the kinase Akt, which is in turn required for inhibiting the transcription factor FoxO3 which drives Bim expression. By inhibiting Akt, mycolactone directly causes the derepression of FoxO3, leading to upregulated Bim expression. Bim induces the apoptotic response in mitochondria; thus, via the mTORC2-Akt-FoxO3 signalling pathway, mycolactone can directly cause apoptotic cell death [40].

**iii. Cytoskeleton derangements: WASP and N-WASP**

The Wiskott - Aldrich syndrome protein (WASP) and neuronal WASP (N-WASP) are a family of scaffold proteins important in cytoskeleton remodelling. N-WASP is found in all cells while WASP is expressed exclusively in haematopoietic cells. When activated, these actin-nucleating proteins link signals transduced from the cell surface to the cytoskeleton, resulting in actin polymerisation. Both WASP and N-WASP are auto-inhibited until they are bound by a GTPase Cdc42, and the activated proteins then bind to the Arp2/3 complex, resulting in actin polymerisation [41].

Mycolactone has been shown to mimic the (N)-WASP activating GTPases, leading to rearrangements in the actin cytoskeleton. This leads to rounding up and detachment of cells from the basement support. Adherent cells (e.g. fibroblasts) that become detached from the surrounding extracellular matrix typically die by anoikis, a form of programmed cell death [42]. Thus, mycolactone can result in cell death by engaging WASP/N-WASP. In addition, mycolactone-induced changes in the cytoskeleton in non-adherent cells

results in downregulation of their functions; for example, macrophages are unable to undergo chemotaxis and phagocytose [41, 42].

#### iv. Neuronal signalling: $AT_2R$

Buruli ulcers are remarkably painless, or only associated with minimal pain, a phenomenon that was thought to be caused by mycolactone-induced neuronal death. However, it was later shown that this analgesia was already apparent early in the infection before neuronal death. Mycolactone-induced analgesia was shown to occur via the type 2 angiotensin II receptor ($AT_2R$), which is part of the renin-angiotensin system and, amongst other functions, is involved in neuronal signalling and tissue repair. Mechanistically, mycolactone binds to $AT_2R$, leading to the release of the G protein Gα coupled to it. Gα triggers a signalling cascade that involves the phospholipase $A_2$ ($PLA_2$)-mediated release of arachidonic acid from membrane phospholipids, and eventually leads to increased potassium ion ($K^+$) efflux via the TRAAK channels. This $K^+$ efflux leads to membrane hyperpolarisation and a decrease in neuronal excitability. Thus, mycolactone induces a decrease in local pain sensation [42 – 46].

#### v. Membrane perturbation

Mycolactone is an amphiphilic molecule, with a hydrophobic lower acyl side chain and a more hydrophilic lactone core and upper chain [47]. This makes it structurally similar to detergent molecules, and like detergents, mycolactone forms micellar-like aggregates when present in aqueous environments. It was recently shown that monomeric mycolactone could insert into lipid mono- and bilayers, which is necessary for the passive diffusion of the toxin into cells. This membrane insertion was also shown to destabilise the packing of lipid rafts, particularly around cholesterol molecules [48, 49]. Given that all the aforementioned mycolactone targets are membrane proteins, and the organisation of membrane proteins and lipid rafts into microdomains is important for

protein activation and signalling, it is possible that mycolactone-induced membrane perturbation may synergise with the other cellular effects of the toxin [48].

#### vi. Pro-inflammatory effects: Induction of IL-1β

Unlike the oft-reported downregulation of inflammatory cytokines attributed to mycolactone (via Sec61 blockade), a recent report showed induction of the pro-inflammatory cytokines IL-1β and IL-18 in macrophages by inducing the NLRP3 and NLRP1 inflammasomes [50]. Here, both the presence of sublethal levels of mycolactone and the recognition of *M. ulcerans* TLR-2 agonists were necessary for inducing the production and secretion of IL-1β, and to a lesser extent IL-18, by macrophages. This was thought to be more relevant in later stages of the disease, contributing to ulcer chronicity.

## 1.4 Diagnosis

The WHO BU control strategy is the early diagnosis and treatment of every case. Currently, diagnosis is done by a combination of clinical and laboratory diagnoses. Laboratory diagnosis of BU relies heavily on resource-intensive and relatively sophisticated techniques such as PCR, although less sensitive but more facile techniques are also in routine use. The typical clinical samples are swabs taken from the undermined edges of ulcers and fine needle aspirates of non-ulcerated lesions. Tissue biopsies are currently considered too invasive for routine diagnosis, but may be collected in exceptional cases [51].

#### i. Clinical diagnosis

In high endemic settings, experienced clinicians can often diagnose BU based on the typical hallmarks of the lesions, such as the undermined edges and whitish necrotic base

of ulcers, and the general painlessness of the lesions. The accuracy of clinical diagnosis can, however, range from 30 – 70%, depending on the skill and experience of the clinician, therefore, laboratory diagnosis is necessary [21, 52].

**ii. Direct microscopy**

Direct microscopy of smears from BU lesions following Ziehl-Neelsen staining is the only laboratory diagnosis routinely performed in the periphery in low-resource settings. It is often used as a confirmation for clinical diagnosis. Ziehl-Neelsen staining does not allow the differentiation of acid-fast bacilli (AFB) as all AFB will give positive reactions in this technique. Therefore, this technique is not specific to BU, with sensitivity ranging from 26 – 67% [19, 21, 53].

**iii. Culture**

Traditionally, culturing an aetiological agent from a clinical sample is regarded as gold standard. *M. ulcerans* can be cultivated on routine media, including Middlebrook media and egg-based media such as Löwenstein-Jensen and Ogawa media. The organism grows optimally at 28 – 33°C in a microaerophilic environment. However, *M. ulcerans* is an extremely slow-growing microorganism, requiring a minimum of 4 weeks of incubation – and up to six months for samples with low inoculum doses – for visible colonies to form on solid growth media. Therefore, culture as a means of BU diagnosis is not practical, with an overall sensitivity ranging from 20 – 60% [51].

**iv. PCR**

PCR aimed at detecting the abundant insertion sequence 2404 (IS*2404*) present in the *M. ulcerans* chromosome is the current diagnostic gold standard, owing to its high sensitivity (98%) and specificity (>98%) [54]. A major drawback to this assay is the need for sophisticated instruments and highly trained personnel, which precludes its routine

use in low-resource settings that lack the necessary instrumentation. In addition, the assay carries a relatively high risk of contamination, hence, stringent quality control is needed at all steps of the assay [51, 55].

**v. Histology**

BU lesions have characteristic histopathological hallmarks, such as the necrotic core with bacteria found extracellularly amid fat cell ghosts in the destroyed subcutaneous adipose tissue. Histological examination of fixed and stained tissue biopsies can be used as a means of diagnosis. However, a high amount of expertise and instrumentation is required for this process. Moreover, more invasive sampling is needed for histological analysis, which has been discouraged in recent times. As such, histology is predominantly used as a research tool [51, 53, 56].

**vi. Mycolactone detection**

Given its uniqueness to *M. ulcerans*, mycolactone makes for an ideal diagnostic target. The lipid nature of the cytotoxin, however, necessitates more cumbersome methods for its detection in clinical samples. For instance, mass spectrometry can be used to detect mycolactone in a sample [57], although the cost of the requisite machinery is prohibitive for routine diagnosis even in resource-rich BU endemic regions. Thin layer chromatography (TLC), another method of detecting mycolactone, can also be used, however, the difficulties in assay performance and interpretation has so far limited its routine use for BU diagnosis [58].

Newer methods of detecting mycolactone are currently being developed, and in later chapters of this thesis, we describe two new immunoassays we have developed for this purpose.

## 1.5 Treatment

Rifampicin (RIF) is the only highly active antibiotic against *M. ulcerans* in standard use. To forestall the development of drug resistance, RIF is given in combination with a companion drug, ideally one that also has activity against the bacteria. Up until 2017, this companion drug was streptomycin (STREP), and the standard antibiotic treatment for BU was an 8-week regimen of oral RIF (10 mg/kg once daily) and intramuscular STREP (15 mg/kg once daily). The problems associated with the daily administration of an injected medication are quite obvious, especially considering that the majority of BU patients are children <15 years old in remote, rural, resource-limited settings. Moreover, persistent hearing loss has been associated with the use of STREP, which like other aminoglycoside antibiotics is ototoxic [59], with nephrotoxicity and hepatotoxicity also being side effects of this therapy. Consequently, the WHO currently advocates an all-oral regimen of RIF and clarithromycin (CLAR, 7.5 mg/kg twice daily or 15 mg/kg once daily) for 8 weeks [60]. Other companion drugs are also used in BU treatment. For instance, in Australia, the typical regimen is a combination of RIF and moxifloxacin (MOXI, 400 mg once daily) or other fluoroquinolone [19]. BU patients in China and Japan are typically treated with a triple combination therapy of RIF, CLAR, and levofloxacin [15].

Antibiotic combination therapy for BU is, however, still less than optimal, owing to considerable drug-drug interactions. The RIF-CLAR therapy for BU suffers from drug interactions showing up about 2 weeks into treatment, because RIF increases the elimination of CLAR by the liver, thus reducing the plasma concentrations of the latter drug [61, 62]. Drug-drug interactions are also a non-trivial problem in cases of BU-HIV coinfection. RIF reduces the plasma concentrations of recommended antiretrovirals used for HIV treatment, necessitating adjustments in dosing strategies for concomitant BU and HIV treatment. For instance, since RIF reduces dolutegravir plasma concentrations by approximately half, double-dosing of the latter drug is needed for

adequate control of HIV [16, 17]. RIF also significantly reduces the levels of protease inhibitors, making them contraindicated in BU-HIV coinfection [63]. Efavirenz, a non-nucleoside reverse transcriptase inhibitor, is less sensitive to increased elimination caused by RIF, however, efavirenz reduces the levels of CLAR, and the combination of both drugs (CLAR and efavirenz) could lead to increased toxicity (with 46% of patients reporting a rash) [16, 17]. Given that CLAR is included in the BU combination therapy to protect RIF from possible resistance, having its concentration decreased by RIF itself and additional ARTs could make resistance development more likely, although RIF-resistant *M. ulcerans* seem to only be rarely encountered [64].

RIF is, therefore, a crucial part of successful BU chemotherapy and it is prudent to develop alternatives for it, in the event of resistance development, or in cases where its use is contraindicated. Being an NTD, there are limited resources for *de novo* drug development (which typically is a multibillion-dollar endeavour), therefore repurposing existing drugs, or tapping into the development pipeline of other drugs (e.g. anti-tuberculosis drugs), are the only feasible means of developing new BU treatment regimens. As a result of such efforts, the recently developed first-in-class anti-tuberculosis drugs bedaquiline and Q203 (telacebec) were also seen to be highly active against *M. ulcerans* [65]. Studies aimed at developing alternative, and possibly shorter, treatment regimens based on these new drugs are underway [66, 67, 68].

In addition to antibiotic chemotherapy, surgical intervention is often necessary for complete resolution of BU, especially in cases of Category III disease. This typically involves debridement or excision of necrotic tissues, skin grafting, prevention of contractures, and amputations [19, 69]. Since recovery is typically faster and less costly when patients present with smaller lesions, there is need for improved diagnostics and community awareness to ensure that cases are diagnosed as early as possible for treatment to be initiated in the earlier stages of disease.

## 1.6 Transmission and Prevention

The transmission route(s) of BU are still unknown. Although there are strong correlations between exposure to suspected environmental reservoirs, such as slow-flowing or stagnant water bodies, and the development of Buruli lesions, it is not yet known how the bacterial infection begins and takes hold. *M. ulcerans* DNA can be detected with some regularity in soils, waterbodies, and the associated flora and fauna. For instance, Hemipteran waterbugs such as *Naucoridae, Gerridae,* and *Belostomatidae* have proven capable of harbouring *M. ulcerans* [70, 71, 72], although they are typically not human-seeking insects, and reported biting events are rare, making them unlikely to be the major drivers of the transmission in BU-endemic areas. *M. ulcerans* DNA has also been detected in some mosquito species in Australian BU-endemic regions [73, 74], however, no such association with mosquitoes in the African context has been established [75]. There have also been sporadic reports of BU infection in other mammalian species such as possums and rodents [76, 77, 78], goats and other farm animals [79, 80, 81], as well as companion animals such as dogs and cats [79, 82, 83]. It is not clear if human reservoirs lead to contamination of these animals, or if the animals are reservoirs for human infections. The proposed transmission cycle is summarised in Fig. 9 below.

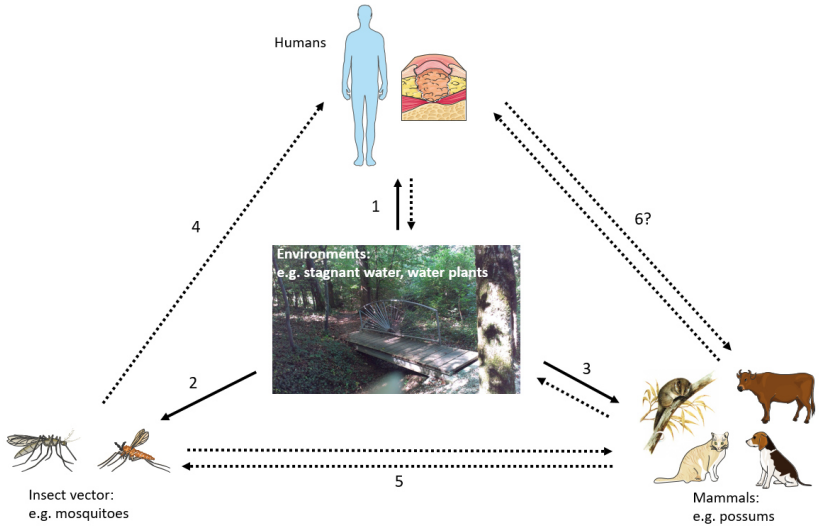


**Figure 9. Proposed transmission pathways of *M. ulcerans*.** (1) Human infection following contamination from environmental sources. Shedding of live bacteria from open ulcers may in turn seed the environment. (2) Insect vectors possibly becoming contaminated following exposure to environmental sources. (3) Infection of other mammals following contamination from environmental sources. Shedding of live bacteria from open ulcers may seed the environment. (4) Insect vectors may transmit *M. ulcerans* to humans via bites. (5) Insect vectors may transmit *M. ulcerans* to other mammals via bites, and in turn may be contaminated via animal faeces. (6) Open lesions from humans may contaminate animals and vice versa. (Graphic generated using icons from SMART Servier Medical Art, www.servier.com)

It has proven incredibly difficult to culture the bacteria from these hypothesised environmental reservoirs. One reason for this is the extremely slow growth of the bacteria, which allows the overgrowth of faster-growing microorganisms. Consequently, stringent decontamination methods are necessary when attempting to isolate *M. ulcerans* from environmental sources. However, although mycobacteria are more resistant to such decontaminants as oxalic acid and concentrated sodium hydroxide owing to their waxy cell wall, Palomino and Portaels showed that all routinely used

decontamination methods still reduced *M. ulcerans* growth by up to 98% [84], with *M. ulcerans* still being overshadowed by faster-growing environmental mycobacteria. As a result, there have only been a couple of reports of successful isolation and stabilisation of *M. ulcerans* cultures from environmental sources.

Consequently, given the difficulty in conclusively linking environmental exposure to BU disease, prevention by breaking transmission chains is not easily done. Nevertheless, preventing insect bites (e.g. by using insect repellent) and mechanical trauma (e.g. by wearing protective clothing) have been correlated with protection [1].

No vaccines exist for the prevention of BU. The only routinely used vaccine for mycobacterial disease – the Bacillus Calmette-Guérin (BCG) vaccine – offers only partial short-term protection from BU, although it appears to reduce the chances of developing osteomyelitis [85]. As such, vaccine development is still an attractive area of BU research, and a variety of targets are currently being explored as vaccine candidates.

## 1.7 Objectives

The main objectives of this thesis were:

- To develop and optimise immunoassays for mycolactone detection in a variety of samples, as potential simple new tools for BU diagnosis.
- To elucidate new treatment modalities by screening a variety of scaffolds and antimicrobials to identify promising new BU drug candidates.
- To explore possibilities for BU prevention via vaccine development.

Section I covers aspects of this thesis dedicated to diagnostics development. Chapters 2 – 4 describe two immunoassays developed for the detection of mycolactone in a variety of laboratory and clinical samples. Chapter 5 describes the development of a qPCR-based protocol for the discrimination of viable and non-viable *M. ulcerans*.

Section II comprises aspects of this thesis dedicated to BU treatment. Chapter 6 details the *in vitro* evaluation of a commercial, sprayable acid-oxidising solution (AOS) as an adjunct to BU chemotherapy. Chapter 7 describes the *in vitro* evaluation of five compound scaffolds for activity against *M. ulcerans*.

Section III covers aspects of this thesis dedicated to BU prevention. In chapter 8, studies on the suitability of mycolactone as a vaccine target are discussed.

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## Section I: Diagnosis

- Development of an ELISA for the quantification of mycolactone, the cytotoxic macrolide toxin of *Mycobacterium ulcerans*.
- Book chapter: Competitive ELISA for the detection and quantification of *Mycobacterium ulcerans* mycolactone.
- An antigen capture assay for the detection of mycolactone, the polyketide toxin of *Mycobacterium ulcerans*.
- A PMA-qPCR method for differentiating viable and dead *Mycobacterium ulcerans* cells.

# Development of an ELISA for the quantification of mycolactone, the cytotoxic macrolide toxin of *Mycobacterium ulcerans*

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**ABSTRACT**

Mycolactones, macrolide cytotoxins, are key virulence factors of *Mycobacterium ulcerans*, the etiological agent of the chronic necrotizing skin disease Buruli ulcer. There is urgent need for a simple point-of-care laboratory test for Buruli ulcer and mycolactone represents a promising target for the development of an immunological assay. However, for a long time, all efforts to generate mycolactone-specific antibodies have failed. By using a protein conjugate of a truncated non-toxic synthetic mycolactone derivative, we recently described generation of a set of mycolactone-specific monoclonal antibodies. Using the first mycolactone-specific monoclonal antibodies that we have described before, we were able to develop an antigen competition assay that detects mycolactones. By the systematic selection of a capturing antibody and a reporter molecule, and the optimization of assay conditions, we developed an ELISA that detects common natural variants of mycolactone with a limit of detection in the low nanomolar range. The mycolactone-specific ELISA described here will be a very useful tool for research on the biology of this macrolide toxin. After conversion into a simple point-of-care test format, the competition assay may have great potential as laboratory assay for both the diagnosis of Buruli ulcer and for the monitoring of treatment efficacy.

**AUTHOR SUMMARY**

The macrolide toxin mycolactone is the key virulence factor of *Mycobacterium ulcerans*, the causative agent of the chronic necrotizing skin disease Buruli ulcer. Mycolactone has cytotoxic activity and is secreted by the mycobacteria, causing tissue necrosis and immunosuppression. For research on Buruli ulcer, there is urgent need for a simple tool to quantify mycolactone. Using the first mycolactone-specific monoclonal antibodies that we have described previously, we have optimized here an antigen competition ELISA that detects common natural variants of mycolactone at a low nanomolar scale. Sensitivity of the assay is sufficient to detect the toxin in *M. ulcerans* culture

supernatants and in the tissue of experimentally infected animals. Converted into a simple point-of-care test format, the competition assay may in future also be suitable as a diagnostic laboratory test for Buruli ulcer.

## INTRODUCTION

*Mycobacterium ulcerans* is the etiological agent of the chronic necrotizing skin disease Buruli ulcer (BU) that primarily affects children in West and Central Africa [1]. Genomic analyses have shown that *M. ulcerans* has emerged from a common ancestor with the fish pathogen *Mycobacterium marinum* [2, 3] by acquisition of a virulence plasmid carrying genes that encode polyketide-modifying enzymes and the giant polyketide synthases responsible for the synthesis of the lipid toxin mycolactone [4]. While *M. marinum* occasionally causes limited granulomatous skin lesions in humans [5], chronic *M. ulcerans* infections are associated with a much more severe pathology. Mycolactone plays a key role in the chronic necrotizing pathogenesis of BU and, in addition, analgesic and immunosuppressive effects are attributed to the toxin [6]. There is evidence of multiple modes of action of mycolactone, including inhibition of Sec61-mediated protein translocation, uncontrolled assembly of actin by binding to the Wiskott-Aldrich syndrome protein (WASP) family, and induction of apoptosis through increased expression of the pro-apoptotic regulator Bim [6, 7]. Mycolactone is an amphiphilic molecule, prone to forming aggregates in aqueous solutions [8, 9], to binding to soluble proteins [10], and to inserting into lipid bilayers [8, 11]. At an air/buffer interface, mycolactone has been shown to have surfactant properties with an apparent surface saturation concentration of 1 μM [8].

Early case detection and rapid initiation of antibiotic treatment are currently the key elements of BU control. The disease presents in a variety of clinical manifestations, complicating the clinical diagnosis [12]. Laboratory tests routinely used for confirmation of clinical diagnosis include primarily the microscopic detection of acid-fast bacilli (AFB) and *M. ulcerans*-specific PCR tests. While microscopy – the only diagnostic test that can currently be performed routinely at hospital level – has limited sensitivity, PCR detecting the insertion sequence IS*2404* is highly sensitive and specific [13]. However, PCR requires sophisticated laboratory infrastructure and well-trained personnel and is not reliable

without strict quality control [14]. In resource-poor BU endemic countries, the test is only available at a few reference centres, which poses major logistical problems. Therefore, there is urgent need for a simple and rapid diagnostic test for BU that can be performed at local hospital level or in the field [13]. Mycolactone represents an ideal target for such an assay, since it seems to be unique to *M. ulcerans*. A mycolactone-specific assay may also be highly suitable for monitoring treatment efficacy and to diagnose relapses, since mycolactone levels in the affected tissue decline during successful specific therapy [15, 16].

Mycolactones consist of a core structure, a short C-linked upper side chain, and a longer C5-O-linked lower acyl side chain. Geographical lineages of *M. ulcerans* produce different pools of molecular variants of mycolactone, which differ in the structure of the lower polyunsaturated side chain but are otherwise structurally conserved [17]. For the generation of mycolactone-specific monoclonal antibodies (mAbs), we have used a truncated synthetic mycolactone in which the lower polymorphic side chain was replaced by a linker molecule [18]. Therefore, the epitopes recognized by these mAbs appear to comprise determinants of the conserved core and upper side chain. Consequently, common natural molecular species of mycolactone were recognized [18]. Here, our aim was to use the mAbs as antigen capture reagents to develop an immunological assay for the quantification of mycolactones.

## MATERIALS AND METHODS

### Ethical statement

Animal experiments performed were approved by the animal welfare committees of the Canton of Basel (authorization number 2375) and the Canton of Bern (authorization number BE95/17)**.** They were conducted in compliance with the Swiss Animal Welfare Act (TSchG), Animal Welfare Ordinance (TSchV), and the Animal Experimentation Ordinance (TVV).

### Preparation of mycolactone stock solutions

Production of synthetic mycolactones and mycolactone derivatives (Fig1) has been described elsewhere [7, 17, 19, 20]. All compounds were HPLC-purified.

### Monoclonal antibodies

Generation of the 12 mouse mAbs designated JD5.1 to JD5.12 has been described previously [18]. MAbs were purified from hybridoma culture supernatants by affinity chromatography using a HiTrap Protein A HP column (GE Healthcare).

### Initial competition ELISA

MaxiSorp immunoassay plates (Thermo Scientific) were coated with 100 µL mAb (10 µg/ml) overnight at 4°C. After washing the plate twice with PBS-0.05% Tween-20 (PBST), the wells were blocked with SuperBlock T20 (TBS) blocking buffer (Thermo Scientific) for 1 h at 37°C. After another washing step, serial dilutions of the sample were made in PBST and added to the plate (100 µL/well), and then incubated in the dark for 2 h at 37°C. Without washing, 100 µL/well of a 200 pg/ml solution of the reporter molecule PG-204 (Fig1) was added to the plate and incubated for an additional 30 min. Subsequently, plates were washed four times, and bound PG-204 was detected using alkaline

phosphatase-coupled streptavidin (SouthernBiotech)/para-nitrophenylphosphate (pNPP) detection after 1 h incubation at 37°C.

**Optimized competition ELISA**

For assay optimization phosphate-, tris(hydroxymethyl)-aminomethan (TRIS)- and triethanolamine (TEA)-based buffers with different concentrations of dimethyl sulfoxide (DMSO) were compared. Furthermore, different reporter molecules, reagent concentrations, detection systems, and the use of detergents (Tween-20, Triton X-100, Triton X-114, IGEPAL, Brij 35, CHAPSO; Sigma-Aldrich) were evaluated. Following these multiple optimization steps, a new standard protocol was defined: MaxiSorp immunoassay plates were coated with 5 µg/ml of mAb JD5.1 overnight at 4°C. After washing the plate twice with PBST, the plate was blocked with SuperBlock T20 (TBS) blocking buffer (Thermo Scientific) for 1 h at 37°C. After another washing step, serial dilutions of the samples in LW buffer (0.2 M TEA, pH 7.5, with 20% DMSO) were added to the plates and incubated in the dark for 2 h at 37°C. Without washing, 100 µL of an 80 ng/ml solution of the reporter molecule MG-161 (Fig1) in LW buffer was added to the plate and incubated for an additional 45 min. Subsequently, plates were washed four times, and bound MG-161 was detected after 1 h incubation at 37°C by horseradish peroxidase-coupled streptavidin (SouthernBiotech) diluted 1:5000 in PBST. Signal development was done with 3,3',5,5'-tetramethylbenzidine (TMB) for 5 min after which the reaction was stopped with 0.5 M sulphuric acid.

**Binding of mAbs to reporter molecules in ELISA**

NeutrAvidin-coated plates (Thermo Scientific) were coated with 1 µg/ml of the different biotinylated mycolactone derivatives – MG-158, MG-160, and MG-161 (Fig1). Each of the 12 mAbs was serially diluted in PBST, added to the coated plates, and incubated for 2 h. After a washing step, bound mAbs were detected using horseradish peroxidase-

conjugated goat anti-mouse IgG antibodies (SouthernBiotech) after 1 h incubation at 37°C. Signal development was done with TMB for 7 min after which the reaction was stopped with 0.5 M sulfuric acid.

**Competition ELISA using mycolactone-coated plates**

NeutrAvidin-coated plates (Thermo Scientific) were coated with 1 µg/ml of the biotinylated mycolactone derivative MG-161 (Fig1). A dilution series of mycolactone was prepared to which the mAb JD5.1 was added at a fixed concentration, and both were incubated together at 37°C for 2 hours to allow for binding. Subsequently, the mixture was transferred to the MG-161-coated plates and incubated for 1 hour at 37°C. Bound mAbs were detected using a secondary antibody conjugated with horseradish peroxidase (HRP), and detection was done with TMB.

**Cultivation of *M. ulcerans* and analysis of secreted mycolactone**

An African *M. ulcerans* strain (S1013) and an Australian strain (S1251) were grown at 30°C in BACTEC liquid culture media (Becton Dickinson). Both strains have only been minimally passaged after isolation from BU lesions. For the detection of mycolactones in the bacterial culture supernatants, 500 µl of well-grown *M. ulcerans* cultures were centrifuged at 13,300 x *g* to pellet the bacteria, and the supernatant was filtered using sterile 0.22 µm syringe filters. The sterile-filtered supernatants were used directly in the assay.

**Analysis of *M. ulcerans* in infected mouse footpads**

BALB/c mice, age 7-8 weeks (Janvier Labs, Le Genest-Saint-Isle, France) were inoculated in the left hind footpad with about 5 x $10^3$ CFU of the *M. ulcerans* isolate S1013 from Cameroon. The progression of footpad swelling was tracked for 13 weeks (S1 Fig), when mice were euthanized, and the footpads were collected and stored in absolute ethanol

for 9 weeks. Uninfected right hind footpads served as controls. Subsequently, the ethanol was recovered and concentrated by vacuum centrifugation (SpeedVac, Thermo Scientific). Footpads were minced and homogenized in a bead-beater (Precellys MK-28R) in 1.5 ml of ethyl acetate. The supernatant was filtered and concentrated by vacuum centrifugation. Each extract was re-suspended in 60 µl of DMSO, and half this volume was used in the assay. For the quantification of the concentration of mycolactone detected in the extracts, standard curves were generated with synthetic mycolactone A/B and used to derive a regression equation for the determination of the concentration in the extracts.

## RESULTS

We have tested a panel of 12 anti-mycolactone mAbs (JD5.1 – JD5.12; Table 1) described previously [18] for suitability as capturing reagent for a mycolactone-specific competition assay. The mAbs were generated by immunization of mice with PG-203, a truncated and non-cytotoxic mycolactone derivative coupled to BSA via a linker replacing the C5-O-linked polyunsaturated acyl side chain (Fig1), and by ELISA screening of hybridomas using PG-204 (Fig1), which is a biotinylated derivative of PG-203 [18]. As predicted from the structure of PG-203 and PG-204, the presence/absence or detailed structure of the C5-O-linked acyl side chain does not affect binding of the mAbs and, since the core and upper side chain of mycolactone are completely conserved, all tested mycolactone variants showed binding [18]. While a competition ELISA format with the mAbs was initially only used to assess their fine specificity [18], we describe here the optimization of this assay format for the detection and quantification of mycolactones in biological samples.

**Table 1**: **Binding of the anti-mycolactone mAbs to different reporter molecules**.

**Figure 1: Structures of mycolactone A/B and of synthetic derivatives.**

### Optimization of the reporter molecules

To find a potentially more suitable substitute for the initially used reporter molecule PG-204 (Fig1) for the competition assay, we screened the panel of 12 mAbs for recognition of related derivatives with modification at the C20 position of the upper side chain (MG-158, MG-160, and MG-161; Fig1). While all mAbs showed strong binding to MG-161, which carries an additional hydroxyl group, only one mAb showed limited binding to MG-160 carrying a bulky *p*-methoxybenzyl ether group. A wide variation in binding was observed with the different mAbs for MG-158, carrying an additional acetoxy group

(Table 1). Overall, competition assays with mAb JD5.1, which bound to both MG-158 and MG-161 (Fig2A) showed the highest sensitivity. This mAb appears to have a higher affinity for PG-204 than for mycolactone, which explains why as little as 100 pg/ml of PG-204 can out-compete a large excess of mycolactone for binding to the mAb [18]. We surmised that using a less tightly bound reporter molecule would allow for lower concentrations of mycolactone to be detected in the assay. In fact, MG-161 was found to be the best substitute for PG-204, resulting in an approximately 5-fold increase in the assay sensitivity (Fig2B). However, to obtain optimal sensitivity, a 400-fold higher concentration of MG-161 than of PG-204 (40 ng/ml versus 100 pg/ml) was required.

**Figure 2**: **Binding properties of mAb JD5.1.**

**Optimization of the ELISA conditions**

We attempted to improve sensitivity of the competition assay further by reducing aggregation of mycolactone without denaturing the mAbs used in the ELISA. While ethanol, acetonitrile, and sodium dodecylsulphate improve the dispersion of the toxin in water [9], these solvents were, even in small concentrations, detrimental for the binding of the mAbs to mycolactone. Screening of a panel of non-denaturing detergents (Tween-20, Triton X-100, Triton X-114, IGEPAL, Brij 35, CHAPSO) at various concentrations showed that no other detergent or concentration out-performed 0.05%-Tween-20 previously selected for the PBS-based assay buffer [18]. Further systematic comparisons of phosphate-, TRIS-, and TEA-based buffers with different concentrations of dimethyl sulfoxide (DMSO) and Tween-20 resulted in the definition of the LW buffer, giving the highest assay sensitivity in the low nanogram scale (Fig3). The buffer contains 20% DMSO and 0.2 M TEA, but no detergent. TEA is commonly used in emulsifiers to dissolve compounds that are poorly soluble in water. The sensitivity of the competition assay is only slightly reduced in the presence of serum (S2 Fig).

**Figure 3**: **Improved assay sensitivity upon buffer optimization**.

Small additional improvements of the assay sensitivity were achieved by switching from the alkaline phosphatase-based to the peroxidase-based antibody detection system, and by reducing the JD5.1 mAb coating concentration from 10 µg/ml to 5 µg/ml. A reversed competition format using biotinylated mycolactone bound to NeutrAvidin plates showed a sensitivity comparable to that with antibody-coated plates (Fig4).

**Figure 4: Indirect competition ELISA format.**

**Detection of mycolactone in biological samples**

Sterile-filtered culture supernatants of an African and an Australian *M. ulcerans* strain both gave positive readouts in the assay (Fig5), demonstrating that both mycolactone A/B predominantly produced by African strains and mycolactone C predominantly produced by Australian strains that are together responsible for >95% of reported BU ulcer cases [1] are recognized. These supernatants were used directly in the assay without any prior lipid extraction.

**Figure 5**: **Detection of mycolactone in culture supernatants of *M. ulcerans* strains**.

Mycolactone could also be specifically detected in extracts from mouse footpads infected with *M. ulcerans*. After extended storage (9 weeks) of the infected footpads in ethanol (EtOH), most of the mycolactone was found in the ethanol extract (Fig6A), and only traces had remained in the footpad tissue, which was extracted with ethyl acetate (EtOAc) after homogenization of the footpads (Fig6B). No mycolactone was detected in the extracts from uninfected control footpads (Fig6). Using a standard curve generated

with synthetic mycolactone A/B, the amount of mycolactone in the extracts was calculated (Table 2).

**Fig 6**: **Detection of mycolactone in extracts from experimentally infected mouse footpads.**

**Table 2: Quantification of mycolactone in infected and uninfected mouse footpads.**

**DISCUSSION**

BU control measures rely mainly on early diagnosis of cases, preferably in the WHO Category I and II stages, and the prompt initiation of treatment. The current gold standard diagnostic test is the highly specific and sensitive PCR assay based on the detection of the high copy number insertion sequence IS*2404*. The main drawback in implementing this assay for routine diagnosis is the indispensability of well-equipped laboratories, skilled and experienced personnel, and rigid adherence to quality control measures. As a result, efforts have been made to develop BU diagnostics that would be low-cost and require simple instrumentation, while still giving results in a short time. Immunodiagnostic assays such as ELISA, which depend on the interactions between antibodies and their corresponding antigens, are viable alternatives to genetic test systems and are comparatively simpler to perform even at the primary healthcare or field levels. For BU, mycolactone makes an ideal antigen for specific immunodiagnosis given its uniqueness to the mycolactone-producing mycobacteria (MPM). Also, since the levels of mycolactone in tissues are reported to decline during specific treatment [15, 16], an assay quantifying mycolactone in BU lesions would be useful in monitoring treatment efficacy.

However, efforts to generate antibodies against this small, lipid-like, cytotoxic and immunosuppressive polyketide have long been unsuccessful. Using a novel approach which involved the use of a modified non-toxic variant of mycolactone coupled to a protein carrier to immunize mice, we have recently described for the first time the generation of mAbs against mycolactone [18]. Here, we describe the optimization of a competition ELISA based on these mAbs for the detection and quantification of mycolactone in biological samples. We sought to systematically optimize each step of the multi-step assay procedure, leading to modest gains per step but an overall 30-fold gain in sensitivity, allowing for the detection of nanogram levels of mycolactone in biological samples.

One key difficulty in detecting mycolactone by ELISA is its inherent hydrophobicity, largely due to the lower acyl side chain. ELISA, by default, is designed for hydrophilic molecules, typically proteins. Special methods have to be devised for the detection of hydrophobic molecules, such as the chloroform-ethanol coating method described for lipids [21, 22, 23]. However, we were aiming for more facile methodologies such that the resultant assay protocol would be relatively straightforward for the development of rapid diagnostic tests that can be performed in peripheral settings. As an amphiphilic molecule, mycolactone is thought to form micelle-like structures in aqueous solutions, with the acyl side chain sequestered within the core of these structures [9]. We attempted to disperse these structures using a variety of methods with the main challenge to find a solvent that would lead to improved mAb binding to the mycolactone molecules without denaturing the mAbs used in the ELISA. While detergents are routinely used to disperse lipid aggregates, none of the non-protein-denaturing detergents we tested led to an improvement in the sensitivity of our assay. In contrast, using 0.2 M TEA as the ELISA running buffer improved assay sensitivity compared to phosphate- and TRIS-based ELISA buffers. TEA is widely used as a buffering agent with surfactant properties in consumer products [24, 25] and in biomedical research [25, 26, 27, 28].

Switching the reporter molecule from PG-204 to MG-161, which is less tightly bound by the capturing mAb, also improved the overall assay sensitivity. Here, a drawback is that a 400-fold higher concentration of the synthetic mycolactone derivative is needed when switching to MG-161.

Conformational changes associated with the binding of mAbs to a plastic surface can lead to drastic changes in their antigen binding properties [29]. Therefore, we also explored the possibility of rearranging the assay set-up to allow the capturing mAb and mycolactone to react in solution, rather than at a solid-liquid interface with immobilized

mAb. However, we found that this indirect competition format had no added benefits, and the need to use NeutrAvidin-coated plates for the coating with a biotinylated mycolactone derivative for mAb capturing increases the overall costs of the assay.

The utility of the improved competition ELISA as a research tool was assessed by measuring mycolactone concentrations directly in *M. ulcerans* culture supernatants and in footpads of experimentally infected mice. The amounts found in the infected footpads (50 to 100 ng/footpad) are comparable to those (49 ng/footpad) that were measured with thin-layer chromatography at a slightly earlier time point after infection [30]. The mycolactone ELISA thus is, in its optimized format, a valuable research tool that will allow to quantify mycolactone in large series of biological specimens. The assay may also be suitable as a diagnostic test for BU at the district hospital level. Reagents and assay conditions developed here may also be instrumental for the development of a simple point-of-care diagnostic test format, such as a lateral flow assay. While basic laboratory equipment and technical expertise is required to perform an ELISA, a simple lateral flow assay could be performed directly with wound exudate obtained by swabbing of ulcerative BU lesions or fine needle aspiration from closed lesions. While it has been shown, that mycolactone is extracted from serum samples with low efficacy [31], the sensitivity of the competition assay is only slightly reduced in the presence of serum. Our preliminary results indicate that no extraction with organic solvents may be required to perform these immunological tests.

In summary, the diagnosis of BU is still problematic, and development of BU diagnostics has not kept pace with the implementation of antibiotic treatments. Here, we describe a simple immunoassay for the specific and sensitive detection of mycolactone in biological samples. The generation of the first-ever described mAbs specific for mycolactone and chemical synthesis of mycolactone derivatives suitable as reporter molecules led to the development of a competition ELISA that we have systematically

optimized here. While representing a valuable research tool for high-throughput quantification of mycolactone, this ELISA may also have potential as diagnostic assay for BU at district hospital level. Furthermore, the developed reagents and protocols may also enable development of a simple point-of-care test by converting the ELISA format into a lateral flow assay.

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**TABLES**

**Table 1**: **Binding of the anti-mycolactone mAbs to different reporter molecules**. NeutrAvidin-coated plates were coated with the different reporter molecules at 1 µg/ml and 3-fold dilution series of the different mAbs starting from 5 µg/ml were added and allowed to bind. Bound mAbs were detected with an HRP-conjugated secondary antibody and TMB. Binding was graded based on the absorbance (OD) values measured at 450 nm as follows: (-) OD < 0.5, (+) OD 0.5 – 1, (++) OD >1 – 1.5, (+++) OD > 1.5.

| | PG-204 | MG-161 | MG-158 | MG-160 |
|---|---|---|---|---|
| **JD5.1** | +++ | +++ | +++ | - |
| **JD5.2** | +++ | +++ | - | - |
| **JD5.3** | +++ | +++ | + | - |
| **JD5.4** | +++ | +++ | ++ | - |
| **JD5.5** | +++ | +++ | ++ | - |
| **JD5.6** | +++ | +++ | + | - |
| **JD5.7** | +++ | +++ | - | - |
| **JD5.8** | +++ | +++ | ++ | - |
| **JD5.9** | +++ | +++ | +++ | - |
| **JD5.10** | +++ | +++ | +++ | - |
| **JD5.11** | +++ | +++ | +++ | + |
| **JD5.12** | +++ | +++ | + | - |

**Table 2**: Quantification of mycolactone in infected and uninfected mouse footpads.

| Sample | EtOH fraction (ng) | EtOAc fraction (ng) |
|---|---|---|
| **Mouse 1 footpad** | 55.9 | 1.0 |
| **Mouse 2 footpad** | 82.8 | 0.9 |
| **Mouse 3 footpad** | 53.5 | 1.4 |
| **Uninfected footpad** | 0 | 0 |

**FIGURES**

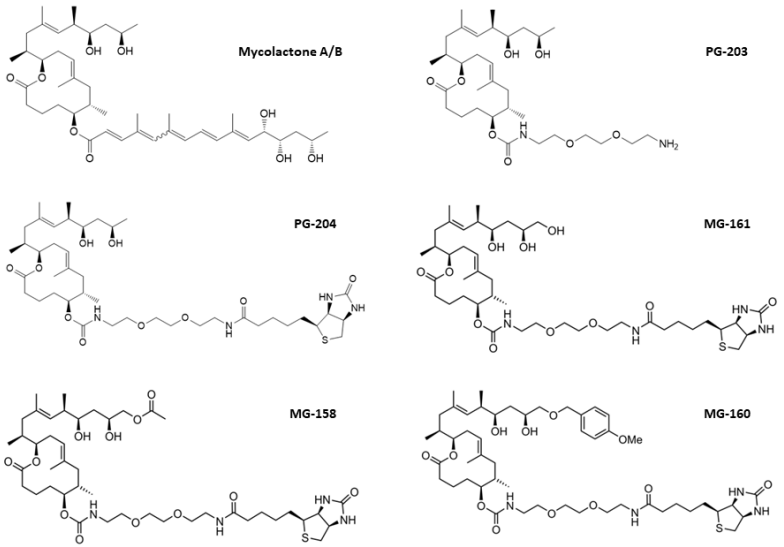



**Figure 1: Structures of mycolactone A/B and of synthetic derivatives.**

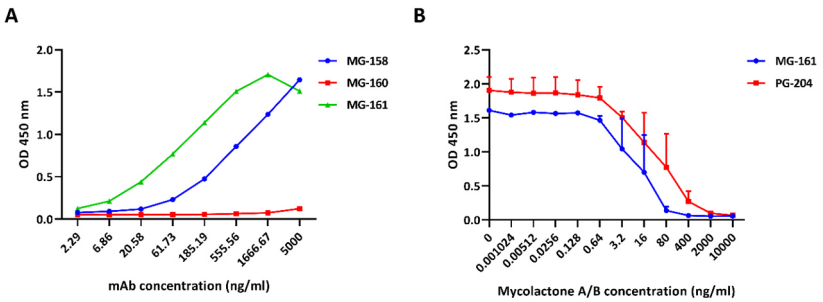



**Figure 2**: **Binding properties of mAb JD5.1.** (A) Binding of mAb JD5.1 to the different reporter molecules. NeutrAvidin-coated plates were coated with the different reporter

molecules (1 µg/ml), a serial dilution of mAb JD5.1 was allowed to bind, and bound mAb was detected with an HRP-conjugated secondary antibody. (B) Sensitivity of the JD5.1-based competition assay with PG-204 (100 pg/ml) or MG-161 (40 ng/ml) as reporter molecules. JD5.1 bound to MaxiSorp plates was allowed to react with dilution series of mycolactone A/B for 2 hours after which PG-204 or MG-161 was added for 45 min; bound reporter was detected with HRP-conjugated streptavidin and TMB. Experiments were done in duplicate (two separate runs on different days) and the results shown are the mean of both runs, with error bars indicating the range.

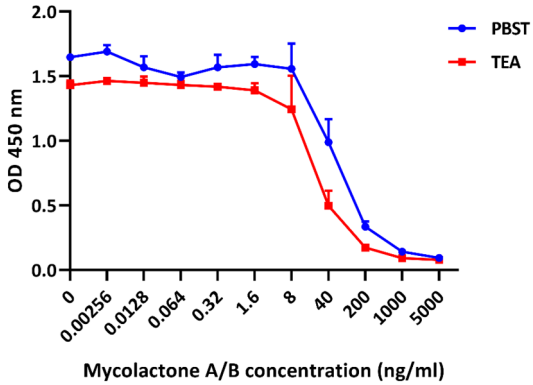



**Figure 3**: **Improved assay sensitivity upon buffer optimization**. Compared is the performance of the ELISA with the TEA-based LW buffer (0.2 M TEA with 20% DMSO) or with a PBST-based buffer (PBST with 20% DMSO). JD5.1 bound to MaxiSorp plates (coating concentration 5 µg/ml) was allowed to react with dilution series of mycolactone A/B in PBST buffer or TEA-based buffer for 2 hours after which MG-161 (40 ng/ml) was added for 45 min; bound reporter was detected with HRP-conjugated streptavidin and TMB. Results shown are the mean of duplicate experiments and error bars indicate the range.

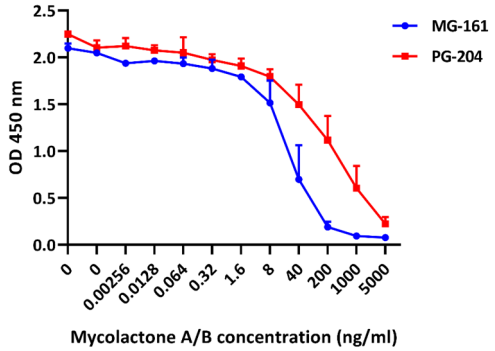



**Figure 4: Indirect competition ELISA format.** Serial dilutions of mycolactone were mixed with fixed concentrations of mAb JD5.1 and allowed to react for 2 h at 37°C after which the mix was transferred to NeutrAvidin plates coated with the target molecules MG-161 or PG-204 at 1 µg/ml. MAb bound to the plate was detected with an HRP-conjugated secondary antibody and TMB. Results shown are the mean of duplicate experiments and error bars indicate the range.

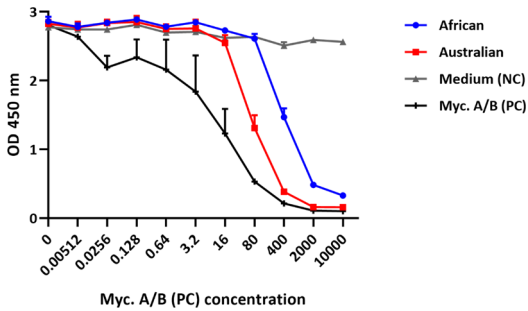



**Figure 5**: **Detection of mycolactone in culture supernatants of *M. ulcerans* strains**. Dilution series of culture supernatants of an African and an Australian *M. ulcerans* isolate, synthetic mycolactone A/B as positive control (PC), or plain medium as negative

control (NC) were made in LW buffer and allowed to react with mAb JD5.1 bound to MaxiSorp plates for 2 hours after which MG-161 (40 ng/ml) was added for 45 min; bound reporter was detected with HRP-conjugated streptavidin and TMB. Results shown are the mean of duplicate experiments and error bars indicate the range.

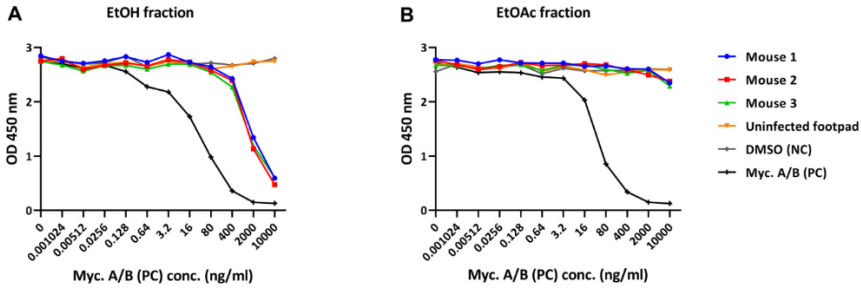
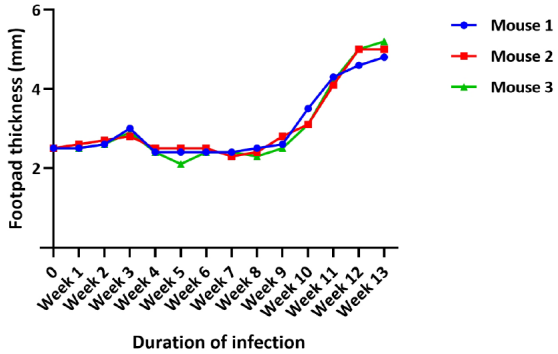
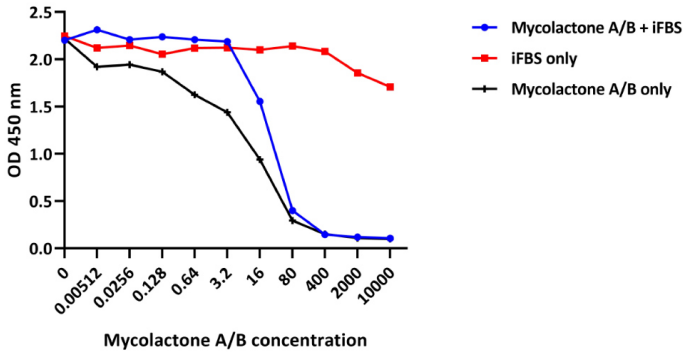



**Figure 6**: **Detection of mycolactone in extracts from experimentally infected mouse footpads.** Extracts were prepared by vacuum-centrifugation of the ethanol in which the footpads were stored (Fig6A; EtOH fraction), or of the ethyl acetate in which the footpads were homogenized (Fig6B; EtOAc fraction). Dilution series of the extracts or synthetic mycolactone A/B were made in LW buffer and allowed to react with mAb JD5.1 bound to MaxiSorp plates for 2 hours after which MG-161 (40 ng/ml) was added for 45 min; bound reporter was detected with HRP-conjugated streptavidin and TMB.

**SUPPORTING INFORMATION**




**S1 Fig.** Time course of footpad swelling in *M. ulcerans* infected mice.




**S2 Fig.** Sensitivity of the assay in the presence or absence of fetal bovine serum. Dilution series of synthetic mycolactone A/B were done in LW buffer alone or LW buffer containing 50% fetal bovine serum (FBS) were allowed to react with mAb JD5.1 bound to MaxiSorp plates for 2 hours; plain FBS was included as negative control. Subsequently, MG-161 (40 ng/ml) was added for 45 min, and bound reporter was detected with HRP-conjugated streptavidin and TMB.

# Competitive ELISA for the Detection and Quantification of *Mycobacterium ulcerans* Mycolactone

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## Summary/Abstract

Lipids and other hydrophobic analytes are difficult to quantify by routine immunoassays due to the need to use aqueous buffers. Here, we describe an ELISA protocol suitable for the detection of mycolactone, the polyketide toxin of *Mycobacterium ulcerans*, the causative agent of Buruli ulcer (BU). Given that mycolactone is unique to this species and has been found in all *M. ulcerans* lineages, the assay herein described has the potential to be useful both as a research tool and as a diagnostic test, even in low-resource BU endemic regions. Furthermore, the triethanolamine buffer described here may also be useful in the specific detection of other lipid analytes by ELISA.

**Key Words**

*Mycobacterium ulcerans,* mycolactone, competitive ELISA, triethanolamine buffer, polyketides

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# Chapter 3: Competitive ELISA for Mycolactone Quantification (Protocol)

## Introduction

Enzyme-linked immunosorbent assays (ELISAs) have become routine methods for the detection and quantification of analytes in a sample. The underlying principle is specific recognition of the analytes by antibodies and the revelation of this interaction by an enzyme-linked colour change [1]. Most ELISAs are designed for the detection of specific proteins in biological samples. However, other biomolecules, including carbohydrates and lipids, can also be detected by ELISA [2, 3, 4].

Buruli ulcer – or *Mycobacterium ulcerans* disease – is a neglected tropical disease with highest prevalence in low-resource settings in Africa [5, 6]. *M. ulcerans* causes this disease largely through the production of a macrolide exotoxin known as **mycolactone** (Fig. 1A). Mycolactone is a small lipid-like molecule and is unique to *M. ulcerans* [7]. Thus, its detection in biological samples can be a means of specific diagnosis. However, existing techniques for the detection of lipid analytes cannot be routinely applied in the rural, low-resource settings that suffer the greatest disease burden. To this end, immunoassays that can detect mycolactone would be much better suited owing to their low cost and less need for sophisticated equipment.

We describe here a competitive ELISA that works for the detection and quantification of this lipid-like molecule [8]. A competitive ELISA detects an analyte by using analyte-specific mAbs in the presence of a reporter molecule. This reporter molecule competes with the analyte for binding to the mAb [9] and, in the case of mycolactone, can be linked to a detectable label, such as biotin (Fig. 1B). In a direct competitive ELISA, microtitre plates are coated with the mAbs, the analyte present in the sample competes with the reporter for mAb binding, and the replacement of the reporter molecule by the analyte is measured.

# Chapter 3: Competitive ELISA for Mycolactone Quantification (Protocol)

## 1 Materials

All reagents should be prepared with ultrapure water and stored at ambient temperature unless otherwise stated. It is recommended to prepare sufficient volumes of stock reagents for consistency. Follow all local waste disposal regulations when disposing any generated wastes. Use proper personal protective equipment (PPE).

### 1.1 Buffers:

1.1.1 PBS (10x): Weigh 80 g NaCl, 2 g KCl, 35.8 g $Na_2HPO_4.12H_20$ (see **Note 1**), and 2.4 g $KH_2PO_4$ into a 1 L graduated measuring cylinder (see **Note 2**). Add water to the 900 ml mark and mix thoroughly on a magnetic stirrer. When all the solids have dissolved, adjust the pH to 7.4 with concentrated sodium hydroxide (see **Note 3**), and then make up to 1 L with water. Store at ambient temperature.

1.1.2 PBST (1x): Add 100 ml of 10x PBS to 900 ml of water. Add 0.05 % Tween-20 and mix thoroughly (see **Note 4**). Store at ambient temperature.

1.1.3 Triethanolamine buffer (0.2 M, pH 7.5). Measure 11.92 g of triethanolamine (see **Notes 5 and 6**) into a measuring cylinder. Add water to the 300 ml mark and mix thoroughly on a magnetic stirrer. When all the solids have dissolved, adjust the pH to 7.5 (see **Note 7**), and then make up to 400 ml with water (see **Note 8**). Store at ambient temperature (see **Note 9**).

### 1.2 ELISA:

1.2.1 MaxiSorp 96-well immunoassay plate (see **Note 10**).

1.2.2 NUNC Low-retention 96-well dilution plate.

1.2.3 Coating buffer: PBS (1x).

1.2.4 Wash buffer: PBST.

1.2.5 Blocking buffer: SuperBlock® (TBS) T20 buffer (Thermo Scientific) (see **Note 11**).

1.2.6 Running buffer: 0.2 M TEA plus 20% dimethyl sulphoxide (DMSO) (see **Notes 12 and 13**).

1.2.7 3,3',5,5'-tetramethylbenzidine (TMB): Ready-made mix (see **Note 14**).

1.2.8 Stop solution: 0.5 M sulphuric acid (see **Note 15**).

1.2.9 ELISA plate washer.

1.2.10 Plate reader.

**1.3 Antigens and Conjugates:**

1.3.1 Purified anti-mycolactone monoclonal antibody (JD5.1) in PBS ([3]; see **Note 16**).

1.3.2 Sample to be analysed, dissolved in DMSO.

1.3.3 Biotin-conjugated mycolactone molecule (reporter molecule, MG-161, Fig. 1B) in DMSO.

## 2 Methods

**Sample preparation**

The typical samples obtained from ulcerated BU lesions are swabs collected from the undermined edges of the wound. Fine needle aspirates (FNAs) are typically taken from non-ulcerated BU lesions. Mycolactone can also be extracted from bacterial pellets or colonies for research purposes.

1. Swabs can be directly extracted in the running buffer. For each swab sample, prepare a 5 ml Eppendorf tube containing 250 µl of running buffer (see **Notes 17** and **18**). Carefully break off the swab, approximately 50 – 80 mm from the tip, into the Eppendorf tube (see **Note 19**). Vortex vigorously for 30 s and then recover the liquid (see **Notes 20** and **21**). This can then be directly used in the assay.

2. Depending on the method of FNA collection, the sample may have to be concentrated to a low enough volume for all of it to be used in the assay. This can be achieved by drying the sample in a vacuum centrifuge (e.g. SpeedVac from Thermo), or by using a stream of nitrogen (see **Note 22**). The dried sample pellet is then resuspended in 150 µl of running buffer for the assay.
3. Ethanolic extracts of bacterial pellets or colonies can be used in the assay. Resuspend the pellet/colony in 500 µl of absolute ethanol and vortex vigorously for 30 s. Incubate the sample at 4°C overnight (see **Note 23**). Centrifuge at 13,000 rpm for 10 min to pellet the bacteria. Carefully remove the supernatant and filter it using sterile 0.22 µm syringe filters (see **Note 24**). Dry the sample by vacuum centrifugation (SpeedVac, Thermo) or under a nitrogen stream. Resuspend the pellet in 30 µl of DMSO.

**ELISA**

All incubation steps are at 37°C unless otherwise stated. Bring all reagents to ambient temperature before use.

1. Determine the number of samples to be analysed. If the sample is in sufficient quantity, we recommend testing in triplicate rows per experiment. Be sure to include adequate controls (see **Note 25**).
2. Coat each well of the required number of rows of a 96-well MaxiSorp plate with 5 µg/ml of the anti-mycolactone mAb (JD5.1) (see **Note 26**). For a full 96-well plate, prepare 10 ml of this coating solution and dispense 100 µl into each well using a multi-channel pipette. Seal the plate and incubate overnight at 4°C (see **Note 27**).
3. After the coating time has elapsed, discard the coating solution by flicking it sharply out of the upended plate. Wash the plate three times (see **Note 28**).

4. Block the plate with blocking buffer (200 µl/well) for 1 h (see **Note 29**).
5. While the plate is blocking, prepare the dilution series for the samples to be analysed. We use 5-fold dilution series for this assay, although higher or lower dilutions may be done. For a full 96-well plate, fill all the wells of a Nunc low-binding plate with 120 µl/well of running buffer (see **Note 30**). For the controls, add 150 µl/well for the first wells in the rows. Add the samples to the appropriate rows (see **Note 31**), for each sample row, the total volume in the first well should be 150 µl. Add enough of positive control to get a concentration of 10 µg/ml into the first well (with 150 µl of running buffer) of the row for the positive control (see **Note 32**). Add only DMSO to the first well of the negative control row. Make the 5-fold dilution series by transferring 30 µl from the first well into the second well, mixing by pipetting up and down 5 times, then transferring 30 µl from the second well into the third well, and on till the eleventh well. Leave the twelfth well without any sample (see **Note 33**).
6. After the blocking time has elapsed, discard the blocking solution and wash the plate thrice as before.
7. Transfer 100 µl from every well of the Nunc low-binding dilution plate into the corresponding well of the ELISA plate, in the dark. Incubate for 2 h in the dark.
8. Prepare a solution of the reporter molecule to a concentration of 80 ng/ml by diluting in the running buffer (see **Note 34**).
9. Do not discard the ELISA plate contents (see **Note 35**)! Carefully add the reporter molecule into each well of the ELISA plate (see **Note 36**). Return to the incubator for another 45 min.
10. Prepare a solution of the streptavidin-horseradish peroxidase in PBST (see **Notes 37 and 38**).

11. After the incubation time has elapsed, discard the plate contents and wash the plate four times (see **Note 39**). Add the diluted streptavidin-HRP to each well of the ELISA plate (100 µl/well). Incubate for 1 h.
12. Prepare the TMB solution (see **Note 40**). Dispense 70 µl of TMB into each well using a multichannel pipette. Incubate at ambient temperature for no more than 5 min (see **Note 41**).
13. Stop the reaction with 0.5 M sulphuric acid. Dispense 70 µl of the stop solution into each well with a multichannel pipette (see **Note 42**).
14. Read the absorbance at 450 nm and save the data in a suitable software (e.g. Microsoft Excel).

## 3 Analysis

1. If samples were tested in duplicates, calculate the mean absorbance of each duplicate well. Do likewise for the controls.
2. The curve of a competitive ELISA is as shown in Fig. 2A below. As shown, the absorbance increases as the concentration of mycolactone in the sample decreases (see **Note 43**). This is a classic example of a curve with an equation $y = m/x$ or $y = m/x^2$. Thus, one can obtain a linear graph by plotting y against the inverse of x $(1/x)$ or $x^2$ $(1/x^2)$, as shown in Fig. 2B below (see **Note 44**).
3. The regression equation can then be used to calculate the concentration of mycolactone in the test sample by substituting "x" with the inverse of the mean absorbance $(1/x)$ or the square of the mean absorbance $(1/x^2)$ – depending on what was done for the standard – for the first well in the dilution series (see **Note 45**).

## 4 Notes

1. Pay attention to the water of crystallisation present in the sodium hydrogenphosphate ($Na_2HPO_4$). This is the amount for the dodecahydrate ($12H_20$) salt. If using the dihydrate ($2H_20$) or heptahydrate ($7H_20$) salt, this amount must be adjusted accordingly. For example, use 26.8 g for the heptahydrate salt.
2. Owing to the amount of salts to be dissolved, we recommend using a wider vessel to prepare the buffer. We use 2 L reagent bottles and measure in the water with a measuring cylinder. This gives the magnetic stirrer bar allowance to rotate freely.
3. Adjust pH with concentrated (at least 5 M) sodium hydroxide. It is best to use a more concentrated solution to raise or lower pH to prevent the addition of too much of the solution as this could alter the concentration of ions in the buffer.
4. Cut the end of a blue tip for ease of aspiration. Draw up the Tween-20 slowly to prevent pipetting errors. Flush the tip thoroughly in the PBS.
5. Handle triethanolamine with care. A solution of the pure compound has a pH of 10.5 – 11.5, and is therefore quite corrosive. We find that the hydrochloride salt can be safely substituted.
6. Be sure to adjust the amount accordingly if the hydrochloride salt is used, e.g. 14.88 g of TEA hydrochloride for 400 ml of water.
7. Adjust pH with concentrated (at least 5 M) hydrochloric acid if pure TEA was used. If the hydrochloride salt was used, the pH would be around 5.5 and then needs to be raised to 7.5 using concentrated (at least 5 M) sodium hydroxide (NaOH).
8. We recommend making this buffer in relatively smaller quantities. Unlike the saline buffers, TEA buffer is prone to microbial contamination, even at higher concentrations.

9. Provided smaller quantities are made, TEA buffer can be stored at room temperature for up to two months. Storage at 4°C is also an option; it then just needs to be brought to room temperature before use.
10. MaxiSorp plates are designed for high-affinity binding of hydrophilic molecules such as antibodies. Other immunoassay plates designed for the adsorption of hydrophilic proteins can be used, e.g. Immulon 4HBX, Microlon, or Corning plates. Use whichever plate is readily available to you.
11. SuperBlock® is a biotin-free blocking buffer useful for quick blocking steps. We find, however, that 5% milk in PBST works just as well in this assay and is a much cheaper alternative. To make 5% milk in PBST, add 1 g of skim milk for every 20 ml of PBST (for example, use 5 g of milk for 100 ml of PBST). It can be stored at 4°C, although it is recommended to prepare it fresh in enough quantities for a single experiment.
12. Be sure to wear proper PPE when handling DMSO as it can be absorbed through the skin and acts as a vehicle for any chemical dissolved in it. Change your gloves if DMSO spills on them!
13. You can prepare this buffer in relatively larger amounts as the DMSO helps deter microbial growth, although it is recommended to prepare not more than 200 ml at a time. Once prepared, store at ambient temperature as DMSO will freeze at 4°C. The solution will feel warm to the touch when freshly made. Allow to cool to ambient temperature before use.
14. We use the KPL 2-component TMB kit, where all that is needed is to mix both provided solutions in a 1:1 ratio. However, pre-mixed alternatives are also available. Whichever is used, it is important to protect from light as the TMB solution is very sensitive to light.
15. Handle with extreme caution. It is highly recommended to prepare under a fume cupboard. Remember to always add acid to water, never the other way around.

Absolute sulphuric acid has a molarity of approximately 18.4, thus, to prepare 1 L of 0.5 M sulphuric acid, you would need 28 ml of acid. Add this quantity of acid slowly to the water while stirring.

16. Do not use mAb solutions containing azide if you would use horseradish peroxidase in the ELISA, because the azide will react unfavourably with the peroxidase.
17. Use larger tubes to accommodate the stem of the swab. We recommend using 5 ml snap-cap Eppendorf tubes.
18. Use smaller volumes of running buffer to allow the sample be as concentrated as possible. If flocked swabs are used for sample collection, approximately 150 µl of the running buffer can be recovered after the extraction. If cotton swabs are used, particularly the loosely woven ones, more fluid may be retained by the swab, in which case it might be better to use 300 µl of running buffer for the extraction.
19. For flocked swabs, the break point is usually indicated. For cotton swabs, aim for approximately 30 – 50 mm above the cotton tip; this would allow for enough room for the swab to move in the capped tube during the vortexing.
20. Mycolactone is light-sensitive, therefore the tubes should be manipulated in the dark during the extraction. To recover more fluid from cotton swabs, pierce the cotton tip with the pipette tip and aspirate as much fluid as possible. Repeat until no more fluid can be recovered from the swab.
21. Longer incubation durations will ensure more complete mycolactone extraction. We recommend starting the extraction before coating the plates for the ELISA. This ensures more time for mycolactone extraction to occur. The extraction can be done at 4°C or at ambient temperature, protected from light.
22. We recommend vacuum centrifugation as it gives more reliable concentration of the sample. Drying under a stream of nitrogen (or a suitable inert gas) is also

possible, although the aqueous buffers used for FNA collection would require a longer time to dry. Air-drying would take too long to be practical, and it is not advisable to use elevated temperatures to speed up drying due to the heat-labile nature of mycolactone. Whichever method is used, ensure that the samples are kept in the dark.

23. The samples can also be incubated for up to 3 d, depending on the size of the pellet of colony – the larger the size, the more time would be needed for complete extraction.

24. *M. ulcerans* is in some countries a biosafety level 3 (BSL-3) organism, therefore it is necessary to sterilise the samples before taking them out of the BSL-3 environment for the assay.

25. For this assay, we use synthetic mycolactone in DMSO as positive control and plain DMSO as negative control. Positive controls should ideally be in duplicates, as we would need them to do the quantification. Negative controls do not need to be in duplicates.

26. Reusable plate covers are indispensable for routine ELISA experiments. It is advisable to have a few of these on hand. If disposable plate covers are not available, parafilm is a good substitute. The plates can be stacked allowing only one cover to be sufficient for each stack of plates. We recommend stacking no more than three plates, especially when incubation at 37°C would be done.

27. In the event that a shorter coating duration is needed, 2 h at 37°C is also sufficient for this assay. Adequate care must be taken to seal the plates to prevent evaporation.

28. An ELISA washer allows for easy and consistent plate washing. However, if this is unavailable, manual washing is possible. We find that this is easily done using a 2 ml or 5 ml syringe (without the needle!) containing the wash buffer. Use a syringe with a plunger that moves freely, otherwise you risk overfilling a well and

thus contaminating adjacent wells. To manually wash a full 96-well plate, fill the syringe with the wash buffer and add 2 – 3 drops into each well; only add enough wash buffer to fill approximately three-quarters of the well. Wash the plate three columns at a time. Flick out the buffer and repeat twice for a total of three washes per well. A general rule of thumb when washing manually is to use one extra wash than stipulated.

29. The amount of blocking buffer used depends on the amount of the developing solution that will be used at the end of the ELISA. We recommend using at least 50 µl more blocking buffer than the total development solution. For example, here we use 70 µl of TMB solution and then 70 µl of stop solution, which gives a total of 140 µl, hence the 200 µl of blocking solution used. If less of the developing solution will be used (e.g. 50 µl and 50 µl), then less blocking solution is required, especially when using more expensive blocking solutions.
30. It is not recommended to make serial dilutions directly in the ELISA plate to prevent dislodging the coat.
31. Aim for 20% DMSO in the first well of the sample row. For instance, if there are 30 µl of sample in DMSO, then add this to 120 µl of plain TEA buffer to bring it to the correct amount of DMSO. We find that DMSO concentrations higher than 25% are detrimental to the mAbs.
32. Our stock solutions of synthetic mycolactone are typically at 1 mg/ml therefore 1.5 µl of this is needed for 150 µl of running buffer to give a concentration of 10 µg/ml.
33. Mycolactone is light-sensitive, therefore these steps must be done in the dark. There should be no overhead fluorescent/incandescent light or direct sunshine on the plate while it is being manipulated.
34. Our stock solution of biotinylated mycolactone (MG-161) is typically at 1 mg/ml, therefore 0.8 µl of this is needed for 10 ml (sufficient for one full 96-well plate)

of running buffer to give a concentration of 80 ng/ml. It may be better to make a ten-fold (or higher) dilution of the stock to avoid errors that could arise from pipetting small volumes.

35. One underlying principle of a competitive ELISA is the equilibrium between the analyte and the target or reporter molecule. For this reason, it is necessary for both to be present in the same reaction medium so that the equilibrium interaction with the mAb can occur. Inadvertently washing the plate in-between will not invalidate the assay but will, however, make it more difficult to interpret. Should this be the case, reduce the incubation time for the reporter molecule and keep all other downstream steps the same.
36. Take care with the plate since it would be twice as full after this step. It is not mandatory to mix in the wells, but mixing can, anyway, be carefully done by pipetting up and down a few times. Take care not to disturb the coated mAbs with the pipette tips.
37. We find plain PBST to be sufficient. If SuperBlock was used as blocking buffer, this can also be used here as well. Do not use any buffer containing DMSO!
38. You would need to have done a prior titration experiment to determine the right dilution to use. We find a 1:5000 dilution to be sufficient but this is subject to the supplier.
39. DMSO is detrimental to the coupling of streptavidin and horseradish peroxidase, therefore, it is imperative that the plate be washed thoroughly. Remember the general rule of thumb in **Note 18**.
40. Only prepare this right before use and keep protected from light, as TMB is very light-sensitive.
41. The timing is crucial as the level of background increases with longer incubation. We recommend starting the timer once the first row has been filled. Fill each

row sequentially, and if multiple plates are being tested, these should also be filled in a defined order.

42. Once the timer reaches 5 min, stop the reaction in the same order that the wells were filled with TMB. This would ensure that no well would be developed much longer than the others would.

43. This is because the absorbance seen is caused by the binding of the biotinylated structure to the coated antibody. Recall that this is just a biotinylated version of mycolactone, therefore, both can be recognised by the coating mAb. Also, the biotinylated structure is added at a constant concentration while the mycolactone is in different concentrations in the dilution series. Thus, the more mycolactone is present, the less of the biotinylated structure can be bound and, therefore, the less signal is seen. This is the underlying principle of a competitive ELISA.

44. The curves for the equations $y = m/x$ and $y = m/x^2$ look very similar. Thus, you would need to try plotting y against the inverse of x ($1/x$) or $x^2$ ($1/x^2$) to see which one gives you a linear curve.

45. Bear in mind that the concentration you would get would be that of the sample that was in the ELISA plate. To get the concentration of the undiluted sample, you would need to account for the dilution of the sample. For instance, 30 µl of sample in 150 µl is a 1-in-5 dilution, therefore the concentration of mycolactone in the undiluted sample would be 5 times the value calculated from the regression equation. If, however, the entire 150 µl of sample was used for the assay, then the mycolactone concentration is the value directly calculated from the regression equation.

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## Figures

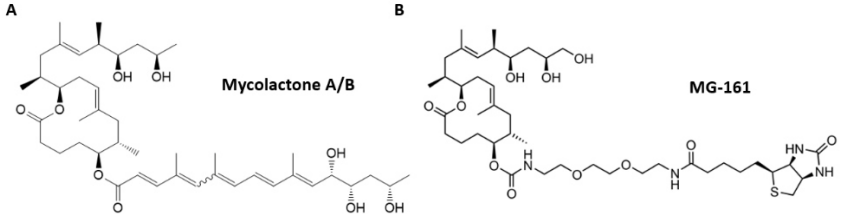


**Figure 1**. (A) Structure of mycolactone A/B. (B) Structure of the reporter molecule MG-161; the lower side chain of mycolactone A/B has been replaced with biotin and a linker.

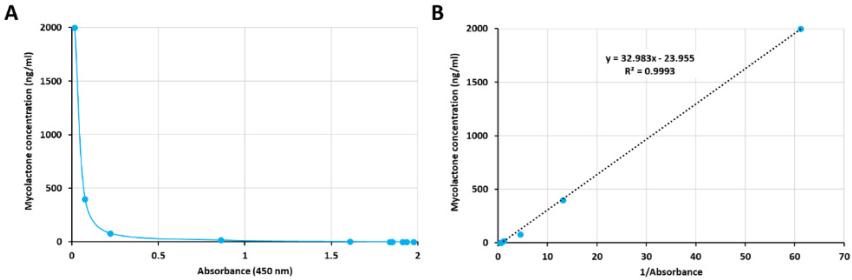


**Figure 2**. (A) Example of a mycolactone competitive ELISA standard curve. (B) Linearized mycolactone competitive ELISA standard curve. Linearization was achieved by plotting the concentration of the standard against the reciprocal of the mean absorbance as a scatterplot. A linear trendline was added; the resultant regression equation (i.e. y = 32.983x – 23.955) and R-squared confidence value (i.e. 0.9993) are indicated on the graph.

# An antigen capture assay for the detection of mycolactone, the polyketide toxin of *Mycobacterium ulcerans*

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**ABSTRACT**

Mycolactone is a cytotoxin responsible for most of the chronic necrotizing pathology of *Mycobacterium ulcerans* disease (Buruli ulcer). The polyketide toxin consists of a 12-membered lactone ring with a lower O-linked polyunsaturated acyl side chain and an upper C-linked side chain. Mycolactone is unique to *M. ulcerans* and an immunological antigen capture assay would represent an important tool for the study of Buruli ulcer pathogenesis and for laboratory diagnosis. When testing sets of mycolactone-specific mouse mAbs, we found that antibodies against the hydrophobic lower side chain only bind mycolactone immobilized on a solid support, but not when present in solution. This observation supports previous findings that mycolactone forms micellar structures in aqueous solution with the hydrophobic region sequestered into the inner core of the aggregates. Although an antigen capture assay typically requires two antibodies which recognize non-overlapping epitopes, our search for matching pairs of mAbs showed that the same mAb could be used both as capture and as detecting reagent for the detection of the mycolactone aggregates. However, the combination of a core-specific and a core/upper side chain specific mAb constituted the most sensitive ELISA with a sensitivity in the low nanogram range. Results of a pilot experiment showed that the sensitivity of the assay is sufficient to detect mycolactone in swab samples from Buruli ulcer lesions. While the described capture ELISA can serve as a tool for research on the biology of mycolactone, the assay system will have to be adapted for use as diagnostic tool.

**Key points**

Sets of mAbs specific for the macrolide toxin mycolactone have been generated

Two selected mAbs enabled development of a highly sensitive capture ELISA

The assay has potential for conversion into a rapid diagnostic test for Buruli ulcer

## INTRODUCTION

Buruli ulcer (BU) – or *Mycobacterium ulcerans* disease – is a chronic necrotizing disease of skin and soft tissue [1] whose pathology is mediated by a plasmid-borne cytotoxin known as mycolactone [2, 3]. Acquisition of the pMUM plasmid, which encodes the machinery necessary for mycolactone biosynthesis, led to the divergence of *M. ulcerans* from a common ancestor with the closely related *M. marinum*, and is a defining characteristic of the species [4, 5]. As such, all pMUM-containing mycobacteria are referred to as the mycolactone-producing mycobacteria (MPMs) [6]. Mycolactone is a polyketide cytotoxin with immunosuppressive and analgesic properties, and at least six different variants of mycolactones have been described, all of which comprise an invariant lactone core with a C-linked short upper side chain, which is esterified with a longer lower acyl side chain [2, 3]. As mycolactone is unique to the MPMs, it is an ideal target for the specific diagnosis of BU. Monoclonal antibodies (mAbs) targeting the core and/or the short upper side chain could allow detection of all known mycolactone variants, due to the structural invariance in these parts of the molecules.

BU control is contingent upon early diagnosis and prompt treatment initiation. BU diagnosis currently hinges on the PCR detection of the *M. ulcerans*-specific insertion sequence (IS) *2404* in clinical samples. Clinical diagnosis is complicated by the myriad differential diagnosis characteristic of the clinical picture [7, 8]. The only decentralized laboratory diagnosis for the confirmation of clinical diagnosis currently available is the microscopic detection of acid-fast bacilli in wound swab samples or fine needle aspirates [9]. While this is a low-cost and relatively easy to perform method, it has low sensitivity. The highly specific and sensitive IS*2404* PCR requires sophisticated laboratory equipment and well-trained laboratory personnel and is, in low-resource settings, typically only available in few reference centers. This in turn leads to delays in diagnosis and treatment, thus thwarting BU control. Moreover, PCR analysis is unreliable unless performed under strict quality control [10]. An easier-to-use rapid diagnostic test (RDT)

is, therefore, in urgent need for use in peripheral and field settings in low-resource BU endemic regions.

We recently reported the generation of mAbs capable of specific binding to mycolactone and developed an antigen competitive immunoassay based on these mAbs [11, 12]. Although this assay is highly specific and sensitive, competitive assays do have a few shortcomings compared to antigen capture assays. For one, by using only one mAb, competitive assays have a higher probability of cross-reactivity (i.e. lower specificity) compared with antigen capture assays which usually use two mAbs of different fine specificities [13, 14]. Also, the need to have a suitable reporter molecule for competitive assays, like a biotinylated mycolactone variant [12], increases the complexity of scale-up for such an assay. Mycolactone is a notoriously difficult molecule to synthesize, even though several total syntheses strategies have been reported [15 – 18]. Finally, conversion of immunoassays into point-of-care lateral flow formats is more facile for antigen capture than for competitive assays. For these reasons, we set out to develop a capture assay for mycolactone detection, which could, in future, be converted into an RDT.

## MATERIALS AND METHODS

### Ethical statement

Approval for the collection of swab samples for BU diagnosis was obtained from the Cameroonian Comité National D'Ethique de la Recherche pour la Santé Humaine. Immunogenicity studies in mice and generation of mAbs were performed under the approval by the animal welfare committee of the Canton of Basel-Stadt (authorization number BE95/17). All animal experimentation were conducted in compliance with the Swiss Animal Welfare Act (TSchG), Animal Welfare Ordinance (TSchV), and the Animal Experimentation Ordinance (TVV).

### Preparation of synthetic mycolactones

The chemical synthesis of mycolactone and mycolactone derivatives (Fig. 1) has been described elsewhere [15, 16, 19]. All synthetic products were HPLC-purified and dissolved in dimethyl sulfoxide (DMSO) to give 1 mg/ml stock solutions.

### Monoclonal antibody generation

Mouse immunization and hybridoma generation were essentially done as described previously [11]. Mouse immunization was done either with PG-180 or PG-203 (Fig. 1) coupled to BSA with the carbodiimide cross-linker EDC. Hybridoma selection was done by ELISA using a panel of biotinylated mycolactone derivatives comprising MG-158, MG-160, MG-161, PG-183, and PG-204 as target antigens (Fig. 1). For the selection tests, NeutrAvidin-coated plates (Thermo Scientific) were coated with each mycolactone derivative at a concentration of 1 µg/ml (100 µl/well) overnight at 4°C. Plates were washed three times with washing buffer (dd$H_2O$ with 0.3% Tween-20) and blocked with SuperBlock T20 (Thermo Scientific) for 1 h at 37°C. Hybridoma culture supernatants were added and incubated for 2 h at 37°C, and after washing as described above, bound antibodies were detected by incubating with goat anti-mouse IgG antibodies coupled to

horseradish peroxidase (HRP, SouthernBiotech) for 1 h at 37°C. Plates were again washed and signal development was done with 3,3',5,5'-tetramethylbenzidine (TMB; KPL SeraCare, catalog number 5120-0047) for 7 min, after which the reaction was stopped with 0.5 M sulfuric acid. Selected hybridoma lines were cloned twice by limiting dilution and mAbs were purified from culture supernatants by affinity chromatography using HiTrap Protein A HP columns (GE Healthcare) and a low-pressure liquid chromatography system (Model EP-1 Econo pump; Bio-Rad Laboratories). All the mAbs were of $IgG_1$ isotype, except for LW1.1b, which was $IgG_{2b}$. Purified mAbs were dialyzed against PBS in Slide-A-Lyzer dialysis cassettes (Thermo Scientific) for 24 h and then sterile-filtered and stored at 4°C until needed, or at -80°C for long-term storage.

**Analysis of the binding pattern of mAbs**

Binding of purified mAbs to the panel of biotinylated mycolactone derivatives used for hybridoma selection (Fig. 1) was assessed by ELISA. NeutrAvidin-coated plates (Thermo Scientific) were coated with each mycolactone derivative at a concentration of 1 µg/ml (100 µl/well) overnight at 4°C. Plates were washed three times with washing buffer ($ddH_2O$ with 0.3% Tween-20) and blocked with SuperBlock (Thermo Scientific) for 1 h at 37°C. Meanwhile, 5-fold serial dilutions of each purified mAb from a starting concentration of 10 µg/ml were prepared in PBST. The ELISA plate was washed as described above and the mAb dilutions were then added in. After incubating for 2 h at 37°C, plates were washed and bound antibodies were detected by incubating with goat anti-mouse IgG antibodies coupled to HRP (SouthernBiotech) for 1 h at 37°C. Plates were again washed and signal development was done using TMB (KPL SeraCare) for 7 min after which the reaction was stopped with 0.5 M sulfuric acid.

**Biotinylation of mAbs**

mAbs at a concentration of 0.5 mg/ml were biotinylated using EZ-link Sulfo-NHSLC-Biotin (Thermo Scientific). Briefly, a Sulfo-NHSLC-Biotin solution at 5.7 mg/ml was prepared by dissolving the appropriate amount of Sulfo-NHSLC-Biotin powder in DMSO. For every 100 µl of mAb, 3 µl of the Sulfo-NHSLC-Biotin solution was added and the mixture was incubated at ambient temperature for 30 min on a shaker, and then dialyzed against PBS for 24 h. The biotinylated mAb was recovered and stored at 4°C until needed.

**Mycolactone extraction from *M. ulcerans* cultures**

Ethanolic extracts were prepared from eight-week old cultures of an African *M. ulcerans* strain (S1013) cultivated on Middlebrook 7H9 agar plates supplemented with 0.2% glycerol and 10% OADC [20]. Colonies were scraped from 4 well-grown plates with a sterile inoculating loop, transferred into 15 ml of absolute ethanol (Sigma), and incubated at ambient temperature for 3 days protected from light. Afterwards, the suspension was vortexed for 1 min and centrifuged at 4,000 x *g* for 10 min to pellet the bacterial debris. The supernatant was filtered through sterile 0.22 µm syringe filters and dried by vacuum centrifugation (SpeedVac, Thermo Scientific). The resulting mycolactone preparations were resuspended in DMSO and stored at -20°C until needed.

**Mycolactone extraction from swab samples collected from BU lesions**

Swab samples were taken from ulcerative BU lesions as described [21] and were extracted, after long-term storage and transport, in 500 µl of PBS by vigorous bead-vortexing. qPCR analysis was done with DNA extracted from 50 µl of the sample, as described [22]. Lipid extracts were prepared from 400 µl of the sample as described previously [11]. Briefly, each sample was divided into 50 µl aliquots, and 950 µl of a chloroform-methanol (2:1, v/v) solution was added to each aliquot. Samples were incubated for 2 h at 25°C with shaking, after which 200 µl of dd$H_2O$ was added to induce

a phase separation, and the samples were vigorously vortexed. After centrifuging for 10 min at 13,300 x *g*, the lower organic phase was transferred to a fresh tube and dried by vacuum centrifugation (SpeedVac, Thermo Scientific). The dried pellets were resuspended in 200 µl of ice-cold acetone, vigorously vortexed, and centrifuged at 13,300 x *g*. The supernatants for all aliquots of each sample were pooled into a fresh tube and again dried by vacuum centrifugation (SpeedVac, Thermo Scientific). The resulting lipid extract was stored at -20°C until needed.

**Mycolactone capture ELISA**

**i. For synthetic or extracted mycolactones**

MaxiSorp plates (Thermo Scientific) were coated with a capturing mAb at a concentration of 4 µg/ml (100 µl/well) in PBS and incubated overnight at 4°C. Plates were then washed three times with washing buffer (dd$H_2O$ with 0.3% Tween-20) and blocked with SuperBlock T20 (Thermo Scientific) for 1 h at 37°C. Serial dilutions of (synthetic or extracted) mycolactone were prepared in a triethanolamine (TEA) buffer (0.2 M TEA, pH 7.5, with 20% DMSO). After washing the plates as described above, the mycolactone dilution series were added to the blocked plate and left to incubate for 2 h at 37°C. Plates were washed again, biotinylated mAb (2 µg/ml) in the TEA buffer was added, and incubated for 1.5 h at 37°C. Bound biotinylated mAb was detected using HRP-conjugated streptavidin (SouthernBiotech) diluted 1:5,000 in PBST and incubated for 1 h at 37°C. After a final washing step, plates were developed with TMB incubated at ambient temperature for 7 min, after which the reaction was stopped with 0.5 M sulfuric acid. Absorbance was measured at 450 nm with an ELISA microplate reader (Tecan Sunrise), and results were illustrated using GraphPad Prism version 8 (GraphPad Software, San Diego, CA) or R (version 3.6.1, package tidyverse).

**ii. For *M. ulcerans* culture filtrates**

Culture filtrates of two *M. ulcerans* strains isolated from BU lesions of patients from Cameroon (strain S1013) and Australia (strain S1251) were tested [23]. *M. ulcerans* strains were cultured in BacT/Alert® MP liquid medium (Biomerieux) for at least 8 weeks. Cultures were harvested and spun at 13,300 x *g* to pellet the bacteria, and the supernatants were filtered through sterile 0.22 µm syringe filters. Culture filtrates were used directly in the assay without any mycolactone extraction. Briefly, MaxiSorp plates (Thermo Scientific) were coated with a capturing mAb at a concentration of 4 µg/ml (100 µl/well) in PBS incubated overnight at 4°C. Plates were then washed three times with washing buffer (dd$H_2O$ with 0.3% Tween-20) and blocked with SuperBlock T20 (Thermo Scientific) for 1 h at 37°C. Serial dilutions of culture filtrates were prepared in a TEA buffer (0.2 M TEA, pH 7.5, with or without 20% DMSO), starting from the undiluted filtrate and proceeding in 2-fold dilutions down the series. The rest of the assay was done as described above.

**iii. For samples containing serum**

The assay was optimized using a modified TEA buffer containing varying concentrations of the chaotropic salts magnesium chloride ($MgCl_2$), magnesium sulfate ($MgSO_4$), or ammonium thiocyanate ($NH_4SCN$), in the presence of up to 50% fetal bovine serum (FBS) or human serum. Where necessary, tyramide amplification was done to improve signals. Tyramide amplification is a signal enhancement method used for assays involving HRP, including ELISA and Western blots. In principle, HRP in the presence of hydrogen peroxide catalyzes the activation of labelled tyramide substrates (in this case, biotinylated tyramide), which then rapidly bind to nearby tyrosine residues (e.g. in HRP). This, therefore, increases the number of biotin molecules present in the reaction, thus increasing the overall signal gotten at the end of the assay.

Assays involving tyramide amplification were done as outlined below. MaxiSorp plates (Thermo Scientific) were coated with a capturing mAb at a concentration of 4 µg/ml (100 µl/well) in PBS incubated overnight at 4°C. Plates were then washed three times with washing buffer (dd$H_2O$ with 0.3% Tween-20) and blocked with PBST containing 3% bovine serum albumin (3% BSA-PBST) for 1 h at 37°C. Serial dilutions of extracted or synthetic mycolactone were prepared in a TEA buffer without DMSO (0.2 M TEA, pH 7.5), containing the different chaotropic salts and up to 50% serum from healthy donors. After washing the plates as described above, the dilutions were added to the blocked plate and left to incubate for 2 h at 37°C. Plates were washed again, biotinylated mAb (2 µg/ml) in the test assay buffer was added, and incubated for 1.5 h at 37°C. The plate was washed and HRP-conjugated streptavidin (SouthernBiotech) diluted 1:50,000 in 1% BSA-PBST was added and incubated for 1 h at 37°C. After washing, biotinyl tyramide (Sigma) prepared at a concentration of 1 µg/ml (70 µl/well) in a citrate buffer containing 0.02% hydrogen peroxide (KPL Seracare, catalog number 5120-0047) was added to the plate and incubated at ambient temperature for 15 min. The plate was again washed and HRP-conjugated streptavidin (SouthernBiotech) diluted 1:50,000 in 1% BSA-PBST was added and incubated at ambient temperature for 15 min. After a final washing step, plates were developed with TMB (KPL SeraCare, catalog number 5120-0047) incubated at ambient temperature for 5 min, after which the reaction was stopped with 0.5 M sulfuric acid. Absorbance was measured at 450 nm with an ELISA microplate reader (Tecan Sunrise), and results were illustrated using GraphPad Prism version 8 (GraphPad Software, San Diego, CA).

For extracts from swab samples, MaxiSorp plates (Thermo Scientific) were coated with a capturing mAb at a concentration of 4 µg/ml (100 µl/well) in PBS incubated overnight at 4°C. Plates were then washed three times with washing buffer (dd$H_2O$ with 0.3% Tween-20) and blocked with 3% BSA-PBST for 1 h at 37°C. Lipid extracts from swabs were

resuspended in 150 µl of assay buffer (0.2 M TEA with 0.2 M $MgCl_2$, pH 7.5) containing a mixture of mouse antibodies (1 mg/ml), and added to blocked plates. After incubating for 2 h at 37°C, plates were washed and biotinylated mAb (2 µg/ml) in assay buffer was added, and incubated for 1.5 h at 37°C. Plates were washed and HRP-conjugated streptavidin (SouthernBiotech) diluted 1:5,000 in 1% BSA-PBST was added and incubated for 1 h at 37°C. After washing, biotinyl tyramide (Sigma) prepared at a concentration of 1 µg/ml (70 µl/well) in a citrate buffer containing 0.02% hydrogen peroxide (KPL Seracare, catalog number 5120-0047) was added to the plate and incubated at ambient temperature for 15 min. The plate was again washed and HRP-conjugated streptavidin (SouthernBiotech) diluted 1:25,000 in 1% BSA-PBST was added and incubated at ambient temperature for 15 min. After a final washing step, plates were developed with TMB (KPL SeraCare, catalog number 5120-0047) incubated at ambient temperature for 7 min, after which the reaction was stopped with 0.5 M sulfuric acid. Absorbance was measured at 450 nm with an ELISA microplate reader (Tecan Sunrise), and results were illustrated using GraphPad Prism version 8 (GraphPad Software, San Diego, CA).

## RESULTS

### mAb generation and analysis of their fine specificity

For the design of an antigen capture assay usually a capture and a detecting mAb which recognize two non-overlapping epitopes on the analyte are required. We, therefore, aimed at generating mAbs with different fine specificity for the relatively small mycolactone A/B molecule, which has a molecular mass of 743 Da. Two different immunogens – PG-180 with intact lower side chain and an upper side chain that is extended by a linker, and PG-203 with unmodified upper side chain and the lower chain replaced by a linker (Fig. 1) – were used to generate sets of mAbs with varying fine specificities (Table I). One of these sets of mAbs (JD5.1 – JD 5.12) has been previously described [11, 12].

Immunizing mice with PG-180 led to the generation of two subsets of mAbs: subset 1 mAbs recognized PG-183 – the biotinylated variant of PG-180 – but none of the derivatives which had the lower side chain replaced by a linker moiety. Subset 2a mAbs, recognized all mycolactone variants with an intact core (MG-158, MG-160, MG-161, PG-183 and PG-204). Binding was not influenced by modifications of the upper chain with the exception of PG-183 (with the upper chain being extended by the linker), which was only recognized with low affinity. Immunization with PG-180 thus seems to have generated mAbs that recognized primarily the hydrophobic lower part of mycolactone.

Three subsets of mAbs were obtained from PG-203 immunized mice. As expected, all of them recognized PG-204 – the biotinylated variant of PG-203 – and also MG-161, a PG-204 derivative with only a minor modification of the upper side chain (Fig. 1). Two mAbs (LW7.1 and 7.2) showed the same fine specificity as the subset 2a mAbs obtained after PG-180 immunization. Fine specificity of subset 2b mAbs resembled subset 2a mAbs in that they recognized all mycolactone variants with an intact core, but in contrast to the latter, their binding to PG-183 was also strong. Subset 3 mAbs were obtained from two

different PG-203-immunised mice, and include mAbs JD5.1 – JD5.12, which have been described before [11, 12]. This subset recognized PG-204 and MG-161, and to varying degrees, the mycolactone derivatives MG-158 and MG-160 with strongly modified upper side chain (Fig. 1). Seven mAbs within this subset (JD5.2, JD5.3, JD5.6, JD5.7, JD5.12, LW7.10, and LW7.11) were likely recognizing parts of the upper side chain distal from the core, as evidenced by their recognition of MG-161 and PG-204 (with little or no modification of this side chain), and lack of or only slight recognition of the remaining derivatives (which have more extensive modifications of this side chain). Immunization with PG-203 thus seems to have generated mAbs that recognized either primarily the core or the upper side chain of mycolactone.

Further analyses revealed that subset 1 mAbs, i.e. all those which primarily recognize the hydrophobic side chain of mycolactone, were only able to bind to PG-183 (which has the hydrophobic side chain intact) when it was affixed to a solid support, but not when it was in aqueous solution. In contrast, all other mAbs, i.e. those which primarily recognized the lactone core and/or the upper side chain of mycolactone, were able to recognize mycolactone derivatives both on solid support and in solution. This is consistent with findings that indicate that due to its amphiphilic structure, mycolactone forms aggregates in aqueous solutions, with the hydrophobic side chain sequestered within the interior of these aggregates [24]. Consequently, mAbs recognizing the hydrophobic side chain are unable to recognize aggregated mycolactone. Only by affixing mycolactones with an unmodified lower side chain to a solid support, thereby preventing aggregation, can they be recognized by subset 1 mAbs.

**Selection of matched pairs of mAbs suitable for mycolactone capture assay development**

Mycolactone extracts were used to screen mAb pairs to select appropriate matched pairs. Typically, a capture assay is designed using two different mAbs each recognizing non-overlapping epitopes on the analyte in question. Thus, we were initially aiming for mAb pairs, where one mAb would bind to the lactone core while the other would recognize the upper side chain of mycolactone. However, mycolactone aggregates could potentially present epitope repeats, making it possible for the same mAb to function both as capturing and as detecting reagent. Therefore, we performed a screen with all feasible mAb pairs to identify both "mixed-pairs" (with each of the two mAbs recognizing a different part of the mycolactone structure) and "same-pairs" (with both mAbs recognizing parts of the same global region of the mycolactone structure). Indeed, when screening 1,360 mAb pairs to identify matched pairs suitable for an antigen capture assay, we confirmed that both "mixed-pairs" and "same-pairs" could function as matched pairs (Fig. 2). Of the 1,360 mAb pairs, 213 potentially suitable matched pairs were identified.

Generally, these 213 matched pairs featured mAbs from subsets 2b and 3, and no pairings involving mAbs from subsets 1 and 2a functioned as matched pairs. There were 14 mAbs, comprising 9 subset 2b and 5 subset 3 mAbs, which were able to function both as capturing and detecting mAb in the same reaction. Except for mAb LW7.15, which gave high background signals, and mAbs JD5.11 and LW7.16, all subset 2b and 3 mAbs were present in at least one matched pair. Altogether, 9 of the 12 subclass 2b and 6 of the 16 subset 3 mAbs were involved in at least 10 matched pairs. Some mAbs showed marked differences in their suitability as detecting versus coating mAbs, as for example the subset 3 mAb JD5.10, which was present in 20 matched pairs as detecting mAb (more than any other mAb), but only in 8 pairs as coating mAb.

Of the 213 matched mAb pairs, 144 gave strong signals ($OD_{450}$>1.5) and did not show a prozone effect at relatively low concentrations of mycolactone and were thus selected for the next screening step. In this step, matched pairs that could detect the potentially non-aggregating mycolactone molecule PG-120 with a truncated lower side chain (Fig. 3A) were selected. Both intact mycolactone and mycolactone fragments that have lost the light-sensitive lower side chain may be present in biological samples and matched pairs that recognize PG-120 should be able to detect both.

Following this screen (Fig. 3), matched pairs comprising the subset 3 mAb LW7.11 as a capturing mAb and any of the (core-specific) subset 2b mAbs LW7.5 – LW7.9 as detecting mAb were prioritized for further tests. We have previously shown a TEA-based buffer to be beneficial for a mycolactone competitive ELISA, yielding improved assay sensitivity compared to a PBST-based buffer [12]. Interestingly, in the current mycolactone capture ELISA, the TEA-based buffer was crucial for mycolactone detection as little or no signals were obtained with a PBST-based buffer. Including DMSO into the buffer (i.e. 0.2 M TEA buffer with 20% DMSO) worked well for most mAb pairs and, in general, mAb pairs recognizing the mycolactone core were more likely to require added DMSO than pairs where one mAb was binding to the short upper side chain of mycolactone. However, some pairs (notably those comprising mAbs LW7.10 and LW7.11) performed better without addition of DMSO (Supplemental Fig 1), leading to the selection of the TEA buffer without DMSO for further analyses involving these mAbs.

**Sensitivity of mycolactone capture assays with different pairs of mAbs**

The sensitivity of capture assays with selected pairs of mAbs was estimated using synthetic mycolactone A/B. While most tested pairs could detect as little as about 2 ng of mycolactone in the assay volume of 75 µl, other matched pairs (such as LW7.12-LW7.5) showed a lower sensitivity (Fig. 4). Based on these results, the matched pair

comprising LW7.11 as capturing mAb and LW7.5 as detecting mAb was selected for further assays.

**Detection of mycolactone in *M. ulcerans* culture filtrates**

In a next step, the LW7.11-LW7.5-based assay was used to detect mycolactone in biological samples without prior lipid extraction. Mycolactone could be detected directly in culture filtrates of African (S1013) and Australian (S1251) *M. ulcerans* strains, which produce primarily mycolactone A/B or mycolactone C, respectively (Fig. 5).

**Serum compatibility of the mycolactone capture assay**

Given that diagnostic samples collected from BU lesions are serum-rich wound exudates or aspirates, and since mycolactone is known to be bound by serum proteins [24], we assessed whether the capture assay is able to recognize mycolactone in the presence of serum proteins. Matrix interference by serum proteins is a common finding during capture ELISA development and may be ascribed to a variety of reasons. Potential causes include the crosslinking of the capturing and detecting mAbs, or the binding of the target antigen to serum components, thus preventing its interaction with one or both mAbs [13, 25].

Typical ways of removing matrix interference include purifying the antigen or diluting the sample, both of which help to remove the interferents from the assay. However, diluting the sample may drive the mycolactone concentration below the limit of detection of the assay, and performing lipid extraction for every sample is an added complication. A more straightforward way of dealing with matrix interference is to use suitable chaotropic agents that are capable of breaking the (typically) low-affinity interactions of the interferents [25]. We modified our assay buffer by adding different concentrations of magnesium chloride ($MgCl_2$), magnesium sulfate ($MgSO_4$), or ammonium thiocyanate ($NH_4SCN$), all of which are commonly used chaotropic agents.

Although the addition of $MgSO_4$ gave better signals than $MgCl_2$, the former resulted in an unfavourable signal-to-noise ratio when tyramide amplification was done. Therefore, following extensive testing (Fig. 6), we defined a modified version of the assay buffer – comprising 0.2 M TEA and 0.2 M $MgCl_2$, pH 7.5 – as best suited for the detection of mycolactone in the presence of serum.

Human serum presents an extra challenge to antigen capture assays owing to the presence of anti-animal antibodies in the serum. Here, human anti-mouse antibodies (HAMA) led to crosslinking of the mycolactone-specific mAbs used in the assay, and this crosslinking could not be prevented by any of the chaotropic salts at any concentration tested. To this end, a mixture of mouse antibodies were instead added to the reaction mix to prevent the crosslinking of the mycolactone-specific mAbs used in the assay. Both the addition of the chaotropic salts and the addition of extraneous mouse antibodies to the reaction mix were crucial for preserving the mycolactone recognition in the assay in the presence of human serum. A final concentration of about 1 mg/ml of mouse antibodies was sufficient to reduce the crosslinking of the mycolactone-specific mAbs caused by HAMA. In addition, tyramide amplification was used to improve the signals obtained when the assay was performed in the presence of serum (Fig. 7).

In a pilot experiment, the utility of the assay in detecting mycolactones present in swabs obtained from IS*2404* qPCR-positive BU lesions was shown (Fig. 8). Five of seven qPCR-positive swab samples tested, with Ct values ranging from 15.5 – 26.5, yielded an absorbance above the 0.2 threshold of the ELISA. qPCR-negative controls tested negative.

**DISCUSSION**

The current gold standard assay for BU diagnosis is the highly specific and sensitive IS*2404*-detecting PCR. Although exquisitely sensitive owing to the high copy number of this insertion sequence in the *M. ulcerans* genome, routine application of this test is hampered by the necessity for sophisticated instrumentation with experienced personnel and rigorous quality control. As such, immunodiagnostic assays have been increasingly considered as practicable surrogates for molecular tests, as they have the advantage of being comparatively easier to perform and potentially low-cost, while still being able to give timely and reliable results [9]. Mycolactone makes an ideal target for the specific diagnosis of BU as it is unique to the MPMs, and an immunoassay that can reliably detect mycolactone could enable point-of-care laboratory diagnosis of BU.

We have generated panels of mAbs able to recognize mycolactone via a novel approach using modified synthetic non-toxic mycolactone variants with amine groups permitting the coupling of the polyketide to a carrier protein [11, 12]. Here, we have explored the use of mAbs with different fine specificities in the generation of an antigen capture assay for mycolactone detection.

That a macrolide can be detected in a sandwich ELISA with two full-sized mAbs is not unheard of. Indeed, such an assay was developed for the macrolide drug tacrolimus, which has a similar molecular mass (804 Da) as mycolactone (743 Da). For the generation of anti-tacrolimus mAbs, the use of truncated structures to constrain recognition to the desired epitopes was applied [26], a similar strategy which we have used for the generation of the anti-mycolactone mAbs used in this report. Selection of mAbs during hybridoma generation based on their reactivity with different mycolactone derivatives reinforced establishment of sets of mAbs with diverse fine specificity.

We used two different derivatives of mycolactone to generate the mAbs described here, each with modifications to either the upper side chain (PG-180) or the lower hydrophobic side chain (PG-203). In both mycolactone derivatives, the core is present in its native form, therefore, it was unsurprising to see that immunizing mice with either derivative gave rise to mAbs recognizing the lactone core of mycolactone. As expected, mAbs recognizing only PG-183 – the biotinylated form of PG-180 – were generated only when mice were immunized with PG-180. In contrast, immunizing mice with PG-203 gave rise to mAbs which recognized structures with modified upper chains to varying degrees. Taken together, we could cover a broad range of epitopes on the mycolactone structure.

The antigen capture assay herein described was able to recognize different natural variants of mycolactone, could recognize mycolactones secreted into *M. ulcerans* culture filtrates without prior lipid extraction, and could recognize mycolactone in the presence of serum. Although we only assessed the recognition of secreted mycolactones A/B and C, we would expect all natural variants of mycolactone to also be recognized in the assay, since they all share the same core and upper side chain structures. The prior development of a more efficient ELISA running buffer was crucial to this assay, since little or no signals could be obtained in the typically used PBS-based buffers [12]. We found that some mAb pairs performed better in the presence of 20% DMSO in the assay buffer, whilst others were indifferent to or impaired by its presence.

With its propensity to form aggregates (which present epitope repeats) in aqueous solutions, we could show that mycolactone can not only be detected by pairs of capturing and detecting mAbs with different fine specificity, but also by “same pairs” with the same specificity. In fact, 14 mAbs yielded mycolactone detection signals, when they were used both as capturing and as detecting reagents in the same reaction. However, careful selection of optimal capturing and detecting mAbs led to the

identification of a small number of combinations, where capturing and detecting mAbs were not identical.

Following systematic selection, we found that the best performing pairs contained the subset 3 mAbs LW7.10 or LW7.11 as capturing mAbs, and the subset 2b mAbs LW7.5 – 7.9 as detecting mAbs. Based on their binding patterns to mycolactone derivatives, these capturing mAbs most likely recognize parts of the mycolactone short upper side chain distal from the core. This is evidenced by the abrogation of their binding with increasing modification of this part of the mycolactone molecule. On the other hand, the detecting mAbs LW7.5 – LW7.9 most likely recognize the core of the mycolactone structure as they could recognize all derivatives containing this substructure to almost identical levels, independent of modifications of the upper side chain. Not surprisingly, therefore, such matched pairs were also able to recognize the derivative PG-120 with truncated lower side chain, potentially a non-aggregated mycolactone. This is an important characteristic, given that the lower side chain is known to be light-sensitive due to its conjugated double bonds [24, 27]. Changes in this hydrophobic side chain could yield structures similar to PG-120, therefore, it was expedient to identify matched pairs that could still recognize mycolactone molecules which have lost the hydrophobic side chain.

Mycolactone has been reported to be bound by serum proteins [24], and this can hamper its recognition by antibodies. Human serum, in addition, is rich in anti-animal antibodies causing matrix interferences well-known in the development of antigen capture assays [28]. We could get around these challenges by (i) incorporating a chaotropic salt into the assay system and (ii) adding extraneous mouse antibodies to suppress crosslinking of the mycolactone-specific mAbs by human anti-mouse antibodies (HAMA). The reduction of signal experienced in the presence of serum could be restored at least partially by including a tyramide amplification step into the protocol.

Further optimization could possibly be done by chimerizing the mAbs used as reagents in this assay to remove the chances of mAb crosslinking by HAMA.

The mycolactone capture assay could recognize as little as about 2 ng of mycolactone. This is comparable to what has been described for other mycolactone-detecting assays, such as 2 ng for f-TLC [29] and 1 ng for the competitive ELISA we recently described [12]. The amounts of mycolactone present in fine needle aspirates or tissue biopsies collected from different types of BU lesions have been estimated by liquid chromatography with tandem mass spectrometry (LC-MS/MS). Detected amounts ranged from 0 – 1970 ng/ml [30]. Samples from nodules and plaques on average had slightly higher detectable mycolactone than those from ulcers and oedema forms of the disease. Thus, the antigen capture assay herein described appears suitable for detection of mycolactone in clinical samples. This was confirmed in a pilot experiment by testing a small number of swab samples from BU patients. The swabs tested had been stored for an extended period of time, potentially leading to partial degradation of mycolactone. Nevertheless, the sensitivity of the present assay was sufficient to detect mycolactone in the majority of the qPCR-positive samples. However, for the use as diagnostic test with serum-protein-containing swab samples and fine needle aspirates from BU lesions, the ELISA conditions will have to be adapted further and validated in comparison to the current diagnostic gold standard for BU, IS*2404* PCR testing. Subsequent conversion of the ELISA into a lateral flow format that could be used as a RDT in field settings is envisioned.

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**FIGURE LEGENDS**

**Figure 1**. Mycolactone A/B and mycolactone derivatives used for mouse immunization and hybridoma selection.

**Figure 2. Screening for matched pairs of mAbs suitable for capture assay development**. Matched pairs shown in green (n = 213) were those pairs giving typical decaying ELISA curves and may thus have potential for capture assay development. Pairs with non-specific interactions shown in yellow (n = 183) were those which gave high signals even in the absence of mycolactone. These were mainly pairs, where subset 1 mAbs recognizing the hydrophobic lower side chain of mycolactone or the subset 2b mAb LW7.15 were involved. Pairs giving no signals are shown in red (n = 964). Subset 1 mAbs were only evaluated as detecting mAbs as they were not expected to function well as capturing mAbs. Two individual replicates of each ELISA was performed.

**Figure 3. Screening for recognition of mycolactones with complete or truncated hydrophobic side chain**. (A) Structure of PG-120. (B) Recognition of PG-120 and extracted mycolactone. Capturing mAbs coated on MaxiSorp plates were allowed to react with five-fold serial dilutions of PG-120 (starting from 5 µg/ml) and extracted mycolactone (starting from 0.1 µl) prepared in a TEA buffer. Detecting mAbs were then added and allowed to react. Bound mAbs were detected with HRP-conjugated streptavidin and TMB. Of the 144 tested pairs, results with the best performing 30 pairs are shown. Each ELISA was done once.

**Figure 4. Determination of the sensitivity of capture assays with synthetic mycolactone A/B for selected pairs of mAbs.** Capturing mAbs coated on MaxiSorp plates were allowed to react with two-fold serial dilutions of synthetic mycolactone A/B prepared in TEA buffer ± 20% DMSO (depending on mAb preference). Detecting mAbs were then

added in the respective test buffers and allowed to react. Bound mAbs were detected with HRP-conjugated streptavidin and TMB. The mean of two independent tests, with error bars indicating the range is shown.

**Figure 5. Recognition of native and synthetic mycolactones.** Capturing mAb LW7.11 coated on MaxiSorp plates was allowed to react with two-fold serial dilutions of *M. ulcerans* culture filtrates (starting from undiluted filtrate), extracted mycolactone, or synthetic mycolactone A/B (starting from 4 µg/ml) prepared in a 0.2 M TEA buffer. Detecting mAb LW7.5 was then added in and allowed to react. Bound mAbs were detected with HRP-conjugated streptavidin and TMB. The mean of two independent tests, with error bars indicating the range is shown.

**Figure 6. Effect of chaotropic salts on mycolactone recognition in the presence of serum.** Capturing mAbs LW7.10 or LW7.11 coated on MaxiSorp plates were allowed to react with two-fold serial dilutions of extracted mycolactones prepared in a 0.2 M TEA buffer (without DMSO) containing 50% FBS and chaotropic salts in varied concentrations. The biotinylated detecting mAb LW7.5 was then added in and allowed to react. Bound mAbs were detected with HRP-conjugated streptavidin and TMB. The mean of two independent tests, with error bars indicating the range is shown.

**Figure 7. Recognition of extracted mycolactones in the presence of human serum.** Capturing mAb LW7.11 coated on MaxiSorp plates was allowed to react with two-fold serial dilutions of extracted mycolactones, prepared in modified a TEA buffer containing 40% human serum and a mixture of mouse mAbs (1 mg/ml). Detecting mAb LW7.5 was then added in and allowed to react. Bound mAbs were detected with HRP-conjugated streptavidin and TMB. The mean of two independent tests, with error bars indicating the range is shown.

**Figure 8. Detection of mycolactones in clinical samples.** Capturing mAb LW7.11 coated on MaxiSorp plates was allowed to react with lipid extracts of qPCR-positive and qPCR-negative swab samples taken from BU lesions. Extracts were prepared in a modified TEA buffer containing a mixture of mouse mAbs (1 mg/ml). Serum samples (50% v/v with assay buffer) with and without extracted mycolactone were included as positive and negative controls, respectively. Detecting mAb LW7.5 was then added and allowed to react. Bound mAbs were detected with HRP-conjugated streptavidin and TMB. The results of seven qPCR-positive (P1 – P7) and two qPCR-negative (N1 – N2) are shown. The Ct values of the qPCR-positive samples were P1 (26.5), P2 (15.5), P3 (23.7), P4 (26), P5 (26.1), P6 (26), and P7 (21.2). Each ELISA was done once.

**Supplementary figure 1. Effects of buffer composition on mycolactone recognition by selected pairs of mAbs.** Capturing mAbs coated on MaxiSorp plates were allowed to react with two-fold serial dilutions of extracted mycolactone prepared in different assay buffers: TEA buffer or PBST ± 20% DMSO. Detecting mAbs were then added in the respective test buffers and allowed to react. Bound mAbs were detected with HRP-conjugated streptavidin and TMB. The mean of two independent tests, with error bars indicating the range is shown.

## TABLES

**Table 1. Recognition of different mycolactone derivatives by the generated sets of mAbs**. NeutrAvidin-coated plates were coated with the different biotinylated derivatives (1 µg/ml), serial dilutions of the different mAbs were allowed to bind, and bound mAbs were detected with an HRP-conjugated secondary antibody. Binding of mAbs at 10 µg/ml was graded based on the measured absorbance (OD) at 450 nm viz: (-) OD < 0.5, (+) OD 0.5-1, (++) OD >1-1.5, (+++) OD > 1.5.

| | Immunogen | mAb | PG-183 | MG-160 | MG-158 | MG-161 | PG-204 |
|---|---|---|---|---|---|---|---|
| Subset 1 | PG-180 | LW1.1a | +++ | - | - | - | - |
| | | LW1.1b | +++ | - | - | - | - |
| | | LW2.4a | +++ | - | - | - | - |
| | | LW2.4b | +++ | - | - | - | - |
| | | LW2.5a | +++ | - | - | - | - |
| | | LW2.5b | +++ | - | - | - | - |
| Subset 2a | PG-180 | LW2.1a | + | +++ | +++ | +++ | +++ |
| | | LW2.1b | + | +++ | +++ | +++ | +++ |
| | | LW2.2a | + | +++ | +++ | +++ | +++ |
| | | LW2.2b | + | +++ | +++ | +++ | +++ |
| | PG-203 | LW7.1 | + | +++ | +++ | +++ | +++ |
| | | LW7.2 | + | +++ | +++ | +++ | +++ |
| Subset 2b | PG-203 | LW7.3 | +++ | +++ | +++ | +++ | +++ |
| | | LW7.4 | +++ | +++ | +++ | +++ | +++ |
| | | LW7.5 | +++ | +++ | +++ | +++ | +++ |
| | | LW7.6 | +++ | +++ | +++ | +++ | +++ |
| | | LW7.7 | +++ | +++ | +++ | +++ | +++ |
| | | LW7.8 | +++ | +++ | +++ | +++ | +++ |
| | | LW7.9 | +++ | +++ | +++ | +++ | +++ |
| | | LW7.14 | +++ | +++ | +++ | +++ | +++ |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | **LW7.15** | +++ | +++ | +++ | +++ | +++ |
| | | **LW7.16** | ++ | +++ | +++ | +++ | +++ |
| | | **LW7.17** | +++ | +++ | +++ | +++ | +++ |
| | | **LW7.18** | +++ | +++ | +++ | +++ | +++ |
| **Subset 3** | **PG-203** | **JD5.1** | - | - | +++ | +++ | +++ |
| | | **JD5.2** | - | - | - | +++ | +++ |
| | | **JD5.3** | - | - | + | +++ | +++ |
| | | **JD5.4** | - | - | ++ | +++ | +++ |
| | | **JD5.5** | - | - | ++ | +++ | +++ |
| | | **JD5.6** | - | - | + | +++ | +++ |
| | | **JD5.7** | - | - | - | +++ | +++ |
| | | **JD5.8** | - | - | ++ | +++ | +++ |
| | | **JD5.9** | - | - | +++ | +++ | +++ |
| | | **JD5.10** | - | - | +++ | +++ | +++ |
| | | **JD5.12** | - | - | + | +++ | +++ |
| | | **LW7.10** | - | + | + | +++ | +++ |
| | | **LW7.11** | - | + | + | +++ | +++ |
| | | **JD5.11** | - | + | +++ | +++ | +++ |
| | | **LW7.12** | - | +++ | +++ | +++ | +++ |
| | | **LW7.19** | - | +++ | +++ | +++ | +++ |

## FIGURES

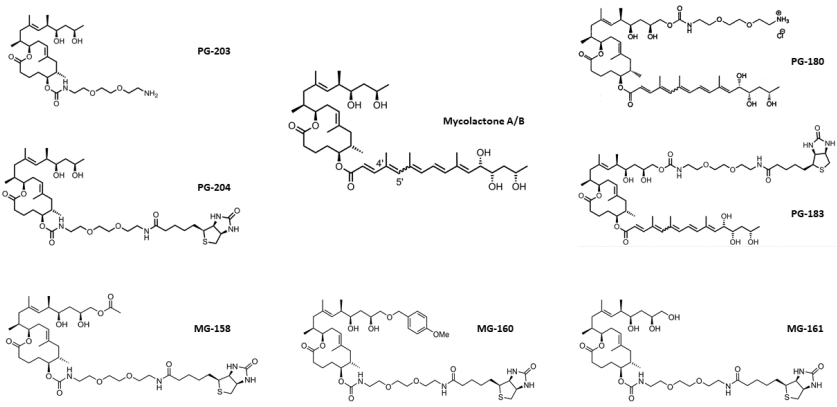
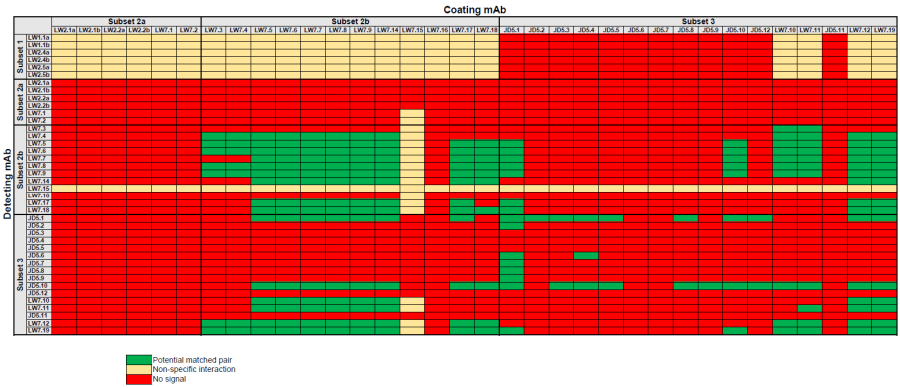



**Figure 1**.

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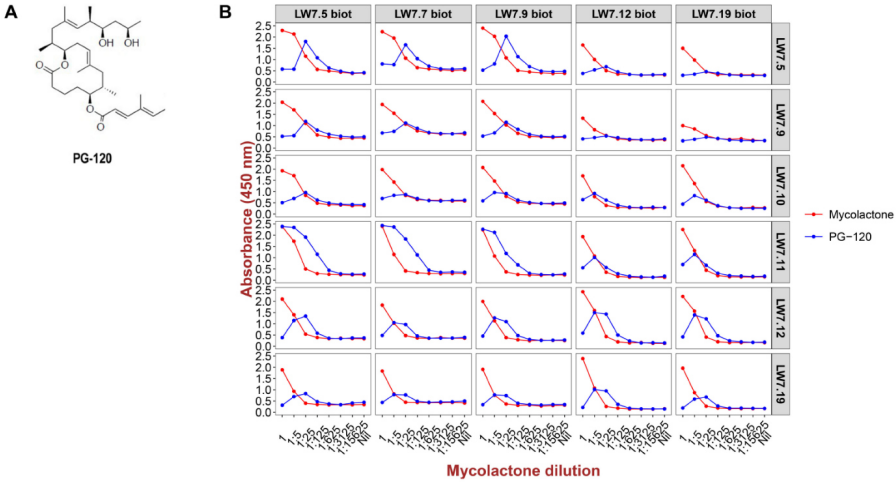

A
PG-120
B
LW7.5 biot
LW7.7 biot
LW7.9 biot
LW7.12 biot
LW7.19 biot
LW7.5
LW7.9
LW7.10
LW7.11
LW7.12
LW7.19
Absorbance (450 nm)
Mycolactone dilution
Mycolactone
PG−120

**Figure 3.**

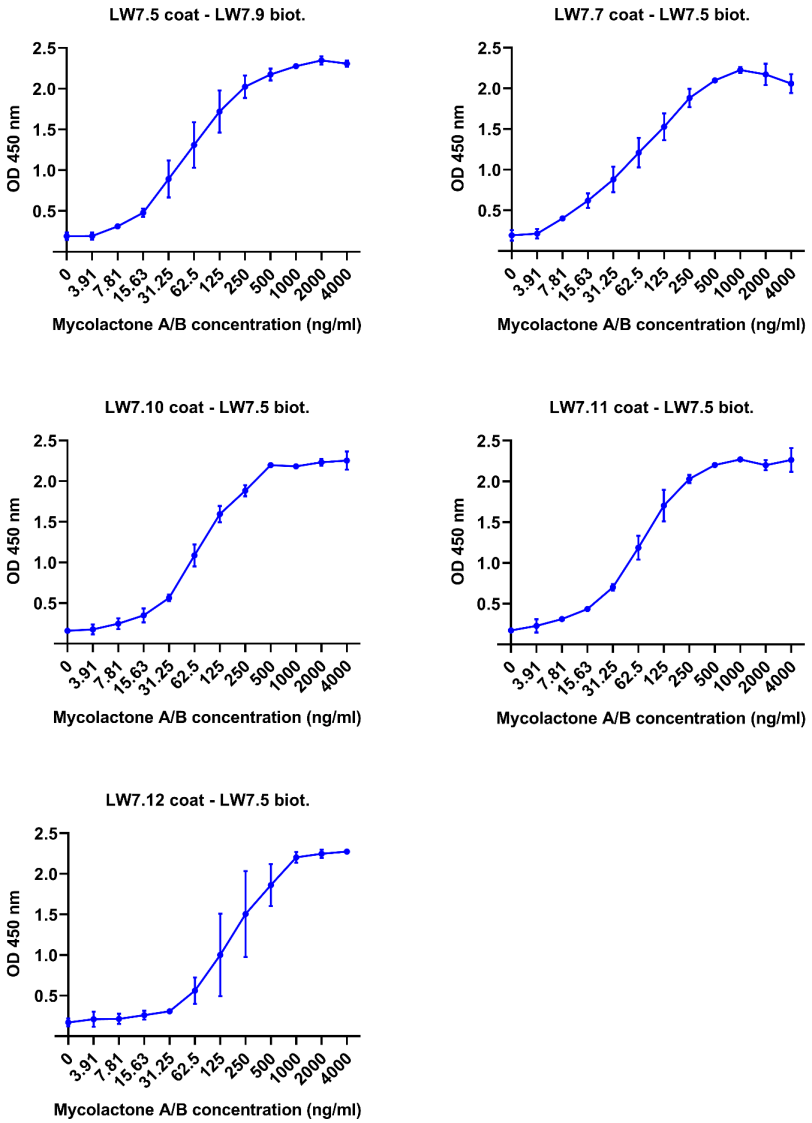

LW7.5 coat - LW7.9 biot.
LW7.7 coat - LW7.5 biot.
LW7.10 coat - LW7.5 biot.
LW7.11 coat - LW7.5 biot.
LW7.12 coat - LW7.5 biot.
OD 450 nm
2.5
2.0
1.5
1.0
0.5
0
3.91
7.81
15.63
31.25
62.5
125
250
500
1000
2000
4000
Mycolactone A/B concentration (ng/ml)

**Figure 4.**

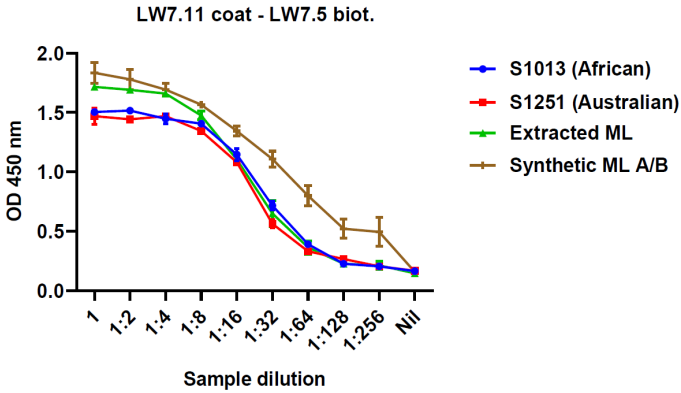
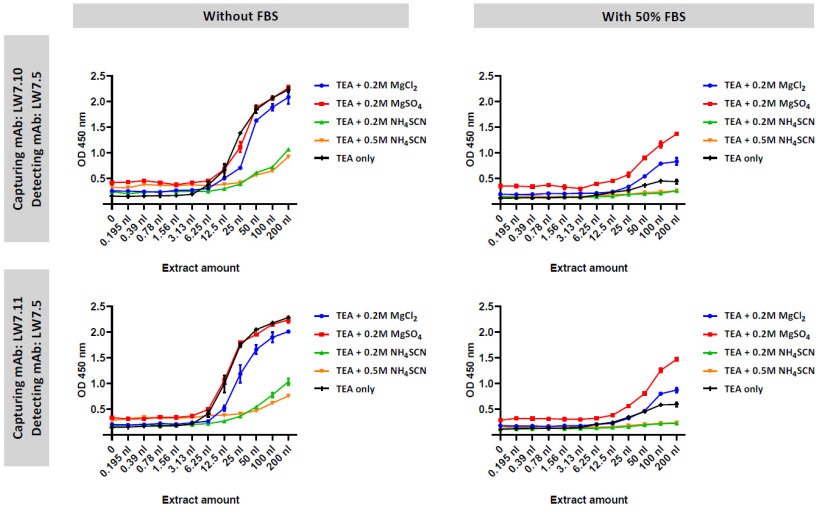



**Figure 5.**

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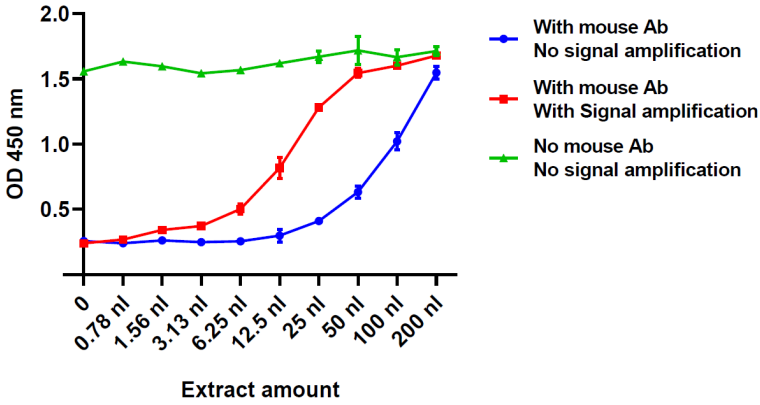



**Figure 7.**

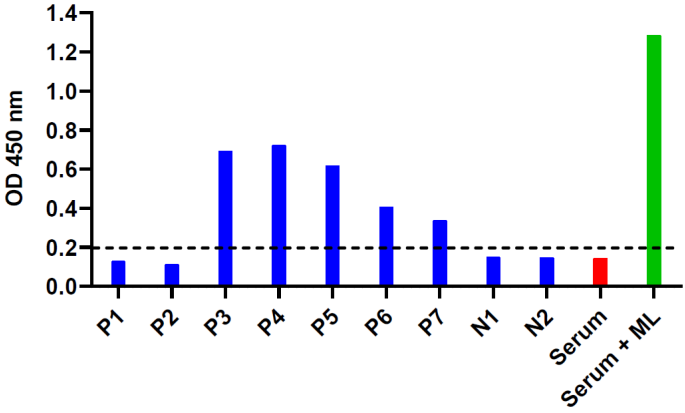
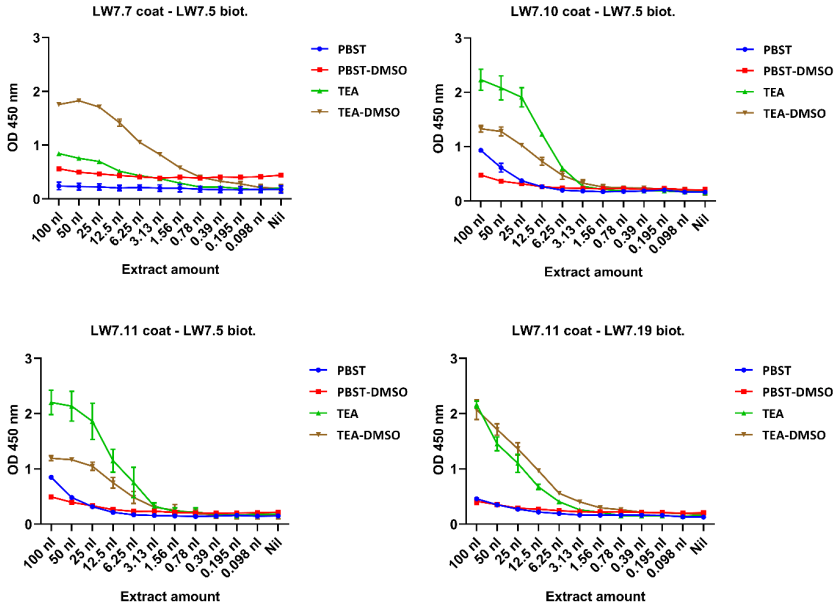



**Figure 8.**




**Supplementary figure 1.**

# A PMA-qPCR method for differentiating viable and dead *Mycobacterium ulcerans* cells

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Working manuscript

# Chapter 5: PMA-qPCR technique for *M. ulcerans*


## ABSTRACT

*Mycobacterium ulcerans* is an extremely slow-growing microorganism which makes experimentation time-consuming. Viability qPCR is increasingly used as one alternative to culture for the discrimination of live and dead microorganisms, especially for difficult-to-culture microorganisms. Such methods have been applied to a variety of cell types, including mycobacteria. Here, we have described a propidium monoazide-coupled qPCR (PMA-qPCR) methodology for the differentiation of live and dead *M. ulcerans*. The method herein described builds onto the well-established qPCR detecting the *M. ulcerans* insertion sequence 2404 (IS*2404*) and is therefore readily applicable in settings where the qPCR is already in use. Additionally, the method works reliably with the IS*2606* and ketoreductase (KR) biomarkers, making it potentially useful for studying *M. ulcerans* ecology. Furthermore, the PMA pretreatment can be applied to other genomic methods in development such as LAMP, making it a versatile alternative to microbial culture for sensitive live/dead discrimination of *M. ulcerans*.


## INTRODUCTION

The neglected tropical disease Buruli ulcer (BU) is a chronic necrotising disease of skin and soft tissue reported in more than 33 countries worldwide, with the majority of cases found in West and Central Africa and parts of Australia [1]. *Mycobacterium ulcerans*, the aetiological agent of BU, is an extremely slow-growing bacterium, with a doubling time of >48 h [2]. Consequently, there are significant difficulties in studying *M. ulcerans* via culture-based methods, with 6 – 24 weeks of incubation needed for colony formation on solid media, resulting in slow turnaround time for experiments [3]. The slow growth rate also makes it difficult to study the bacteria in its hypothesised environmental niches owing to overgrowth of faster-growing microorganisms. Mycobacteria are hardier than

most Gram-positive and Gram-negative bacteria owing to their thick waxy cell wall, therefore, harsh decontamination techniques have been employed to isolate *M. ulcerans* from environmental sources [4]. However, as Palomino and Portaels [4] showed, whilst effective at reducing the overgrowth of most competing bacteria, such decontamination techniques also adversely affect the viability of *M. ulcerans*, and do not prevent its been overrun by faster-growing environmental mycobacteria.

Owing to their greater speed and higher sensitivity compared to culture-based methods, molecular methods of detecting *M. ulcerans* DNA are routinely applied both in the diagnosis of BU and in the evaluation of environmental samples to assess the epidemiology of the bacteria. Such methods, however, cannot discriminate between viable and non-viable cells, since DNA persists much longer after cells die [5], limiting the usefulness of molecular methods for the study of *M. ulcerans* in the environment. Compared to DNA, RNA is much more labile and its presence in a sample is typically used as a proxy for the presence of viable organisms. Consequently, reverse transcriptase quantitative PCR (RT-qPCR) has been applied to the detection of *M. ulcerans* in biological samples [6, 7]. However, RNA extraction from *M. ulcerans* is a cumbersome process, likely due to the difficulty in lysing the thick cell wall and dissociating the cells from the abundant ECM in which they are typically encased [6, 8]. As such, RT-PCR has not been extensively applied in the study of *M. ulcerans* in the environment.

One method that can potentially combine the speed and sensitivity of genomic methods with the live/dead discrimination of culture-based methods for detection and quantification of cells is viability (q)PCR. This is based on the ability of certain DNA intercalating dyes – such as ethidium monoazide (EMA) and propidium monoazide (PMA) – to bind to and inhibit the amplification of DNA following their preferential penetration of dead cells (i.e. cells with a compromised cell membrane). EMA and PMA are photoreactive versions of the routinely used fluorescent DNA intercalating dyes ethidium bromide (EB) and propidium iodide (PI), respectively. Like EB and PI, EMA and

PMA can penetrate cells with compromised cell membranes and modify their DNA. The intact cell membranes of live cells exclude the dyes, therefore their DNA is not modified. Cells are incubated with EMA/PMA protected from light (dark incubation) to allow for dye penetration, after which they are exposed to light of wavelength 464 nm to activate the dyes (light incubation). Subsequent DNA extraction and PCR allows for discrimination of live cells from dead cells, since DNA from dead cells would not be amplified.

PMA is used more often than EMA owing to its greater discrimination of live and dead cells, as well as its lower toxicity [9]. PMA-qPCR techniques have been developed for a variety of cell types, including Gram-positive bacteria (*S. aureus, Bacillus, Listeria*) [10 – 13], Gram-negative bacteria (*E. coli, Legionella, Salmonella, Pseudomonas*) [11, 14, 15, 16, 12], archaea [17], phytoplankton [18], fungi [19], as well as bacterial endospores [20], and parasite eggs and cysts [21 – 23]. The technique has also been applied to mycobacteria such as *M. fortuitum* [24]*, M. avium* [11]*, M. paratuberculosis* [25, 26]*,* and *M. tuberculosis* [27 -29].

Lu *et al.* (2018) developed a PMA-qPCR technique for the discrimination of viable and non-viable *M. tuberculosis* in sputum samples [27]. One major difference in their technique from those reported for other mycobacteria and other cell types was the need for a longer dark incubation time to allow penetration of PMA through the cords and cell wall of *M. tuberculosis*. Indeed, compared to other bacteria which only required 10 min or less of dark incubation, *M. tuberculosis* required 120 min of dark incubation for optimum live/dead discrimination. Hence, we surmised that a similar protocol would be necessary for *M. ulcerans* given that it also produces an abundant ECM [30], which could hamper the penetration of the dye. Therefore, building on this protocol, we developed a PMA-qPCR method for discriminating viable from non-viable *M. ulcerans*, and applied this protocol in different contexts. A PMA pre-treatment step could be successfully combined with the established qPCR methodology described for *M. ulcerans* insertion sequence 2404 (IS*2404*) detection [31]. The IS*2606* and ketoreductase (KR) markers also

gave similar results, suggesting that the protocol described here could also be useful in discriminating *M. ulcerans* from other IS*2404*-containing environmental mycobacteria.

## MATERIALS AND METHODS

PMA dye (Biotium) was dissolved in cell culture grade DMSO (Sigma) to give a stock solution of 20 mM, and stored at -20°C protected from light until needed.

### Preparation of bacterial samples

The *M. ulcerans* strain S1013, a low-passage Cameroonian clinical isolate [32], was grown in Middlebrook 7H9 medium (Becton Dickinson) supplemented with 0.2% glycerol (Sigma) and 10% OADC (Becton Dickinson) for 8 weeks before being used in the tests. Bacteria were killed by heat-inactivating aliquots of *M. ulcerans* culture at 95°C for 45 min. Bacterial samples were prepared by diluting the live or dead cultures in buffer (PBS or PBS-0.2% Tween-20 (PBST)) to give an optical density measured at 600 nm ($OD_{600}$) of 0.001 – 0.1 as required.

### PMA-qPCR optimisation experiments

For every condition tested, quadruplicate samples were prepared (two live samples and two dead samples). PMA was added to one live and one dead sample to give the desired final concentration (50 µM – 150 µM). The equivalent amount of DMSO was added to samples not treated with PMA. All samples were incubated for 120 min at ambient temperature with gentle shaking protected from light. Thereafter, samples were exposed to blue light-emitting diodes (LEDs) for 40 min using the PMA-Lite™ LED Photolysis Device (Biotium). PMA-treated bacteria were washed twice with nuclease-free water to remove residual dye, and stored (in 50 µl of PBST) at 4°C if DNA extraction was performed the same day, or at -20°C if DNA extraction was performed at a later time.

DNA extraction and qPCR were done as described [31]. For each condition tested, the ΔCt was derived by subtracting the Ct of live PMA-treated cells from the Ct of dead PMA-treated cells.

**PMA-qPCR for soil samples**

High clay content soil (approx.100 g) was collected from the banks of a slow-flowing pond, mixed with 200 ml of $ddH_2O$ to give a slurry, and sterilised by autoclaving. Sterility was confirmed by inoculating into Middlebrook 7H9-OADC medium and incubating at ambient temperature for up to six months. Soil slurry was stored at 4°C until needed.

Each 500-µl aliquot of soil slurry was inoculated with 100 µl of a suspension of live or dead bacteria ($OD_{600}$ 0.1). Uninoculated soil samples were included as controls. Additionally, PBST inoculated with the cell suspensions were processed in parallel. Samples were transferred to MK28 bead-beating tubes (Precellys; Bertin Technologies, Montigny-le-Bretonneux, France) containing 2.8 mm stainless steel beads and homogenised (3 x 20 s, at 5 000 rpm). After sedimenting soil debris using low-speed centrifugation (10 min, 500 rcf), supernatants were centrifuged at high speed (10 min, 13 300 rcf, 4°C) to pellet the bacteria. The pellets were resuspended in 200 µl PBS and carefully layered over 1 ml of 70% (1.09 g/ml) Percoll. Samples were centrifuged at 13 000 rcf for 25 min at 4°C, after which the upper phases containing bacterial cells were carefully transferred to fresh sterile screw-capped tubes. Samples were washed once with PBS to remove residual Percoll, and then resuspended in PBST for PMA treatment.

PMA (50 µM) or the equivalent amount of DMSO was added to each sample. All samples were incubated at ambient temperature for 120 min with gentle shaking protected from light. Thereafter, samples were exposed to blue LEDs for 30 min using the PMA-Lite™ LED Photolysis Device (Biotium). PMA-treated bacteria were washed twice with

nuclease-free water to remove residual dye, and stored (in 50 µl of PBST) at 4°C until DNA extraction was performed.

DNA extraction and qPCR were done as described [31]. For each condition tested, the ΔCt was derived by subtracting the Ct of PMA-treated cells from the Ct of PMA-untreated cells.

**RESULTS**

Preliminary experiments indicated that a dark incubation time of 120 min was best suited for optimal live/dead discrimination of *M. ulcerans*, consistent with what was reported for *M. tuberculosis* [27]. These experiments also indicated that increased light incubation periods (>60 min) were not more discriminatory, therefore, for all subsequent tests, we incubated PMA-treated *M. ulcerans* cells for 120 min in the dark, followed by blue LED light exposure for 40 min.

It was suggested that the addition of detergents could reduce the dark incubation period needed for *M. tuberculosis* by improving penetration of the dye [27]. Inclusion of 0.2% Tween-20 into the PBS buffer did not reduce the dark incubation period for *M. ulcerans*, however, it resulted in slightly higher ΔCt values with better discrimination between PMA-treated and PMA-untreated cells (Fig. 1). The three *M. ulcerans* markers – IS2404, IS2606, and KR – gave comparable results. All subsequent tests were, therefore, performed using PBS containing 0.2% Tween-20 (PBST).

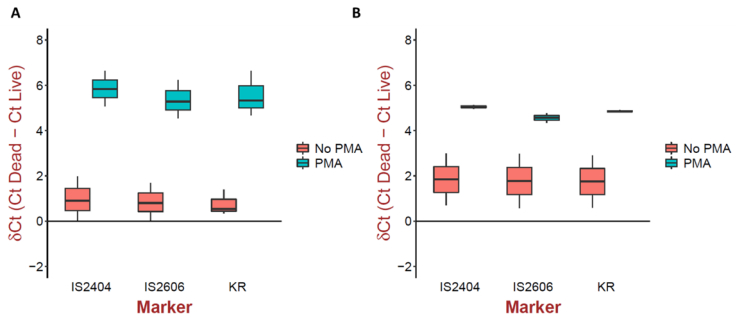


**Figure 1**. Live/dead discrimination of *M. ulcerans* by PMA-qPCR in the presence (A) or absence of (B) Tween-20. Assays were performed in three independent replicates. Parameters: dark incubation = 120 min; light incubation = 40 min; PMA concentration = 50 µM.

Improved differentiation of live and dead cells can be achieved by increasing the concentration of PMA used, to increase the suppression of DNA from dead cells. Therefore, different concentrations of PMA (from 50 – 150 µM) were tested to determine the optimum amount needed for consistent live/dead discrimination of *M. ulcerans*. However, none of the higher concentrations of PMA tested were better than the recommended 50 µM, therefore, 50 µM was retained as the optimal PMA concentration for the assay.

The amount of cells being treated has a bearing on the discriminatory power of PMA-qPCR, as higher amounts of dead cells require more dye for complete DNA suppression. To evaluate this, we treated *M. ulcerans* samples with different cell density (i.e. $OD_{600}$ 0.1 vs. 0.001), whilst retaining the PMA concentration of 50 µM. As shown in Fig. 2 below, there was less variation when high bacterial numbers were tested compared to low bacterial numbers, likely due to the inherent difficulties in serially diluting the typically clumpy *M. ulcerans* cultures.

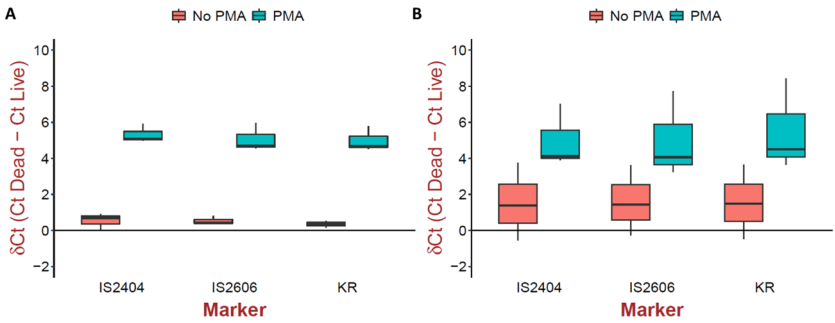


**Figure 2**. Live/dead discrimination of *M. ulcerans* by PMA-qPCR using (A) high ($OD_{600}$ = 0.1) or (B) low ($OD_{600}$ = 0.001) inoculum sizes. Assay was performed in three independent replicates. Parameters: dark incubation = 120 min; light incubation = 40 min; PMA concentration = 50 µM.

Suitability of the optimised assay conditions was tested for the detection of viable *M. ulcerans* in spiked soil samples. Sterile soil slurries were inoculated with live or heat-inactivated *M. ulcerans* and evaluated by PMA-qPCR (Fig. 3). In parallel, PBST inoculated with the same bacterial suspensions were processed to assess the effect of the presence of soil on the assay.

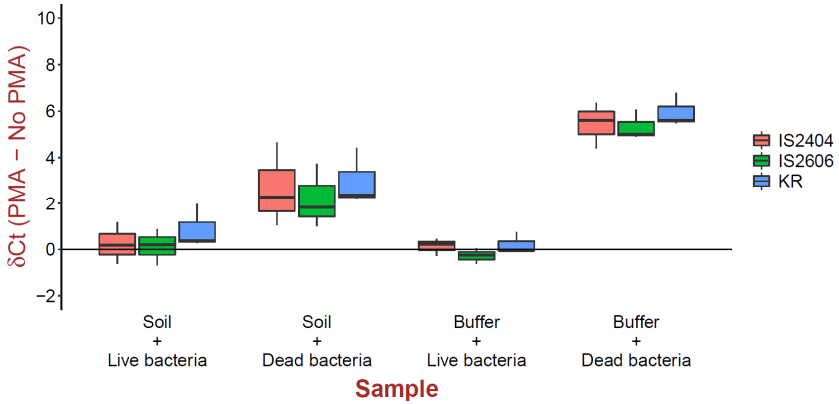


**Figure 3**. Application of PMA-qPCR to detect *M. ulcerans* in a spiked soil sample. Suspensions of live or heat-killed bacteria ($OD_{600}$ = 0.1) were inoculated into 500 µl of sterile soil slurry or PBST buffer and all samples were processed immediately in parallel. Assay was performed in three independent replicates. Parameters: dark incubation = 120 min; light incubation = 40 min; PMA concentration = 50 µM. No amplification of any of the three markers was seen in the uninoculated soil included as contamination controls.

**DISCUSSION AND CONCLUSION**

DNA intercalating dyes have been widely applied in combination with PCR techniques to distinguish viable from non-viable cells in a variety of matrices, ranging from laboratory culture media to food products such as milk and minced meat and clinical samples such as sputum [16, 21, 33, 34, 35]. Here we have optimised conditions for a PMA-qPCR-based method to distinguish live and dead *M. ulcerans* cells, and applied it to the detection of the viable bacteria in spiked soil samples. This method imparts a live/dead discriminatory ability to the existing qPCR protocol for detecting *M. ulcerans* DNA, thus circumventing the problems inherent in the cultivation of the extremely slow-growing organism.

Similar to reports of PMA-qPCR application for *M. tuberculosis* [27], we found a prolonged dark incubation time to be necessary for optimal *M. ulcerans* live/dead discrimination. This is unlike the situation for most other microorganisms, which typically require under 10 min of dark incubation with the PMA dye for adequate penetration into cells. On the other hand, we found that the light activation step for *M. ulcerans* could be considerably shorter than the dark incubation period, allowing us to define optimal assay parameters of 120 min of dark incubation followed by 40 min of light incubation. With these parameters, we were able to consistently distinguish between live and dead cultures of *M. ulcerans*.

The PMA-qPCR technique was applied to the detection of viable *M. ulcerans* in a spiked soil sample. Soil samples spiked with live *M. ulcerans* could be differentiated from those spiked with dead bacteria with an average signal reduction of 2.5 cycles. On the whole, this was a smaller signal reduction compared to that gotten from buffer spiked with the same inocula (≈5.5 cycles). One reason for this could be the low efficiency of the Percoll purification step used in this protocol. Additionally, the abundance of DNA in soil – so called “relic DNA”, which is persistent even following autoclaving – has been reported to result in underestimation of viable cells in soils following PMA-qPCR [5, 9]. Further

optimisation of the protocol would be necessary, especially to ensure maximal recovery of cells from different soil matrices, and to suppress the interference caused by relic DNA (e.g. by repeated PMA treatment as reported by [25]).

Unlike as reported for other microorganisms, we were not able to achieve ΔCt values up to 10 using PMA-qPCR with *M. ulcerans*, although there was still a clear difference in the Ct values of live and dead *M. ulcerans*. This lower ΔCt value was not due to inadequate concentrations of the dye, since higher dye concentrations were not more discriminatory than lower concentrations. A likelier reason for this is the amplicon sizes of the qPCR for the three target sequences tested. Whilst qPCR benefits most from shorter amplicons, PMA-qPCR benefits from longer amplicons, because there is a higher chance of PMA deposition on longer stretches of DNA and therefore of inhibition of DNA polymerase activity [9]. Accordingly, it is recommended that the targeted DNA fragment for optimal PMA discrimination should be above 100 bp, with shorter fragments yielding signal reduction of less than 6 cycles [9, 36]. The amplicon sizes of the three *M. ulcerans* targets IS*2404*, IS*2606*, and KR are 59 bp, 58 bp, and 65 bp, respectively [37], which while optimal for the qPCR are probably too short to achieve higher signal reduction by PMA-qPCR.

A second reason for the lower signal reduction is the copy number of the target genes. Logically, the fewer the copies of the target gene, the more likely they are to be completely suppressed by PMA [9]. However, the *M. ulcerans* qPCR targets IS*2404*, IS*2606*, and KR are estimated to be present in up to 209, 98, and 30 copies per cell, respectively. The high copy number of the IS*2404* target is the reason for the high sensitivity of the qPCR, although it probably results in less likelihood of complete inhibition by PMA. Thus, the combined effects of short amplicons and high copy numbers of the *M. ulcerans* qPCR resulted in lower signal reduction following PMA pretreatment. Nevertheless, the PMA-qPCR technique we have described here would be readily applicable since it builds onto qPCR protocols widely established in laboratories working with *M. ulcerans*.

In summary, we have shown the feasibility of applying PMA-coupled qPCR to differentiate live from dead *M. ulcerans* cells. This technique is faster than *M. ulcerans* culture and more straightforward to perform than reverse transcriptase PCR for *M. ulcerans,* which are the only currently available techniques that can detect the viable bacteria in samples. The PMA-qPCR technique herein described has the potential to be useful for ecological studies of *M. ulcerans* since it circumvents the problems of cultivating the bacteria from environmental samples. It could also be helpful in monitoring treatment success in BU chemotherapy, and avoiding the cumbersome RNA-based PCR methods for *M. ulcerans*. Additionally, PMA pretreatment can be readily combined with other genomic tests such as LAMP, which could be beneficial for field applications.

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## Section II: Treatment

- Efficacy of an acid-oxidising solution (AOS) against *Mycobacterium ulcerans*.
- Prospects for the repurposing of scaffolds with anti-tuberculosis activity for the treatment of Buruli ulcer.

## Efficacy of an acid-oxidising solution (AOS) against *Mycobacterium ulcerans*

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**ABSTRACT**

Proper wound care is an important element in the management of severe cases of the chronic necrotising skin disease Buruli ulcer. For the treatment of chronic wounds, acid-oxidising solutions (AOSs) have been developed that have broad-spectrum microbicidal activity without disturbing the formation of the granulation tissue. Here we show that AOS formulations are efficient in killing *Mycobacterium ulcerans,* the causative agent of Buruli ulcer, which is able to survive harsh decontamination treatments. Topical treatment of Buruli ulcer lesions with AOS may support the recommended antibiotic therapy with oral rifampicin and clarithromycin, prevent contamination of the environment by the mycobacteria, and control secondary infections, which are a prevalent wound management problem in resource-poor Buruli ulcer endemic settings.

**INTRODUCTION**

*Mycobacterium ulcerans* disease – or Buruli ulcer (BU) – is a chronic necrotising infectious disease afflicting skin and soft tissue [1]. The pathology of the disease is primarily attributed to the production of the polyketide cytotoxin mycolactone by *M. ulcerans* [2]. Buruli ulcer disease typically starts out as innocuous nodules, plaques, or oedema that can all devolve into ulcerated forms if treatment is not initiated early. In areas of West and Central Africa, which have the highest BU endemicity worldwide, patients often present with advanced stages of the disease when chronic ulceration has set in. Although BU is treatable by an 8-week regimen of daily rifampicin and clarithromycin [3], the destruction to skin and soft tissue may necessitate adjunct surgical intervention for complete resolution. In this regard, BU is similar to other chronic wounds, such as diabetic foot ulcers, needing long-term dedicated wound care and physiotherapy to complete healing [4]. Secondary colonisation of BU lesions by other bacteria, including *Staphylococcus aureus* and *Pseudomonas aeruginosa* is common [5]. This may delay wound healing and cause other complications, and in BU endemic areas

of Africa, an abundance of antimicrobial agents is therefore commonly prescribed to treat suspected secondary infections [6].

Chronic wounds are wounds that do not progress through the typical phases of healing in a timely fashion. Although they have a variety of aetiologies, there are some features common to all of them. For instance, chronic wounds tend to have an alkaline pH and a bioburden in the form of biofilms [7]. They also have an imbalance in the levels of matrix metalloproteinases (MMPs), which degrade damaged tissues in wounds, and their inhibitors, the tissue inhibitors of matrix metalloproteinases (TIMPs) [8]. Higher MMP levels relative to TIMP levels contribute to wound chronicity. Studies have shown that a reduction of pH from alkaline to acidic levels is necessary for healing to occur [7]. Consequently, efforts have been made to develop new wound care regimens that acidify chronic wounds to facilitate healing.

An acid-oxidising solution (AOS, Applied Pharma Research, Balerna, Switzerland) has been developed for the treatment of chronic wounds. This AOS formulation is based on hypochlorous acid (which represents >95% of the total free chlorine species in the solution) with a low pH (2.5 – 3.0) and high reduction-oxidation (redox) potential. As such, it has a three-pronged approach to promoting wound healing: (i) hypochlorous acid, which is broadly microbicidal [9], (ii) a low pH that is refractory to microbial growth in wounds [10], and (iii) the high redox potential, which destabilises the membrane potential of microorganisms and facilitates their killing [11]. In addition, the combination of low pH and high redox potential aids in the inhibition of MMPs [12]. The AOS has been shown to be efficacious in the resolution of chronic wounds, such as diabetic foot ulcers, venous ulcers, and pressure ulcers, both in inpatient and outpatient conditions [13, 14, 15]. Additionally, it was found to have broad-spectrum microbicidal activity, to induce morphological changes in biofilms (thereby facilitating the local accessibility of microorganisms) and to prevent their formation in an *in vitro* 3-dimensional model of

human epidermis [16]. Preclinical tests showed no indication of cytotoxicity related to the AOS and, in clinical reports, its topical application to wounds had no sensitising effect nor did it irritate the skin, mucosal membranes, or eyes [13, 14, 15].

Improving BU healing by way of wound acidification has been explored previously. Acidified nitrate was shown to be bactericidal to *M. ulcerans in vitro* [17] and to aid wound size reduction in BU lesions [18]. Neither acidic pH alone nor nitrite alone was found to lead to bacterial killing [17]. Therefore, in the present study, we assessed *in vitro* killing of *M. ulcerans* following exposure to two different AOS formulations and found them to be rapidly microbicidal against *M. ulcerans in vitro.* Importantly, this activity was preserved in the presence of human serum.

## MATERIALS AND METHODS

### AOS formulations

Two different formulations of AOS, produced with a patented Tehclo Technology™, were tested: AOS formulation 1 contains 40 – 70 mg/L of stabilised hypochlorous acid with a redox potential between 1000 and 1200 mV, while AOS formulation 2 contains 70 – 100 mg/L of stabilised hypochlorous acid with a redox potential between 1000 and 1300 mV. Both AOS solutions have low pH (2.5 – 3.0).

AOS formulation 1 is approved as medical device class III with ancillary antimicrobial action in Europe and under 510k regulation in the US with the indication of debridement, irrigation, cleansing and moistening of acute and chronic wounds (e.g. diabetic foot ulcers, pressure ulcers, lower leg ulcers, vascular ulcers), post-surgical wounds, cuts, abrasions, burns and other lesions.

### Bacteria

The *M. ulcerans* strain S1013, a low-passage Cameroonian clinical isolate [19], was grown for 8 weeks in Middlebrook 7H9 medium (Becton-Dickinson), supplemented with 0.2% glycerol (Sigma) and 10% OADC (oleic acid, albumin, dextrose, and catalase; Becton-Dickinson), before being used in the tests.

### Determination of the antimicrobial activity of AOS

Cultures (approximately $10^6$ CFU/ml) were exposed to the test solutions in a 1:20 ratio (50 µl of culture to 950 µl of AOS) for varying lengths of time, after which the suspension was centrifuged at 13,300 x *g* for one minute to pellet the bacteria. The supernatant was immediately removed and the bacterial pellet resuspended in 200 µl of Middlebrook 7H9 liquid medium supplemented with 0.2% glycerol and 10% OADC. For resazurin tests, 20 µL of a resazurin solution (0.125 mg/mL; Sigma) was added to the treated cells, the culture was incubated at 30°C for three days after which the fluorescence was measured,

and the metabolic activity was calculated with respect to the included controls. Alternatively, the treated cells were plated out on Middlebrook 7H9 agar medium supplemented with 0.2% glycerol and 10% OADC, and incubated at 30°C for up to six months. CFUs were counted monthly and the final count was done at the end of the experiment.

Efficacy of AOS formulation 1 was further assessed by adding 950 µl of AOS to 50 µl of *M. ulcerans* cultures containing different amounts of human serum, from no serum up to 50% serum. Additionally, *M. ulcerans* cultures of $5 \times 10^5 – 4 \times 10^6$ CFU/ml were tested to see the efficacy of AOS formulation 1 against particularly heavy doses of the bacteria. The resazurin assay was used for both these tests, and the bacteria were exposed to the AOS test solution for a total of 10 min prior to plating.

**RESULTS**

Initial resazurin tests revealed a time-dependent reduction in *M. ulcerans* metabolic activity upon exposure to both AOS formulations tested (Fig. A). A 2-minute exposure to AOS formulations 1 and 2 resulted in a 70.5% and 84.3% reduction, respectively. Increasing the exposure time resulted in over 85% reduction with AOS formulation 1, and over 95% reduction with AOS formulation 2.

CFU count-based analyses revealed a similar picture with an 82.6% and 84.5% reduction in CFUs following a 2-minute exposure to AOS formulations 1 and 2, respectively, relative to unexposed bacteria. Increasing the exposure time to 10 minutes resulted in >99% reduction in *M. ulcerans* CFUs with both AOS formulations (Fig. B). Although the AOS formulation 2 had a slightly higher activity against *M. ulcerans* than AOS formulation 1, formulation 1 was selected for further tests as this formulation is approved both in the EU and the USA for chronic wound management.

While the inoculum dose of $10^6$ CFU/ml selected for the previous analyses is well above the range routinely used for drug screens [19], we assessed whether even larger doses are also eliminated by the AOS formulation. As expected, there was some dose-dependent reduction in AOS efficacy with increasing inoculum sizes (Fig. C). Nevertheless, a >90% reduction in metabolic activity was achievable even with a starting inoculum of 2 x $10^6$ CFU/ml. At the highest inoculum dose tested (4 x $10^6$ CFU/ml), metabolic activity was still reduced by 62.83%.

BU lesions, like many other chronic wounds, can have varying amounts of exudate, which are serum-rich. Therefore, we also assessed the efficacy of the AOS in the presence of human serum. The presence of serum reduced the efficacy of the AOS formulation 1 slightly, but there was still over 75% reduction in metabolic activity even in bacterial suspensions containing 50% human serum (Fig. D). Interestingly, the efficacy of the AOS formulation did not decline much with increasing serum concentrations.

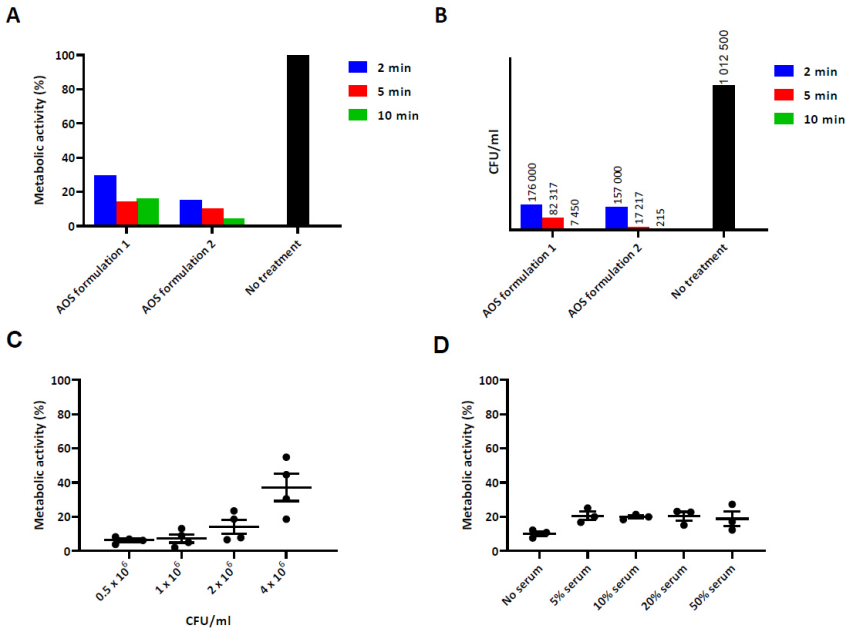


**Figure**. (A) Reduction in *M. ulcerans* metabolic activity as measured by the resazurin assay following exposure to AOS formulations 1 and 2 for 2, 5, or 10 min; untreated bacteria were included as controls. (B) Reduction in *M. ulcerans* CFU following exposure to AOS formulations 1 and 2 for 2, 5, or 10 min; untreated bacteria were included as controls. The CFU counts are displayed above the bars. (C) Metabolic activity of bacterial suspensions containing increasing amounts of *M. ulcerans* following a 10-minute exposure to AOS formulation 1; quadruplicate results are shown with the plotted means. (D) Metabolic activity of bacterial suspensions containing different concentrations of human serum following a 10-minute exposure to AOS formulation 1; triplicate results are shown with the plotted means. For the experimental results shown in A, B and D bacterial suspensions tested contained $10^6$ CFU/ml.

**DISCUSSION**

Although, specific chemotherapy for BU exists, effective wound care is still required for proper resolution of more extensive ulcers. Such wound care could be in the form of simple cleansing and proper dressing of the wound, or more sophisticated measures such as wound debridement and skin grafting. In BU, similar to chronic ulcers of other aetiologies, typical wound healing phases often do not progress in an orderly and timely manner, leading to aberrant or stagnated wound healing processes [20]. Secondary bacterial infections of BU lesions are common complications especially in settings where proper wound management is not ensured. Therefore, measures developed for the treatment of chronic wounds of other aetiologies are also suitable for BU treatment.

Here, we report the assessment of the bactericidal activity of two formulations of an AOS against *M. ulcerans*. Both AOS formulations resulted in >99% reduction of *M. ulcerans* CFU count following only 10 min of exposure. AOS formulation 1 was also shown to be efficacious for bacterial suspensions containing up to 50% human serum. The highest inoculum of *M. ulcerans* tested could still be reduced by >60%, and it is conceivable that repeated applications of the formulation would have improved efficacy.

Clinical reports have demonstrated the efficacy of AOS formulation 1 when incorporated into the management of chronic wounds such as diabetic foot ulcers, venous ulcers, and pressure ulcers. Ricci (2016) and Strohal *et al.* (2018) showed improved clinical outcomes in patients whose chronic wounds were treated with the AOS in a hospital setting [13, 14], while Iacobi *et al.* (2018) showed that similar improvement could also be achieved even when patients self-administered the AOS at home [15].

The well-established antimicrobial properties of the AOS components (i.e. low pH, hypochlorous acid, and high redox potential) undoubtedly account for its broad-spectrum activity against microorganisms, including *M. ulcerans* which is able to survive

harsh decontamination procedures [21], with recent reports indicating efficacy of the AOS against SARS-CoV-2, the aetiological agent of the ongoing COVID-19 pandemic [22]. In addition, the observed clinical efficacy of AOS formulation 1 could be partly explained by its reported *in vitro* activity. D'Atanasio *et al.* (2015) described the activity of the AOS in controlling MRSA biofilms in a 3-dimensional *in vitro* model of human epidermis, with the AOS preventing new formation of biofilms while causing deterioration of already formed biofilms [16]. It degraded the extracellular polymeric substrate of the biofilm, releasing planktonic forms of the bacteria, while exhibiting minimal cytotoxicity to the tissue [16]. It is well known that biofilms are more recalcitrant to both host defences and chemotherapy, and that chronic wounds are often colonised by biofilm forming microorganisms. By releasing the bacteria from the protective extracellular matrix (ECM), thereby making them more accessible to antimicrobial substances, the AOS aids in clearing the infection. Since *M. ulcerans* also produces an abundant ECM and adopts biofilm-like structures [23], the anti-biofilm activity of the AOS could also aid in the clearance of *M. ulcerans* from BU lesions.

Taken together, AOS treatment could be a valuable adjunct to the WHO recommended standard combination chemotherapy, complementing antibiotic treatment by directly killing *M. ulcerans*, and improving wound healing by eliminating secondary infections and stimulating a favourable wound microenvironment that fosters healing. While direct human-to-human transmission of *M. ulcerans* seems to be very rare, it has been suggested that spread of the bacteria from large chronic human BU lesions to environmental reservoirs may contribute to transmission. AOS treatment could reduce the spread of the pathogen into the environment. The simplicity of use of the AOS (thanks to the spray formulation) could allow patients to be treated at home, either by self-administration or by a health worker, thus reducing the need for repeated hospital visits for those patients in remote areas who reside faraway from a primary health post.

Clinical studies are needed to assess how translatable these results are into routine BU treatment.

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# Prospects for the repurposing of scaffolds with anti-tuberculosis activity for the treatment of Buruli ulcer

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# Chapter 7: Efficacy of compounds against *M. ulcerans*


## ABSTRACT

Repurposing of new anti-tubercular drug candidates for the treatment of *Mycobacterium ulcerans* disease (Buruli ulcer) is an attractive strategy to reduce development costs. Here we have evaluated 167 compounds, representing five scaffolds, for activity against *M. ulcerans*. Highly active compounds with $MIC_{50}$ values between 0.02 and 1 µg/ml were found among all scaffolds, which included arylvinylpiperazine amides, pyrrolo[3,4-c]pyridine-1,3(2H)-diones, quinolone carboxamides, quinazolines, and bismuth-thiols. Future efficacy testing in the experimental *M. ulcerans* mouse model could guide selection of scaffolds for further medicinal chemistry optimisation.


## INTRODUCTION

Repurposing of drugs to treat rare diseases is an attractive approach because costs and attrition rates of new drug discovery and development activities are prohibitively high [1]. In the case of drugs for the treatment of *Mycobacterium ulcerans* disease (Buruli ulcer; BU), repurposing of new scaffolds under development for the treatment of tuberculosis, is a particularly attractive strategy, as it is potentially associated with lower overall development costs and shorter development timelines. This approach opens the possibility to use pharmacological, formulation, and safety data generated by previous research and development efforts for tuberculosis, and new candidate therapies could therefore progress more rapidly to clinical efficacy testing for BU. As *M. ulcerans* is closely related to the *Mycobacterium tuberculosis* complex, drug targets may be conserved between the two species. However, *M. tuberculosis* active compounds are often inactive or only weakly active against *M. ulcerans* [2]. Loss of drug target structures during genome reduction [3], the extremely slow growth rate of *M. ulcerans*, and the

expression of a highly hydrophobic extracellular matrix [4] may all contribute to resistance of these mycobacteria to many scaffolds.

While drug candidates with high activity against *M. tuberculosis,* therefore, are not always active against *M. ulcerans,* imidazopyridine amide (IPA) compounds, including the anti-tuberculosis clinical development candidate Telacebec (Q203) [5], have shown outstanding activity against *M. ulcerans* [6]. Telacebec is an anti-tuberculosis drug candidate targeting cellular energy production through inhibition of the mycobacterial respiratory cytochrome $bc_1$:$aa_3$ (cyt-$bc_1$:$aa_3$) supercomplex. Although the cyt-$bc_1$:$aa_3$ complex is the primary terminal oxidase in the *M. tuberculosis* electron transport chain, a functional alternative *bd*-type terminal oxidase is also present in these bacteria. Hence, Telacebec is only bacteriostatic to *M. tuberculosis*, since the bacteria can switch to the alternative terminal oxidase following Telacebec-mediated cyt-$bc_1$:$aa_3$ inhibition [6]. Similarly, Telacebec is bacteriostatic rather than bactericidal to ancestral lineage *M. ulcerans* strains (e.g. those found in Japanese BU-endemic regions), since these strains also have a functional *bd*-type terminal oxidase. In contrast, *M. ulcerans* strains of the classical lineage (i.e. those found in African and Australian BU-endemic regions) have lost this alternative terminal oxidase in the course of reductive evolution, therefore, Telacebec has a bactericidal effect on these strains, with $MIC_{50}$ values <1 nM. Accordingly, a single dose of Telacebec was curative in a mouse model of BU [7]. Thus, Telacebec could potentially greatly simplify the current WHO recommended BU chemotherapy, which entails daily administration of rifampicin and clarithromycin for eight weeks [8].

BU chemotherapy involves the administration of a combination of antibiotics, as is the case with most infectious diseases. Typically, synergy or at least additivity of activity is the goal when designing such combination drug regimens, and combinations of drugs with differing targets are desired [9]. Clinical use of Telacebec in a two-drug combination

therapy would benefit strongly from the identification of a second drug with comparable pharmacological properties, including a long half-life. Therefore, in the current study, we have screened new compound classes with activity against *M. tuberculosis* or other bacteria for activity against *M. ulcerans.* Energy metabolism of mycobacteria as a drug target has received broad attention in recent years, as it is not only the target of Telacebec, but also of the approved tuberculosis drug Bedaquiline [10]. Here we have included derivatives of the arylvinylpiperazine amide AX-35 (GW861072X), which was identified by screening of a large panel of compounds against *M. bovis* BCG and *M. tuberculosis* [11]. AX-35 targets the *b* subunit of cyt-$bc_1$:$aa_3$ [12], has a MIC of 0.3 µM against both species, and is also active against *M. ulcerans* [13]. Like for Telacebec, the alternative *bd* oxidase has a compensatory role in *M. tuberculosis*. Another novel class of antimycobacterials targeting mycobacterial energy generation included in our analysis are the pyrrolo[3,4-c]pyridine-1,3(2H)-diones [14]. As for AX-35 and Telacebec, *M. tuberculosis* cytochrome *bd* oxidase mutants are hypersensitive to these compounds indicating that they also represent inhibitors of cyt-$bc_1$:$aa_3$. Furthermore, we have included quinolone carboxamides and quinazoline analogues with antituberculosis activity, as well as the bismuth-based drug Pravibismane and other bismuth-thiols (also thought to target energy generation in a variety of bacteria [15]) in the analysis. Fig. 1 shows the known or presumed molecular targets of the compound classes tested.

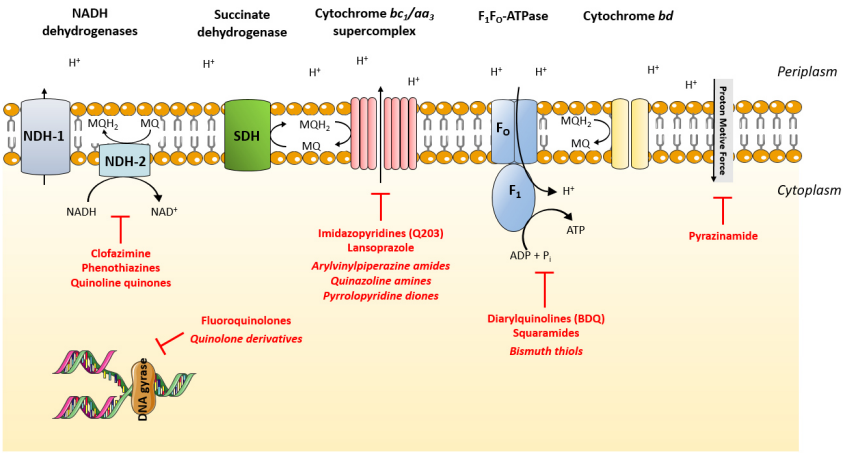



**Figure 1. Schematic showing targets of antimycobacterial drugs/compounds**. Examples of drugs known to inhibit targets in the electron transport chain and DNA replication are indicated; compound classes evaluated in the current work are italicised. Abbreviations: Type I NADH dehydrogenase (NDH-1), Type II NADH dehydrogenase (NDH-2), Menaquinone (MQ), Bedaquiline (BDQ). (Graphic generated using icons from SMART Servier Medical Art, www.servier.com)

**MATERIALS AND METHODS**

**Compounds tested**

Three bismuth-thiol (BT) compounds, 15 arylvinylpiperazine amides, 14 quinazoline amines, 32 pyrrolo-[3,4-c]pyridine-1,3(2H)-diones, and 103 quinolone derivatives were tested. All compounds were dissolved in sterile cell culture grade DMSO (Sigma) to give stock solutions of 4 – 10 mg/ml.

***M. ulcerans* strains**

The *M. ulcerans* strains S1013, a low-passage Cameroonian clinical isolate, and S1326, a low-passage Japanese clinical isolate [6], were grown for 8 weeks in Middlebrook 7H9 medium (Becton-Dickinson), supplemented with 0.2% glycerol (Sigma) and 10% OADC (oleic acid, albumin, dextrose, and catalase; Becton-Dickinson), before being used in the tests.

**Resazurin assay**

The resazurin assay was done as previously described [16]. Briefly, cultures of the *M. ulcerans* strain S1013 (and S1326 where indicated) were incubated in triplicates with each test compound at a concentration of 10 µg/ml for 8 d at 30°C, after which 10% v/v of a resazurin solution (0.125 mg/ml; Sigma) was added. Cultures were incubated for an additional 3 d, following which the fluorescence was measured. The metabolic activity was determined relative to the included *M. ulcerans* drug-free control culture. Compounds which reduced viability of *M. ulcerans* by ≥50% were further assessed to determine the minimum inhibitory concentration (MIC).

The MIC was defined as the concentration required to reduce *M. ulcerans* metabolic activity by 50% ($MIC_{50}$). Here, *M. ulcerans* strain S1013 cultures (and S1326 cultures, where indicated) were incubated in duplicates with 2-fold serial dilutions of test

compounds, from 10 µg/ml to 19.5 ng/ml. The rest of the resazurin assay was done as described above.

**Time-kill assay**

Selected test compounds were assessed for microbicidal activity against *M. ulcerans*. Cultures were exposed to varying concentrations of test compounds (0.5x MIC, 1x MIC, 2x MIC, or 4x MIC) for different lengths of time (At start, Week 1, Week 2, Week 3, Week 4). At each time point, ten-fold serial dilutions of the cultures were plated out on Middlebrook 7H9 agar medium supplemented with 0.2% glycerol and 10% OADC, and incubated at 30°C for up to six months. CFUs were counted monthly and the final count was done at the end of the experiment. In parallel, the undiluted treated cultures were also evaluated via the resazurin assay to give an early indication of expected CFU counts, by incubating for 3 d with 10% v/v resazurin and then determining metabolic activity as described above.

## RESULTS AND DISCUSSION

We evaluated 167 compounds, representing five scaffolds, for activity against *M. ulcerans*. For all scaffolds, resazurin assays were used for an initial screen to identify highly active compounds, and for subsequent determination of their $MIC_{50}$ values. One highly active compound from each group was further evaluated in time-kill assays to assess its bactericidal effect.

### *i. Bismuth-thiol compounds*

We assessed three bismuth-thiol (BT) compounds for activity against *M. ulcerans* (Table 1).

**Table 1**: Bismuth-thiol compounds tested against *M. ulcerans*.

| Name | Abbreviation | Composition (Bismuth : thiol molar ratio) |
|---|---|---|
| MB-1-B3, Pravibismane | BisEDT | Bismuth-1,2-ethanedithiol (2 : 3) |
| MB-2 | BisBAL | Bismuth-2,3-dimercaptopropanol (2 : 3) |
| MB-6 | BisBDT | Bismuth-2,3-butanedithiol (2 : 3) |

Of the three BTs tested (Table 1), MB-1-B3 (BisEDT) was found to be the most active against *M. ulcerans* with a $MIC_{50}$ of about 0.3 µg/ml (Fig. 2A). The compound was active against strains from both the classical and the ancestral lineages of *M. ulcerans* (Fig. 2B). MB-1-B3 (BisEDT) was rapidly bactericidal against *M. ulcerans*, as all concentrations of the drug tested reduced colony counts by ≥99% after only two days of exposure (Fig. 2C).

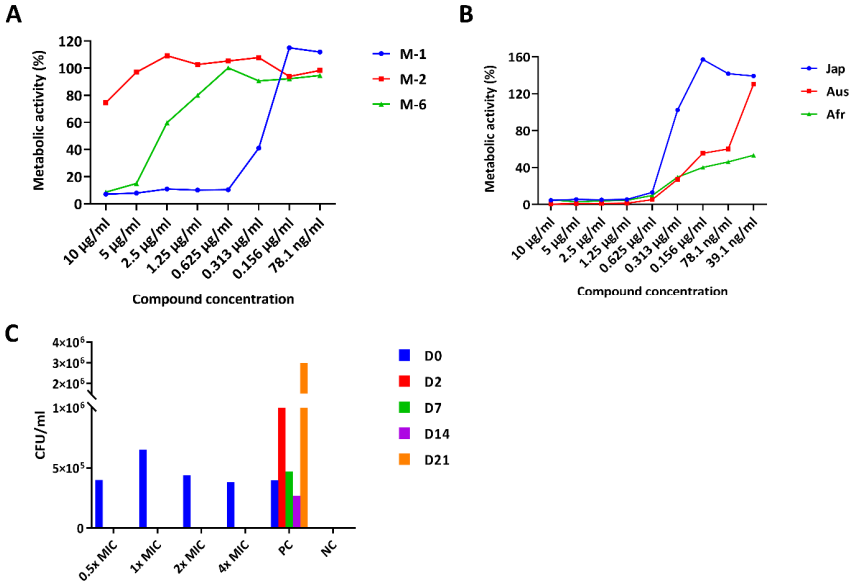



**Figure 2. Activity of bismuth-thiols against *M. ulcerans***. (A) Resazurin test showing activity of three BTs against *M. ulcerans*. (B) Resazurin test showing activity of MB-1-B3 (BisEDT) against *M. ulcerans* strains of the classical (African and Australian) and ancestral (Japanese) lineages. (C) Bactericidal activity of MB-1-B3 (BisEDT) against African *M. ulcerans* strains as indicated by determination of CFU counts. Concentrations of MB-1-B3 (BisEDT) used were 0.15 µg/ml (0.5x MIC), 0.3 µg/ml (1x MIC), 0.6 µg/ml (2x MIC), and 1.2 µg/ml (4x MIC). Included controls were untreated bacteria (positive control, PC) and uninoculated medium (negative control, NC).

Bismuth has been used medically for centuries, being one of the first drugs used for the treatment of syphilis [17]. Compared to other heavy metals in medical use (e.g. antimony still used to treat leishmaniasis, and arsenic used to treat a form of leukaemia), bismuth is relatively non-toxic, with reported cases of toxicity being mainly associated with

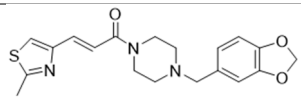
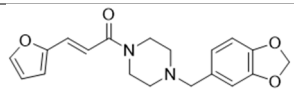
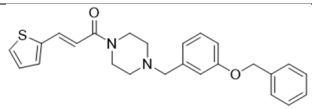
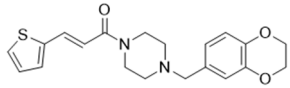
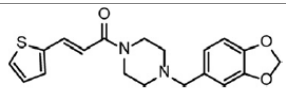
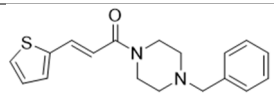
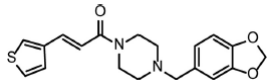
overdoses [17, 18]. Currently, bismuth is predominantly used in formulations for the treatment of gastrointestinal ailments such as gastritis and diarrhoea, and it is highly efficacious against *Helicobacter pylori* [17]. Combination with thiols imparts an increased lipophilicity to bismuth, greatly enhancing its uptake by bacterial cells [19]. Mechanistically, bismuth is regarded as a metabolic poison, able to inhibit a variety of enzymes given its propensity for targeting thiol-containing enzymes, as well as preventing slime/capsule expression (thus inhibiting biofilm formation) in a range of Gram-positive and -negative bacteria. Notably, it is known to inhibit $F_1$ (the ATP-synthesising portion of the $F_1F_O$-ATPase), leading to disruption of energy generation in bacteria [17]. Given that the mycobacterial electron transport chain also comprises an $F_1F_O$-ATPase, which is validated as drug target of Bedaquiline, it is conceivable that BTs could also specifically target energy metabolism in *M. ulcerans*. Furthermore, BTs can act synergistically with other antibiotics, such as rifampicin (against staphylococci [15, 20]) and tobramycin (against *Burkholderia cepacia* and *Pseudomonas aeruginosa* [15, 19]). BTs were also shown to be highly active *in vitro* against drug-sensitive *M. tuberculosis* as well as strains resistant to both isoniazid and rifampicin [21]. Therefore, BTs could potentially constitute parts of novel regimens against *M. ulcerans*.

Pravibismane has been developed as a topical agent for the treatment of chronic ulcers. A topical formulation has the added benefit of being safe to use, since it avoids the risk of toxicity that could arise from oral or parenteral administration of bismuth [18]. It is therefore conceivable that a topical BT formulation could complement wound management of Buruli ulcers.

***ii. Arylvinylpiperazine amides***

We evaluated 15 arylvinylpiperazine amides – comprising the prototype AX-35 and derivatives – for activity against *M. ulcerans.* The top 7 compounds are shown in Table 2 and the full list of activities in Table S1.

**Table 2**. Arylvinylpiperazine amides tested against *M. ulcerans*.

| Compound ID | Structure | $MIC_{50}$ (µg/ml) |
|---|---|---|
| DM-344 |  | 0.27 |
| JW-011 |  | 0.57 |
| FH-007 |  | 0.68 |
| DM-359 |  | 0.76 |
| AX-35 |  | 1 |
| JW-010 |  | 2.47 |
| AX-36 |  | 3.1 |

As shown in Table 2, there were four derivatives with higher activity than the prototype arylvinylpiperazine amide compound AX-35. However, AX-35 was found to be superior on further comparison to the most active derivative DM-344 (Fig. S1), and was, therefore, selected for further evaluation. Similar to Q203, AX-35 has been reported to target the *b* subunit of cyt-$bc_1$:$aa_3$, therefore we compared its activity against classical and ancestral strains of *M. ulcerans* to observe the difference in efficacy in the absence

or presence of the alternative *bd*-type cytochrome. Consistent with reports for Q203 [6], AX-35 was bactericidal against an *M. ulcerans* strain of the classical lineage (S1013), regardless of the concentration tested. Conversely, although the classical and ancestral *M. ulcerans* strains showed similar metabolic activity following one week of exposure to AX-35, the ancestral strain (S1326) regained full metabolic activity with prolonged exposure to the compound (Fig. 3A). All concentrations of AX-35 ≥1 µg/ml were bactericidal against the classical *M. ulcerans* strain S1013, with >99% reduction in CFU count following four weeks of exposure to the compound (Fig. 3B).

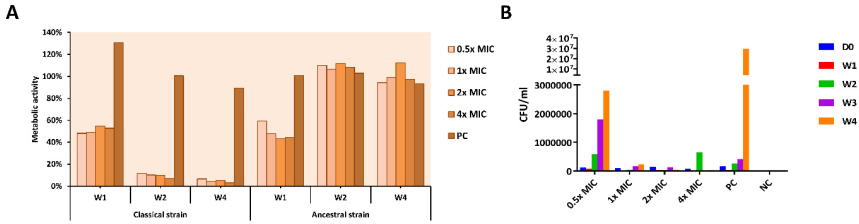



**Figure 3. Activity of AX-35 against *M. ulcerans***. (A) Resazurin test comparing activity of AX-35 against classical (S1013) and ancestral (S1326) *M. ulcerans* strains. (B) Bactericidal activity of AX-35 against the classical *M. ulcerans* strain S1013. For both assays, concentrations of AX-35 used were 0.5 µg/ml (0.5x MIC), 1 µg/ml (1x MIC), 2 µg/ml (2x MIC), and 4 µg/ml (4x MIC). Included controls were untreated bacteria (positive control, PC) and uninoculated medium (negative control, NC).

Arylvinylpiperazine amides were identified as novel inhibitors of mycobacterial energy generation. The prototype compound GW861072X (AX-35) was identified by GSK following phenotypic screening of a diverse library of low molecular weight compounds. Synthesis and further derivatisation were subsequently done, yielding compounds with varied activities against *M. tuberculosis* and favourable safety profiles in cultured human hepatocytes [13]. Like the imidazopyridine amide Q203 (Telacebec), arylvinylpiperazine

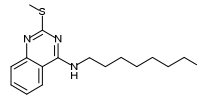
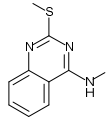
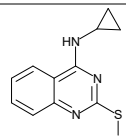
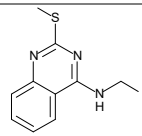
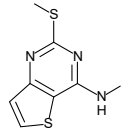
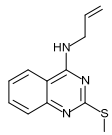
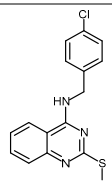
amides were shown to target QcrB in the *M. tuberculosis* cyt-$bc_1$:$aa_3$, albeit via a different interaction mechanism compared to Q203. Indeed, while Q203-resistant mutants were also resistant to AX-35, the reverse was not always the case, with some AX-35-resistant mutants being susceptible to Q203. Additionally, ATP levels were not affected in AX-35-resistant mutants in the presence of AX-35, but were depleted in the presence of Q203, indicating that both compounds differed in their interaction with QcrB [13]. That AX-35 targets mycobacterial energy generation seems to be corroborated by findings in the current study of differential susceptibility of *M. ulcerans* strains of the classical and ancestral lineages. Indeed, the ancestral lineage strains, with their functional alternative *bd*-type cytochrome, were practically insensitive to all the concentrations of AX-35 tested, whereas their classical lineage counterparts, which lack a functional alternative *bd*-type cytochrome, were effectively killed by the compound.

Hitting multiple targets in the electron transport chain is increasingly shown to be a useful strategy in tackling mycobacterial infections. Recently, in mouse models of BU, drug combinations including Bedaquiline (which targets the ATPase), Q203 (which targets the $bc_1$:$aa_3$ cytochrome), and clofazimine (which targets the NADH dehydrogenase), were shown to be superior to the standard combination of rifampicin and streptomycin [22]. Thus, compounds active against the mycobacterial respiratory chain, such as AX-35 could inspire novel drug regimens for BU chemotherapy.

### *iii. Quinazoline amine derivatives*

We tested 14 quinazoline amines with antituberculosis activity against *M. ulcerans*. The seven compounds with the highest activity ($MIC_{50}$ <10 µg/ml) are shown in Table 3 (the full list of activities is shown in Table S2).

**Table 3**. Quinazoline amines with activity against *M. ulcerans* ($MIC_{50}$ <10 µg/ml).

| Compound | Lab ID | Structure | $MIC_{50}$ (µg/ml) |
|---|---|---|---|
| **5931399** | B-A02 |  | 0.019 |
| **5931398** | B-A03 |  | 0.025 |
| **6152102** | B-A05 |  | 0.076 |
| **5968230** | B-B03 |  | 0.28 |
| **5965917** | B-A08 |  | 0.40 |
| **5931400** | B-A07 |  | 0.81 |
| **5931401** | B-A04 |  | 3.34 |

One highly active quinazoline amine, compound 5931398 (B-A03) was selected for further evaluation (Fig. 4A). Time-kill assays indicated a dose-dependent bactericidal effect of B-A03 against *M. ulcerans*, which was more pronounced with prolonged exposure (Fig. 4B).

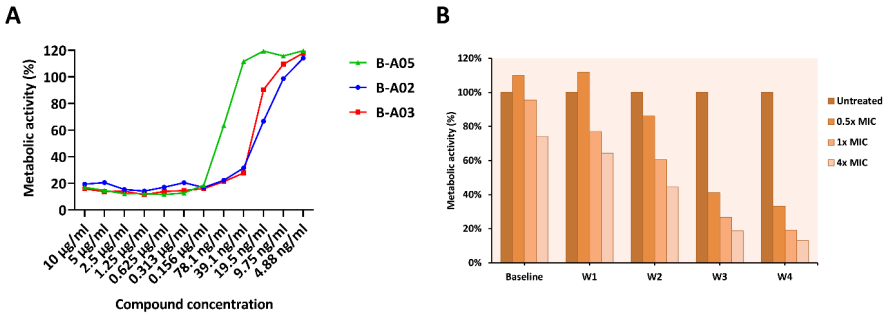



**Figure 4. Activity of quinazoline amines against *M. ulcerans***. (A) Resazurin test showing activity of the three most active quinazoline amines against *M. ulcerans* strain S1013. (B) Time-kill assay showing activity of compound B-A03 against *M. ulcerans* strain S1013. Concentrations of B-A03 used were 0.0125 µg/ml (0.5x MIC), 0.025 µg/ml (1x MIC), and 0.1 µg/ml (4x MIC). Included controls were untreated bacteria (positive control, PC) and uninoculated medium (negative control, NC).

Quinazoline derivatives have been shown to have myriad biological activities, including antibacterial, antifungal, antiviral, antimalarial, antihelminthic, diuretic, anticancer, and antidepressant functions, amongst others [23]. The quinazoline backbone is versatile and lends itself readily to medicinal chemistry efforts, with the resultant compounds having a variety of cellular targets. Recently, 2-ethylthio-4-methylaminoquinazoline derivatives, which are quinazolines structurally similar to the quinazoline amines described in the current study, were shown to be active against *M. tuberculosis*, with $MIC_{50}$ values between 0.011 – 0.013 µg/ml [12]. Those quinazolines were active against

drug-sensitive and drug-resistant mycobacteria in the *M. tuberculosis* complex, as well as the closely related *M. marinum*, but were inactive against other bacteria tested (including both Gram-positive and Gram-negative bacteria).

The close structural similarity of the quinazoline amines evaluated in the current study to the 2-ethylthio-4-methylaminoquinazoline derivatives suggests that both groups of compounds could act via similar mechanisms. The 2-ethylthio-4-methylaminoquinazoline derivatives target QcrB, and possibly QcrA, of the $bc_1$:$aa_3$ cytochrome [12]. Classical lineage *M. ulcerans* strains, which account for the majority of BU cases worldwide, are repeatedly reported to be hypersensitive to cyt-$bc_1$:$aa_3$ inhibitors, owing to their lack of a functional alternative *bd* cytochrome. Thus, quinazoline derivatives such as those evaluated in the current study, could potentially contribute to new treatment paradigms for BU.

#### *iv. Pyrrolo-[3,4-c]pyridine-1,3(2H)-diones*

We evaluated 32 pyrrolo-[3,4-c]pyridine-1,3(2H)-diones for activity against *M. ulcerans*. The seven pyrrolopyridine diones with the lowest $MIC_{50}$ values are shown in Table 4 below (the full list of activities is shown in Table S3).

**Table 4**. Pyrrolo-[3,4-c]pyridine-1,3(2H)-diones with activity against *M. ulcerans* ($MIC_{50}$ <10 µg/ml).

| Compound | Lab ID | Structure | $MIC_{50}$ (µg/ml) |
|---|---|---|---|
| H3D-001520-01-02 | Z-17 | | 0.20 |
| H3D-001121-01-02 | Z-04 | | 0.25 |
| H3D-001509-01-02 | Z-07 | | 0.27 |
| H3D-001511-01-02 | Z-09 | | 0.43 |
| H3D-001507-01-02 | Z-05 | | 0.45 |

| | | |
|---|---|---|
| H3D-001513-01-02 | Z-11 | 0.54 |
| H3D-001508-01-02 | Z-06 | 0.87 |

One highly active pyrrolopyridine dione, compound H3D-001121-01-02 (Z-04) was selected for further evaluation (Fig. 5A). Time-kill assays indicated that Z-04 was bactericidal against *M. ulcerans*, with increased activity following prolonged exposure (Fig. 5B).

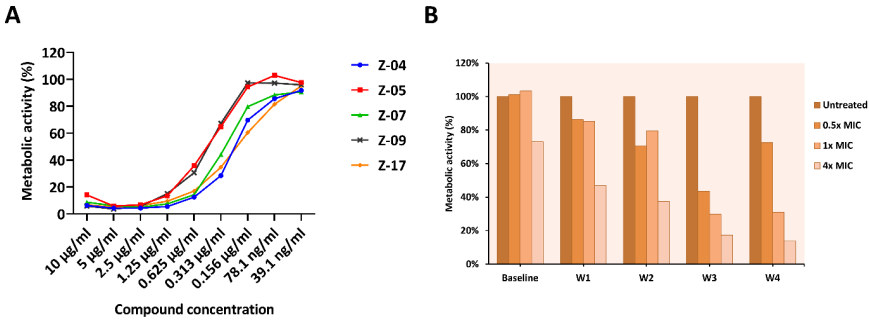



**Figure 5. Activity of pyrrolo-[3,4-c]pyridine-1,3(2H)-diones against *M. ulcerans***. (A) Resazurin test showing activity of the five most active pyrrolopyridine diones against *M. ulcerans* strain S1013. (B) Time-kill assay showing activity of compound Z-04 against *M. ulcerans* strain S1013. Concentrations of Z-04 used were 0.125 µg/ml (0.5x MIC), 0.25 µg/ml (1x MIC), and 1 µg/ml (4x MIC). Included controls were untreated bacteria (positive control, PC) and uninoculated medium (negative control, NC).

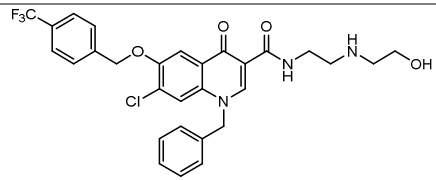
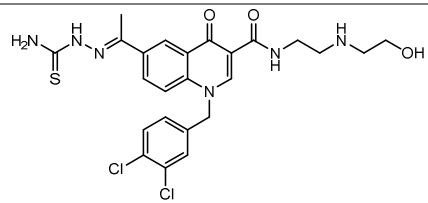
Pyrrolopyridine diones were identified as potent inhibitors of mycobacterial respiration. The parent hit compound was identified at the Novartis Institute for Tropical Diseases following high-throughput phenotypic screening of a large library of up to 6000 compounds occupying a chemical space where typical anti-tuberculosis drugs reside [14]. This hit compound, although active against *M. tuberculosis*, was found to have poor *in vitro* metabolic stability. Subsequent hit optimisation resulted in potent derivatives

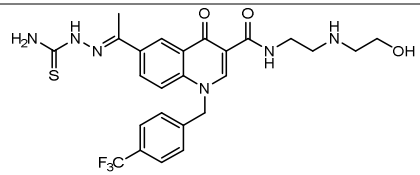
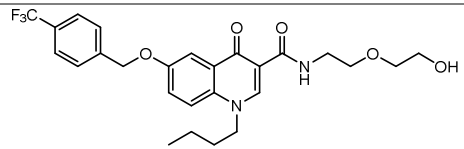
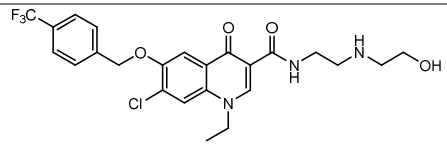
with improved *in vitro* stability in liver microsomes, albeit with poor *in vivo* stability in mice. Nevertheless, pyrrolopyridine diones are a promising class of antimycobacterial compounds. Like the imidazopyridine amide Q203 and the arylvinylpiperazine amide AX-35, the pyrrolopyridine diones were found to target QcrB, and could therefore be valuable additions to the development of new combination chemotherapy for BU.

***v. Quinolone derivatives***

We tested 103 quinolone derivatives for activity against *M. ulcerans*. The seven compounds with highest activity against *M. ulcerans* are shown Table 5 below (the full list of activities is shown in Table S4).

**Table 5.** Quinolone derivatives with activity against *M. ulcerans*.

| Compound | Structure | $MIC_{50}$ (µg/ml) |
|---|---|---|
| 18Qn14 |  | 0.7 |
| RB038 |  | 1.7 |

| | | |
|---|---|---|
| RB026 |  | 3.0 |
| 18Qn32 |  | 4.2 |
| 18Qn16 |  | 5 – 10 |

The most highly active quinolone derivative, compound 18Qn14, was selected for further evaluation (Fig. 6A). Time-kill assays indicated that 18Qn14 was bactericidal against *M. ulcerans* at higher concentrations (≥4x MIC) but bacteriostatic at lower concentrations (Fig. 6B).

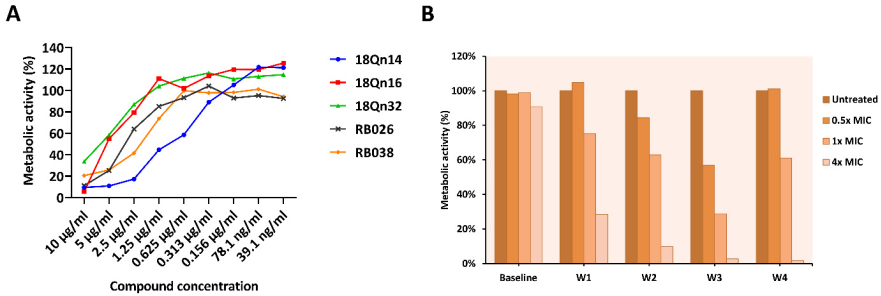



**Figure 6. Activity of quinolone derivatives against *M. ulcerans***. (A) Resazurin test showing activity of the five most active quinolone derivatives against *M. ulcerans* strain S1013. (B) Time-kill assay showing activity of compound 18Qn14 against *M. ulcerans* strain S1013. Concentrations of 18Qn14 used were 0.35 µg/ml (0.5x MIC), 0.7 µg/ml (1x

MIC), and 2.8 µg/ml (4x MIC). Included controls were untreated bacteria (positive control, PC) and uninoculated medium (negative control, NC).

Quinolones are a versatile compound class, with a wide range of activities, including broad-spectrum antibacterial activity, as well as antifungal, antiparasitic, and other activities. They target DNA topoisomerases, with a predilection for prokaryotic topoisomerases [24]. During DNA synthesis, topoisomerases relax supercoiled DNA by transiently nicking the sugar-phosphate backbone of DNA and resealing the nick after the DNA strand has unravelled at the break point. The prokaryotic Type IIA topoisomerases (DNA gyrase and topoisomerase IV) are the targets of quinolones, including fluoroquinolones. Quinolones act by binding to and stabilising the topoisomerase-DNA complex, resulting in prolonged DNA nicking, and ultimately causing DNA fragmentation and cell death [24, 25].

The quinolone derivatives found to be active against *M. ulcerans* were all based on a 4-quinolone-3-carboxyl pharmacophore. This pharmacophore features in many useful drugs, including the fluoroquinolone antibiotics that are important components of the tuberculosis second-line treatment regimen. Compound 18Qn14, and the other quinolone carboxamides with antimycobacterial activity, were derivatised to be lipophilic since highly lipophilic drugs are known to more readily penetrate the lipid-rich mycobacterial cell wall [26, 27]. In addition to its anti-tubercular activity, 18Qn14 was also active against the protozoa *Plasmodium falciparum* and *Trypanosoma brucei brucei*, which cause significant disease and mortality in sub-Saharan Africa [26]. Given the overlap in the endemicity of BU with these diseases, it is an attractive approach to develop such a drug that would be especially beneficial in cases of coinfections.

**CONCLUSION**

Drug development for BU treatment is still a necessity, and repurposing candidates from the drug development pipeline for other diseases is a viable strategy for overcoming the inherent difficulties in *de novo* drug development for neglected diseases. We have evaluated five scaffolds for activity against *M. ulcerans* and discussed the known or proposed drug targets of each compound class. Further studies would help elucidate the therapeutic potentials of these scaffolds.

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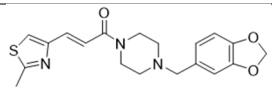
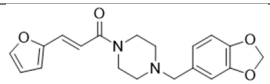
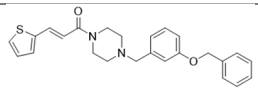
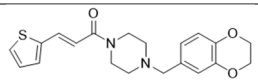
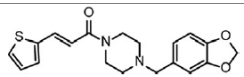
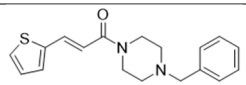
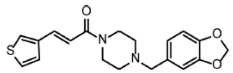
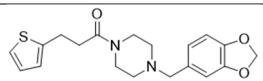
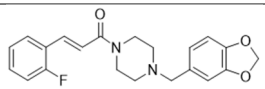
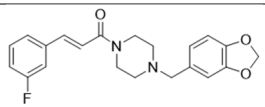
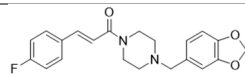
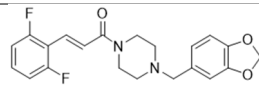
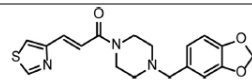
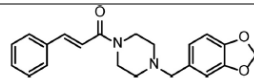
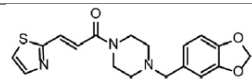
## Supplementary Data

**Table S1. Arylvinylpiperazine amides tested against *M. ulcerans***

| Compound ID | Structure | $MIC_{50}$ (µg/ml) | $MIC_{50}$ (µM) |
|---|---|---|---|
| DM-344 |  | 0.27 | 0.73 |
| JW-011 |  | 0.57 | 1.66 |
| FH-007 |  | 0.68 | 1.63 |
| DM-359 |  | 0.76 | 2.04 |
| AX-35 |  | 1 | 2.81 |
| JW-010 |  | 2.47 | 7.9 |
| AX-36 |  | 3.1 | 8.71 |
| JW-003 |  | >3.58 | >10 |
| DM-368 |  | >3.68 | >10 |
| JW-058 |  | >3.68 | >10 |

| | | | |
|---|---|---|---|
| DM-257 |  | >3.68 | >10 |
| DM-369 |  | >3.86 | >10 |
| AX-37 |  | >5 | >14 |
| AX-38 |  | >10 | >28.6 |
| AX-39 |  | >10 | >28.01 |

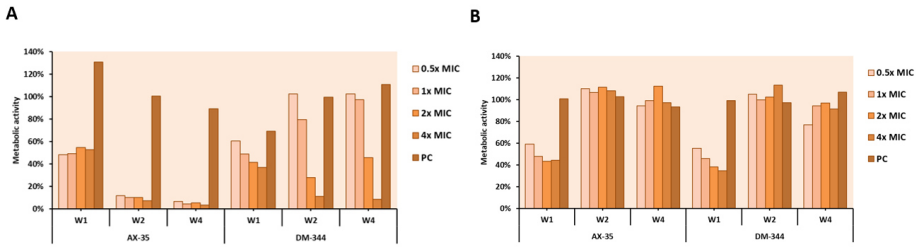
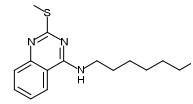
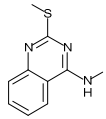
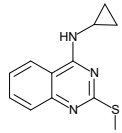
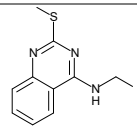
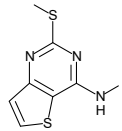
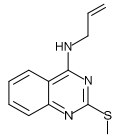
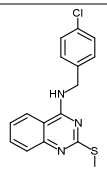



**Figure S1. Activity of arylvinylpiperazine amides against *M. ulcerans***. The long-term activity of the prototype compound AX-35 and a highly active derivative DM-344 against classical (A) and ancestral (B) lineage *M. ulcerans* strains were compared using the resazurin assay. The classical lineage strain was completely inhibited by AX-35 at all concentrations of the compound tested following prolonged incubation, while DM-344 was only completely inhibitory at the highest concentration tested. The ancestral lineage strain was only transiently inhibited by all concentrations of both compounds tested. Concentrations of AX-35 used for both strains were 0.5 µg/ml (0.5x MIC), 1 µg/ml (1x

MIC), 2 µg/ml (2x MIC), and 4 µg/ml (4x MIC). Concentrations of DM-344 used for both strains were 0.37 µM (0.5x MIC), 0.73 µM (1x MIC), 1.46 µM (2x MIC), and 2.92 µM (4x MIC). Untreated bacteria were included as positive controls (PC).

**Table S2. Quinazoline amines tested against *M. ulcerans***

| Compound | Lab ID | Structure | $MIC_{50}$ (µg/ml) |
|---|---|---|---|
| **5931399**<br>2-methylsulfanyl-N-octylquinazolin-4-amine | B-A02 |  | 0.019 |
| **5931398**<br>2-methylsulfanylquinazolin-4-amine | B-A03 |  | 0.025 |
| **6152102**<br>N-cyclopropyl-2-methylsulfanylquinazolin-4-amine | B-A05 |  | 0.076 |
| **5968230**<br>N-ethyl-2-methylsulfanylquinazolin-4-amine | B-B03 |  | 0.28 |
| **5965917**<br>N-methyl-2-methylsulfanylthieno[3,2-d]pyrimidin-4-amine | B-A08 |  | 0.40 |
| **5931400**<br>2-methylsulfanyl-N-prop-2-enylquinazolin-4-amine | B-A07 |  | 0.81 |
| **5931401**<br>N-[(4-chlorophenyl)methyl]-2-methylsulfanylquinazolin-4-amine | B-A04 |  | 3.34 |

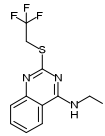
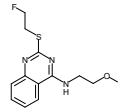
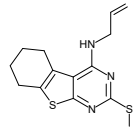
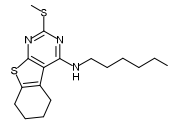
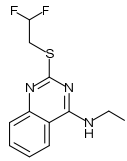
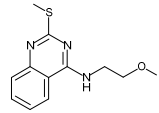
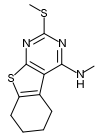
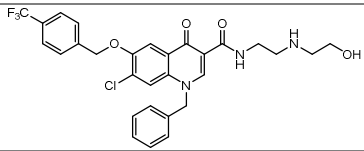
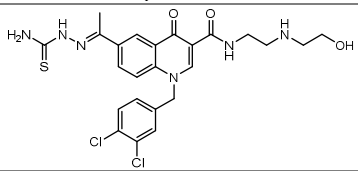
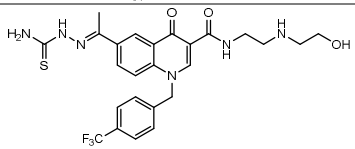
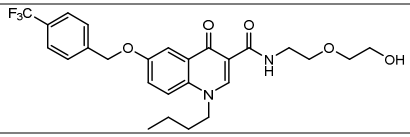
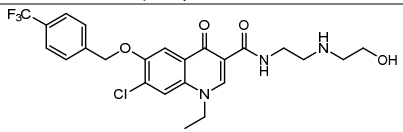
| | | | |
|---|---|---|---|
| **6152830**<br>N-ethyl-2-(2,2,2-trifluoroethylsulfanyl)quinazolin-4-amine | B-B01 |  | 5 – 10 |
| **6151935**<br>N-(2-methoxyethyl)-2-fluoroethylsulfanylquinazolin-4-amine | B-A01 |  | >10 |
| **6152873**<br>N-allyl-2-(methylsulfanyl)-5,6,7,8-tetrahydro[1]benzothieno[2,3-d]pyrimidin-4-amine | B-A06 |  | >10 |
| **5966608**<br>N-hexyl-2-methylsulfanyl-5,6,7,8-tetrahydro-[1]benzothiolo[2,3-d]pyrimidin-4-amine | B-A09 |  | >10 |
| **6152827**<br>2-(2,2-difluoroethylsulfanyl)-N-ethylquinazolin-4-amine | B-A10 |  | >10 |
| **5272947**<br>N-(2-methoxyethyl)-2-methylsulfanylquinazolin-4-amine | B-A11 |  | >10 |
| **6153079**<br>N-methyl-2-methylsulfanyl-5,6,7,8-tetrahydro-[1]benzothiolo[2,3-d]pyrimidin-4-amine | B-B02 |  | >10 |

**Table S3. Pyrrolopyridine diones tested against *M. ulcerans***

| Compound | Lab ID | Structure | $MIC_{50}$ (µg/ml) |
|---|---|---|---|
| H3D-001520-01-02 | Z-17 | | 0.20 |
| H3D-001121-01-02 | Z-04 | | 0.25 |
| H3D-001509-01-02 | Z-07 | | 0.27 |
| H3D-001511-01-02 | Z-09 | | 0.43 |
| H3D-001507-01-02 | Z-05 | | 0.45 |
| H3D-001513-01-02 | Z-11 | | 0.54 |
| H3D-001508-01-02 | Z-06 | | 0.87 |
| H3D-001522-01-02 | Z-18 | | 1.32 |
| H3D-001526-01-02 | Z-22 | | 2.37 |
| H3D-001525-01-02 | Z-21 | | 2.44 |
| H3D-001538-01-02 | Z-25 | | 2.51 |
| H3D-001523-01-02 | Z-19 | | 4.3 |
| H3D-001048-01-02 | Z-03 | | 5 – 10 |
| H3D-000863-01-02 | Z-01 | | >10 |
| H3D-000872-01-02 | Z-02 | | >10 |
| H3D-001510-01-02 | Z-08 | | >10 |
| H3D-001512-01-02 | Z-10 | | >10 |
| H3D-001514-01-02 | Z-12 | | >10 |
| H3D-001515-01-02 | Z-13 | | >10 |
| H3D-001516-01-02 | Z-14 | | >10 |
| H3D-001518-01-02 | Z-15 | | >10 |
| H3D-001519-01-02 | Z-16 | | >10 |
| H3D-001524-01-02 | Z-20 | | >10 |
| H3D-001527-01-02 | Z-23 | | >10 |
| H3D-001535-01-02 | Z-24 | | >10 |
| H3D-001540-01-02 | Z-26 | | >10 |
| H3D-001541-01-02 | Z-27 | | >10 |

| | | |
|---|---|---|
| H3D-001542-01-02 | Z-28 | >10 |
| H3D-001543-01-02 | Z-29 | >10 |
| H3D-001544-01-02 | Z-30 | >10 |
| H3D-001552-01-02 | Z-31 | >10 |
| H3D-001575-01-02 | Z-32 | >10 |

**Table S4. Quinolone derivatives tested against *M. ulcerans***

| Compound | Structure | $MIC_{50}$ (µg/ml) |
|---|---|---|
| 18Qn14 |  | 0.7 |
| RB038 |  | 1.7 |
| RB026 |  | 3.0 |
| 18Qn32 |  | 4.2 |
| 18Qn16 |  | 5 – 10 |
| 18Qn29 | | >10 |
| RB025 | | >10 |

| | |
|---|---|
| 18Qn31 | >10 |
| 18Qn34 | >10 |
| RB012 | >10 |
| RMB006 | >10 |
| INQ006 | >10 |
| E1 | >10 |
| C5 | >10 |
| B5 | >10 |
| A5 | >10 |
| AA | >10 |
| G | >10 |
| B1 | >10 |
| B3 | >10 |
| C1 | >10 |
| C2 | >10 |
| C3 | >10 |
| D1 | >10 |
| D2 | >10 |
| D3 | >10 |
| A4 | >10 |
| E2 | >10 |
| E3 | >10 |
| B4 | >10 |
| F1 | >10 |
| F2 | >10 |
| F3 | >10 |
| C4 | >10 |
| D4 | >10 |
| EC001 | >10 |
| EC004 | >10 |

| | |
|---|---|
| EC009 | >10 |
| EC014 | >10 |
| EC015 | >10 |
| EC019 | >10 |
| EC020 | >10 |
| EC021 | >10 |
| EC023 | >10 |
| EC024 | >10 |
| EC027 | >10 |
| EC031 | >10 |
| EC032 | >10 |
| EC036 | >10 |
| EC037 | >10 |
| EC040 | >10 |
| EC041 | >10 |
| EC042 | >10 |
| 18Qn3 | >10 |
| 18Qn4 | >10 |
| 18Qn5 | >10 |
| 18Qn6 | >10 |
| 18Qn7 | >10 |
| 18Qn8 | >10 |
| 18Qn9 | >10 |
| 18Qn10 | >10 |
| 18Qn11 | >10 |
| 18Qn12 | >10 |
| 18Qn13 | >10 |
| 18Qn15 | >10 |
| 18Qn17 | >10 |
| 18Qn18 | >10 |

| | |
|---|---|
| 18Qn19 | >10 |
| 18Qn22 | >10 |
| 18Qn23 | >10 |
| 18Qn25 | >10 |
| 18Qn26 | >10 |
| 18Qn28 | >10 |
| 18Qn35 | >10 |
| 18Qn37 | >10 |
| 18Qn38 | >10 |
| 18Qn39 | >10 |
| 18Qn40 | >10 |
| 18Qn41 | >10 |
| INQ003 | >10 |
| INQ4b | >10 |
| INQ005 | >10 |
| INQ007 | >10 |
| INQ008 | >10 |
| RB002 | >10 |
| RB005 | >10 |
| RB009 | >10 |
| RB010 | >10 |
| RB011 | >10 |
| RB014 | >10 |
| RB027 | >10 |
| RB029 | >10 |
| RB030 | >10 |
| RB031 | >10 |
| RB036 | >10 |
| RB037 | >10 |
| RB037 | >10 |

| | |
|---|---|
| RMB003 | >10 |
| RMB013 | >10 |
| RMB041 | >10 |
| RMB042 | >10 |
| RMB073 | >10 |
| EC003 | >10 |

## Section III: Prevention

- Generation of toxin neutralising antibodies by immunisation with mycolactone conjugate vaccines: Perspectives for vaccine development.

# Generation of toxin neutralising antibodies by immunisation with mycolactone conjugate vaccines: perspectives for vaccine development

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Working manuscript

**ABSTRACT**

Buruli ulcer control is currently dependent on the diagnosis and treatment of all cases. Vaccine development is still an attractive means of lasting control and is therefore the subject of continued research. Thus far, most vaccine candidates have failed to offer robust protection in mouse models of BU, although they engender high antibody titres. The polyketide cytotoxin mycolactone is an ideal vaccine candidate given its central role in the pathogenesis of BU. Mycolactone consists of a 12-membered lactone ring with a lower O-linked polyunsaturated acyl side chain and an upper C-linked side chain. We recently described the generation of sets of mycolactone-specific monoclonal antibodies using protein conjugates of non-toxic mycolactone derivatives, and have reported on the toxin-neutralising ability of some of these antibodies. In the current study, we have explored the utility of these protein conjugates as toxoid vaccines, extensively characterising the generated mAbs, including elucidating the differential neutralisation potency. We found that the most potent neutralisation was afforded by mAbs recognising the upper C-linked side chain or the lactone ring; mAbs recognising the lower O-linked acyl side chain were non-neutralising. Sequence analyses indicated differences in antigen binding sites that could account for some of the differences in binding specificity and antibody affinity. Thus, we have defined a minimum truncated mycolactone derivative capable of eliciting potent toxin-neutralisation.

# Chapter 8: Evaluation of BU vaccine candidates

## INTRODUCTION

*Mycobacterium ulcerans* disease, more commonly known as Buruli ulcer (BU), is the third most common mycobacterial disease, after tuberculosis and leprosy. BU is a neglected tropical disease endemic in tropical and subtropical regions of the world [1]. The characteristic lesions of this chronic necrotising skin disease are slow-healing ulcers, which can be quite expansive and destructive, sometimes affecting whole limbs or covering large swaths of the torso. Without treatment, the ulcers heal very slowly, often taking many months to years to heal, with the formation of contractile scars that can lead to permanent disability because they often pull the affected body parts into unnatural positions. Severe infection involving critical body sites such as the face, external genitalia, and underlying bone, may result in amputations and loss of function in the affected sites. These are typical endpoints of the disease in resource-limited endemic regions [2]. Uncomplicated BU is rarely fatal, although disfigurements and disabilities are common. More worrisome is the high risk of sepsis due to secondary infection of poorly managed ulcers, which could very well lead to fatal outcomes [3]. Other manifestations of the disease are nodules, plaques, and widespread oedema, but all these forms typically devolve into ulcers without treatment.

*M. ulcerans* diverged from a common ancestor with *M. marinum* partly by the acquisition of the large pMUM plasmid that encodes machinery for the synthesis of a polyketide macrolide toxin known as mycolactone [4]. Mycolactone is responsible for the key features of BU [5, 6], and has been shown to cause the widespread tissue destruction seen in BU, due to its cytotoxicity to different cell types. Even minute quantities of mycolactone have been reported to be cytotoxic *in vitro* to such cells as fibroblasts, endothelial cells, adipocytes, macrophages, neurons, and osteocytes at low µg/ml concentrations [7]. At sublethal concentrations, mycolactone causes derangements in cellular function, resulting in local immunosuppression, local analgesia, amongst other

known effects [5]. Due to its central role in the pathogenesis of BU and its uniqueness to *M. ulcerans*, mycolactone has been the focus of a great deal of research both to understand the pathology of the disease and to develop specific diagnostic tools. Additionally, understanding the immunological response to mycolactone could help in the development of vaccines, which could lead to the generation of neutralising antibodies able to disarm the bacteria and ultimately lead to their clearance.

Several attempts have been made to generate antibodies against mycolactone that could be used to develop immunodiagnostic tools for BU. The cytotoxic nature of mycolactone prevents use of the unmodified toxin in the generation of monoclonal or polyclonal antibodies using the typical hybridoma technology. Therefore, following extensive SAR studies that showed that the hydrophobic side chain of mycolactone was crucially important for the cytotoxicity of the molecule [8], we previously reported the successful generation of the first-ever anti-mycolactone monoclonal antibodies (mAbs) using the hybridoma technology [9]. By linking synthetic variants of mycolactone lacking this side chain to carrier proteins, a robust immune response could be generated on inoculation of this construct into mice, with the eventual generation of mAbs able to specifically and sensitively detect mycolactone in lipid extracts of *M. ulcerans* cultures [9, 10]. Phage display is an alternative method that has been used to generate recombinant antibodies that can recognise mycolactone [11].

Interestingly, we found that the anti-mycolactone mAbs were also able to neutralise the toxin to varying extents and protect cultured mouse L929 fibroblasts from the cytotoxicity of mycolactone [9]. This brings up the possibility of utilising such mAbs therapeutically, and opens the door to the development of vaccination paradigms. Using the same methodology, we have generated additional sets of anti-mycolactone mAbs and utilised these mAbs to develop an antigen capture assay for mycolactone detection [12]. In the current study, we describe the differential mycolactone neutralising abilities

of these mAbs in comparison to those previously generated, and discuss the suitability of modified mycolactones as targets for BU vaccine development.

## MATERIALS AND METHODS

### Ethical statement

Mouse immunogenicity studies and mAb generation were performed under the approval by the animal welfare committee of the Canton of Basel-Stadt (authorisation number BE95/17). All animal experimentation were conducted in compliance with the Swiss Animal Welfare Act (TSchG), Animal Welfare Ordinance (TSchV), and the Animal Experimentation Ordinance (TVV).

### Preparation of synthetic mycolactones

The chemical synthesis of mycolactone and mycolactone derivatives (Fig. 1) has been described elsewhere [8, 9, 13]. All synthetic products were HPLC-purified and dissolved in dimethyl sulfoxide (DMSO) to give 1 mg/ml stock solutions.

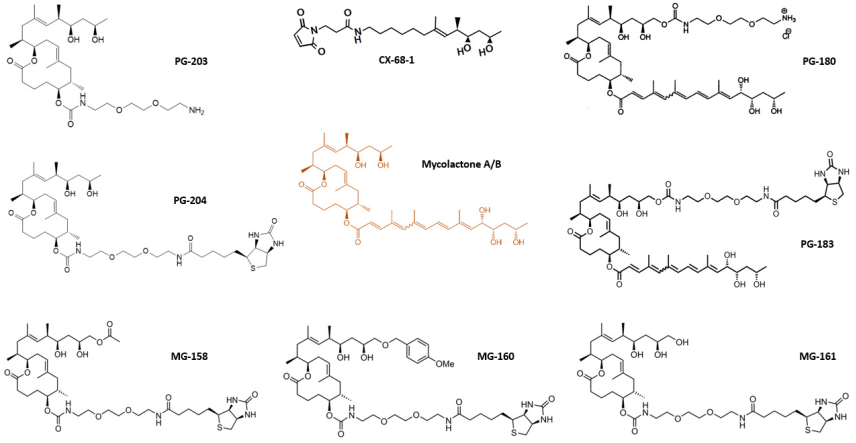


**Figure 1**. Structure of mycolactone A/B and modified mycolactones used for mouse immunisations and mAb selection.

**Generation of mAbs and analyses of mycolactone binding patterns**

The generation and binding pattern analyses of anti-mycolactone mAbs have been fully outlined elsewhere [12]. Briefly, mice were immunised in a prime-boost approach with any one of the modified mycolactones PG-203, PG-180, or CX-68-1 that had been coupled to BSA (Fig. 1). Hybridomas were selected by ELISA using a panel of biotinylated mycolactones comprising PG-204, MG-161, MG-158, MG-160, and PG-183 (Fig. 1). Selected hybridomas were cloned by limiting dilution, and mAbs were purified from culture supernatants by affinity chromatography.

The binding patterns of purified mAbs to mycolactone were elucidated by ELISA with the same panel of biotinylated mycolactones that was used for hybridoma screening. For this, biotinylated mycolactones were coated onto NeutrAvidin-coated plates (Thermo Scientific) at a concentration of 1 µg/ml. Coated plates were then blocked with SuperBlock® T20 (Thermo Scientific) after which serially diluted mAbs (from 10 µg/ml – 0.64 ng/ml) were added in and allowed to react. Bound mAbs were detected with horseradish peroxidase-coupled goat anti-mouse IgG (SouthernBiotech), with 3,3',5,5'-tetramethylbenzidine (TMB; KPL SeraCare) used for signal development.

**Preparation of ethanolic extracts from *M. ulcerans* cultures**

A low-passage *M. ulcerans* strain S1013 cultivated on Middlebrook 7H9 agar plates supplemented with 0.2% glycerol and 10% OADC was used to prepare ethanolic extracts. Eight-week old cultures were scraped off the agar surface with a sterile inoculating loop and transferred into 15 ml of absolute ethanol (Sigma). Samples were vortexed for 1 min and then incubated at ambient temperature for 3 days in the dark. Samples were again vortexed for 1 min and then centrifuged at 4,000 x *g* for 10 min to pellet the bacteria. The supernatant was filter-sterilised using 0.22 µm syringe filters and dried by vacuum centrifugation (SpeedVac, Thermo Scientific). The extracts were resuspended in sterile

cell culture-grade DMSO (Sigma) and stored at -20°C until needed. Mycolactone contents of the extracts were quantified by competitive ELISA as previously described [10].

**Mycolactone neutralisation assays**

The resazurin assay was used to evaluate the mycolactone neutralising ability of the mAbs. Neutralisation of ethanolic extracts of *M. ulcerans* cultures was assessed first, and the results were confirmed using synthetic mycolactone A/B. Mouse L929 fibroblasts (20,000 cells/well) were grown in 24-well tissue culture plates (TPP) containing RPMI medium (Sigma) supplemented with 2 mM L-glutamine (Gibco), and incubated for 24 h at 37°C in 5% $CO_2$. The medium was then removed and replaced with fresh medium containing 15 ng/ml of mycolactone (either synthetic or extracted), as well as different molar ratios of each mAb to be tested. Positive control wells received medium containing 1% DMSO only. Negative control wells were cell-free and contained only fresh medium. Also included was an isotype-match mAb JD4.1 as negative comparator. All plates were incubated for 48 h at 37°C in 5% $CO_2$. Thereafter, 10% v/v of a 0.125 mg/ml resazurin solution (Sigma) was added to each well and plates were incubated for a further 2 h at 37°C in 5% $CO_2$. To quantify the metabolic activity of the fibroblasts, the fluorescence of the negative control wells was subtracted from that of all other wells, and the difference was normalised for the DMSO control. Each assay was performed in duplicates in at least two independent replicates.

## RESULTS

### mAb generation and mycolactone recognition patterns

Three different conjugate vaccines incorporating synthetic mycolactone derivatives were used as mouse immunogens for mAb generation (Fig. 1). Three sets of anti-mycolactone mAbs were generated from two of the immunogens used – PG-180 and PG-203. No mAbs could be generated using CX-68-1 as immunogen. The mycolactone recognition patterns of the generated mAbs are shown in Table 1 below. Subset 1 mAbs were generated using PG-180 as immunogen, and consequently only recognised PG-183, the sole biotinylated mycolactone with an intact hydrophobic lower side chain. These mAbs are therefore thought to recognise epitopes on the lower side chain of mycolactone. Subset 2 mAbs recognised all biotinylated mycolactones with a lactone core, regardless of the degree of modification to either side chain. These mAbs, generated using either PG-180 or PG-203 as immunogen, are thought to recognise the lactone core of mycolactone. The binding of Subset 3 mAbs decreased to varying degrees with increasing modifications to the upper side chain of mycolactone. Consequently, these mAbs, generated using PG-203 as immunogen, are thought to recognise the upper side chain of mycolactone.

**Table 1**. Binding patterns of anti-mycolactone mAbs (adapted from [12])

| | Immunogen | mAb | PG-183 | MG-160 | MG-158 | MG-161 | PG-204 |
|---|---|---|---|---|---|---|---|
| Subset 1 | PG-180 | LW1.1a | +++ | - | - | - | - |
| | | LW1.1b | +++ | - | - | - | - |
| | | LW2.4a | +++ | - | - | - | - |
| | | LW2.4b | +++ | - | - | - | - |
| | | LW2.5a | +++ | - | - | - | - |
| | | LW2.5b | +++ | - | - | - | - |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Subset 2 | PG-180 | LW2.1a | + | +++ | +++ | +++ | +++ |
| | | LW2.1b | + | +++ | +++ | +++ | +++ |
| | | LW2.2a | + | +++ | +++ | +++ | +++ |
| | | LW2.2b | + | +++ | +++ | +++ | +++ |
| | PG-203 | LW7.1 | + | +++ | +++ | +++ | +++ |
| | | LW7.2 | + | +++ | +++ | +++ | +++ |
| | | LW7.16 | ++ | +++ | +++ | +++ | +++ |
| | | LW7.3 | +++ | +++ | +++ | +++ | +++ |
| | | LW7.4 | +++ | +++ | +++ | +++ | +++ |
| | | LW7.5 | +++ | +++ | +++ | +++ | +++ |
| | | LW7.6 | +++ | +++ | +++ | +++ | +++ |
| | | LW7.7 | +++ | +++ | +++ | +++ | +++ |
| | | LW7.8 | +++ | +++ | +++ | +++ | +++ |
| | | LW7.9 | +++ | +++ | +++ | +++ | +++ |
| | | LW7.14 | +++ | +++ | +++ | +++ | +++ |
| | | LW7.15 | +++ | +++ | +++ | +++ | +++ |
| | | LW7.17 | +++ | +++ | +++ | +++ | +++ |
| | | LW7.18 | +++ | +++ | +++ | +++ | +++ |
| Subset 3 | PG-203 | JD5.1 | - | - | +++ | +++ | +++ |
| | | JD5.2 | - | - | - | +++ | +++ |
| | | JD5.3 | - | - | + | +++ | +++ |
| | | JD5.4 | - | - | ++ | +++ | +++ |
| | | JD5.5 | - | - | ++ | +++ | +++ |
| | | JD5.6 | - | - | + | +++ | +++ |
| | | JD5.7 | - | - | - | +++ | +++ |
| | | JD5.8 | - | - | ++ | +++ | +++ |
| | | JD5.9 | - | - | +++ | +++ | +++ |
| | | JD5.10 | - | - | +++ | +++ | +++ |
| | | JD5.12 | - | - | + | +++ | +++ |

| | | | | | | |
|---|---|---|---|---|---|---|
| | **LW7.10** | - | + | + | +++ | +++ |
| | **LW7.11** | - | + | + | +++ | +++ |
| | **JD5.11** | - | + | +++ | +++ | +++ |
| | **LW7.12** | - | +++ | +++ | +++ | +++ |
| | **LW7.19** | - | +++ | +++ | +++ | +++ |

**Mycolactone neutralisation**

**i. mAbs recognising the hydrophobic acyl side chain of mycolactone (Subset 1)**

None of the mAbs that recognised the polyunsaturated hydrophobic side chain of mycolactone was able to neutralise the toxin. Even when tested at forty-fold excess molar ratio, no mycolactone neutralisation was observed (Fig. 2).

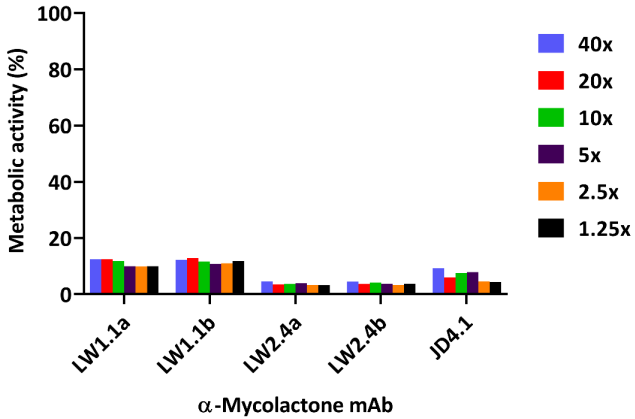


**Figure 2. Mycolactone neutralising ability of Subset 1 mAbs**. Mouse L929 fibroblasts were exposed to 15 ng/ml of synthetic mycolactone with different molar ratios of mAbs recognising epitopes on the hydrophobic acyl side chain of mycolactone. Isotype-

matched mAb JD4.1 was included as negative comparator. No mycolactone neutralisation was evident. The assay was performed in two independent replicates.

**ii. mAbs recognising the lactone core of mycolactone (Subset 2)**

All but three of the mAbs with specificity for the lactone core of mycolactone were able to neutralise the toxin to varying degrees. mAbs LW7.1, LW7.2, and LW7.16 – all of which were generated using PG-203 – were unable to neutralise mycolactone. The four mAbs in this subset generated using PG-180 as immunogen (LW2.1a, LW2.1b, LW2.2a, and LW2.2b) were required in a molar excess ≥40x for full neutralisation (Fig. 3a). In contrast, those mycolactone neutralising mAbs in this subset that were generated using PG-203 could achieve complete neutralisation at much lower molar excesses (≥5x) (Fig. 3b).

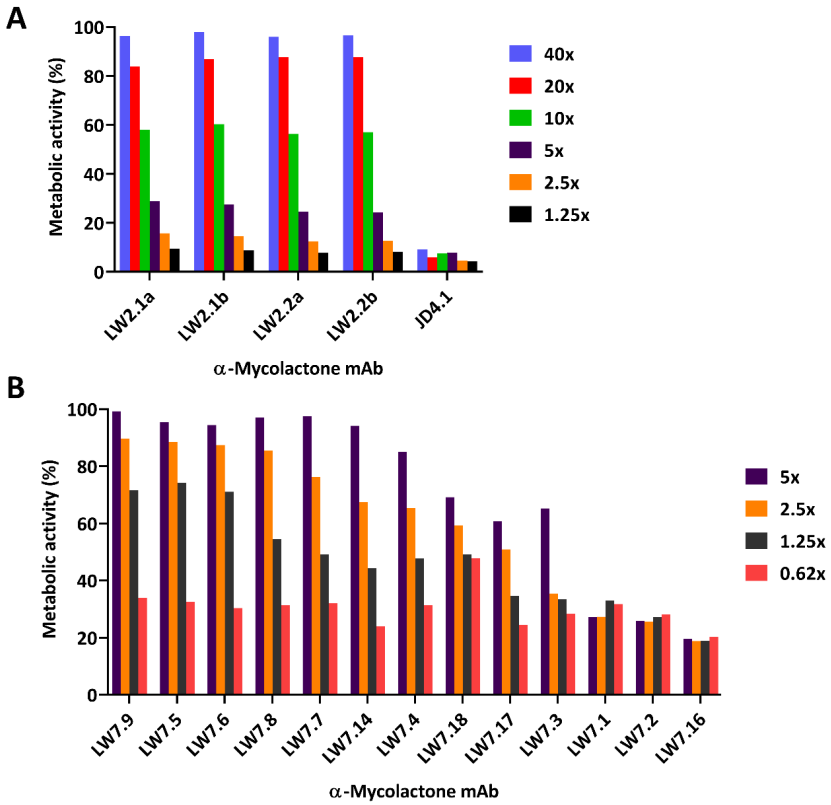


**Figure 3. Mycolactone neutralisation by Subset 2 mAbs.** Mouse L929 fibroblasts were exposed to 15 ng/ml of synthetic (A) or extracted (B) mycolactone with different molar ratios of mAbs recognising the lactone core of mycolactone. Isotype-matched mAb JD4.1 was included as negative comparator. The assay (A) was performed in two independent replicates.

### iii. mAbs recognising the upper side chain of mycolactone (Subset 3)

Most of the mAbs in this subset were able to neutralise mycolactone to varying degrees. The neutralising abilities of mAbs JD5.1 – JD5.12 have been reported previously [9]. The mAbs LW7.12 and LW7.19 were unable to neutralise the toxin, as shown in Fig. 4 below.

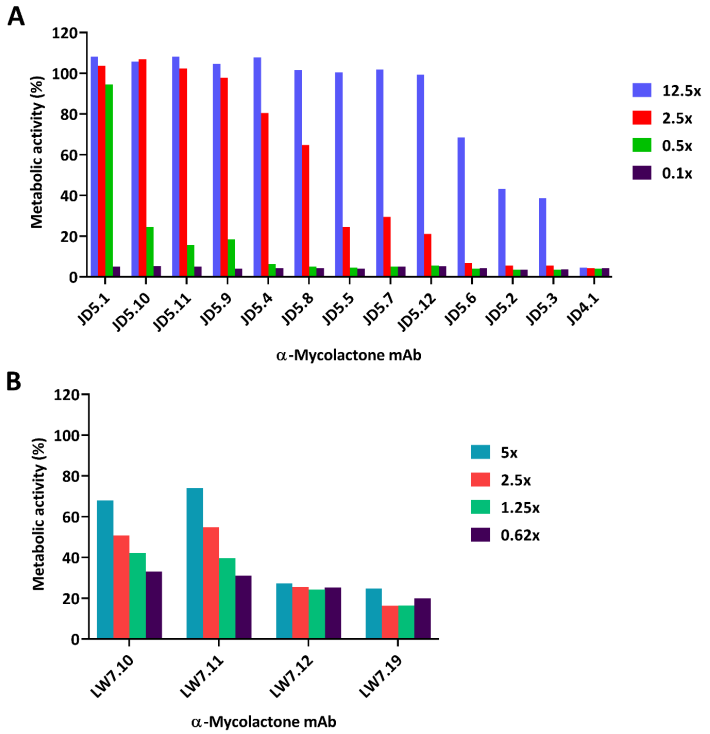


**Figure 4. Mycolactone neutralisation by Subset 3 mAbs.** Mouse L929 fibroblasts were exposed to 15 ng/ml of synthetic (A) or extracted (B) mycolactone with different molar ratios of mAbs recognising the upper side chain of mycolactone. Isotype-matched mAb JD4.1 was included as negative comparator. (A) was adapted from [9].

## DISCUSSION

The transmission routes of BU are yet to be ascertained, and there are currently no effective vaccines against BU. Consequently, BU control relies on early detection and treatment of all cases. However, as control efforts would benefit greatly from prevention of infection, the development of efficacious vaccines against BU remains a priority. The Bacillus Calmette-Guérin (BCG) vaccine, the only vaccine available for mycobacterial disease, offers only partial, short-term protection from severe BU, but does not prevent the disease [14]. Several different approaches have been applied to develop vaccines against BU, including the use of inactivated whole cells [15], cell surface proteins [16, 17, 18], proteins in the mycolactone synthesis machinery [19], amongst others. However, like BCG, most of these generate an immune response that does not fully protect against the disease.

Another possible approach could be the development of a toxoid vaccine, similar to what is used for tetanus and diphtheria prevention. Mycolactone, the polyketide toxin of *M. ulcerans*, is thought to be constitutively produced and secreted by the bacteria, forming a protective cloud that shields them from host immunity [20]. Therefore, even though antibody or cell-mediated immune responses may be generated by the aforementioned vaccine candidates, the effects of the toxin prevents host immune cells from reaching the infection foci and clearing the bacteria. It is conceivable, therefore, that neutralising the toxin could prevent the build-up of mycolactone, thus preserving the antimicrobial functions of host immunity. This is similar to what transpires during antibiotic treatment of BU, where immune reconstitution occurs following killing of the bacteria with concomitant cessation of mycolactone production [21, 22].

In the current study, we have highlighted the feasibility of developing a BU vaccine based on detoxified mycolactones. We previously reported the neutralisation of mycolactone

by mAbs generated using PG-203, a mycolactone derivative lacking the polyunsaturated acyl side chain that mediates the cytotoxicity of the molecule [9]. Using a similar approach, we applied two other modified mycolactones – PG-180 and CX-68-1 – to generate mAbs [12]. PG-180, although containing the polyunsaturated acyl side chain, was found to be about four times less cytotoxic compared to mycolactone A/B [8]. CX-68-1 comprises the upper side chain of mycolactone tethered to the carrier protein via a linker. Consistent with previous findings, immunisation with PG-203 resulted in potent mycolactone-neutralising antibodies, whether by recognising the lactone core (Subset 2 mAbs) or by targeting epitopes on the upper side chain (Subset 3 mAbs). Antibodies were able to neutralise both mycolactones in lipid extracts of *M. ulcerans* cultures as well as synthetic mycolactone. Neutralising antibodies could also be generated using PG-180 as immunogen, although such antibodies were much less efficient at neutralising the toxin, compared to those generated using PG-203. All attempts to generate mAbs using CX-68-1 as immunogen were unsuccessful, although inoculated mice had transient humoral responses to the antigen (antibody titres produced by priming were lost upon boosting).

Given the consistent generation of highly active neutralising antibodies using PG-203, this antigen, of all three that were tested in the current study, appears best suited as a candidate for further vaccine development. Efforts are ongoing to assess the efficacy of combinations of mAbs, as well as to define parameters for challenge studies in a mouse model of BU. Should results remain favourable, we envisage that a toxoid vaccine based on PG-203 could be developed and may contribute to the lasting control of BU.

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# General Discussion and Conclusions

## 9. General remarks

Over a century since BU was first described, and 23 years since its addition to the list of NTDs (since 1998), there are still considerable gaps in the current understanding of this debilitating disease. Ideally, disease control is by prevention, whether by interrupting transmission chains, or by vaccination to prevent disease in case transmission cannot be prevented. However, despite a considerable amount of effort, the transmission routes of *M. ulcerans* are still not known, and there are no effective vaccination paradigms available for BU, with BCG – the only available vaccine for mycobacterial infections – giving only modest, short-term protection from BU [1]. Therefore, BU can currently not be effectively prevented. Consequently, the WHO BU control strategy revolves around the diagnosis and treatment of every case [2, 3].

While a number of diagnostic methods and treatment options are in use, BU control is far from complete. Due to years of underreporting, owing to the lack of access to reliable healthcare by patients in remote rural settings with the highest endemicity, global BU incidence has been reportedly reducing steadily, which conveys an erroneous view of the disease being successfully controlled. As a counterpoint, BU incidence has steadily increased in resource-rich endemic settings in parts of Australia, which belies the documented decreasing global incidence [4, 5, 6]. Additionally, the disease is being increasingly reported from areas in which it historically was not present [7 – 10]. This points to BU being a potentially emerging or re-emerging disease, making it all the more imperative for effective BU control measures to be strengthened.

Within the framework of this thesis, we have attempted to fill some of the gaps still existing in aspects of BU control. The immunoassays herein described have the potential for development into simple diagnostics that are germane to both low- and high-resource BU-endemic regions. New treatment modalities evaluated, including a wound

disinfection device, could be developed into ancillary tools for clinical management of BU. Lastly, success in generating and characterising mycolactone-neutralising mAbs strengthens the suitability of mycolactone as a target for BU vaccine development.

## 9.1 Improving BU diagnosis

Traditionally, cultivating live microorganisms from a clinical sample is considered gold standard for diagnosis. *M. ulcerans*, however, is an extremely slow-growing bacterium requiring at least six weeks of cultivation for colonies to form on solid growth media [11, 12]. This makes cultivation as a means of BU diagnosis impractical. Consequently, detecting *M. ulcerans* DNA by (q)PCR is the current diagnostic gold standard [13]. However, whilst the IS*2404*-based (q)PCR is highly sensitive and specific, difficulties in its application in low-resource BU-endemic settings limit its widespread uptake [14]. As a result, less sensitive/specific diagnostics, such as clinical diagnosis and microscopy to detect AFB, are still the only available means of diagnosis in many low-resource settings [12]. Given that early diagnosis and prompt initiation of treatment are the cornerstones of BU control, there is urgent need for more easily applied diagnostics.

The WHO defines ideal diagnostics as so-called ASSURED tests, which means they should be Affordable, Sensitive, Specific, User-friendly, Rapid and Robust, Equipment-free, and Deliverable to the end user [15]. The current diagnostic gold standard – although sensitive, specific, and rapid – does not have all the features of an ideal assay. In the framework of this thesis, we have described two immunoassays that detect mycolactone, making them specific for *M. ulcerans*. Both the competitive (Chapters 2 and 3) and antigen capture assays (Chapter 4) had similar limits of detection, being able to detect as low as 1 – 2 ng of mycolactone. Although both assays are still, on the whole, less sensitive than the IS*2404* qPCR, their limits of detection make them suitable for use

with clinical samples, which have been estimated to contain between 0 – 1970 ng/ml of mycolactone [16, 17]. Immunoassays are viable alternatives to molecular tests, and are closer to being ideal tests than molecular tests [15], therefore, it is conceivable that our assays could be further developed into useful point-of-care diagnostics.

We also describe a cultivation-independent method of detecting viable *M. ulcerans* that takes advantage of the high sensitivity of the qPCR (Chapter 5). Such a technique could be directly applicable in settings where the IS*2404* qPCR is already established, since it an extension of the existing qPCR. A consistent finding upon treatment follow-up is the continuous detection of *M. ulcerans* DNA in BU lesions by (q)PCR [18, 19]. Since DNA can persist long after the bacteria are dead, it is often difficult to decide if the presence of *M. ulcerans* DNA in patients undergoing BU-specific chemotherapy is an indication of treatment failure and therefore the possibility of relapse, or just the remains of successfully killed bacteria. The picture is further blurred by the paradoxical reaction, or the apparent worsening of lesions, that is often seen upon treatment initiation [19, 20, 21]. Culture-based methods are currently the only means of ascertaining treatment success, however, the slow growth of *M. ulcerans* precludes the routine use of culture to monitor treatment success [12]. With the development of the culture-independent PMA-qPCR herein described, it may be possible to readily decipher relapsing disease from healing lesions.

## 9.2 Improving BU treatment

Rifampicin (RIF) is currently the only highly active antibiotic against *M. ulcerans* in clinical use, and the standard BU treatment is an 8-week regimen of daily RIF and clarithromycin (CLAR). Efforts are ongoing to develop alternative highly-active antibiotics against *M. ulcerans* to augment the BU drug armamentarium, and reduce the overreliance on RIF.

Shorter treatment regimens could also be formulated, to improve patient adherence and ease treatment [22, 23, 24].

The activity of the wound care solutions against *M. ulcerans*, described in chapter 6, may complement antibiotic therapy by directly killing the bacteria and fostering a wound microenvironment conducive to healing. Their broad-spectrum antimicrobial activity and stimulatory effects may also help speed up wound healing. Buruli ulcers have typical hallmarks of other chronic wounds such as diabetic foot ulcers and venous ulcers, therefore measures applied to chronic wound management are also applicable to BU treatment. The medical device evaluated in Chapter 6 was developed for the management of chronic wounds of other aetiologies, but we have shown that it could also be useful for BU treatment, since it efficiently and rapidly killed the bacteria *in vitro*. The broad-spectrum antimicrobial activity of the device would be advantageous in BU management given that secondary infections of Buruli ulcers are a common occurrence with poorly managed wounds [25]. Further studies would be necessary to show its feasibility as adjunctive BU treatment.

Energy generation/metabolism in mycobacteria is again becoming an attractive target for drug development. Bedaquiline and Telacebec (Q203) are examples of new drugs developed to target this pathway of mycobacteria, with clofazimine (an anti-leprosy drug) being suggested as a repurposed drug for BU treatment [22, 23, 24, 26]. Most of the compound classes evaluated in chapter 7 also target energy generation in mycobacteria, with the exception of the quinolone derivatives, which typically target DNA synthesis. Further development of the identified compounds with activity against *M. ulcerans* may result in the definition of new BU treatment regimens.

## 9.3 Can BU be prevented?

As aforementioned, BU control is currently contingent upon early diagnosis and treatment of the disease. However, forestalling infection could also aid in the control of the disease. Currently, there are no effective vaccines against BU. The Bacillus Calmette-Guérin (BCG) vaccine, the only vaccine available for mycobacterial disease, offers only partial, short-term protection from severe BU, and does not prevent the disease [1]. Efforts have been made to develop vaccines against BU, using a variety of routes, including using inactivated whole cells [27], cell surface components [28, 29], as well as improved adjuvants targeting TLR-2 [29], and parts of the mycolactone synthesis machinery [30]. However, like BCG, most of these generate an immune response that does not protect against the disease. From what is known about the pathogenesis of *M. ulcerans*, the bacteria appear to constitutively produce mycolactone, which forms a protective cloud around the bacteria shielding them from infiltrating immune cells [31]. Thus, although the vaccine candidates stimulate antibody responses (and cell-mediated responses in some cases), the effects of mycolactone prevents the host immune response from reaching the bacteria and clearing the infection.

Given that mycolactone is the only known virulence factor of *M. ulcerans*, it follows that incapacitating this toxin could allow for the bacteria to be efficiently cleared by host immune cells. Indeed, this is likely the case during antibiotic treatment, where cessation of mycolactone production upon bacteria killing by the antibiotics allows the immune system to clear the infection. We show that a variety of mAbs generated against a synthetic mycolactone derivative could neutralise the effects of mycolactone and protect cultured fibroblasts from its deleterious effects (Chapter 8). We have previously reported this antibody-mediated neutralisation of mycolactone [32], and in the current thesis, have reiterated this finding. In generating the additional panels of mAbs described in Chapter 8, we utilised three different immunogens for the mouse immunisations: PG-

203, PG-180, and CX-68-1. Functional analyses of the resultant mAbs helped to streamline the choice of immunogen for further vaccine development. Thus, whilst it was possible to generate potent mycolactone-neutralising mAbs following PG-203 immunisation of mice, mAbs generated following PG-180 immunisation only modestly neutralised mycolactone. No mAbs could be generated using the CX-68-1 immunogen as its use was found to result in lethal outcomes in some mice. Hence, PG-203 appears best suited as a candidate for further vaccine development. Further development would be necessary before vaccine candidates based on PG-203 would be ready for human trials, but if the results remain favourable, it is feasible that such a vaccine could be of great benefit to the lasting control of BU.

## 9.4 Outlook

We have begun initial attempts to assess the utility of our immunoassays in real-world settings. We previously reported the development of an antigen capture immunoassay for the detection of MUL_3720, a surface protein of *M. ulcerans* [33]. While this immunoassay is highly sensitive, the MUL_3720 protein is not unique to *M. ulcerans,* being found in other environmental mycobacteria (e.g. *M. fortuitum, M. gordonae, M. smegmatis*) but not in typical pathogenic mycobacteria (e.g. *M. tuberculosis* and *M. leprae*). Nonetheless, further development of this assay has continued and we have applied it, together with the competitive mycolactone assay (described in Chapters 2 and 3), to the detection of *M. ulcerans* in clinical samples from BU lesions. Most of these samples had been extendedly stored in unknown but possibly less-than-optimal conditions, although a few tests on-site have also been done. In both scenarios, it has been possible to detect these analytes in BU samples, pointing to the applicability of both assays to BU diagnosis after optimisation.

We have, thus far, tested both immunoassays with four sets of swabs collected from patients in three BU-endemic countries in West Africa, and once on-site in a small cohort of patients. In all these testing rounds, both analytes, that is mycolactone and the MUL_3720 surface protein, could be detected in a sizable proportion of qPCR-positive samples. Interestingly, some qPCR-negative samples were clearly positive for mycolactone and/or MUL_3720, indicating that the immunoassays could complement the qPCR. More importantly, in the on-site assessment, results could be obtained on the same day the samples were collected, which is a considerable improvement over the current situation where PCR diagnosis is often not received weeks after sample collection, in low-resource BU endemic areas.

That a Buruli lesion could give a qPCR-negative result has been reported, and is thought to be due to sampling insufficiencies [34]. It is equally plausible that samples containing more wound exudate (which theoretically contains secreted mycolactone) than bacterial cells (which contain the DNA detected by PCR) could be qPCR-negative but mycolactone-positive. The reasons behind qPCR-negative but MUL_3720-positive samples are less clear, since both tests detect bacterial cells and the qPCR is the more sensitive test in this pairing, but may be linked to the fact that the MUL_3720 protein is not specific to *M. ulcerans* [33]. Further evaluation would be necessary to determine how best to combine these various tests, with adequate sample acquisition and storage, for optimal BU diagnosis. Nevertheless, implementing such immunoassays as complements to the gold-standard qPCR could allow for a triaging of samples in the periphery, thus helping to reduce the number of samples that need to be sent to the central diagnostic centres for qPCR testing. Being able to promptly diagnose BU would also help in treatment efforts, since it would be possible to initiate treatment as soon after patient presentation as possible. This is important in light of findings that patients from locations distant from health posts are likely to be lost to treatment if they needed to return at a later date to receive their diagnostic results [15].

Both the MUL_3720 capture assay and the mycolactone competitive assay are currently being converted to lateral flow formats for use as rapid diagnostic tests (RDTs). It may also be possible to develop combined RDTs for the simultaneous detection of mycolactone and the MUL_3720 protein in a sample, similar to what is obtainable for RDTs for malaria (detecting both *Plasmodium falciparum* and *P. vivax*) and HIV (detecting both HIV-1 and HIV-2) diagnoses. This could help mitigate the uncertainties due to sampling and ensure better concordance between the different immunoassays. For ease of interpretation, both immunoassays to be converted into a combined lateral-flow assay would have to be antigen capture assays. This is because whilst antigen capture assays show a signal to indicate the *presence* of an analyte, competitive assays show a signal to indicate its *absence*. Therefore, it is more intuitive to design lateral flow RDTs based on antigen capture immunoassays, particularly when attempting to combine two immunoassays into one RDT. Fortunately, the mycolactone capture assay described in Chapter 4 has similar sensitivity to the competitive assay, and could therefore be readily paired with the MUL_3720 assay for simultaneous detection of both analytes. We have preliminary data that indicates that such assay combination is feasible, at least in a 96-well ELISA format. More extensive testing would help to show the utility of this approach, and it is expected that both assays could work synergistically, resulting in a combined assay of higher sensitivity.

As a final point regarding the transmission of *M. ulcerans*, the various *M. ulcerans*-detecting assays herein described may be put to good use to the ecological study of the bacteria. As aforementioned, culture of *M. ulcerans* from environmental sources is incredibly difficult, and has only been successfully done a couple of times [35, 36]. This is largely due to its extremely slow growth that allows faster-growing microorganisms to overshadow *M. ulcerans* upon cultivation on laboratory media. Decontamination protocols can be done prior to cultivation to decrease the chances of this happening, but faster-growing environmental mycobacteria are also able to survive these treatments,

and all reported decontamination methods drastically reduce the growth of *M. ulcerans* [11]. Consequently, it is not easy to determine if *M. ulcerans* can thrive in the environment. Detecting the bacterial DNA in environmental sources is often used as an indication of its presence; however, there is no way to determine if the DNA originated from viable bacteria growing in the environment or from dead bacteria that had been shed into the environment from open lesions.

As reported in Chapter 5, it is possible to utilise the PMA-qPCR protocol to discern if bacteria present in soil samples are dead or alive, and it is feasible that this method could be applicable to other environmental samples. In addition, detection of mycolactone can also be a proxy for the presence of viable *M. ulcerans* in the environment. Mycolactone is thought to be constitutively produced and secreted by the bacteria, and has been shown to be rather unstable [37, 38]. Therefore, the presence of mycolactone in a sample could indicate the presence of viable bacteria. It is worth mentioning that both these methods do not suffer from contaminating microorganisms, since the PMA-qPCR uses *M. ulcerans*-specific primers, and mycolactone is specific to *M. ulcerans*. Accordingly, they could bypass the main drawbacks of culture-based methods for detecting the bacteria in environmental sources.

In a pilot study, we could show the accumulation of mycolactone over a 12-week period in sterilised soil samples spiked with *M. ulcerans*, as quantified by the competitive assay (described in Chapters 2 and 3). Hence, a combination of the PMA-qPCR and the mycolactone-detecting immunoassays could be used to study the carriage of *M. ulcerans* in the environment in an expedited manner. Such a study is currently underway, and the results would help address the question of whether *M. ulcerans* can thrive as a free-living organism in the environment, or if it requires carriage in a biotic reservoir. Either way, the different assays described in this thesis could be valuable tools in studying the bacteria in its proposed environmental niches.

Last but not least, in Appendix 1 of this thesis, we describe our observations regarding the use of commercial *Mycobacterium tuberculosis* culture media for the cultivation of *M. ulcerans*. Although time-intensive, *M. ulcerans* culture is still necessary both for research and for diagnostic confirmation. Currently, *M. ulcerans* is cultivated *in vitro* using typical mycobacterial growth media, such as egg-based solid media (Löwenstein-Jensen and Ogawa media) and the Middlebrook media (7H9, 7H10, and 7H11). In comparing commercial liquid culture media developed for *M. tuberculosis* cultivation, we observed that they were not all directly convertible to *M. ulcerans* cultivation. For instance, some commercial *M. tuberculosis* growth systems are designed for incubation at 37°C, which is higher than the optimum incubation temperature of 28 – 33°C for *M. ulcerans*. Some of our observations are outlined in Appendix 1.

## 9.5 Conclusion

In the framework of this thesis, we have described a variety of tools with the potential to improve BU control by simplifying diagnosis, aiding wound management, and possibly facilitating BU vaccine development. The main outputs of this thesis are:

1. Optimising a mycolactone competitive assay for quantifying the toxin in a variety of samples. The assay is able to detect as low as 1 ng of mycolactone and is tolerant of serum, making it suitable for use with clinical samples. First attempts to utilise this assay to detect mycolactone in clinical samples have been successfully done, and further developments of the assay are envisaged.

2. Developing a mycolactone capture assay for detecting the toxin in a variety of samples. This is the first report of such an assay being developed for the hapten-like mycolactone molecule. Careful selection of mAbs allowed for the development of a highly sensitive assay, able to detect as low as between 1 – 2 ng of mycolactone. Initial attempts to make the assay tolerant of the presence of human serum allowed for its use with clinical samples. Further improvements to simplify the assay parameters are envisioned.

3. Development of a culture-independent qPCR protocol to discriminate live and dead *M. ulcerans* in a variety of samples. This assay is an extension of the diagnostic gold-standard qPCR, and combines the high sensitivity and specificity of the qPCR with the live/dead discriminatory ability of microbial culture. The assay has the potential for use during treatment monitoring, and could help in the ecological study of *M. ulcerans*.

4. Assessing a wound care solution as a possible adjunct to BU treatment. Proper wound management is crucial to the resolution of BU, particularly with severe disease. The wound care solution described could complement the recommended BU treatment and wound management.

5. Identifying anti-*M. ulcerans* compounds from the drug discovery pipelines for tuberculosis and other diseases with the potential for repurposing for BU drug development.

6. Assessing proposed BU vaccine candidates targeting mycolactone. Extensive characterisation of mAbs generated following mouse immunisations with the different candidates helped streamline the choice of a suitable vaccine candidate for further development.

7. Evaluating existing *M. ulcerans* cultivation methods to outline guidelines for successful culture of the bacteria. This would be useful for laboratory research.

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# APPENDIX

## Defining growth media for *Mycobacterium ulcerans* cultivation

*Mycobacterium ulcerans* – the aetiological agent of Buruli ulcer (BU) – is a slow-growing organism with doubling time of >48 hours, typically as long as 7 – 9 days [1]. Consequently, bacterial culture is not typically used for routine BU diagnosis, owing to the long-term incubation of between 4 weeks – 6 months necessary for colony formation on solid culture media. Nevertheless, culture is still applicable as a confirmation for the diagnostic gold standard, the IS*2404* PCR. Culture is also important in research, for example for *in vitro* drug screening, to study genetic manipulations of the bacteria, as well as to produce inocula for animal experiments. *M. ulcerans* is not a particularly fastidious microorganism, and it can be routinely propagated both on solid and in liquid culture media. Solid culture media are useful for qualitative and quantitative assessments of bacterial growth; for example, colony-forming units (CFUs) are typically counted in time-kill assays during drug screening. Solid culture media can, however, not be readily scaled-up, therefore, liquid culture media are typically used when larger bacterial inocula are needed, e.g. for animal experimentation.

Commercial culture media designed for laboratory detection of the related bacterium *Mycobacterium tuberculosis* are routinely used to cultivate *M. ulcerans*. The Middlebrook media (7H9, 7H10, and 7H11) can be used both for solid and liquid cultivation of *M. ulcerans*, for which they are typically supplemented with glycerol, ADC (albumin, dextrose, and catalase), and oleic acid or Tween-80. Egg-based media such as Löwenstein-Jensen (LJ) and Ogawa media, are also used for culturing mycobacteria, including *M. ulcerans*. The Mycobacteria Growth Indicator Tube (MGIT®), based on supplemented Middlebrook medium, is a commonly used commercial growth system, with growth determined either visually by observing mycobacterial clumps in the medium, or via fluorescence. The MGIT system is mainly a diagnostic method and is not typically used as a means of long-term cultivation of mycobacteria. A number of culture systems, similar in principle to the MGIT culture system, have been designed for *M. tuberculosis* detection in clinical samples, and are intended for use with proprietary instrumentation for automated growth monitoring. These include the BacT/Alert® MP (BioMérieux), the BD BACTEC™ Myco/F Lytic (Becton Dickinson), and the VersaTREK Myco (Thermo Scientific) culture systems. However, since the culture vials can be obtained independently of the instrumentation, it is possible to use them as standardised liquid growth media for mycobacterial cultivation.

The choice of culture medium for *M. ulcerans* cultivation is not a trivial matter. Although efforts have been made to modify the Middlebrook media with a variety of supplements to speed up *M. ulcerans* growth, some unexpected consequences have resulted. For instance, when glucose was supplied as an energy source, the bacteria were found to produce significantly less mycolactone and more of mycobactin (an orange-pigmented siderophore). Subsequent *in vivo* experimentation

using such cultures resulting in lower infection rates in a mouse model of BU [2]. This underscores the need for careful selection of *M. ulcerans* growth media, with the ideal choice being one that supports quick growth whilst being versatile enough for a variety of downstream applications.

To facilitate consistency in the cultivation of *M. ulcerans* for research purposes, we set out to compare growth in a number of available commercial growth media designed for *M. tuberculosis* cultivation. We assessed the growth of an African *M. ulcerans* strain – S1013 – in a variety of commercial and in-house liquid culture media (Table 1). Growth was compared in the following media:

i. BacT/Alert® MB (BioMérieux) with its supplement (Enrichment fluid)
ii. BacT/Alert® MP (BioMérieux) with its supplement (Reconstitution fluid)
iii. BD BACTEC™ Myco/F Lytic (Becton Dickinson)
iv. VersaTREK Myco (Thermo Scientific) with its supplement (VersaTREK Supplement)
v. In-house prepared Difco™ Middlebrook 7H9 medium (Becton Dickinson) supplemented with 0.2% glycerol (Sigma) and 10% OADC (Becton Dickinson)

Middlebrook 7H9 was prepared in 15-ml McCartney bottles, while commercial media were assessed in their original vials. Metrics for growth were fortnightly OD measurements at 600 nm ($OD_{600}$) and analysis of metabolic activity as determined by the resazurin assay [3].

**Table 1.** Growth media tested

| Medium | Supplier | Ready-to-use Supplement | Volume in assay |
|---|---|---|---|
| **BacT/Alert® MB** | BioMérieux | Enrichment fluid | 20 ml |
| **BacT/Alert® MP** | BioMérieux | Reconstitution fluid | 10 ml |
| **Middlebrook 7H9** | Becton Dickinson | OADC + 0.2% Glycerol | 10 ml |
| **BD BACTEC™ Myco/F Lytic** | Becton Dickinson | - | 20 ml |
| **VersaTREK Myco** | Thermo Scientific | VersaTREK Supplement | 12.5 ml |

The BacT/Alert® MP culture medium was designed as the replacement for the discontinued BacT/Alert® MB culture medium. However, these initial assays indicated that it was not as suitable for *M. ulcerans* growth as its predecessor was. Indeed, *M. ulcerans* exhibited a much longer lag phase in the BacT/Alert® MP medium than in the BacT/Alert® MB medium, and *M. ulcerans* metabolic activity in the former was significantly lower than that in the latter medium (Fig. 1). Conversely, the Middlebrook 7H9 medium outperformed all the currently available commercial growth media tested, and was therefore selected for further analyses.

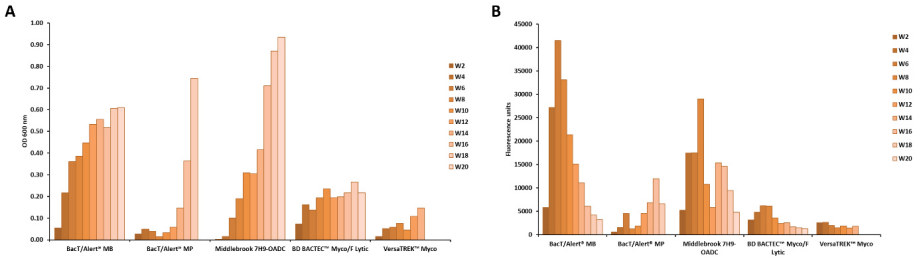


**Figure 1.** Assessment of long-term *M. ulcerans* growth in a variety of culture media as determined by $OD_{600}$ measurements (A) and the resazurin assay (B). Cultures were sampled fortnightly for 20 weeks; the VersaTREK Myco medium could not be assessed beyond 14 weeks for logistical reasons.

To ascertain the versatility of the Middlebrook 7H9 medium for our purposes, we assessed mycolactone production, MUL_3720 surface protein expression, and bacterial CFU counts, as these are typical metrics that we utilise in our lab. We sampled the Middlebrook 7H9-OADC culture fortnightly for 14 weeks and plated out ten-fold serial dilutions on Middlebrook 7H9-OADC agar plates, for CFU counting. At weeks 6 and 14 of incubation, we determined the expression of the MUL_3720 surface protein by ELISA, as previously described [4]. *M. ulcerans* grew appreciably and expressed the MUL_3720 protein, as shown in Fig. 2 below.

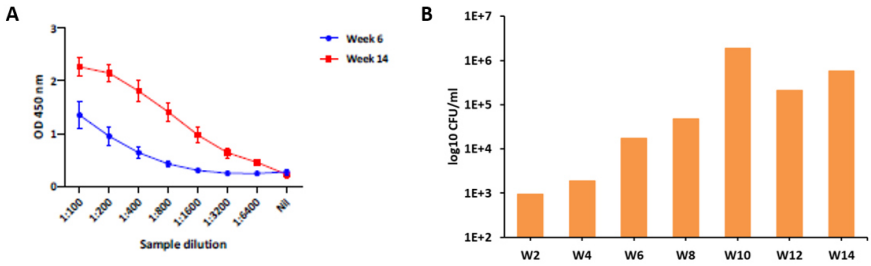


**Figure 2**. *M. ulcerans* growth in Middlebrook 7H9-OADC as determined by expression of the MUL_3720 surface protein (A) and by CFU counts (B). Cultures were sampled fortnightly for 14 weeks, and plated out on Middlebrook 7H9-OADC agar plates; MUL_3720 expression was measured by ELISA at weeks 6 and 14.

Regarding mycolactone production, we found that the toxin was more associated with the bacterial cells and/or the extracellular matrix, rather than being released into the culture supernatant. This necessitated the use of lipid extraction methods to recover the toxin, although we also found that the incorporation of a detergent into the culture medium was sufficient to release mycolactone into the supernatant. Indeed, we could detect higher amounts of mycolactone when 0.2% saponin was included in the Middlebrook 7H9-OADC medium, or when organic extraction was done (Fig. 3).

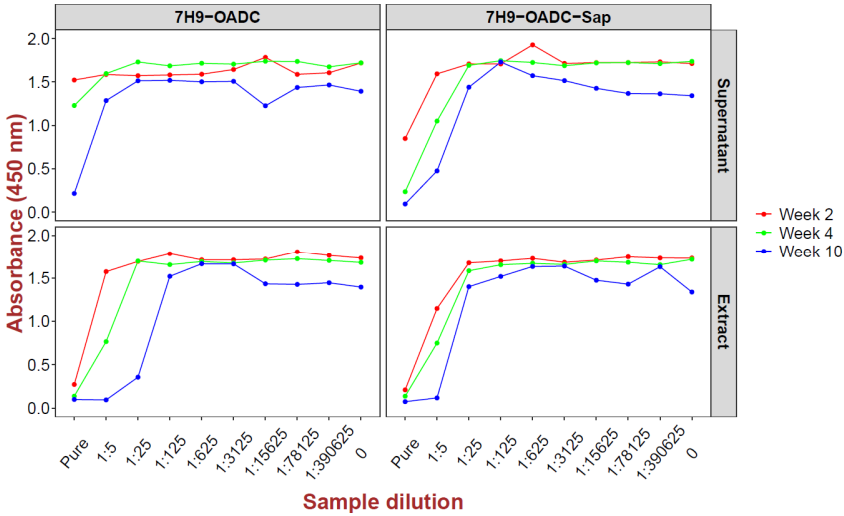


**Figure 3**. Mycolactone detection in culture supernatants or ethanolic extracts by competitive ELISA [5]. Culture filtrates ("Supernatant") and ethanolic extracts ("Extract") of cultures in Middlebrook 7H9-OADC medium ("7H9-OADC") and Middlebrook 7H9-OADC medium with 0.2% saponin ("7H9-OADC-Sap") were prepared. Five-fold dilution series of culture filtrates or extracts prepared in a TEA buffer (0.2 M TEA with 20% DMSO) were allowed to react with mAb JD5.1 coated onto MaxiSorp plates for 2 h. The reporter molecule MG-161 was then added in and allowed to react for a further 45 min. Bound reporter molecule was detected by HRP-conjugated streptavidin and TMB.

In conclusion, we found that *M. ulcerans* grew comparably better in Middlebrook 7H9-OADC medium than in available commercial media designed for *M. tuberculosis* cultivation, as determined by OD measurements, CFU counts, and the resazurin assay. Importantly, this medium was versatile enough for our routine downstream applications, with the bacteria expressing appreciable amounts of the surface protein MUL_3720 and producing good quantities of mycolactone. We have not

dissected particulars of *M. ulcerans* metabolism in this study, but rather focused on the overall health of the bacteria as measured by the parameters applied in this study. Further studies would be necessary to determine the effects of selected growth media on particular biomarkers of interest.

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# Curriculum vitae

## Louisa Omotayo WARRYN

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## Education

Swiss Tropical and Public Health Institute, University of Basel 2017 – 2021
**PhD. Microbiology (6.0/6.0)**
*Thesis*: *Development of tools for the control of* Mycobacterium ulcerans *disease (Buruli ulcer)*

Swiss Tropical and Public Health Institute, University of Basel 2015 – 2017
**MSc. Infection Biology (5.5/6.0)**
*Thesis*: *Generation and characterisation of monoclonal antibodies against* Mycobacterium ulcerans *mycolactone*

University of Lagos 2006 – 2011
**BSc. Microbiology (4.75/5.0)**
*Thesis*: *Antimicrobial resistance patterns of some food pathogens and clinical isolates*
***Award****: Best graduating student in Faculty of Science*

## Research Experience

**Postdoctoral Scientific Collaborator –** Molecular Immunology Unit, Swiss TPH June 2021 - present

Develop and validate immunoassays
Perform mAb sequencing from hybridomas
Manage a biosafety level 3 laboratory (for *M. ulcerans* cultivation)

**PhD Student** – Molecular Immunology Unit, Swiss TPH Aug 2017 – May 2021

Generated mAbs and developed immunoassays (ELISAs)
Performed low-throughput screening to identify compounds active against *M. ulcerans*
Performed genomic techniques (DNA extractions and purification, PCR)

**Intern** – Molecular Immunology Unit, Swiss TPH Mar – Aug 2017

Cultivated mammalian hybridoma cell lines for mAb production
Performed conventional immunological tests (ELISA, Western blot, IFA)
Performed genomic techniques (DNA extractions and purification, PCR)

**Medical Microbiologist** – Prenatal Diagnosis and Therapy Centre, College of Medicine, University of Lagos (CMUL) Nov 2014 – Mar 2015

Performed microbiological diagnosis of bacterial and fungal pathogens
Performed immunologic diagnosis of TORCH, STIs, and viral infections
Performed routine haematological tests on blood samples
Gave weekly lectures to new patients

**Medical Laboratory Scientist (Trainee)** - Havana Specialist Hospital Apr – Sep 2009

Performed routine diagnostic tests on a variety of patients' samples
Assisted in the daily running of the laboratory

# Curriculum vitae

## Teaching experience

| | |
|---|---|
| Supervision of a master student (Swiss TPH) | Feb 2020 – Feb 2021 |
| Classroom teaching (Swiss TPH)<br>Delivered lectures once yearly to master students | 2018/2019/2020/2021 |
| Block course organisation and supervision (Swiss TPH)<br>Organised block courses for undergraduate students<br>Demonstrated lab techniques, supervised experiment execution and result analyses | 28 Nov – 06 Dec 2017<br>02 – 20 Dec 2019 |
| Chemistry teacher (GSS Agbamu, Kwara state, Nigeria)<br>Delivered chemistry lessons to secondary school students<br>Organised and supervised laboratory sessions | Apr 2012 – Feb 2013 |

## Skills

| | |
|---|---|
| **Laboratory skills**: | Immunology (Monoclonal antibody generation, ELISA, Western blot, IFA)<br>Molecular biology (DNA and RNA extraction and purification, conventional and real-time PCR)<br>Microbiology (working with biosafety level 1 – 3 microorganisms)<br>Cell biology (Adherent and suspension cell line cultivation, routine haematological assays) |
| **Software**: | Microsoft Office (Word, Excel, PowerPoint), R, GraphPad Prism |

## Publications


**Warryn L**, Dangy J-P, Gersbach P, Gehringer M, Altmann K-H, and Pluschke G. 2021. An antigen capture assay for the detection of mycolactone, the polyketide toxin of *Mycobacterium ulcerans*. J. Immunol. 206: 2753 – 2762.

**Warryn L**. (in press). Competitive ELISA for the detection and quantification of *Mycobacterium ulcerans* mycolactone. In Pluschke G. ed., *Mycobacterium ulcerans*: Methods and Protocols Springer Nature.

**Warryn L**, Dangy J-P, Gersbach P, Gehringer M, Schäfer A, Ruf M-T, Ruggli N, Altmann K-H, and Pluschke G. 2020. Development of an ELISA for the quantification of mycolactone, the cytotoxic macrolide toxin of *Mycobacterium ulcerans*. PLoS. Negl. Trop. Dis. 14(6): e0008357.

Pinho-Nascimento CA, Bratschi M, Höfer R, Soares CC, **Warryn L**, Pečerska J, Minyem JC, Paixão ICNP, Baroni de Moraes MT, Um Boock A, Niel C, Pluschke G, and Röltgen K. 2018. Transmission of hepatitis B and D viruses in an African rural community. mSystems 3(5): e00120-18.

Röltgen K, Rose N, Ruggieri A, **Warryn L**, Scherr N, Pinho-Nascimento CA, Tamborrini M, Jaenisch T and Pluschke G. 2018. Development of dengue virus serotype-specific NS1 capture assays for the rapid and highly sensitive identification of the infecting serotype in human sera. J. Immunol. 200(11): 3857 – 3866.

## Presentations

**Talk** (*Developing mycolactone-based immunoassays for Buruli ulcer diagnosis*)
World Microbe Forum 2021

**Talk** (*Diagnosing Buruli ulcer*)
Swiss TPH Research Seminar, Basel, Switzerland

**Talk** (*Development of tools for Buruli ulcer control*)
NTD Flash Talks, Swiss TPH, Basel, Switzerland
(**Award**: Third best speed talk)

**Talk** (*Towards the development of a simple diagnostic test for Buruli ulcer*)
Swiss TPH Research Seminar, Basel, Switzerland

**Talk and Poster** (*Improving the diagnosis and control of* Mycobacterium ulcerans *disease*)
1st One Health PhD/Postdoc Summer School, University of Bern, Switzerland

**Poster** (*A simple ELISA for the detection of mycolactone in biological samples*)
WHO Meeting on Buruli ulcer and other skin neglected tropical diseases
World Health Organisation, Geneva, Switzerland

**Talk** (*Development of an antigen-capture assay for mycolactone detection*)
SSTMP PhD Student Meeting, Sigriswil, Switzerland
(**Award**: First place, best presentation)

## Languages

| | |
|---|---|
| English | Native |
| Italian | A2 |
| German | A2 |