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Spécialités : Pathologies Humaines - Maladies Infectieuses.

Réservoirs de *Mycobacterium ulcerans*: développement de nouvelles techniques de laboratoire

Membres du Jury de la Thèse:

Prof.	Florence FENOLLAR	Présidente
Prof.	Sylvain GODREUIL	Rapporteur
Prof.	Emmanuel BOTTIEAU	Rapporteur
Prof.	Michel DRANCOURT	Directeur de Thèse

Laboratoire d'accueil
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Que le Seigneur soit toujours à l'écoute de ses enfants

Table of content

AVANT PROPOS

Le format de présentation de cette thèse correspond à une recommandation à la spécialité Pathologie Humaine, Maladies infectieuses, à l'intérieur du Master des Sciences de la Vie et de la Santé qui dépend de l'Ecole Doctorale des Sciences de la Vie de Marseille.

Le candidat est amené à respecter les règles qui lui sont imposées et qui comportent un format de thèse utilisé dans le Nord de l'Europe et qui permet un meilleur rangement que les thèses traditionnelles. Par ailleurs, la partie introduction et bibliographie est remplacée par une revue envoyée dans un journal afin de permettre une évaluation extérieure de la qualité de la revue et de permettre à l'étudiant de commencer le plus tôt possible une bibliographie sur le domaine de cette thèse.

Par ailleurs, la thèse est présentée sur article publié, accepté, ou soumis associé d'un bref commentaire donnant le sens général du travail. Cette forme de présentation a paru plus en adéquation avec les exigences de la compétition internationale et permet de se concentrer sur des travaux qui bénéficieront d'une diffusion internationale.

Professeur Didier RAOULT

Résumé

L'ulcère de Buruli est une maladie infectieuse tropicale présente dans des foyers endémiques et atteignant surtout les populations pauvres en zones rurales. En 1997, l'Organisation Mondiale de la Santé (OMS) a reconnu l'ulcère de Buruli comme une maladie ré-émergente et en 1998, un programme mondial de lutte contre cette infection (Global Buruli Ulcer initiative « GBUI ») a été mis en route lors de la Conférence de Yamoussoukro en Côte d'Ivoire. Cette infection essentiellement cutanée est causée par *Mycobacterium ulcerans*, dérivé d'un ancêtre commun avec *Mycobacterium marinum*, et est la troisième mycobactériose la plus prévalente dans le monde, après la tuberculose et la lèpre. *M. ulcerans* produit la toxine mycolactone qui est responsable du pouvoir pathogène du bacille. Cependant, *M. ulcerans* est un pathogène opportuniste dont le réservoir est environnemental. Notre revue de la littérature a répertorié les sources et hôtes potentiels de cette mycobactérie avec une proéminence du réservoir hydro-tellurique. L'ADN de *M. ulcerans* a été détecté dans le sol, l'eau, les biofilms, les végétaux, les mousses, les fèces ainsi que chez les mollusques d'eau, les punaises d'eau, les poissons d'eau douce, la tortue, les crevettes , les batraciens et les petits mammifères. L'ADN a été détecté également chez le chat, le cheval, le chien, l'alpacas, les possums et le koala. Cependant, seulement cinq souches de *M. ulcerans* ont été isolées à partir de prélèvements de l'environnement en zones d'endémie de l'ulcère de Buruli et l'absence d'une large collection de souches est un frein important à l'investigation des sources et vecteurs de cette mycobactérie. Nous avons montré qu'il existe une corrélation inverse entre réchauffement climatique dans les pays endémiques et incidence de l'ulcère de Buruli, peut-être liée à la sensibilité intrinsèque de *M. ulcerans* aux variations de température et de la lumière, ou bien à des modifications de son écosystème. Dans la perspective d'améliorer les protocoles d'isolement et de culture de *M. ulcerans* à partir de l'environnement, nous avons entrepris une analyse phénotypique à haut débit des substrats carbonés métabolisés par *M. ulcerans* et le profil obtenu nous a orientés après une recherche bibliographique des principales sources

environnementales de ces substrats, vers des interactions plus spécifiques de *M. ulcerans* avec les autres bactéries, les algues, les mollusques et les champignons. Les résultats de ce premier travail ont servi de base pour la mise au point de milieux de culture innovants qui, combinés à une méthode originale de décontamination par la chlorhexidine, nous ont permis d'isoler pour la première fois, une microcolonie de *M. ulcerans* à partir de fèces d'agouti collectés en Côte d'Ivoire. Cette microcolonie a été correctement identifiée par amplification de séquences spécifiques et par analyse de son profil peptidique par spectrométrie de masse grâce à un protocole développé au cours de notre thèse. Egalement, nous avons mis au point une méthode de lecture automatisée des échantillons colorés par la coloration de Ziehl-Neelsen à la recherche des mycobactéries, dans une perspective de lecture haut-débit. Notre travail de thèse a produit des protocoles qui ont pour objectif d'être mis en œuvre dans les pays d'endémie Africains dont le Burkina Faso dont nous sommes originaires, pour préciser les sources et modes de transmission de *M. ulcerans* aux populations, par une approche basée sur l'isolement, la culture et la caractérisation des souches.

Mots clés : Ulcère de Buruli, *Mycobacterium ulcerans*, *Mycobacterium marinum*, réchauffement climatique, sources carbonées, culture, agouti, environnement, sources.

Summary

Buruli ulcer is a tropical infectious disease present in endemic foci and mostly affects poor populations in rural areas. In 1997, the World Health Organization (WHO) recognized Buruli ulcer as a re-emerging disease and in 1998 a global program to combat this infection (Global Buruli Ulcer initiative « GBUI ») was launched at the Yamoussoukro Conference in Côte d'Ivoire. This mainly cutaneous infection is caused by *Mycobacterium ulcerans*, which has a common ancestor with *Mycobacterium marinum*, and is the third most prevalent mycobacterial disease in the world after tuberculosis and leprosy. *M. ulcerans* produces mycolactone toxin which is responsible for the pathogenicity of the bacillus. However, *M. ulcerans* is an opportunistic pathogen from the environment. Our literature review has listed the potential sources and hosts of this mycobacterium with a prominence of the hydro-telluric reservoir. The DNA of *M. ulcerans* was detected in soil, water, biofilms, plants, mosses, faeces as well as in water molluscs, water bugs, freshwater fish, turtle, shrimp, amphibians and small mammals. DNA was also detected in cat, horse, dog, alpacas, possums and koala. However, only five strains of *M. ulcerans* have been isolated from environmental sampling in endemic areas of Buruli ulcer and the absence of a large collection of strains is a major obstacle to investigation of the sources and vectors of this mycobacterium.

We have shown that there is an inverse correlation between global warming in endemic countries and incidence of Buruli ulcer, possibly related to the intrinsic sensitivity of *M. ulcerans* to temperature and light changes, or to changes in its ecosystem. In order to improve the isolation and culture protocols of *M. ulcerans* from the environment, we conducted a high-throughput phenotypic analysis of the carbon substrates metabolized by *M. ulcerans* and the profile obtained oriented us afterwards a bibliographic search of the main environmental sources of these substrates, towards more specific interactions of *M. ulcerans* with other bacteria, algae, molluscs and fungi. The results of this first work served as a basis for the development of innovative culture media which, combined with an original method of chlorhexidine decontamination, allowed us to isolate for the first time a microcolony from feces of agouti collected in Côte d'Ivoire. This microcolony was correctly identified by amplification of specific sequences and by analysis of its peptide profile by mass spectrometry through to a protocol developed during our thesis. We also developed a method for automated reading of samples stained by Ziehl-Neelsen staining for mycobacteria in a high-throughput reading perspective. Our thesis work has produced protocols that are intended to be implemented in African endemic countries, including Burkina Faso, in order to clarify the sources and modes of transmission of *M. ulcerans* to populations, an approach based on the isolation, culture and characterization of strains.

Keywords: Buruli ulcer, *Mycobacterium ulcerans*, *Mycobacterium marinum*, global warming, carbon sources, culture, agouti, environment, sources.

Introduction

Buruli ulcer is an infectious disease caused by *Mycobacterium ulcerans* which releases mycolactone, a cytotoxine and the major factor responsible for the pathogenicity of the bacillus. The disease was first identified in 1897 by Sir Albert Cook, a British physician working at Mengo Hospital in Kampala (1) and formally described in Australia in 1935 and named after the Buruli county in Uganda (now called Nakasongola) where many cases occurred in the 1960s (2). MacCallum and his colleagues in Australia were the first scientists to identify *M. ulcerans* as the pathogen causing Buruli ulcer in 1948 (3). Buruli ulcer is a World Health Organization (WHO)-notifiable disease which has been reported in 33 countries over the last twelve years. The infection occurs in well-defined areas throughout the world, mostly in tropical and sub-tropical countries.

Buruli ulcer is one of the 17 tropical diseases classified as neglected diseases by the WHO which recognized Buruli ulcer as an emerging public health problem in 1998 at the Yamoussoukro Conference (4). Between 5,000 -6,000 cases

have been reported every year by 15 of the 33 declarant countries, predominantly in rural regions across West and Central Africa (5). The incidence of Buruli ulcer is highest in Africa especially in coastal countries of West Africa where the emergence of the disease has rapidly escalated since the 1980s, though cases also occur in Asia, South America, Papua New Guinea and Australia. However starting in 2010, the incidence of Buruli ulcer has been regularly falling without a definitive explanation for that favourable trend.

M. ulcerans is an environmental mycobacterium and the related disease is usually found in communities near rivers, swamps and wetlands (ponds, swamps, marshes, impoundments, backwaters), especially in areas prone to human-made disturbance and flooding (6, 7).

Currently available diagnostic laboratory tests include operator-dependant microscopic examination, culture, molecular detection and histopathological analysis (8-10). By contrast and despite the frequent detection of *M. ulcerans* short DNA sequences in environmental samples, isolation of *M. ulcerans* from environmental specimens remains tedious

and the first isolation of *M. ulcerans* from the environment was obtained by Portales and colleagues in 2008 after a laborious 24-month work (11). The intrinsic fastidious growth of *M. ulcerans* with a 3.3 ± 0.56 days doubling time observed in currently available culture media (18) and the rapid overgrowth of contaminants have been pointed as two limits to be overpassed in order to improve the culture-based investigation of environmental *M. ulcerans*.

Since 2004, Buruli ulcer has been treated with eight weeks of intramuscular injection of streptomycin and oral rifampicin according to the WHO protocol of treatment with antibiotics plus surgical excision and skin grafting (12).

The compliance to the recommended eight-week treatment is difficult to maintain, particularly in rural settings where health facilities are rare. The daily injection with streptomycin is problematic, as most patients live in remote areas with limited acces to health care facilities; and potentially expose patients to toxicity and injection-borne infections. A fully oral intermittent regimen avoiding intramuscular injections would greatly simplify Buruli ulcer

treatment on the field and facilitating supervision by health workers (13). The current definitive treatment of Buruli ulcer with antibiotics makes the issue of antimicrobial drug resistance an unavoidable one. This is as a result of drug misuse by health personnel and patients' noncompliance to treatment regimen (14). There is a need of alternative oral antimicrobials treatment to existing treatment against Buruli ulcer. Parenteral and potentially toxic streptomycin-rifampicin is to be replaced by oral clarithromycin or fluoroquinolone combined with rifampicin. Recent developments toward a fully oral therapy, not including a quinolone but rather a combination of rifampicin and clarithromycin, were presented at the WHO Buruli ulcer meeting in March 2017. The oral regimen with rifampicin and clarithromycin is already recommended by the WHO and regularly administered in West African countries.

It was shown that unbreached skin was impermeable to *M. ulcerans*. The pathogen has to be inoculated through previously breached skin or biting and it has been recently shown that 2.6 colony-forming units.small inoculums of *M.*

ulcerans is sufficient to provoke the disease in experimental model (15). Several insects harboring *M. ulcerans* have been proposed as potential vectors, some of which being acknowledged vectors of other pathogens (16,17). In a recent study, it was noticed in Benin an absence of *M. ulcerans* at both pupae and adult stages, certainly revealing the low ability of infected or colonized mosquitoes to vertically transmit the bacteria to their offspring (19) It follows that the mosquitoes play much more a role of formation of breaches on the skin which will then be entrance doors for *M. ulcerans*. Alternatively, an animal model showed that the mechanical disruption of *M.ulcerans*-contaminated skin allowed for the development of Buruli ulcer (15). However, no studies have been carried-out on the skin carriage of *M. ulcerans* in healthy subjects in endemic and non-endemic regions to understand the ultimate modes of transmission of the pathogen.

All these facts led us to orient our thesis work according to the following key points: (i) a contribution to the elucidation of the spectrum of potential reservoirs by studying the main

carbons substrates metabolized by *M. ulcerans* strains by high-throughput carbon substrate profiling; (ii) the development of growth promoters' media and decontamination method in order to improve the culture-based detection of *M. ulcerans* from environmental sources (iii) the molecular detection of *M. ulcerans* in environmental samples (agouti feces, water, water plants debris) collected in Côte d'Ivoire; (iv) the Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass-Spectrometry (MALDI-TOF MS) identification of *M. ulcerans* and other mycobacteria ;(v) the automated Ziehl Neelsen detection of mycobacteria by virtual microscopie; (vi) the study of *M. ulcerans* dormancy; and (vii) Skin carriage of *M. ulcerans* in healthy subjects.

Thus, this Thesis manuscript is composed by five chapters presented as following:

- **Chapter I:** This section was devoted to:
    1. **Article 1 (in revision)**, a review entitled « Buruli ulcer: a prototype for ecosystem-related infection, caused by *Mycobacterium ulcerans* » and;

2. **Article 2 (in review)**, entitled "Global warming correlates with decreasing incidence of Buruli ulcer in Africa"

- **Chapter II:** We present in this chapter, the use of the Biolog Phenotype MicroArray (Biolog Inc.) for the high-throughput carbon substrate profiling of *Mycobacterium ulcerans* and elucidation of the spectrum of potential reservoirs of the bacillus (**Article 3)**
- **Chapter III:** In this chapter, we described new growth promoter media for *M. ulcerans* culture of environmental samples in which *M. ulcerans* DNA were detected by Real-Time-PCR (IS*2404*, IS*2606*, KR-B) **(Article 4**). "A protocol for culturing environmental strains of the Buruli ulcer agent, *Mycobacterium ulcerans"*
- **Chapter IV:** In this chapter we present the use of MALDI-TOF-MS for direct identification of mycobacteria from colonies. The **Article 5** untiltled "Direct matrix-assisted laser desorption ionisation time-of-flight mass spectrometry identification of

mycobacteria from colonies “. This work led us to the writing one short communication on the subject. The **Article 6** untitled “Emerging of Matrix-assisted laser desorption ionization-time of flight mass spectrometry for identification of mycobacteria in a clinical microbiological laboratory” is a review that discusses the various technical approaches used for the identification of mycobacteria by MALDI-TOF.

- **Chapter V:** In this chapter, we present the results of automated reading of smears stained by the Ziehl-Nielsen method for the detection of acid fast bacilli (AFB). Virtual microscopy for the detection and automatic counting of acid fast bacilli by the Zeiss Axio Z1 microscope has been developed (**Article 7** (in review)Automatic microscopic numeration of mycobacteria in sputum: a proof-of-concept)

Reference List

1. Grant A Jenkin MS, Mark Fairley and Paul D R Johnson. Acute, oedematous Mycobacterium ulcerans infection *in a farmer from far nort*h Queensland. Med J Aust 2002;176(4):180-1.

2. Demangel C, Stinear TP, Cole ST. Buruli ulcer: reductive evolution enhances pathogenicity of Mycobacterium ulcerans. Nat Rev Micro. 2009;7(1):50-60.

3. Johnson PD, Veitch MG, Leslie DE, Flood PE, Hayman JA. The emergence of Mycobacterium ulcerans infection near Melbourne. Med J Aust. 1996;164(2):76-8.

4. Asiedu K, Sherpbier R, Raviglione M. Buruli Ulcer: Mycobacterium ulcerans infection. WHO Global Buruli Ulcer initiative. Association Française Raoul Follereau, 2000.

5. WHO. Buruli ulcer (Mycobacterium ulcerans infection).Fact Sheet. N°199. World Health Organization2014.

6. Wagner T, Benbow ME, Brenden TO, Qi J, Johnson RC. Buruli ulcer disease prevalence in Benin, West Africa: associations with land use/cover and the identification of disease clusters. Int J Health Geogr. 2008;7(25):7-25.

7. Walsh DS, Portaels F, Meyers WM. Buruli ulcer (Mycobacterium ulcerans infection). Transactions of the Royal Society of Tropical Medicine and Hygiene. 2008;102(10):969-78.

8. Herbinger K-H, Adjei O, Awua-Boateng N-Y, Nienhuis WA, Kunaa L, Siegmund V, et al. Comparative Study of the Sensitivity of Different Diagnostic Methods for the Laboratory Diagnosis of Buruli Ulcer Disease. Clinical Infectious Diseases. 2009;48(8):1055-64.

9. WHO. Guidance on sampling techniques for laboratory-confirmation of Mycobacterium ulcerans infection (Buruli ulcer disease).2013.

10. Zingue D, Flaudrops C, Drancourt M. Direct matrix-assisted laser desorption ionisation time-of-flight mass spectrometry identification of mycobacteria from colonies. European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology. 2016;35(12):1983-7.

11. Portaels F, Meyers WM, Ablordey A, Castro AG, Chemlal K, de Rijk P, et al. First Cultivation and Characterization of Mycobacterium ulcerans from the Environment. PLoS neglected tropical diseases. 2008;2(3):e178.

12. WHO. Global Buruli Ulcer Initiative. Provisional guidance on the role of specific antibiotics in the

management of Mycobacterium ulcerans disease (Buruli ulcer). Geneva: World Health Organization. 2004.
13. Chauffour A, Robert J, Veziris N, Aubry A, Jarlier V. Sterilizing Activity of Fully Oral Intermittent Regimens against Mycobacterium Ulcerans Infection in Mice. PLoS neglected tropical diseases. 2016;10(10):e0005066.

14. Owusu E, Newman MJ, Addo KK, Addo P. In Vitro Susceptibility of Mycobacterium ulcerans Isolates to Selected Antimicrobials. The Canadian journal of infectious diseases & medical microbiology = Journal canadien des maladies infectieuses et de la microbiologie medicale. 2017;2017:5180984.

15. Wallace JR, Mangas KM, Porter JL, Marcsisin R, Pidot SJ, Howden B, Omansen TF, Zeng W, Axford JK, Johnson PDR, Stinear TP. 2017. Mycobacterium ulcerans low infectious dose and mechanical transmission support insect bites and puncturing injuries in the spread of Buruli ulcer. PLoS Negl Trop Dis 11:e0005553.

16. Meyin AES, Garcia-Pena GE, Pluot-Sigwalt D, Marsollier L, Le Gall P, et al. (2017) Ecology and Feeding Habits Drive Infection of Water Bugs with Mycobacterium ulcerans. Ecohealth.
17. Zogo B, Djenontin A, Carolan K, Babonneau J, Guegan J-F, et al. (2015) A Field Study in Benin to Investigate the Role of Mosquitoes and Other Flying Insects in the

Ecology of Mycobacterium ulcerans. PLoS Negl Trop Dis 9: e0003941.

18. Marsollier L, Stinear T, Aubry J, Saint Andre JP, Robert R, et al. (2004) Aquatic plants stimulate the growth of and biofilm formation by Mycobacterium ulcerans in axenic culture and harbor these bacteria in the environment. Appl Environ Microbiol 70: 1097-1103.

19. Djouaka R, Zeukeng F, Daiga Bigoga J, #x, golo Coulibaly D, Tchigossou G, Akoton R, Aboubacar S, Tchebe SJ-E, Nantcho Nguepdjo C, Adeoti R, Djegbe I, Tamo M, Mbacham WF, Kakou-Ngazoa SE, Ablordey A. (2017). Evidences of the Low Implication of Mosquitoes in the Transmission of Mycobacterium ulcerans, the Causative Agent of Buruli Ulcer. Canadian Journal of Infectious Diseases and Medical Microbiology 2017: 12

Chapter 1: A review of the literature on Buruli ulcer in the world: epidemiology, infection ecology and advances in disease control

Article 1: "Buruli ulcer: a prototype for ecosystem-related infection, caused by *Mycobacterium ulcerans*."

Clin Microbiol Rev 2017 (in revision)

Review:

Article 1 (in revision): "**Buruli ulcer: a prototype for ecosystem-related infection, caused by *Mycobacterium ulcerans*.**"

Bairnsdale ulcer or Buruli ulcer is a chronic, indolent, necrotizing, debilitating subcutaneous skin, soft tissue and rarely bone disease that can lead to disfigurement and disability. The ulcers usually appear on the legs or arms. Buruli ulcer is listed among the neglected tropical diseases by World Health Organization. It's caused by mycolacton toxin-producing mycobacteria, *Mycobacterium ulcerans.* At least 33 countries with tropical, subtropical and temperate climates have reported Buruli ulcer in Africa, South America and Western Pacific regions. The majority of cases are reported from West and Central Africa, including Côte d'Ivoire, Ghana, Benin, Cameroon and Democratic Republic of the Congo. Australia has been reporting a higher number of cases during the last decade. Since 2010 the incidence of the disease has decreased in Africa. The exact

cause of decline is unknown. Buruli ulcer is the third mycobacteriosis after tuberculosis and leprosy. Most Buruli ulcer patients are children aged less than 15 years old. The cases of the disease are endemic around the marshy areas and the rice fields. Buruli ulcer rages in a hydrotelluric ecosystem of tropical and subtropical countries. *Mycobacterium ulcerans* needs a temperature between 28–33 °C and a low 2.5% oxygen concentration to grow. We have shown in this review that lesions of the disease in specific areas of the human body are correlated with body temperature.

M. ulcerans is an environmental mybacteria; the exact mode of transmission is unknown but may initially be confused with insect bite, wound and contact with the mud. There is no prevention treatment against the disease. Early diagnosis and curative treatment are the main strategy to minimize morbidity, costs and prevent long-term disability. In this review we have done a history of the epidemiology of Buruli ulcer in the world since its first descriptions in the 1940s. A summary of the *M. ulcerans* DNA detection studies and

attempts to culture the bacteria from the environmental samples was done. A well-supplied listing of the potential reservoirs of *M. ulcerans* was conducted, taking into account certain factors related to their chitin content. This review also shows in a somewhat exhaustive way the investigative pathways of *M. ulcerans* in the environment, the risk factors of the Buruli ulcer and leads to a significant questonner which is whether there is a carriage of *M. ulcerans* in apparently healthy subjects.

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REVIEW

Buruli Ulcer, a Prototype for Ecosystem-Related Infection, Caused by *Mycobacterium ulcerans*

AQ: au

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SUMMARY Buruli ulcer is a noncontagious disabling cutaneous and subcutaneous mycobacteriosis reported by 33 countries in Africa, Asia, Oceania, and South America. The causative agent, *Mycobacterium ulcerans*, derives from *Mycobacterium mari-*

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D.Z. and A.B. contributed equally to this review.

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num by genomic reduction and acquisition of a plasmid-borne, nonribosomal cytotoxin mycolactone, the major virulence factor. *M. ulcerans*-specific sequences have been readily detected in aquatic environments in food chains involving small mammals. Skin contamination combined with any type of puncture, including insect bites, is the most plausible route of transmission, and skin temperature of <30°C significantly correlates with the topography of lesions. After 30 years of emergence and increasing prevalence between 1970 and 2010, mainly in Africa, factors related to ongoing decreasing prevalence in the same countries remain unexplained. Rapid diagnosis, including laboratory confirmation at the point of care, is mandatory in order to reduce delays in effective treatment. Parenteral and potentially toxic streptomycin-rifampin is to be replaced by oral clarithromycin or fluoroquinolone combined with rifampin. In the absence of proven effective primary prevention, avoiding skin contamination by means of clothing can be implemented in areas of endemicity. Buruli ulcer is a prototype of ecosystem pathology, illustrating the impact of human activities on the environment as a source for emerging tropical infectious diseases.

KEYWORDS *Mycobacterium ulcerans*, *Mycobacterium marinum*, environmental mycobacteria, Buruli ulcer

INTRODUCTION

AQ: A

Large ulcers compatible with the diagnosis of Buruli ulcer were described by Sir Albert Cook in 1897 and by Kleinschmidt in northeastern Congo during the 1920s (1–4), but the causative agent, *Mycobacterium ulcerans*, was not isolated until 1948 in the Bairnsdale region of Victoria, Australia, by MacCallum et al. (5). The disease was finally named after Buruli (now called Nakasongola) County in Uganda, where the disease was described (6). The same infection has also been described under local names, according to the place where it occurred or was observed: Bairnsdale ulcer, Daintree ulcer, Mossman ulcer, and Searl ulcer in Australia, Tora and Mexican ulcer in Mexico (7), and mbasu, Kasongo ulcer, Kakerifu ulcer, La maladie mystérieuse de Daloa, and Mputa ya Luaka in African settings, where this infection has become more prevalent over the last few decades (8). Over the last decade, osteomyelitis has been an increasingly described form of the infection (9, 10). Still a query infection, Buruli ulcer is now known as a mycobacteriosis of the cutaneous and subcutaneous tissues caused by the nontuberculous bacterium *Mycobacterium ulcerans* (5, 11–18).

Buruli ulcer is a World Health Organization (WHO) reportable disease, reported in 33 countries in Southeast Asia, Australia, Africa, South America, and the Western Pacific, with impoverished rural communities of West and Central Africa being the most affected (Fig. 1) (14, 19, 20). Between 5,000 and 6,000 cases have been reported annually by 15 of the 33 reporting countries (21). Since only half of these countries regularly report data to the WHO, the full extent of the problem is unknown. Nevertheless, Buruli ulcer is regarded as the third-most-common mycobacterial infection in immunocompetent patients (15, 22) and is the second-most-common mycobacterial disease after tuberculosis in some countries with low endemicity for leprosy (23, 24). Buruli ulcer is one of the 17 tropical diseases classified as neglected diseases by the WHO, which recognized Buruli ulcer as an emerging public health problem in 1998 at the Yamoussoukro Conference (25). Starting in 2010, the number of registered cases regularly decreased in Africa, without a definitive explanation for that favorable trend (Fig. 2; Table 1). Causes for the decline in the overall incidence of Buruli ulcer remain purely speculative. Decline may reflect the positive effects of control programs or collateral effects of other health programs (26). In contrast, the incidence rose in Australia, from 32 cases in 2010 to 106 cases recorded in Victoria in 2015 (27). Understanding the epidemiological trends of Buruli ulcer has been obscured by the lack of definite knowledge regarding the reservoirs and modes of transmission of the causative agent, *M. ulcerans*, in every region of endemicity (19, 28, 29). Human-to-human transmission of Buruli ulcer has rarely been reported, suggesting environmental sources, as corroborated by several studies (30). Epidemiological studies have linked

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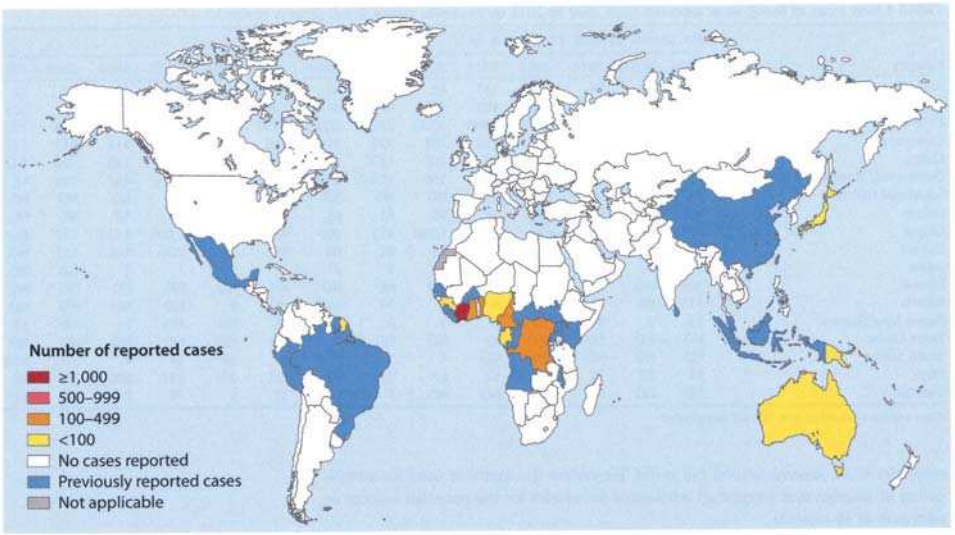

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FIG 1 Global map representing countries that have reported cases of Buruli ulcer disease as of 2014 (344).

Buruli ulcer mainly to low-lying wetland areas and slow-moving rivers, especially in man-made environments (31–33). In West Africa and Central Africa, outbreaks of Buruli ulcer in the 1980s were linked to man-made changes in the natural environment (34, 35). More-recent studies have shown that in aquatic and swampy environments, *M. ulcerans* is detected in biofilms, soil, and aquatic insects (36–40).

The severe morbidity of Buruli ulcer and the high frequency of disabling sequelae contrast with the low mortality associated with the disease. As an example in Ghana, 2 patients of 102 died of sepsis and tetanus within 2 years (41). However, the disabling sequelae of Buruli ulcer have enormous physical and socioeconomic impacts on affected individuals (38).

Therefore, there is still a need for research concerning environmental reservoirs and sources, risk factors, and the contamination cycle in order to invent new protocols to fight Buruli ulcer. With this in mind, we herein review the current state of knowledge on Buruli ulcer in regions of endemicity and the management and environmental

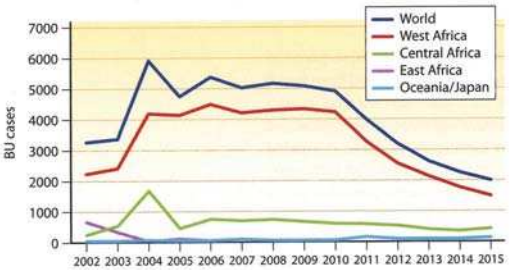

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FIG 2 Cases of Buruli ulcer (BU) reported in major areas of the world during the last decade. The correlation between Buruli ulcer cases in the world and in West Africa is 0.97.

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TABLE 1 New cases of Buruli ulcer reported from 2002 to 2015 by countries where Buruli ulcer is endemic[a]

Country	No. of new cases of Buruli ulcer in:													
	2015	2014	2013	2012	2011	2010	2009	2008	2007	2006	2005	2004	2003	2002
Australia	110	89	74	105	143	42	35	40	61	72	47	34	14	32
Benin	311	330	378	365	492	572	674	897	1,203	1,195	1,045	925	722	565
Cote d'Ivoire	549	827	1,039	1,386	1,659	2,533	2,679	2,242	2,191	1,872	1,564	1,153	768	750
Cameroon	133	126	133	160	256	287	323	312	230	271	265	914	223	132
Congo	ND	ND	6	38	56	107	147	126	99	370	53	235	180	102
Democratic Republic of the Congo	234	192	214	284	209	136	172	260	340	74	51	487	119	17
Equatorial Guinea	ND	ND	ND	ND	0	ND	ND	ND	ND	ND	3	ND	ND	ND
Gabon	40	47	59	45	59	65	41	53	32	54	91	43	ND	ND
Ghana	275	443	550	632	971	1,048	853	986	668	1,096	1,005	1,157	737	853
Guinea	72	46	96	82	59	24	61	80	ND	279	208	146	157	ND
Japan	3	7	10	4	10	9	5	2	3	1	1	1	ND	ND
Liberia	105	ND	8	21	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Nigeria	113	65	23	40	4	7	24	ND	ND	9	ND	ND	ND	ND
Papua New Guinea	11	3	ND	ND	8	5	8	24	26	ND	ND	31	18	13
Sierra Leone	ND	ND	ND	ND	28	ND	ND	1	ND	ND	ND	ND	ND	ND
South Sudan	ND	ND	ND	ND	ND	4	5	3	8	38	24	4	360	568
Togo	81	67	37	51	52	67	52	95	141	40	317	800	38	96
Uganda	ND	ND	ND	ND	ND	ND	3	24	31	5	72	7	10	117

[a]Data are from reference 344. ND, not determined.

reservoirs of *M. ulcerans* around the world. We review the methods used for investigating *M. ulcerans* and suggest an intellectual framework for the potential sources or reservoirs of *M. ulcerans*.

METHODS

We performed a review of the literature through NCBI/PubMed, Google Scholar, published data from the WHO website, and the Web of Knowledge, using the following keywords: "Buruli ulcer," "*Mycobacterium ulcerans*" AND "environment" AND "reservoir" AND "laboratory diagnosis" AND "clinic" and the related names of Buruli ulcer in countries of endemicity. We identified data up to March 2017. The titles and abstracts of the available articles were selected for their relevance to Buruli ulcer epidemiology, Buruli ulcer diagnosis, environmental factors (reservoirs, vehicle, source, *M. ulcerans* host), and the detection and isolation of *M. ulcerans* from environmental samples. The reference lists of the included papers were reviewed for additional references, including Web pages concerning the subject. We compared the geographical, ecological, and demographic characteristics of six West African countries with high rates of prevalence of Buruli ulcer (numbers of cases superior to 1/100,000 inhabitants) with those of six neighboring countries with low rates of prevalence of Buruli ulcer. Then, we downloaded from the Internet photos of farmers working in paddy fields in West Africa to analyze their degree of protective clothing when farming to correlate clothing with the main locations of Buruli ulcer lesions on the body. A comparison of the body temperatures at different points and the main locations of Buruli ulcer lesions was done.

M. ulcerans, the Agent of Buruli Ulcer

M. ulcerans has been shown to meet the four criteria (Koch's postulates) required to establish that an organism causes a disease: (i) it has been regularly isolated from Buruli ulcer-diseased tissues at various stages of the disease, (ii) it has been isolated in pure culture, (iii) its inoculation in appropriate laboratory animals reproduces the clinical and histopathological features of the disease, and (iv) the pathogen has been reisolated from the new host and shown to be the same as the originally inoculated pathogen. However, it must be noted that the absence of isolation from nondiseased skin has never been clearly reported (42–44).

M. ulcerans may date from the Jurassic Period, as its current repartition fits with the breakup of supercontinents 150 million years ago (45). Genome-based and gene-based phylogenetic reconstructions suggest that an ancestor common to *M. ulcerans* and its closest neighbor *Mycobacterium marinum* diverged by 470,000 to 1,200,000 years ago

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(46). *M. ulcerans* should therefore be regarded as a member of an *M. marinum* complex, also comprising *Mycobacterium ulcerans* subsp. *shinshuense*, *Mycobacterium pseudoshottsii* isolated from fish, and "*Mycobacterium liflandii*," which has been isolated from *Xenopus tropicalis* and *Xenopus laevis* frogs (47). These species all produce the toxin mycolactone and form the so-called mycolactone-producing mycobacteria (MPM) but are not necessarily associated with Buruli ulcer (47). All MPM are thought to have evolved directly from *M. marinum* (48). In particular, *M. ulcerans* subsp. *shinshuense* has been described in China and Japan (49). It possesses a 174-kbp virulence plasmid coding for polyketide synthase, producing mycolactone (49). Within the *M. marinum* complex, the evolution of *M. ulcerans* has been marked by a reduction in the chromosome size, from 6.6 Mb in *M. marinum* to 5.8 Mb in *M. ulcerans* (50, 51). It is noteworthy that this region of difference between *M. marinum* and *M. ulcerans* comprises 28 to 22 PE-PPE genes, whose poorly characterized products have been shown to support the survival of *M. marinum* inside phagocytes (52). Proliferation of more than 200 copies of insertion sequence 2404 (IS*2404*) is another mark of genome decay. The genome of *M. ulcerans* Agy 99 (a strain isolated from a single individual in Ghana) contains two prophages, 18-kb phiMU01, encoding 18 coding DNA sequences (CDS) and 24-kb phiMU02, encoding 17 CDS. The two prophages look like other mycobacteriophages described for other *Mycobacterium* species with the same overall structure and contain CDS associated with replication functions. However, phiMU02 is probably nonfunctional due to the proliferation of the IS*2606* insertion sequence, which has inactivated several genes (14). Accordingly, no phage has been reported to be associated with *M. ulcerans* in naturally or experimentally infected cells and tissues or in culture. Moreover, 14 mycobacteriophages have been tested for their ability to infect 18 different *M. ulcerans* strains, including the ATCC 35840 strain (which lacks mycolactone production), a rifampin-resistant strain, and 15 clinical isolates from various geographic origins, along with 2 *M. marinum* strains (53). A later study indicated that four mycobacteriophages, named Bxz2, D29, L5, and TM4, induced plaque formation of *M. ulcerans* but not *M. marinum*. However, plaque formation was not specific to *M. ulcerans*, as plaque formation was also observed in *Mycobacterium tuberculosis* and *Mycobacterium bovis* bacillus Calmette-Guérin (BCG). Furthermore, this study showed that *M. ulcerans* cell wall mycolactone was not involved in mycobacteriophage penetration into *M. ulcerans* (53). A second major genomic evolution event was the acquisition of a 174-kb plasmid called pMUM001, which is required for the synthesis of the major virulence factor mycolactone toxin (51). The replication site of this plasmid is more closely related to the one reported in the cryptic plasmid of *Mycobacterium fortuitum* (51).

M. ulcerans exhibits strong geographic diversity, as first suspected by partial 16S rRNA gene sequencing, which distinguished two subtypes of *M. ulcerans* linked to the Australian and African continents (11, 54). Further analysis of large sequence polymorphism in 12 regions of difference in 30 *M. ulcerans* isolates from diverse geographic origins indicated that *M. ulcerans* was involved in five insertion-deletion haplotypes that separated a so-called "classical lineage," comprising most pathogenic genotypes from Africa, Australia, and Southeast Asia, and a so-called "ancestral lineage," genetically closer to *M. marinum*, comprising isolates from Asia (China/Japan), South America, and Mexico (55).

It is estimated that these two *M. ulcerans* lineages diverged at the time of the emergence of *Homo sapiens* (250,000 to 400,000 years ago) (56), while the African isolates may have arisen in the past 18,000 years (46). Restriction fragment length polymorphism (RFLP) followed by IS*2404* probe hybridization did not produce any band with *M. marinum* and yielded six *M. ulcerans* groups related to six geographic regions, including Africa, Australia, Mexico, South Asia, Asia, and South America (57). All African isolates are genomically extremely closely related in the same cluster, and the classical-lineage *M. ulcerans* isolates from Australia also are all genomically extremely closely related and located in another cluster (58). A further genomic epidemiological study showed that isolates from West Africa (Côte d'Ivoire, Ghana, Togo, Benin) and Central

Africa (Cameroon, Gabon, Congo-Brazzaville, Democratic Republic of the Congo in Bas-Congo, Angola) had identical mycobacterial interspersed repetitive unit-variable number of tandem repeat (MIRU-VNTR) profiles, their genomes differing in a limited number of single nucleotide polymorphisms (SNPs) (59, 60). This strong association between *M. ulcerans* genotype and the geographic origins of strains was interpreted as indicating that the reservoir of *M. ulcerans* was relatively fixed in space (50, 58, 60, 61). A further comparative whole-genome sequencing study of isolates from Africa showed that several distinct clonal complexes of *M. ulcerans* could be found in the same areas where Buruli ulcer is endemic (58, 60, 61). Likewise, two Cameroonian clonal complexes, differing by 828 SNPs, were shared by all members of the respective lineages (60).

These results suggested that some moving reservoir might be responsible for the introduction of *M. ulcerans* into a new area, where it further spread within human populations (58, 61). Recently, Vandelannoote et al. reconstructed the evolutionary history of *M. ulcerans* by comparing 165 *M. ulcerans* clinical isolates recovered between 1964 and 2012 in 11 African regions of endemicity (62). The authors identified two specific *M. ulcerans* lineages within the African continent: lineage Mu_A1, putatively dating from 68 BC, and lineage Mu_A2, which is more closely related to Papua New Guinea isolates (62). Bayesian analysis indicated that the Mu_A2 lineage was probably introduced in Africa as recently as 1800 AD, supporting the hypothesis of a human-mediated introduction in Africa (62). Genome-based analyses further indicated close relationships between the environment and patients' strains; this is true of *M. ulcerans* Agy 99 (51, 63). The DNA of this strain was recovered from a small mammal (*Mastomys*) in Côte d'Ivoire (64). In Ghana, genome types W, X, Y, and Z were found in both human and environmental samples (13, 63). Whole-genome sequencing of an *M. ulcerans* isolate from a ringtail possum isolated in Point Lonsdale, Australia, revealed extremely close genetic relationships with the genome sequence of a human isolate in the same township, suggesting a major role for mammals in the ecology of this mycobacterium (61, 65).

The genomic diversity of *M. ulcerans* is further reflected by the structural diversity of mycolactones, first identified in 1999 (66). Indeed, mycolactones are polyketides comprising a core lactone and a fatty acid side chain and belonging to the family of macrolides (67, 68), and six naturally occurring structural variants named A/B, C, D, E, F, and G have been characterized in the different MPM species (48, 69). *M. liflandii* produces mycolactone E (70, 71), while *M. pseudoshottsii* and *M. marinum* produce mycolactone F (72). Mycolactone F-producing mycobacteria do not culture at a temperature above 30°C, which likely limits their virulence for humans (72). Each *M. ulcerans* isolate produces one type of mycolactone, either A/B, C, or D, and different congeners of mycolactones are produced by the different geographical isolates; mycolactone A/B is produced by the African and Malaysian isolates, the Australian isolates produce toxic mycolactone C, while the Chinese isolates produce mycolactone D (67, 70–72). Indeed, clinical data indicate that *M. ulcerans* isolates collected in Australia, Asia, Central America, and Mexico are less pathogenic than African isolates (48, 71). Mycolactone synthesis is a complex process related to polyketide synthesis (PKS) (51). In brief, mycolactones are synthetized by polyketide synthases encoded by three large genes located in the 174-kb pMUM001 plasmid, *mlsA1* and *mlsA2*, encoding the mycolactone core-producing PKS, and *mlsB*, encoding the side chain enzyme (51). After its synthesis, the toxin is secreted in bacterial-membrane-derived vesicles and concentrated in the extracellular matrix, which acts as a reservoir (68, 73). This synthesis is drastically downregulated by the presence of specific carbohydrates, such as glucose, maltose, and maltopentaose (74). Exposure to sunlight also causes its degradation and a loss of its biological activity. On the other hand, mycolactone preserves its structure and cytotoxic effects even after being heated at 100°C for 6 h. Outside the mycobacteria, mycolactones alter the Wiskott-Aldrich syndrome protein target and related scaffolding proteins (75), altering actin dynamics and cell adhesion with cell death (76). Mycolactone inhibits the function of the Sec61 translocation, which is responsible for protein translocation to the endoplasmic reticulum. This affects 30 to 50% of mammalian

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proteins, including circulating inflammatory mediators and proteins involved in lipid metabolism, coagulation, and tissue remodeling. Buruli ulcer patients have systemic and chronic defects in protein metabolism (77). Research has shown that the hypoalgesic effect observed in Buruli ulcer results from the activation of the angiotensin II type 2 receptor (AT2R), leading to neurite degeneration, cell death, and extensive coagulative necrosis (78). It was also shown that mycolactone decreased thrombomodulin expression on the surfaces of human dermal microvascular endothelial cells and that tissue necrosis might be caused by fibrin-driven ischemia (79). The identification of the Wiskott-Aldrich family proteins as molecular targets of the mycolactones would allow focusing the search for functional inhibitors of the toxins and probably provide the therapeutic tools of tomorrow (75, 76). All the A/B, C, and D mycolactones are toxins responsible for the damage observed in the skin and subcutaneous fat tissue, inducing apoptosis with minimal or no inflammation; unlike in other mycobacterioses, mycolactone does not induce lesions on healthy skin (66, 80).

However, a sole injection of mycolactone through the skin produces ulcers in guinea pigs (81), while a mutant deficient in mycolactone did not cause ulcers (66). Indeed, mycolactones have been shown to elicit a combination of ulcerative, analgesic, and anti-inflammatory effects in human skin by completely blocking the production of lipopolysaccharide (LPS)-dependent proinflammatory mediators posttranscriptionally (82–84). Mycolactone blunts the capacity of immune cells to produce inflammatory mediators by an independent mechanism of protein synthesis blockade (82). It has been demonstrated that mycolactone is sufficient to cause neurological damage (84, 85). Mycolactone can be detected in diseased skin samples from patients with Buruli ulcer by conventional thin-layer chromatography (86). The fact that the immunosuppression stops after removal of infected tissues supports the view that the systemic diffusion of mycolactone is responsible for its immunosuppressive effects (87). Indeed, mice injected by a radiolabeled form of the toxin (88) and clinical studies indicated that mycolactones diffuse from ulcerated lesions in clinically accessible samples. They also diffuse into the peripheral blood of Buruli ulcer patients (89), targeting mononuclear cells in peripheral blood and lymphoid organs, with a particular tropism for the spleen. The capacity of circulating lymphocytes to produce interleukin-2 upon stimulation is then hampered (88). The role of mycolactones during the environmental stages of *M. ulcerans* is unknown.

The study of *M. ulcerans* has been sharply limited by a lack of available isolates; none of the five environmental isolates advocated (39, 90, 91) have been deposited in public collections, and only 18 of 320 reported clinical isolates are available in public collections (see Table S1 in the supplemental material). Also, from 342 strains in the repertoire, only four complete *M. ulcerans* genomes have been reported: in Ghana (*M. ulcerans* Agy 99), the United States (*M. ulcerans* strain Harvey), Benin (*M. ulcerans* S4018), and Japan (*M. ulcerans* ATCC 33728) (Table S1). The lack of isolates may be due to intrinsic fastidiousness, rendering the isolation of *M. ulcerans* particularly susceptible to contaminant overgrowth (91, 92). Indeed, the *M. ulcerans* doubling time of 4.8 ± 0.3 days (93) correlates with the presence of only one chromosomic ribosomal operon, classifying *M. ulcerans* as a slow-growing mycobacterium (46). Optimal growth is obtained at 28 to 33°C under a 2.5 to 5% oxygen atmosphere and a final pH of 6.6 ± 0.2 at 25°C (94–98). The exposition of *M. ulcerans* to 41°C for 24 h kills more than 90% of the inoculum (22). This observation may have unanticipated practical implications for the culture of specimens that should not be exposed to high temperatures, such as the ones frequently encountered in tropical regions of endemicity. Moreover, *M. ulcerans* exhibits sunlight susceptibility, probably due to the lack of light-inducible carotenoids that protect *M. marinum* (46), linked to a stop codon in *crtL*, involved in pigment synthesis (14). This characteristic has been suggested to support the *in vitro* and *in vivo* susceptibility of *M. ulcerans* to purified methylene blue; all other tested mycobacteria, including *M. marinum*, are resistant to this dye (99). *M. ulcerans* was reported to grow in Middlebrook 7H9 broth, Middlebrook 7H10, and Middlebrook 7H11 agar media with oleic acid-albumin-dextrose-catalase (OADC) enrichment and Löwenstein-Jensen (LJ)

medium. The addition of chitin to 7H9 Middlebrook broth was indirectly shown to increase the growth of one strain of *M. ulcerans* (100). Interestingly, the five available *M. ulcerans* genomes encoded a GH18 family member, compatible with a putative chitinase activity. Decontamination of environmental specimens is the key step for the isolation of *M. ulcerans* from environmental sources. F. Portaels and collaborators have tested several decontamination methods, including the Petroff method (101), incorporating sodium hydroxide (NaOH), the reversed Petroff method, and a mild decontamination method using HCl and oxalic acid treatment (102–104). All these methods proved to adversely affect the growth rate of *M. ulcerans*, but incorporation of egg yolk into the culture media limited the cytotoxic effects of these agents, especially the effect of oxalic acid. A recent study compared the effect of clinical sample decontamination with that of NaOH or oxalic acid, followed by inoculation in LJ medium slants with glycerol or inoculation in the same LJ medium slants supplemented with 2% PANTA (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin). The decontamination methods did not differ in their effects on the recovery of *M. ulcerans*, but the use of inoculated media had a significant impact on the recovery of *M. ulcerans*. Indeed, the use of LJ medium slants with glycerol reduced the probability of *M. ulcerans* recovery by 65% (39). In the same study, the authors also compared the effects of the transport media on the growth of *M. ulcerans* and contamination cultures and found no significant difference between 7H9 medium containing PANTA and the antibiotic-free Amies medium Middlebrook (39). Using environmental specimens, the combination oxalic acid-NaOH gave more-effective results than the SDS-NaOH, NaOH-malachite green-cycloheximide, and *N*-acetyl-cysteine–NaOH combinations. Also, LJ medium supplemented with PANTA and mycobactin J best supported the growth of mycobacteria, including *M. ulcerans*, compared to isoniazid- or ethambutol-supplemented LJ medium (39, 40).

In contrast with the hundreds of clinical strains that have been isolated, only five isolates from the environment have been isolated (39, 91, 105) (Table S1). Many attempts to isolate *M. ulcerans* from flora and fauna failed (22, 105). The culture of diverse environmentally collected samples from areas where Buruli ulcer is endemic failed to yield *M. ulcerans* in the past (22), despite the parallel detection of *M. ulcerans* DNA sequences (19, 64, 106–113). Failure to culture *M. ulcerans* from environmental samples may possibly be attributable to inadequate sampling, conditions of transport, inadequate decontamination procedures, and the culture conditions of this fastidious heat-sensitive organism (22, 98). The initial isolation of an *M. ulcerans* strain was obtained from an aquatic Hemiptera from a Beninese sample collected by Portaels et al., who suggested that the disease resulted from exposure to a contaminated environment (91). This isolate was obtained after a 15-day incubation period in Bactec 12b broth and three successive passages in mouse footpads P1, P2, and P3 for 9 months, 6 months, and 12 months, followed by culture on LJ medium for 2 months (91). In contrast, recently, Aboagye et al. set up an efficient protocol and succeeded in obtaining a pure culture of two poorly characterized *M. ulcerans* strains in less than 6 months from soil and moss (39).

Cellular and Animal Models for *M. ulcerans* Infection

The fact that the pathogenesis of *M. ulcerans* is dependent on the temperature of the area where the bacteria were inoculated is the first notable characteristic of *M. ulcerans* (114). The second notable characteristic of *M. ulcerans* is its inability to penetrate intact skin and its inability to infect abraded skin, as demonstrated in an experimental infection of guinea pigs and mice (115). These results suggest that Buruli ulcer is dependent on the passive inoculation of *M. ulcerans* through intact skin as an alternative to ineffective passive passage through abraded skin, with the precise role of "biological needles," such as mosquitoes and other insects, remaining to be studied in comparison with the effectiveness of mechanical needles. The third notable characteristic of *M. ulcerans* pathology is the presence of cell damage in the absence of an acute inflammatory response. Injection of mycolactone in guinea pig skin resulted in exten-

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sive tissue destruction and extensive apoptosis as the size of the lesion expanded (81). Knowing that apoptosis is associated with a lack of inflammatory response, these observations reproduced the observations made on Buruli ulcer lesions (81). In fact, these data indicate that Buruli ulcer is not an infectious disease depending on the multiplication of the pathogen but rather a toxemic disease caused mainly by mycolactone. It has been shown that the use of rifampin and streptomycin in the treatment of Buruli ulcer resulted in a rapid onset of local cellular immune responses associated with the phagocytosis of extracellular *M. ulcerans*. This may be related to declining levels of mycolactone in the tissue, thus leading to an enhanced chemotherapy-induced clearance of the infection (116). Mycolactone A/B causes apoptosis in keratinocyte stem cells (KSC) and transit-amplifying cells (TAC) extracted from human skin biopsy specimens even in small doses of 1 to 10 ng/ml. This apoptosis is dose dependent, as measured by morphological criteria, chromatin condensation, and nuclear fragmentation or as measured by the mitochondrial membrane potential. However, mycolactone A/B was less toxic in human keratinocyte cell lines (HaCaT). Only 25 to 30% of HaCaT cells were affected after treatment with 100 and 1,000 ng/ml of mycolactones A and B, respectively, compared to more than 60% TAC apoptosis at 1 ng/ml and 50% KSC apoptosis at 10 ng/ml. The apoptotic activity of mycolactone A/B was also tested on the human hepatoma cell line HuH7 and on the human epithelial embryonic kidney cell line HEK 293T, since mycolactone has renal and hepatic tropism when it diffuses into the blood (88). No apoptotic cells were detected after treatment with 1 to 1,000 ng/ml of mycolactone (117).

M. ulcerans probably escapes phagocytes during its first steps after intradermal inoculation, behaving as an extracellular pathogen, as observed mainly in cutaneous and subcutaneous lesions (118, 119); this is in opposition to what occurs in XTC2 cells and mice macrophage models, in which an intracellular growth phase for the pathogen has been reported (120, 121). It was shown that *M. ulcerans* bacilli were captured by phagocytes and were predominately intracellular organisms at 24 h postinfection, whereas examination of tissues of infected BALB/c mice harvested at the ulcerative stage (8 weeks postinfection) showed that *M. ulcerans* bacilli were exclusively in the extracellular compartment. This was also characterized by an extensive inflammatory infiltrate and the presence of neutrophils and major histocompatibility complex class II (MHC II) cells surrounding the bacterial foci (122). Accordingly, bone marrow-derived RAW264.7 macrophages, the dendritic cell line FSDC, and neutrophils, but not nonphagocytic L929 fibroblasts, were isolated from BALB/c mice phagocytizing *M. ulcerans* bacilli (123). In the same study, the authors showed that incubating bone marrow-derived macrophages with mycolactone significantly reduced their ability to phagocytize *M. ulcerans* bacilli. Furthermore, macrophages and dendritic cells infected with *M. ulcerans* exhibited alterations in their morphology similar to that after cytotoxicity from exogenously added mycolactone at 6 h postinfection (123). Apoptosis was observed as an important tissue destruction mechanism in human lesions associated with viable *M. ulcerans* cells (124). Nuclear fragmentation indicative of apoptosis was also observed before the death of cells at 24 h postinfection (123). Cells infected with *M. ulcerans* expressed less tumor necrosis factor alpha (TNF-α) and the transforming growth factor β (TGF-β) cytokine than cells infected with the *M. ulcerans* mutant, which does not produce mycolactone (123). In contrast, the macrophage inflammatory protein MIP-2, which is chemotactic and activating for neutrophils, was expressed more in cells infected with wild-type *M. ulcerans* than in cells infected with the *M. ulcerans* mutant. These data demonstrate an upregulation of inflammatory chemokines and a downregulation of inflammatory cytokines during infection with *M. ulcerans* (123). In a subsequent study, Torrado et al. reported that *M. ulcerans* induces the expression of gamma interferon (IFN-γ) at the infection sites of experimentally infected mice (125). Also, IFN-γ-deficient mice are more susceptible to *M. ulcerans* infection than wild-type mice when they are infected with intermediate or avirulent strains (118). In contrast, no difference in the susceptibilities to infection between IFN-γ-deficient and wild-type mice was noted when they were infected by the highly virulent strain, suggesting that

the highly virulent strain of *M. ulcerans* has an adverse effect on the protective activity of IFN-γ on infected macrophages. Accordingly, by using bone marrow-derived macrophages, activated or not with IFN-γ, the authors showed that IFN-γ can activate macrophages to control the intracellular growth of avirulent and intermediate-virulence strains but not that of the highly virulent strain of *M. ulcerans*. The clinical observations of a recent study showed pronounced swelling of the infected footpads of IFN-γ-deficient mice; in contrast, nothing unusual was observed in wild-type mice after 5 weeks of infection (118). Histopathological analysis showed that IFN-γ-deficient mice exhibited more tissue necrosis, more edema, and a significantly greater bacterial load as measured by quantitative PCR (qPCR) than wild-type mice. These results suggest that IFN-γ activated the macrophages to eliminate intracellular bacteria at an early stage of infection (5 weeks) (118). Histological observations of adipose tissues from infected patients showed extensive necrosis of subcutaneous fatty tissues, which was directly correlated with mycobacterial invasion and toxin production (81, 126–128). This feature was also reproduced in infected pig skin with *M. ulcerans* (119). The histopathological analysis showed clusters of extracellular mycobacteria and fat cell ghosts after *M. ulcerans* infection and mycolactone injection (129). Furthermore, the interaction between *M. ulcerans* and adipose tissue was investigated using a human adipose cell model (128). After 24 h of incubation, electron microscopic observations showed an extracellular location of *M. ulcerans* and a cytotoxic effect on cells. Within 3 days, both apoptosis and necrosis were observed. Under the same conditions, cells were incubated with *M. ulcerans* culture filtrate and purified mycolactone. While *M. ulcerans* culture filtrate induced both necrosis and apoptosis, mycolactone induced only necrosis.

Studying the interactions with another phagocytic model, amoebae, brought additional data. It has been reported that *M. ulcerans* persisted inside *Acanthamoeba polyphaga* cells for 2 weeks, with an inoculum declining by 1 to 2 logs, as measured by culture (130). In a subsequent study of *Acanthamoeba castellanii* coculture, the authors showed that the number of *M. ulcerans* cells decreased by 90% over 28 days (29). These data suggest an improbable role of amoebae as sources or reservoirs of *M. ulcerans*. Temperature can partly explain divergent results obtained in animals, macrophages, and amoeba models. Indeed the optimal growth temperature for macrophages used in the experiments cited above is 37°C, while these experiments have been conducted at 32°C to mimic the optimal growth temperature of *M. ulcerans* (131). Using a suboptimal temperature can affect the antimicrobial activities of macrophages, such as cytokine production, antimicrobial peptide secretion, and activities and membrane dynamics required for phagolysosome biogenesis (132–134). Under these conditions, the survival and the multiplication of *M. ulcerans* cells in macrophages are facilitated.

The unique microbiological features of *M. ulcerans* among the species of the genus *Mycobacterium* indicate that Buruli ulcer should be understood as a toxic effect of infection, with major features linked to the activities of the plasmid-encoded mycolactone, rather than to the replication of *M. ulcerans*. Indeed, *M. ulcerans* replication is strongly controlled by the local temperature, which is not the case with mycolactone (114). In the laboratory, the optimal temperature for replication is 30 to 33°C (20). This situation is indeed encountered in the same skin territories where Buruli ulcer lesions are more prevalent (Fig. 3). Accordingly, *M. ulcerans* does not disseminate in the bloodstream, and tissue lesions remain localized, despite the remote immunosuppressive neurotropic activities of mycolactone (88, 135). This is in agreement with animal studies, suggesting that the bacilli remain essentially localized within ulcerative lesions in subcutaneous tissues but not in the blood (123).

M. ulcerans in the Environment

The fact that *M. ulcerans* exhibits a reduction in chromosome size compared to that of *M. marinum* suggests a reduction in the ecological niches, i.e., specialization (14, 136–138). Accordingly, genomic analysis has suggested that *M. ulcerans* may reside inside one or several hosts (14), in agreement with previous observations (22). However,

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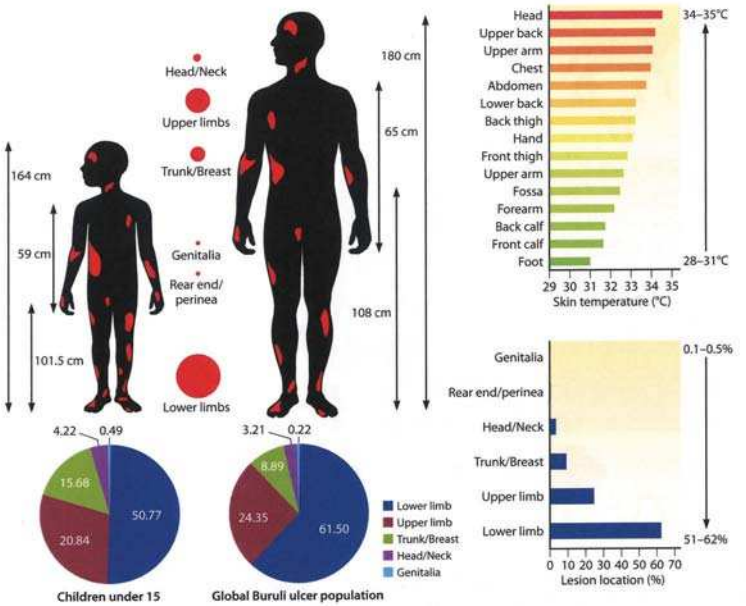

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FIG 3 Pattern of distribution of Buruli ulcer lesions on the bodies of human patients in Africa. This figure is a composite of data from 10 independent studies (17, 23, 26, 155, 226, 235, 241, 242, 244, 253). The histograms show that there is an inverse correlation between the gradient of body temperature and the location of lesions.

it was demonstrated in an experimental study that it can live as a free-living organism in its environmental niches, where it can survive for a long time despite its fragility under certain climatic conditions, such as solar light, temperature elevation, and UV light (14, 29, 37). As discussed above, these aspects have been poorly investigated, as the vast majority of field studies have relied upon molecular biology methods, which gave no clues regarding the viability of the detected mycobacteria. *M. ulcerans* DNA has been detected in inanimate soil and aquatic environments, but most of the attempts to isolate it from these inanimate environments have failed (105, 139).

T2/AQ:E Molecular methods used to detect *M. ulcerans* DNA sequences in environmental specimens are summarized in Table 2. As for molecular targets, the insertion sequence IS*2404* used in previous studies (98, 140, 141) was detected in other MPM (142, 143). The conventional IS*2404* PCR assay alone cannot be relied upon for the specific detection of *M. ulcerans*. To increase the specificity of PCR assays, three independent repeated sequences in the *M. ulcerans* genome, i.e., two multicopy insertion sequences (IS*2404*, IS*2606*) and a multicopy sequence encoding the ketoreductase B domain

TABLE 2 DNA targets for *M. ulcerans* and detection of related mycolactone-producing mycobacteria from environmental samples

Mycobacterium	Presence of: IS*2404* sequence	IS*2606* sequence	KR-B gene	Plasmid type
Mycobacterium ulcerans	Yes	Yes	Yes	pMUM001
Mycobacterium liflandii	Yes	Yes	Yes	pMUM002
Mycobacterium pseudoshottsii	Yes	Yes	Yes	pMUM003
Mycobacterium marinum	No/yes	Yes	Yes	pMM23

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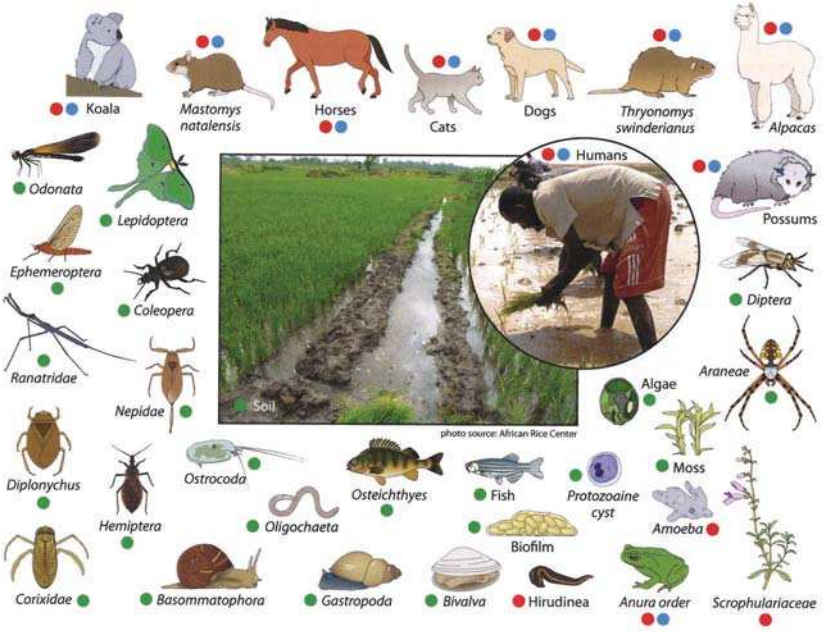

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FIG 4 Buruli ulcer risk factors and *M. ulcerans* reservoirs with chitin sources around paddy fields and swampy areas.

(KR-B), need to be used (39, 113, 144, 145). Moreover, this multiplex PCR can control PCR inhibitors commonly present in environmental samples. Despite these limitations, molecular techniques have provided important clues in revealing the uncertain sources of *M. ulcerans*.

Detection of *M. ulcerans* DNA in Bodies of Water and Moss

In Ghana, *M. ulcerans* DNA was detected in biofilms and water filtrate by amplifying the KR-B gene, which was then confirmed by VNTR-PCR (13), and in a body of water in an area of the Ashanti region where Buruli ulcer is endemic (145). Another study in Ghana detected *M. ulcerans* in biofilm, soil, filters, and detritus (63). Recently, using real-time PCR in samples to amplify the *M. ulcerans* IS*2404* and KR-B genes, *M. ulcerans* was detected in stagnant water specimens, soil, water filtrate residues, and plants in Côte d'Ivoire, thereby confirming that water is a reservoir of *M. ulcerans* in areas of endemicity of Côte d'Ivoire (106, 146). Bodies of water act as vehicles for disseminating *M. ulcerans* strains (Fig. 4). Recently, Aboagye and collaborators detected *M. ulcerans* DNA in moss from Ghana, and then obtained a positive culture of *M. ulcerans* from this sample (39). In French Guiana (South America), *M. ulcerans* DNA was detected for the first time in water (112). In Louisiana (United States), an area where Buruli ulcer is not endemic, *M. ulcerans* DNA was detected in water and biofilms (147).

F4

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Detection of *M. ulcerans* in Insects and Aquatic Animals

Aquatic insects have been implicated in the transmission of *M. ulcerans* and are considered potential vectors (16, 139, 148). Marsollier et al. subsequently carried out an experimental study demonstrating not only that Naucoridae concentrate *M. ulcerans* in their salivary glands but also that their bite transmits the infection to mice (16). Then *M. ulcerans* was detected in the salivary glands of water bugs belonging to the Naucoridae and Belostomatidae families and in snails. They are considered potential transient hosts of *M. ulcerans*, without offering favorable conditions for its growth and replication (16, 40, 141). In Côte d'Ivoire, *M. ulcerans* was detected by PCR in the Planorbidae family (planorbid and bulinini) (40). In Benin, it was demonstrated by the detection of the mycobacterium in the tissue of aquatic bugs captured during their migration toward water points that aquatic insects outside the aquatic context may be vectors of *M. ulcerans* (149). *M. ulcerans* DNA was detected in the tissues of water bugs (genera *Micronecta* and *Diplonychus*) (148), in aquatic insects (Belostomatidae, Hydrophilidae, and Naucoridae), and mollusks, supporting the hypothesis that the fauna in major foci where Buruli ulcer is endemic, especially in swampy areas of tropical and subtropical regions, may be a source of *M. ulcerans* infection (16, 22, 139, 141). *M. ulcerans* was isolated from an aquatic Hemiptera insect collected in Benin, and it was the first isolation of *M. ulcerans* after cultivation (91). It was detected in aquatic insects (Belostomatidae, Naucoridae, Corixidae, Ranatridae, and Nepidae) and in the saliva of *Diplonychus* sp. in Côte d'Ivoire (109) and in Benin (141). In Ghana, *M. ulcerans* was detected in Belostomatidae, Naucoridae, and Nepidae (150), and IS*2404* PCR and VNTR analysis were used to detect *M. ulcerans* or *M. liflandii* in wild amphibians (frogs) and fish (*Hemichromis bimaculatus*) in Ghana (151). In Benin, collected samples of plants (*cyperus*, *panicum*, *eichhornia*) were used for the detection of *M. ulcerans*. The result was unsuccessful, but *M. ulcerans* strains were detected in insects (Naucoridae) dwelling in the plant roots (139). A study conducted in Ghana detected *M. ulcerans* in an invertebrate and vertebrate collection of specimens (13). Aquatic Heteroptera can bite humans and contaminate them with *M. ulcerans*, as well as contaminate water, which would ensure the dissemination of the germ from one pond to another. They can also infect humans outside aquatic environments because of their ability to fly many kilometers away from their source (61, 152). In Cameroon, *M. ulcerans* DNA was detected in communities of aquatic macroinvertebrates and vertebrates (153, 154).

In Benin, *M. ulcerans* was detected in about 8.7% of aquatic insects, but not in mosquitoes (*Mansonia africana*, *Culex nebulosus*, *Culex quinquefasciatus*, *Anopheles pharoensis*, *Aedes vittatus*, *Culex decens*, and *Culex fatigans*) or in other flying insects (107). Mosquitoes may not play a pivotal role in the ecology and transmission of *M. ulcerans* in the areas of endemicity studied (107), although a previous study in Ghana indicated the role of mosquitoes as vectors in the transmission of Buruli ulcer (155). In Australia, *M. ulcerans* DNA was detected in mosquitoes (*Aedes camptorhynchus*, *Coquillettidia linealis*, *Anopheles annulipes*, *Culex australicus*, *Aedes notoscriptus*) in several studies (19, 156). In Benin, several pathogenic free-living amoeba were isolated from water and biofilm specimens taken from protected and unprotected sources of water in villages known to have either high or low endemicity for Buruli ulcer, and no specimen was positive (157).

M. ulcerans strains were detected in aquatic plants in emergent zones from both lotic and lentic bodies of water in regions of endemicity of Ghana (158). These observations support the idea that aquatic plants are a reservoir of *M. ulcerans* and add a new potential link in the chain of transmission of *M. ulcerans* to humans (105). In Benin, *M. ulcerans* DNA was detected in stems and leaves of plants (107). Several plants were implicated as a growth factor for *M. ulcerans* in Côte d'Ivoire. This led to the use of *Crinum calamistratum*, *Eriocephalus africanus*, *Vicia nana*, and *Vicia torta* for the development of a new culture medium to cultivate *M. ulcerans* (159). We can conclude from this study that these aquatic plants contribute to the survival of *M. ulcerans* strains and might even play a central role in biofilm formation (Fig. 4; Table 3).

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TABLE 3 Detection and isolation of *M. ulcerans* strains from environmental samples around the world[a]

Reference	Country(ies)	Type(s) of samples collected	Reservoir(s)	Method(s) used
207	Ghana	Fecal specimens of domestic animals	None	qPCR (IS*2404*, KR-B)
39	Ghana	Soil, water, fungi, snails, moss, vegetation	Soil, moss	Ziehl-Neelsen, culture, heat shock protein 65, IS*2404*, IS*2606*, *rpoB*, ketoreductase gene
150	Ghana	Biting water bugs (Hemiptera: Naucoridae, Belostomatidae, Nepidae)	Belostomatidae, Naucoridae, Nepidae	Amplification of the ER domain in *mlsA*
13	Ghana	Macroinvertebrate/vertebrate, water filtrate, soil, biofilm	Anura order, Araneae, Coleoptera, Diptera, Ephemeroptera, Gastropoda, Hemiptera, Hirudinea, Lepidoptera, Odonata, Oligochaeta, Osteichthyes, Ostracoda, Basommatophora, Bivalva, Diptera, soil, water filtrate, biofilm, fish	ER PCR and IS*2404* PCR, VNTR-PCR, DNA sequencing
145	Ghana	Environmental samples (water, detritus, trunk biofilm, plant biofilm)	Water	RT-PCR (IS*2404*, IS*2606*, KR-B)
63	Ghana	Soil, water filtrands, detritus, biofilm, small mammals	Biofilm, soil, filter, detritus, small mammal (*Mastomys*), mouse	IS*2404*, ER analysis, 16S rRNA and VNTR analysis, sequencing
29	Ghana	FLA from collected aerosols, biofilm plant, biofilm trunk, detritus, water	IS*2404* detected in FLA from biofilm plant, biofilm trunk, water, detritus, aerosols	RT-PCR (IS*2404*, IS*2606*, KR-B)
151	Ghana	Water, fish, amphibians	Amphibian, fish	ER analysis, VNTR
145	Ghana	Environmental samples, organs of small mammals	Water	Real-time PCR
428	Ghana	Fish	Fish	Nested IS*2404* PCR
139	Ghana, Benin	Plants from swamps areas, insects of plants roots	Insects (cyperus, panicum, eichhornia, Naucoridae)	Culture, nested IS*2404* PCR
141	Benin	Belostomatidae (*Appasus* sp.), Dytiscidae, Hydrophilidae, Naucoridae (*Naucoris* sp., *Macrocoris* sp.), molluscs (*Bulinus senegalensis*), fish	Belostomatidae, Hydrophilidae, Naucoridae, molluscs, fish	Nested IS*2404* PCR
91	Benin	Aquatic specimens	Hemiptera (*Gerris* sp.)	Culture positivity on LJ medium, nested IS*2404* PCR
149	Benin	Aquatic insects	*Diplonychus* sp.	PCR (IS*2404*, KR-B)
144	Benin	Water filtrand, macrophytes, soil, excrement, biofilm, aquatic invertebrate taxa, fish, tadpoles	Water filtrand, well filtrand, pond/river filtrand, cistern filtrand, biofilm	PCR (IS*2404*, ER)
107	Benin	Mosquitoes (adults and larvae), vertebrates, aquatic insects and plants	Aquatic insects (Odonatan, Hemiptera, Coleoptera, Diptera), vertebrates (Anura, fish), plants	qPCR (IS*2404*, KR-B)
16	Côte d'Ivoire	Water bugs	Naucoridae	Nested IS*2404* PCR
105	Côte d'Ivoire	Aquatic plants (Scrophulariaceae)	Scrophulariaceae	IS*2404* qPCR, culture
40	Côte d'Ivoire	Snails (*Planorbis* sp., *Bulinus* sp.)	Planorbid, bulin	PCR
148	Côte d'Ivoire			
109	Côte d'Ivoire	Aquatic Heteroptera	*Diplonychus* sp. (Belostomatidae), *Naucoris* sp. (Naucoridae), *Micronecta* sp. (Corixidae), *Ranatra fusca* (Ranatridae), *Laccotrephes ater* (Nepidae), *Anisops* sp. (Notonectidae)	qPCR (IS*2404*, KR-B)
64	Côte d'Ivoire	Small mammals	*Mastomys natalensis*	ER analysis, 16S rRNA, IS*2404* PCR, sequencing
106	Côte d'Ivoire	Soil, stagnant water, plants, animal feces	Stagnant water, feces of *Thryonomys swinderianus* (agouti), soil	qPCR (IS*2404*, KR-B)
146	Côte d'Ivoire	Plant biofilms, water filtrate residues, plant detritus, soils	Plant biofilms, water filtrate residues, plant detritus, soils	ER analysis, 16S rRNA, IS*2404*-PCR, MIRU-VNTR

(Continued on next page)

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TABLE 3 (Continued)

Reference	Country(ies)	Type(s) of samples collected	Reservoir(s)	Method(s) used
108	Japan	Environmental samples from a water channel in the patient's residence	Crayfish	Whole-genome amplification, touchdown PCR, DNA sequencing
208	Japan	Turtles	Turtles	PCR, nucleotide sequence analysis
19	Australia	Mosquitoes	*Aedes camptorhynchus*, *Coquillettidia linealis*, *Anopheles annulipes*, *Culex australicus*, *Aedes notoscriptus*	Real-time PCR (IS*2404*, IS*2606*, KR)
156	Australia	Mosquitoes	*Anopheles* sp.	Real-time PCR (IS*2404*, IS*2606*, KR)
199	Australia	Cats	Cats	Histological examination, Ziehl-Neelsen staining, PCR
113	Australia	Soil, sediment, mosquitos	Soil, sediment, mosquitos	PCR (IS*2404*, IS*2606*, KR)
200	Australia	Horses	Horses	Ziehl-Neelsen, IS*2404* PCR
201	Australia	Dogs	Dogs	Real-time IS*2404* PCR
202	Australia	Alpacas	Alpacas (*Vicugna pacos*)	IS*2404* PCR
204	Australia	Koala	Koalas (*Phascolarctos cinereus*)	
111	Australia	Possums	Ringtail possums (*Pseudocheirus peregrinus*), brushtail possum (*Trichosurus vulpecula*), mountain brushtail possum (*Trichosurus cunninghami*)	IS*2404* PCR
112	French Guiana (South America)	Water, filtered water	Water	qPCR (IS*2404*, KR-B)
147	United States (Louisiana)	Water, biofilms	Water, biofilms	IS*2404* PCR
153	Cameroon	Aquatic communities (vertebrates and small invertebrates)	Vertebrates (Fish, Anura), Insecta (Odonata, Ephemeroptera, Hemiptera, Coleoptera, Diptera, Plecoptera, Lepidoptera), Mollusca, Crustacea (Decapoda, Cladocera), Annelida, Arachnida (Acari, Araneae)	qPCR (IS*2404*, KR-B)
154	Cameroon	Diptera, Hemiptera, Coleoptera, Odonata, Ephemeroptera	Diptera, Hemiptera, Coleoptera, Odonata, Ephemeroptera	qPCR (IS*2404*, KR-B)

[a]ER, enoyl reductase; FLA, ●●●●.

M. ulcerans in Environmental Biofilms

Biofilms are sessile microbial communities growing on surfaces, frequently embedded in a matrix of extracellular polymeric substances (160–162). The nature of the *M. ulcerans* biofilm is not fully elucidated. Chitin may be one component and an important nutrient source for *M. ulcerans*. Chitin is the (1→4)-β-linked homopolymer of *N*-acetyl-D-glucosamine (163). It is one of the most important carbohydrates of the fungal cell wall in the carapace of mud crabs (*Scylla olivacea*), the structural backbone of the exoskeletons of crustaceans (shrimp, crayfish, crabs), shells of *Chelonibia patula*, yeasts and lichens, marine algae (barnacle, Crustacea), rotifer eggshells (*Brachionus plicatilis*), adult females and egg shells of microfilariae (*Onchocerca gibsoni*, *Onchocerca volvulus*), *Ascaris lumbricoides* eggshells, the cuticle of microfilariae of *Wuchereria bancrofti*, the radulae of certain mollusks, insects, fish (zebrafish), lissamphibians, internal shells of cephalopods, some bird guano (penguin guano), and cysts of various protozoans (Fig. 5) (164–176). Although more-complex plants have no chitin, they do secrete chitin-degrading enzymes (chitinase), which is a common plant hydrolase that defends against pathogenic-fungus attacks (177). Chitin synthases (CHS) are widespread among eukaryotes and known to have a complex evolutionary history in some of the groups (178). The functional importance of each CHS in the growth and development of *M.*

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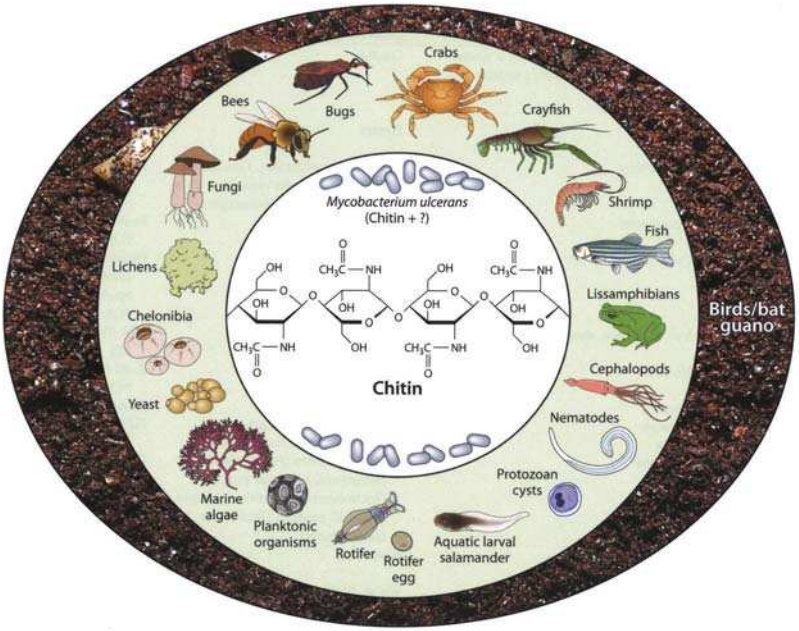

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FIG 5 Sources of chitin in the environment, West Africa. The middle circle includes primary sources, and the outer circle includes secondary sources.

ulcerans should be investigated, because each CHS probably plays particular roles during the different developmental stages of bacteria in the environment (Fig. 4 and 5).

Roles of Salts and Other Nutrients in the Maintenance of Environmental *M. ulcerans*

Salinity is one of the key environmental factors that limit crop growth and agricultural productivity. Hypersalinity is caused by an excessive concentration of soluble salts in the soil. The main ionic salt species are composed of sodium, calcium, and magnesium, appearing as chlorides and sulfates. Sodium chloride (NaCl) is the predominant salt. Salinity conditions occur in coastal, arid, and semiarid areas. In assuming that the salinity of the water and soil is a factor of *M. ulcerans* viability in the environment, we summarized soil and water salinity in Côte d'Ivoire as an example for West African countries, especially since *M. ulcerans* DNA was detected from a soil sample collected near rice paddy fields in Côte d'Ivoire (106, 146). The average salinity of lagoons in Côte d'Ivoire ranges between 4 and 19 mg/liter, whereas the salinity of rivers at their outlet in the south varied between 0 g/liter and 30 g/liter (179–185). The viability of *M. ulcerans* in salty areas has not been established, but in our laboratory, an experimental study proved that *M. ulcerans* strains could grow at a salinity above 20 g/liter (186). Soil salinity can be caused by the type of agriculture practiced in a given region. Therefore, it has been observed that intensive cultivation of rice for a short or long period is the basis of soil salinization and that the pH is below 8.5 in rice fields (187). It was shown that *M. ulcerans* followed seasonal dynamics and was present mainly in waters with a higher pH (188). In the United States, there were positive associations between pH levels and the concentrations of ammonia, dissolved oxygen, nitrate, nitrite, and sulfide

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TABLE 4 River and lagoon salinity in Côte d'Ivoire, West Africa

Site	Geographical position(s)	Avg salinity	pH	Reference
Lagune de Fresco	2°50′, 5°25W	15.69 mg/liter	7.52	179
Lagune Aby	2°51′–3°21E, 5°05′–5°22′N	0.283–1.28 ppt	6.96–7.8	180
Baie des Milliardaires/Lagune Ebrié	4°00′–4°10′W, 5°10′–5°20′N; 3°40′–4°50′W, 5°2′–5°10′N	0.4–6.9‰	7.1–7.7	181
Estuary zone/Grand-Lahou	4°26′–5°20′N, 4°20′–5°20′W			182
	Bac Sicor	12.87‰	7.75	
	Groguida	18.95‰	7.74	
	Kpanda	18.95‰	7.74	
	Braffedon	18.95‰	7.74	
Fleuve Sassandra	Basse Côte d'Ivoire	0–4%	6.8–7.4	183
Fleuve Bandama	Grand-Lahou (coast of Côte d'Ivoire)	0–32‰		184

in freshwater rivers where *M. ulcerans* DNA was detected in water and biofilms (147). Salinity is a major problem in tropical coastal regions having predominantly rice-based farming systems because of the intrusion of brackish water during the dry season through tidal movements and capillary rise from shallow saline groundwater. Salinization of rice paddies can cause a decrease in productivity if adequate irrigation methods are not used (187). Salinity continues to be high at the onset of the wet season, during and after rice transplantation, until sufficient rain washes it from the soil (189). Soil T4 salinity also increases in proportion to sea proximity (Table 4). Recently, we proved that *M. ulcerans* strains could survive in soil for 4 months, suggesting that Buruli ulcer might be acquired through inoculation with watery soil as a transient source of infection (37). The increase in the incidence of Buruli ulcer in West Africa, especially in coastal areas, might be related to the construction of canals to irrigate rice fields. *M. ulcerans* is common in humid rural tropical areas where agriculture is the main activity of the population (190). In Ghana, a spatial relationship was demonstrated between the prevalence of Buruli ulcer and its proximity to drainage channels, farmlands, and the immunosuppressant arsenic found in soil (191). In Ghana, *M. ulcerans* was detected in soil by searching for the KR-B gene only (13) and recently by the use of several PCR systems detecting heat shock protein 65, IS*2404*, IS*2606*, *rpoB*, and the ketoreductase gene (39). Plants, aquatic invertebrates, amphibians, and specific water conditions might allow *M. ulcerans* to grow and persist in the environment (16, 37, 40, 40, 121, 146, 158, 192, 193). Rice fields include all the risk factors for transmission of Buruli ulcer. The environment of rice fields is always wet and muddy. Farmers with their families, including children less than 15 years old, work for several hours with limbs in permanent contact with muddy water and without adequate protection. Consequently, rice fields are the ideal breeding ground and source of *M. ulcerans*, with more potential F6 reservoirs in the tropics (Fig. 4 and 6). Arsenic occurs naturally in the earth's crust, is widely distributed in the environment, and exists at an average concentration of AQ: G approximately 5 mg/kg of soil (194, 195). There are many possible routes of human exposure to arsenic from both natural and anthropogenic sources (195). Natural mineralization and activities of microorganisms enhance arsenic mobilization in the environment, and human intervention has exacerbated arsenic contamination (194). A study conducted in Ghana to statistically quantify landscape characteristics and their relationship with the disease showed that arsenic levels in soil and gold mining areas were significant covariates and related to an increased risk of prevalence in the Amansie West District of Ghana (191). In the Amansie West District, which was one of the worst Buruli ulcer-affected districts, there are arsenic-enriched surface environments resulting from the oxidation of arsenic-bearing minerals occurring naturally in mineral deposits (191, 195). Proximity analyses, carried out to determine spatial relationships between Buruli ulcer in affected areas and arsenic-enriched farmlands and arsenic-enriched drainage channels in the Amansie West District, showed that the mean Buruli ulcer prevalence in settlements along arsenic-enriched drainage areas and

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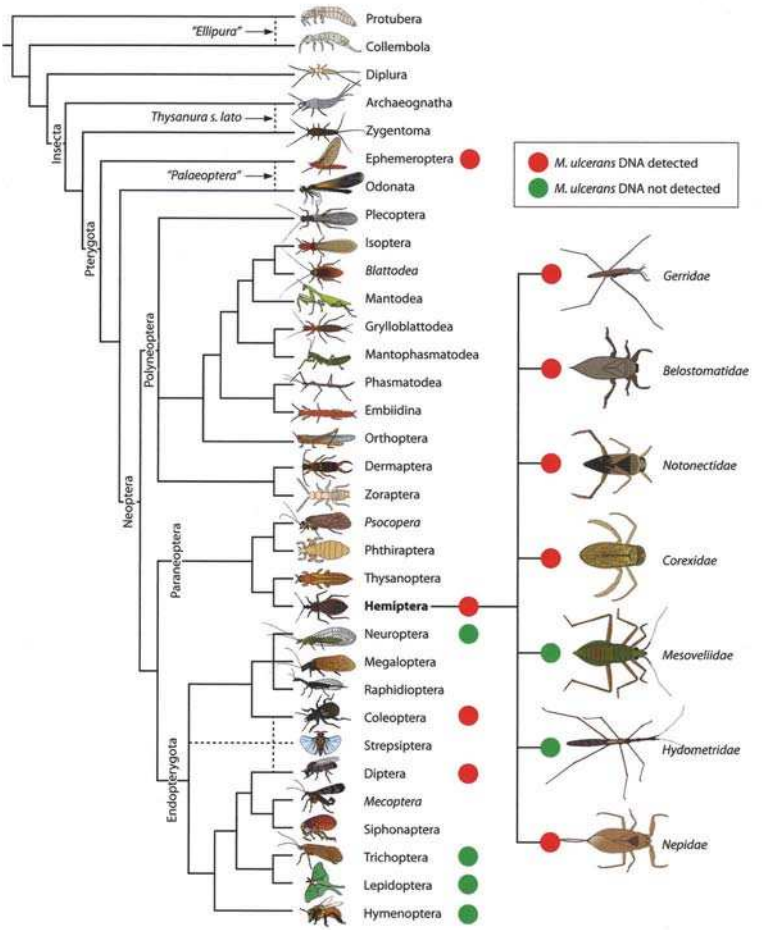

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FIG 6 Cladogram of postulated relationships of extant hexapods, based on combined morphological and nucleotide sequence data showing *M. ulcerans* findings in insects (adapted from the work of Gullan and Cranston [427]).

within arsenic-enriched farmlands is greater than elsewhere (191, 195). Furthermore, the role of arsenic in the prevalence of Buruli ulcer has been questioned, and the results of a Ghanaian study suggest that arsenic in the environment may play a contributory role in *M. ulcerans* infection (191). The Amansie West District, which is drained by the Ofin River, had high caseloads of Buruli ulcer in 1998 (24), but in recent years, hardly any cases have been observed in the area (196).

Buruli Ulcer in Animals

Buruli ulcer is by no means specific for humans, and studying *M. ulcerans* infection in animals may provide clues to the sources and transmission of the pathogen. In Ghana, small mammals within communities of endemicity may be susceptible to *M.*

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ulcerans infection and act as reservoirs; *M. ulcerans* Agy 99 was detected in lesions on *Mastomys* mouse tails caught in houses (63). In Côte d'Ivoire since the 1990s, the fish called tilapia (cichlid fish) has been suspected of being a reservoir of *M. ulcerans* (197), and *M. ulcerans* DNA was detected in fish collected in Benin (141) and in Ghana (13, 151). In Ghana, *M. ulcerans* DNA was also detected in amphibians (151). Recently, *M. ulcerans* DNA has been detected in the carcasses of small mammals, in *Mastomys natalensis*, in the mouse genus *Mastomys*, and in the stools of the small mammal *Thryonomys swinderianus* in Côte d'Ivoire and Ghana (63, 64, 106), suggesting that these animals may shelter and transport *M. ulcerans*. Later results corroborated an experimental study showing that *T. swinderianus* was susceptible to *M. ulcerans* infection (198) (Fig. 4). Small mammals living in close proximity to humans and commonly hunted animals, such as rabbits and rats, may therefore be potential sources of *M. ulcerans* (63). In Australia, *M. ulcerans* was detected in a cat (first known case in a cat) (199), horses (200), dogs (201), alpacas (*Vicugna pacos*) (202), possum species (111), koalas (*Phascolarctos cinereus*) (203–205), and frogs (206). These observations contrast with investigations conducted in Ghana, where *M. ulcerans* DNA was not detected in the feces of domestic animals in rural areas, showing that domestic animals are unlikely to be major reservoirs of *M. ulcerans* (207). In Japan, *M. ulcerans* DNA sequences were detected in turtles (*Lissemys punctata punctata*) and crayfish (108, 208, 209).

Buruli ulcer has also been encountered in aquatic invertebrates, mosquitoes (13, 16, 19, 91, 107, 113, 139, 141, 149, 150, 152–154, 156, 193), crayfish (108), amoeba, mollusks, crustaceans, annelida (29, 40, 141, 153), aerosols, water, biofilm, moss, detritus, feces, plants, and soil (13, 29, 39, 63, 93, 106, 107, 112, 113, 144–147, 210). The hypothesis most advanced to aggregate data issuing from the investigations on the environment is that *M. ulcerans* may be part of a food chain (211, 212).

Buruli Ulcer in Patients: Clinical Aspects

The usual clinical appearance of Buruli ulcer is a deep, rapidly developing chronic ulcer associated with necrosis of subcutaneous fat (34), often causing functional limitations which occur in as many as 25 to 50% of cases (22, 213). Prevention of disabilities and physiotherapy is now accepted as an integral part of therapy (214). The impact of the shift to pharmacological therapy on the occurrence of functional limitations has been studied by Barogui et al. (215). Most often, the diagnosis is made in the presence of a deep, rapidly developing chronic ulcer associated with necrosis of subcutaneous fat (34). Buruli ulcer evolves in three clinical stages, with a mean incubation period of 2 to 3 months but ranging between 3 weeks to almost a year. It includes (i) preulcerative lesions presenting as a nodule, papule, plaque, or edema; (ii) ulcerative lesions enlarging and contaminating underlying tissues, characterized by granulomatous healing and further fibrosis (216); and (iii) scars. A study conducted in the Democratic Republic of the Congo (former Zaire) showed that lesions appeared in body areas having undergone trauma, such as an accidental needlestick-like injury (scorpion stings). Nevertheless, 80% of cases detected early can be cured by an 8-week course of rifampin plus streptomycin, sometimes followed by a skin graft (15, 217, 218). Intact skin completely prevents Buruli ulcer, as *M. ulcerans* is unable to penetrate through intact skin by itself from an external route (115, 219). As for the mechanism of inoculation, two main hypotheses have been suggested. The first is that bacteria are injected into the skin through the bite of an insect or ectoparasite vector, and the second is that bacteria enter previous and open wounds from direct contact with the contaminated environment, aerosols from water surfaces, and water-dwelling fauna (22, 115, 220). An alternative hypothesis is that *M. ulcerans* is inhaled or ingested (220, 221) and reactivated in low-temperature areas of the body at the sites of trauma, but this hypothesis has not been challenged by any model or direct clinical observation (222, 223). An intriguing feature of Buruli ulcer is that 10.34% of patients have several localizations, the most parsimonious explanation being that *M. ulcerans* is inoculated several times (98, 224) (Fig. 7), perhaps by auto-inoculation from an index lesion.

F7

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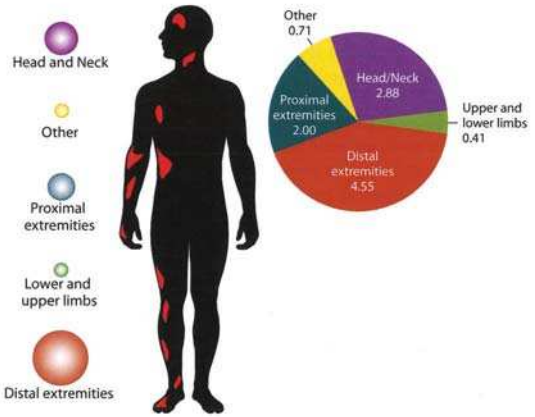

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FIG 7 Percentages of Buruli ulcer occurring in multiple locations on 1,702 patients.

However, human-to-human transmission of *M. ulcerans* is extremely rare (22), with only one reported case after a human bite (225).

After the transcutaneous inoculation of *M. ulcerans*, the clinical presentation includes a papule, nodule, plaque, or edematous form, which eventually leads to extensive skin ulceration within 4 weeks with the classical, undermined borders (98, 226–231). The severe forms include osteomyelitis, reactive osteitis, and bone deformities (232, 233). One rare case of disseminated osteomyelitis has been reported following snake bite in an apparently nonimmunocompromised patient (234). Buruli ulcer is responsible for physical suffering, often leading to considerable disability if treatment has not been initiated quickly (18). The lesions are categorized according to the World Health Organization (WHO) classification as category I, which consist of lesions <5 cm at their widest diameter; category II, which consist of lesions between 5 and 15 cm at their widest diameter; and category III, which consist of lesions >15 cm at their widest diameter, lesions at critical sites, and multiple lesions (216). A comparison of Buruli ulcer clinical forms between African and Australian Buruli ulcer patients according to the WHO classification for lesion size is summarized in Fig. 8. In Africa, Buruli ulcer presents mainly as a disease of the skin and subcutaneous tissues, with rare extension to deeper tissues, including bone (97, 235, 236), and few extensions to muscle and bone, which are much more local (97, 235–237). In human immunodeficiency virus (HIV)-coinfected patients, even though systemic perturbations in the serum metabolome were reported (238) and severe Buruli ulcers were observed in some studies (239, 240), there was no disseminated infection.

F8

Lesion Topography

Lesion topography is not uniform on the body, and the pattern may not be random, as similar patterns have been reported in several countries, including Côte d'Ivoire, Ghana, Benin, Togo, and Nigeria. Neither the sex nor the age of the patient significantly alters the pattern of lesions. Approximately 80% of the lesions are located on the limbs, most commonly on the lower extremities, regardless of the age and sex of patients (Fig. 3) (20, 98). In Ghana, lesions were on the legs in 49% of patients and on the arms in 36% of patients, regardless of gender. Lesions on the distal extremities were observed in 61% of the patients, compared with lesions on the proximal extremities in 28% of patients (155). Males were significantly more likely than females to develop trunk lesions, but there was no gender difference for the extremities (155). A further study in

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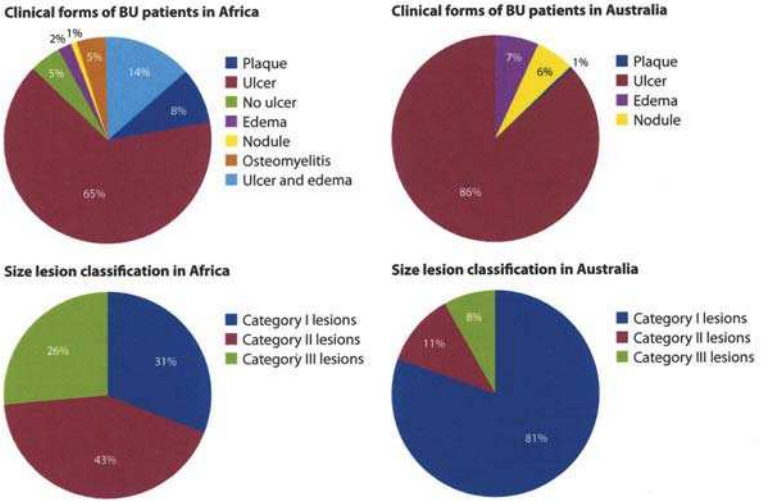

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FIG 8 Comparison of the percentages of Buruli ulcer clinical forms and WHO-classified sizes of lesions between African and Australian Buruli ulcer patients.

Ghana found lesions distributed on the lower limbs (67.9%), upper limbs (21.4%), trunk/breast (0.9%), head/neck (6.2%), and both lower and upper limbs (3.6%) (235). Another study in Ghana found lesions distributed on the head/neck (6.8%), upper limbs (20.3%), trunk (1.7%), and lower limbs (71.2%) (241). In Benin, Capela et al. found that the different locations of Buruli ulcer lesions were the head/neck (1.3%), thorax/abdomen (9.0%), upper limbs (35.9%), and lower limbs (53.8%) (242). In Côte d'Ivoire, lesions were most frequently located on the lower limbs (76.5%) and upper limbs (17.5%) (23). In Togo, the main locations of lesions were upper limbs (39.5%), lower limbs (39.8%), and trunk/head (21.7%) (243). The first description of a large cohort in Nigeria found lesions on the lower limbs (56.7%), upper limbs (28.3%), other locations (5.5%), and disseminated locations (9.45%) (26). In Togo, a hospital study involving 180 patients found lesions on the upper limbs, trunk, head, and neck (244). Consequently, in the different countries where Buruli ulcer is endemic, the observations that lesions are predominantly distributed on the lower limbs (60%), upper limbs (30%), and other body parts (10%) (245, 246) are highly concordant (Fig. 3). These observations, which first indicated that clothed body parts are almost free of lesions, suggest that Buruli ulcer occurs on unclothed body parts and that clothes are sufficient to protect the skin against contamination by *M. ulcerans* or an injury inoculating *M. ulcerans*. By including factors which may moderate the pattern of lesions, we analyzed clothes and shoes worn in West Africa. The analysis of the clothing style used as protection by farmers and children in rice paddies from collected Web photos indicated that, while the majority of farmers wear pants for their farming activities, there remains the fact that the protection is inadequate, because they have to roll up their pants in the mud (Fig. 9). Thus, the extension of lesions is significantly correlated with an unclothed, unprotected skin surface. This observation suggests that Buruli ulcer may not result from the contamination of previous gross wounds by *M. ulcerans* but rather from passive or active transcutaneous inoculation by a plant, soil, water, insect, or small animal unable to penetrate clothing. In Australia, both using insect repellent and wearing long trousers were found to reduce the odds of contracting Buruli ulcer (18). Wearing clothing such as pants in areas with *M. ulcerans* in the environment seems to prevent

F9

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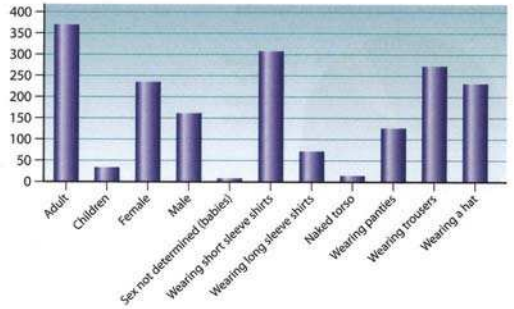

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FIG 9 Clothing protection among farmers working in rice fields in West Africa. Analysis of 90 screenshots from the Internet of a total of 403 farmers.

the disease, and this explains the fact that, despite the presence of *M. ulcerans* in Louisiana wetlands, no cases of Buruli ulcer have been reported in health facilities (147). The most plausible mode of transmission is skin trauma at sites contaminated by *M. ulcerans* strains (31). Then, on unclothed body parts, we observed that the pattern of Buruli ulcer lesions was inversely correlated with the pattern of skin temperature. By comparing the gradient temperature of the body and the location of Buruli ulcer lesions, we found that there was an inverse correlation between the gradient of body temperature and the location of lesions (Fig. 3). Body temperature is maintained by thermoregulation, which depends on heat balance (247). Even if the core temperature of a healthy adult human is 36.8 ± 0.4°C in the normal physiological situation, it should be noted that the body temperature is not uniform and depends on the topography of the body portion (247, 248). The temperature of the skin over the entire body is not 37°C, as in the core, but varies between 28°C and 34.5°C, depending on the location (Fig. 3) (247). Skin temperature is compatible with the growth of *M. ulcerans* in the population living in tropical regions of West Africa, Central Africa, and East Africa (95, 249, 250). *M. ulcerans* can survive but does not grow at 37°C (97). The temperature sensitivity of *M. ulcerans* has long been recognized. It is sensitive to temperatures above 37°C (25, 251). These clinical observations correlate with observations of mice experimentally infected with *M. ulcerans* (114). In that study, Buruli ulcer lesions were observed regardless of the route of inoculation of *M. ulcerans*, demonstrating that the tail temperature was between 24.8 and 25.6°C and 11 to 12°C lower than the general body temperature (114).

As for the limited deep extension, we observed a significant inverse correlation between the prevalence of Buruli ulcer by skin region and the skin regional temperature (Fig. 3). This may be due to the facts that *M. ulcerans* itself lacks the protective pigments encoded by its close relative *M. marinum* and that the key virulence factor, mycolactone, is highly sensitive to solar radiation (68, 252). Reviewing the data indicates that both bare skin and skin temperature under 35°C significantly correlate with the pattern of distribution of Buruli ulcer skin lesions. Among 1,742 cases of Buruli ulcer from eight studies conducted in areas of endemicity of West Africa, multisite lesions were found. There were 49 (2.9%) disseminated lesions in the head and neck, 7 (0.41%) lesions located on the lower and upper limbs, 74 (4.34%) lesions disseminated to the distal extremities, 34 (1.99%) lesions disseminated to the proximal extremities, and 12 (0.7%) other disseminated lesions (23, 26, 155, 235, 241, 242, 244, 253). Because Buruli ulcer is not a systemic disease, the likely explanations for disseminated lesions may be multiple bites from contaminated insects, multiple contacts of wounds with sources or reservoirs of *M. ulcerans*, or body parts being scratched with hands that had been in contact with environmental *M. ulcerans* strains (99).

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HIV Coinfection

Currently, the association between HIV infection and Buruli ulcer is not fully understood (254). In Africa, Buruli ulcer and HIV coinfection management is still a challenge for Buruli ulcer treatment. HIV positivity among Buruli ulcer patients was 8% in Ghana (254) and 2.6% in Benin (240), and in Cameroon the prevalence was approximately 4% in children, 17.0% in males, and 36.0% in females (255), which was higher than in the control population attending health facilities. HIV infection may affect the clinical presentation and severity of Buruli ulcer (254–256). A low CD4 cell count was significantly associated with a larger size of the main lesion (255). Studies have addressed the role of HIV as a risk factor for Buruli ulcer (155, 240, 255). Severe paradoxical reactions, including immune reconstitution inflammatory syndrome, can occur during the treatment of *M. ulcerans*-HIV-coinfected patients (86, 256, 257). As a consequence, the appropriate time to start antiretroviral therapy to minimize paradoxical reactions in relation to Buruli ulcer treatment with streptomycin and rifampin needs to be investigated (254). *Mansonella perstans* coinfection also needs to be considered in the diagnosis and treatment of Buruli ulcer. Nearly 23% of patients with Buruli ulcer in Ghana were coinfected with *M. perstans*, and this rate was higher than in the control population, in which 13% of patients were infected with *M. perstans* (258). Rarely, *M. ulcerans* and *Leishmania braziliensis* coinfection can be observed, and its corollary can be diagnostic confusion if the staff is not well trained and knowledgeable in the management of such diseases (259). At present, no specific underlying condition has been reported to support the development of Buruli ulcer. While hemoglobinopathies (hemoglobin sickle cell disease [HbSS]/sickle cell-hemoglobin C [SC]) were seven times more frequent in patients with Buruli ulcer osteomyelitis than in controls, these hemoglobinopathies were not associated with an increased prevalence of Buruli ulcer (260).

Differential Diagnosis of Buruli Ulcer

Buruli ulcer lesions can be confused with other cutaneous lesions, which is problematic, especially in tropical settings with limited access to laboratory facilities (261). Demographic and clinical criteria, including the age of the patient, the geographical area of residence, the location of lesions, and the presence of pain, help in the differential diagnosis. In Australia and other countries, the initial papular lesions are sometimes confused with insect bites (261). The differential diagnosis includes filariasis, leprosy, yaws, deep fungal infections (such as blastomycosis or coccidioidomycosis), mycetoma, ulcerative squamous cell carcinoma, abscesses, onchocerciasis, elephantiasis, scrofuloderma, mycosis, actinomycosis, herpes, cutaneous leishmaniasis, tropical phagedenic ulcer, venous ulcer, and noma (258, 261, 262).

Laboratory Diagnosis of Buruli Ulcer

In the past, Buruli ulcer was suspected on clinical evidence, but now the diagnosis can be confirmed by direct smear examination for acid-fast bacilli after Ziehl-Neelsen staining, and the test relies upon PCR targeting the genomic region IS*2404*, a test now widely available in regions of endemicity (26, 34, 95, 98, 233, 242, 261, 263). Microbiological diagnosis helps to reduce inappropriate administration of antibiotics also active against *M. tuberculosis*. Additional techniques, including culture of viable bacilli and histological staining, are used rarely. The current management of patients follows WHO recommendations and has been implemented for many years in countries of West and Central Africa (e.g., Côte d'Ivoire, Ghana, Togo, Benin, Cameroon, and the Democratic Republic of the Congo, as well as Nigeria recently and others) (95, 264–266). The quality of sample collection and the quality of the laboratory diagnosis of Buruli ulcer disease with microscopy, PCR, and histopathology have to be ensured by participation in external quality assurance systems (95). As for microscopy, it is possible to implement quality assurance for Ziehl-Neelsen staining, but it is difficult for auramine staining. The development of point-of-care (POC) tests is considered a research priority in order to make diagnosis more accessible to patients (267).

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Useful Clinical Samples

Fine-needle aspiration and swab samples are usually used for the laboratory diagnosis of Buruli ulcer (265). The WHO recommends that a maximum of two swabs or two fine-needle aspirations be taken for each lesion, depending on the experience of the person performing the technique (265). There is no specific recommendation for the transport of specimens for PCR-based diagnosis. However, with regard to isolation and culture, which are no longer routinely practiced, temperatures should never exceed 32°C during specimen transportation (22). Tissue samples that had been placed for up to 21 days in a transport medium, namely, Middlebrook 7H9 broth supplemented with polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin (Becton Dickinson, Sparks, MD), oleic acid, albumin, dextrose, catalase (Difco Laboratories, Detroit, MI), and 0.5% agar (also named semisolid transport medium) were still culture positive (97, 98). The application of harsh decontamination methods on specimens that contain few or rare organisms can be detrimental to the successful culture of *M. ulcerans* (22).

Microscopy

Optical microscopy is the diagnostic method most used in resource-limited settings. Several methods of staining are used; two are Ziehl-Neelsen staining and auramine staining (268). The Ziehl-Neelsen method is time-consuming and less sensitive than auramine staining, which improves sensitivity and turnaround time for the detection of acid-fast bacilli (269). The microscopic examination of skin exudate from an ulcer clinically suspected of being a Buruli ulcer is not the best tool for laboratory diagnosis, due to poor technical sensitivity (40 to 60%) (270). Nevertheless, it remains a good first means of investigation in an area of endemoepidemicity (271). Confirmation of clinically suspected cases of Buruli ulcer by microscopic examination occurs in 29% to 78% of cases (97, 102, 264, 272). Direct smear examination is easy to perform at a local level but has low sensitivity, below 60% (265). Nevertheless, it is the only test usually available in areas of endemicity (273). In general, the overall sensitivity of PCR is significantly higher than that of microscopic examination and culture (264).

Molecular Detection

PCR is considered the most sensitive method for the laboratory confirmation of Buruli ulcer. However, PCR remains expensive and involves reagents unsuitable for use in tropical countries with poor storage conditions, hindering the development of reliable qPCR diagnostic assays (274). It is highly sensitive and specific and is also reasonably rapid, but it requires trained personnel with specific equipment (274). Nevertheless, PCR is routinely performed in hospitals in countries such as Côte d'Ivoire, Ghana, Benin, Nigeria, Cameroon, and Togo, with the strengthening of laboratory capacity supported by national and international programs and nongovernmental organizations. IS*2404* PCR has been used as reference method to confirm the presence of *M. ulcerans* in tissues (102, 272), and a dry-reagent-based PCR formulation has been proposed (261, 273). This procedure is based on the standard diagnostic IS*2404* PCR developed by Stinear et al. (142) and has shown an excellent diagnostic sensitivity, >95% (261, 265, 274). The WHO recommends IS*2404* qPCR amplification for the confirmation of Buruli ulcer diagnosis, because this technique is both the most rapid and the most sensitive (95). The dry-mix qPCR approach can be adapted for other sets of primers and probes, such as the ketoreductase-B (KR) domain of the *M. ulcerans* mycolactone polyketide synthase genes (95). Dry-reagent-based PCR was shown to be a reliable tool for the diagnosis of Buruli ulcer disease, and it is well adapted to tropical conditions (261, 273). The agreement rate between dry-reagent-based PCR and standard PCR was 91.7% for swab specimens and 95% for tissue specimens (273).

The loop-mediated isothermal amplification (LAMP) technique has also proven to be useful for the early diagnosis of Buruli ulcer (275, 276). Recently, LAMP was developed as a simple, robust, cost-effective technology and has been selected as a promising POC test candidate (267). The IS*2404* detection-based LAMP assay employs lyophilized reagents (dry-reagent based, which provides significant advantages for application

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under tropical climate conditions) (267). The requirement of cold chains for transport and storage of reagents is avoided with the development of a dry-reagent-based LAMP assay employing lyophilized reagents (267). The sensitivities of IS*2404* PCR, the conventional LAMP assay (83.22%), and IS*2404* dry-reagent-based PCR (86.79%) were found to be comparable (267). LAMP was inferior in a study by Ablordey et al., but it can be used as a POC diagnostic test for Buruli ulcer (277).

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Culture

Routine diagnosis of Buruli ulcer does not rely on culture, which offers the possibility of strain characterization and antibiotic susceptibility testing. *M. ulcerans* grows better at <35°C, which may explain the finding that bacilli do not disseminate in the blood of experimentally inoculated animals (123). Culture on LJ medium at 32°C is the most discriminatory method but is not very sensitive and takes more than 8 weeks, rendering it of little use to clinicians (274). The primary cultures of clinical specimens from swabs are usually positive within 9 to 12 weeks of incubation at 29 to 33°C, but a much longer incubation period of up to 9 months may be necessary for some isolates (98). Culture detects between 34% and 79% of positive cases but is not useful for immediate patient management (102, 265, 272), though culture is appropriate for the monitoring of antimycobacterial treatment (98, 264) as well as for performance of molecular epidemiology analyses, which are almost impossible to carry out directly from clinical specimens (94).

Histopathological Analysis

Histopathological examination is sensitive but expensive and requires a sophisticated laboratory, well-trained personnel, and invasive procedures (biopsy) (274). Histopathological analysis confirms >90% of clinically diagnosed cases and >70% of clinically suspected cases (102, 272). Its sensitivity is about 90% but requires a sophisticated laboratory and the use of invasive procedures (265), and histopathology is not available in most countries of endemicity for treatment decisions (270).

Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry

Initially, matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) was used as a rapid and highly sensitive technique for the analysis of mycolic acids and hydrolysis products of mycolactone A/B by *M. ulcerans* (91, 278). It has recently been demonstrated that *M. ulcerans* and *M. marinum* can be separately identified directly from colonies by MALDI-TOF MS (279). Colonies of *M. marinum* are always identified as *M. marinum* by MALDI-TOF MS. However, colonies of *M. ulcerans* are identified as *M. ulcerans* but often as *M. pseudoshottsii* or *M. shottsii* (279).

Other Methods

Mycolactones A and B, which are specific to *M. ulcerans* and are present around infection sites, are therefore promising targets for the development of such a test (88). Because these toxins are specific to *M. ulcerans*, they represent a promising marker for developing a new diagnostic test (68). New methods based on detecting mycolactone have been proposed to replace the current WHO gold standard PCR method, which is expensive and not available in most areas of endemicity. Samples taken from the necrotic portion of ulcerative lesions provide mycolactone for analysis (265). One of these methods consists in the detection of mycolactone after its extraction from clinical samples by fluorescent thin-layer chromatography. The sensitivity of this technique is higher than that of microscopy or culture but lower than that of histology and PCR (270). More recently, a new molecular method based on detecting mycolactone by using RNA aptamers, which are an emerging novel class of detection molecules, has been proposed. However, this was a preliminary proof-of-concept report, and more tests must be done to approve this new method in the diagnosis of Buruli ulcer (280).

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BURULI ULCER TREATMENT

Medical Treatment

Ciprofloxacin, sparfloxacin, ofloxacin, and amikacin are effective *in vitro* against *M. ulcerans* at a MIC between 0.5 mg/liter and 2 mg/liter (281). The MICs of rifampin, streptomycin, amikacin, moxifloxacin, R207910 (bedaquiline), linezolid, and PA-824 (pretomanid) were 2, 0.25, 1, 0.06, 0.06, 2, and 16 mg/liter, respectively, against the reference strain of *M. ulcerans* ATCC 19423. They were, respectively, 2, 0.5, 1, 0.25, 0.12, 1, and >16 mg/liter against isolate CU001 (282, 283). Rifamycins such as rifampin, with a MIC of 2 mg/liter, have exhibited the broadest range of activity against clinical and reference strains of *M. ulcerans* (281).

The MICs of clarithromycin ranged from 0.125 to 2 mg/liter at pH 6.6 and from <0.125 to 0.5 mg/liter at pH 7.4 (284). *M. ulcerans* was inhibited by dapsone (4-4′-diaminodiphenyl sulfone), with MICs varying between 0.3 and 0.1 mg/liter (285, 286). The bactericidal activity of rifampin combined with those of moxifloxacin or clarithromycin and of moxifloxacin with clarithromycin equaled that of rifampin combined with streptomycin, and such combinations are validated as orally administered treatments of Buruli ulcer (287). Accordingly, an animal study showed that oral daily administration of rifapentine plus clarithromycin was at least as effective as injected streptomycin plus oral rifampin (288). *In vitro* activity testing against clinical isolates of *M. ulcerans* showed MIC values ranging from 2 to 8 g/liter for milbemycin oxime and from 2 to 4 g/liter for selamectin (289) In the same experiment, ivermectin and moxidectin showed no significant activity, with a MIC of >32 g/liter (289). On the other hand, moxidectin was shown to inhibit the growth of *M. ulcerans* JKD8049 at 4 g/liter, and *M. ulcerans* strains were susceptible to ivermectin at 8 g/liter for *M. ulcerans* JKD8049 and at 4 g/liter for *M. ulcerans* 1117-13 (290). Further *in vivo* susceptibility tests with mice showed the superiority of the benzoxazinorifamycin KRM-1648 over rifampin (291). Likewise, the effectiveness of purified methylene blue against the initial stage of Buruli ulcer in mice was recently proven (99). Ciprofloxacin, sparfloxacin, ofloxacin, amikacin, and rifampin were shown to be effective *in vitro* against primary clinical and reference isolates of *M. ulcerans* in Ghana (281).

Using mouse models, rifampin, streptomycin, amikacin, moxifloxacin, R207910, and linezolid showed various bactericidal activities, while PA-824 failed to reduce the number of CFU in the footpads of infected mice (282). In this model, a few rifampin-resistant *M. ulcerans* mutants were isolated after the results of rifampin monotherapy, leading to the recommendation that rifampin should never be used as monotherapy in humans (90). In addition, Beissner et al. reported a rifampin-resistant clinical isolate from Ghana after monotherapy (292). These data indicate that rifampin should not be used as monotherapy. Accordingly, an 8-week treatment with rifampin-streptomycin sterilized an *M. ulcerans* infection in mice (287). Combined rifampin-amikacin, rifampin-clarithromycin-sparfloxacin, or rifampin-amikacin cured *M. ulcerans*-infected mice and prevented relapse up to 26 weeks after completion of treatment (293). The association of rifampin with moxifloxacin, R207910, or linezolid showed bactericidal effects equal to those of rifampin-streptomycin and rifampin-amikacin (282). Recently, a mouse model indicated that an oral intermittent 8-week regimen of rifapentine combined with clarithromycin was highly bactericidal and had better sterilizing activity than the conventional rifampin-streptomycin regimen (294). These *in vitro* and animal model data supported the proposal to shift from the once-standard streptomycin-based therapy to oral combinations. In Australia, fully oral combinations of rifampin with either clarithromycin or fluoroquinolones were shown to be effective and well tolerated (295). Moreover, a shorter 29-day therapy was shown to achieve an overall 95% success rate (296). All together, these data recently led the WHO to modify its recommendations for the treatment of Buruli ulcer in favor of oral combinations. Accordingly, the provisional guidelines of the WHO were changed and now state that streptomycin-based therapy is no longer the standard of care. Clofazimine has similar MICs against *M. tuberculosis* and *M. ulcerans* of 0.25 to 0.5 g/liter (297). Clofazimine alone blocks the

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TABLE 5 Antibiotic and biocide susceptibility of *M. ulcerans*[a]

Drug (generic name)	Range	MIC_{50}	MIC_{90}
Rifampin	0.12 to 4.0	0.5	2.0
Streptomycin	0.12 to 1.0	0.25	0.5
Amikacin	0.25 to 2.0	0.5	2.0
Moxifloxacin	0.015 to 0.5	0.12	0.5
R207910 (bedaquiline)	0.015 to 0.12	0.03	0.06
Linezolid	0.25 to 4.0	0.5	2.0
PA-824 (pretomanid)	4.0 to 16	16	>16
Saprofloxacin	0.1 to 2	ND	0.5
Ofloxacin	0.1 to 2	ND	2
Ciprofloxacin	0.1 to 2	ND	1
Clarithromycin	0.125 to 4	ND	0.125 to 2
Clofazimine	0.06 to 2	ND	0.25 to 0.5
Ivermectin	0.125 to 64	ND	>64
Milbemycin oxime	0.125 to 64	ND	1 to 8
Moxidectin	0.125 to 64	ND	16 to >64
Selamectin	0.125 to 64	ND	1 to 4
Abamectin	0.125 to 64	ND	>64
Doramectin	0.125 to 64	ND	>64
Emamectin	0.125 to 64	ND	16 to 32
Eprinomectin	0.125 to 64	ND	>64

[a]MICs are given in grams per liter. ND, not determined.

multiplication of *M. ulcerans* in mouse footpads. In combination with rifampin, it eliminates the presence of *M. ulcerans* after 6 weeks of treatment, but its effectiveness is lower than that of the combination of rifampin-streptomycin and rifampin-clarithromycin in mouse footpads after 4 weeks of treatment (297). In this experiment, no relapses were observed in mice treated with rifampin-streptomycin and one relapse (5%) was observed in a mouse treated with rifampin-clofazimine, while relapses were observed in 50% of cases with the rifampin-clarithromycin combination (297) (Table 5).

Early detection and management is very important in reducing morbidity and the disease's disfiguring nature. A key factor contributing to the steady increase of Buruli ulcer in resource-limited settings is improper practice of personal hygiene. Until the introduction of antibiotic therapy, the use of surgery to remove all infected tissue, with a wide safety margin to ensure the complete removal of infected tissues, was regarded as the most effective treatment (218, 298). Recurrence rates after surgical treatment without antibiotics vary from 16% to 28% (299). In addition, the cost of surgical treatment is far beyond the means of those most severely affected (299). Prevention of functional limitations and physiotherapy are now accepted as an integral part of therapy (214). The impact of the shift to pharmacological therapy on the occurrence of functional limitations has been studied by Barogui et al. (215). In this study, no differences in resulting functional limitations were observed between patients treated with surgery, antibiotics, or both. Since 2004, Buruli ulcer has been treated with 8 weeks of intramuscular injections of streptomycin (15 mg/kg) and oral rifampin (10 mg/kg) according to the previous WHO protocol of treatment with antibiotics, plus surgical excision and skin grafting (218, 299, 300). Without antibiotics, recurrence has been reported to be higher: as high as 48% (301). Since the introduction of antibiotic treatment, recurrence rates have receded remarkably (0 to 2%), and the requirement for surgical intervention has diminished (299). The combination of rifampin and streptomycin was effective for most patients with Buruli ulcer and proved to be a highly successful and practical treatment for all forms of *M. ulcerans* disease (217, 218). Streptomycin administration can cause both ototoxicity and nephrotoxicity (302). It was observed that cured patients were more likely to become reinfected rather than relapse (303). Compliance with the recommended 8-week treatment (218) is difficult to maintain, particularly in rural settings where health facilities are rare. The daily injection with streptomycin is problematic, as most patients live in remote areas with limited access to health care facilities. Proper hygiene with these injections is also a concern. Moreover, the antibiotic treatment may be accompanied by a clinical deterioration, known

as a paradoxical reaction, which may be the result of restoration of local and systemic immune responses (304). For these reasons, an oral regimen avoiding intramuscular injections has been developed (294). In Japan, a combination of oral medication composed of rifampin, levofloxacin, and clarithromycin was successful in treating Buruli ulcer and showed better results than other chemotherapies. This treatment increases the probability of patient adherence and needs to be evaluated in a multisite study. It may also be the best way to decentralize patient care in rural areas with fewer resources (305). Combination oral therapy alone has been tested in Australia, and the results demonstrated that Buruli ulcer can be treated effectively using oral antibiotics alone, with an acceptable toxicity profile (295, 296). In Benin, an 8-week oral combination of clarithromycin and rifampin in Buruli ulcer patients was well tolerated, resulting in no treatment failures (306). Recent developments toward a fully oral therapy not including a quinolone but rather a combination of rifampin and clarithromycin were presented at the WHO Buruli ulcer meeting in March 2017. The provisional guideline was changed accordingly, and as of now, fully oral treatment has become standard therapy; streptomycin has been abandoned (306–308). The oral regimen with rifampin and clarithromycin is already recommended by the WHO and regularly administered in West African countries (e.g., Benin, Togo, and Ghana), though its effectiveness has not yet been proven by the ongoing randomized trial in West Africa.

Warming the affected skin at 38 to 39°C may improve the outcome of extensive or relapsing lesions, but observations are anecdotal (309). The theoretical frame for such practice includes the optimal growth of *M. ulcerans* at 32°C and better cellular microbicidy at 39°C (309). Accordingly, in the search for innovative treatments, the efficacy of phase change material (PCM) thermotherapy as local thermotherapy was proven in a phase 2 clinical trial in Cameroon to be a highly effective, simple, inexpensive, and safe treatment for *M. ulcerans* disease. PMC involves applying temperature from 39°C to 42°C to the skin surface. It has potential as a home-based remedy for lesions suspected of being Buruli ulcers at the community level, where laboratory confirmation is not available (251, 298, 310). Phototherapy and UV therapy are sometimes used to treat human skin diseases, such as psoriasis or eczema, but rarely in infectious disease and may be a therapeutic solution for the treatment of Buruli ulcer (68).

It has been established that the standard first-line treatment for tropical ulcers is a combination of penicillin and metronidazole (311). Antibiotics such as beta-lactams (penicillin, ampicillin, cefuroxime, cefixime, flucloxacillin), macrolides (erythromycin, clarithromycin), aminoglycosides (amikacin, gentamicin), quinolones (ciprofloxacin), cyclines (tetracycline), phenicol (chloramphenicol), and sulfamethoxazole-trimethoprim (co-trimoxazole) have been used for the treatment of Buruli ulcer secondary infections, which are often thought to be responsible for the severe complications in Buruli ulcer (283, 284). The role of *Staphylococcus aureus* has recently been investigated (312) with the alternative hypothesis that paradoxical inflammation is causing severe complications.

Traditional Medicine

Traditional treatments remain the first option for poor populations in Africa, who may have restricted access to synthetic products due to their cost and accessibility (313, 314). However, the use of traditional treatment as first-line therapy, lay perception, and self-medication contribute to longer delays in diagnosis and treatment (315, 316). Such treatment is considered devastating, expensive, and ineffective in some cases (317). According to a socio-anthropological study conducted in Benin, the main steps in traditional treatment were diagnosis, removal of necrotic tissue, wound care, and exorcism (314). In the history of the development of new therapeutic molecules, plants have always occupied a preponderant place as sources for new pharmacological molecules (318). In Africa, much effort is spent in the pharmacological study of medicinal plants used in traditional medicine for the treatment of Buruli ulcer. *Ricinus communis*, *Cyperus cyperoides*, *Nicotiana tabacum*, *Mangifera indica*, *Solanum rugosum*, *Carica papaya*, and *Moringa oleifera* have demonstrated clinical efficacy (319). Another study in West Africa showed that active

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extracts from 10 plant species (*Alstonia boonei, Annona reticulata, Annona senegalensis, Bridelia ferruginea, Carica papaya, Eucalyptus globulus, Polyalthia suaveolens, Sorindeia juglandifolia, Spathodea campanulata*, and *Zanthoxylum zanthoxyloides*) and one extract from *Cleistopholis* showed activity against *M. ulcerans* (320). These plants were from different families, namely, Annonaceae, Apocynaceae, Bignoniaceae, Caricaceae, Compositae, Euphorbiaceae, Myrtaceae, Phyllanthaceae, and Rutaceae (320). These medications are used as decoctions, infusions, powders, pomade, and macerations and taken orally or applied to wounds (319). Further studies are required to isolate and characterize the active ingredients in the extracts of these plants. In a study conducted in Benin, it was proven that the extract from aerial parts of *Holarrhena floribunda* had significant antimycobacterial activity against *M. ulcerans* (318). Natural products represent potential alternatives to standard therapies for use as curative medications for *M. ulcerans* disease (319). Plants with medicinal potential should be scrutinized for biologically active compounds by the bioassay-guided fractionation approach to provide new insights for finding novel therapeutics for Buruli ulcer control (319). Given that traditional healers represent a parallel point of entry into the health system to support people suffering from Buruli ulcer with products that have often proven their effectiveness, there is a need for health authorities to better supervise this area. However, the involvement of plants and the possible role of local herbal therapies are not evidence based; it is rather opinion based and speculative and requires special attention by authorities and scientists.

Medical Prevention

There is no proven effective primary prevention of *M. ulcerans* infection. Nevertheless, our partial knowledge of the sources and transmission of environmental *M. ulcerans* does suggest some measures of prevention, the efficacy of which remains to be measured, that are, to date, the most effective methods to reduce disease transmission. Indeed, mandatory early detection through active case finding, early laboratory-confirmed diagnosis, and early initiation of treatment to prevent long-term sequelae do not prevent additional cases of noncontagious Buruli ulcer (321, 322).

Due to the significant reduction in the quality of life of patients presenting with extensive tissue scarring, a Buruli ulcer vaccine would be greatly beneficial to the worldwide community (323). Despite the efforts for the development of vaccines against Buruli ulcer disease, there is still no effective preventive vaccine for Buruli ulcer (324–329). Antibodies to surface antigens of *M. ulcerans* do not seem to have a protective effect (330). BCG vaccination status provides relatively short-term immune protection from *M. ulcerans* infection and prevents osteomyelitis (31, 331). Preliminary data suggest that BCG effectively serves as a vehicle to *M. ulcerans* antigens, warranting further studies to improve efficacy (323). Since prevention is not possible in the absence of either an effective vaccine or a clear understanding of the mode of transmission, a major control strategy for Buruli ulcer consists in early detection and treatment, depending on effective laboratory confirmation of suspected cases (270). Currently, preventive measures include clothing in the course of pastoral work, the quick disinfection of wounds after an injury with running water and soap (30), and a swimming prohibition in the presence of an open wound, as well as the use of insecticides and impregnated mosquito nets in homes.

Buruli Ulcer Prevalence in the Population

Before 2010, the prevalence of Buruli ulcer was increasing in West Africa and Central Africa (34, 35, 332–335). The resurgence of Buruli ulcer in the world has led the scientific community to a better understanding of the disease, including its reservoirs and modes of transmission, as well as risk factors. The most affected countries are in West Africa, with Côte d'Ivoire being among the most affected countries in the world (19, 336) (Fig. 10; Table 1). In 2014, 1,736 of 2,151 (80.7%) cases of Buruli ulcer reported to the WHO by African countries were from West Africa (Table 1). At the beginning of 2014, 12 of the 15 countries regularly reporting data to the WHO reported nearly

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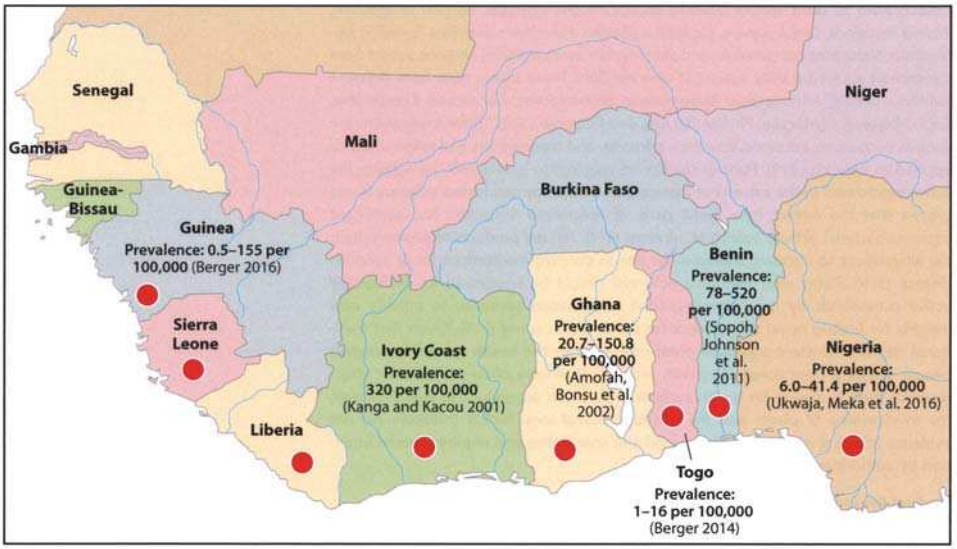

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FIG 10 Countries reporting Buruli ulcer to the WHO and prevalence of Buruli ulcer, 2002 to 2015.

2,200 new cases, which represents a decrease of about 50% compared to the number in 2009, when 5,000 cases were reported. Except in a couple of countries (Japan, Australia), the number of cases has declined since 2010 in most areas of endemicity. The exact cause of this decline is unknown, but it may be a positive side effect of the fight against coreservoirs and covectors of other targeted infections in the tropics (232).

Epidemiology of Buruli Ulcer

More than half of the new cases of Buruli ulcer reported annually around the world are from West Africa (Fig. 2). Among 15 West African countries, countries along the Gulf of Guinea, including Benin (17, 337), Côte d'Ivoire (197, 334), Nigeria (338), Ghana (24, 339, 340), Sierra Leone (341), Togo (342), and Guinea (343), are reporting new cases to the WHO (Fig. 10; Table 1). Eight West African countries declared 83.6% (range, 80.89% to 86.30%) of the total number of cases over the past 10 years (Fig. 2) (34, 35, 332–335, 344), with Côte d'Ivoire being among the most affected countries in the world (19, 336) (Fig. 10; Table 1). Côte d'Ivoire, Ghana, Benin, Togo, Guinea, and Nigeria (343) (Fig. 1 and 10; Table 1) have regularly reported new cases to the WHO during the last 2 decades, and these countries have the highest prevalence of the disease (20 to 158 cases per 100,000) (Fig. 10). Mali is a new potential African country where the disease is endemic, with a recent report of cases (345). Notably, Buruli ulcer has never been reported in Niger, Cabo Verde, Sao Tome, Principe, Chad, and Guinea-Bissau.

In Côte d'Ivoire and Ghana, Buruli ulcer is the second leading cause of mycobacterial infection after tuberculosis (23, 24). Affected populations live in rural areas, and children less than 15 years of age account for about 70% of cases (25, 63). The first probable case of Buruli ulcer in Ghana was reported in the Greater Accra Region in 1971, and more than 2,000 cases were reported between 1991 and 1997 (24). In Côte d'Ivoire, the first detection of Buruli ulcer occurred in 1981, but the number of cases clearly increased in 1987 and then became a national public health problem (334). In Nigeria, Buruli ulcer cases were first reported from Benue in 1967 (346).

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In central Africa, Buruli ulcer foci have been reported in Gabon, Cameroon, Congo, the Democratic Republic of the Congo, South Sudan, Angola, the Central African Republic, and Equatorial Guinea (347–350) but never in Sao Tome, Principe, or Chad. In Cameroon, the first case of Buruli ulcer was reported in 1969 (351), in 1950 in the Democratic Republic of the Congo (352), and in 1998 in Angola (353).

In East Africa during the 1960s, many cases of Buruli ulcer were reported in Uganda, especially in Buruli County, which eventually provided the name for this disease (2). Cases of Buruli ulcer were reported in other countries of East Africa, such as Kenya (354) and Sudan (131). In South Africa, the first cases of Buruli ulcer were reported in 2001 in Malawi (355).

AQ: 1 The true-incidence data of the disease in each of the West, Central, East, and South African countries are difficult to collect because not all patients attend health facilities (because of the lack of information about the disease, because of the lack of financial means, or because of the social stigma associated with chronic wounds), surveillance measures are poor, and there is a lack of case confirmation in health facilities (13, 356). All the African countries where Buruli ulcer is endemic do not necessarily have a systematically organized health system for monitoring and reporting Buruli ulcer cases. Programs are often put in place, but they do not work efficiently in some countries.

In Oceania, Bairnsdale ulcer (Buruli ulcer) was first reported in 1935 as a series of unusual painless ulcers in a patient from Southeast Australia (3). Since 1991, its incidence has progressively increased in Australia (18). However, elderly patients comprise a significant proportion of Buruli ulcer patients in Australian populations (18, 230, 231, 357). In continental Asia, the first reported case of *M. ulcerans* infection in China was described in 2000 (358). In eastern Asia, the first reported culture-documented case occurred in Japan in 1980 (359). An *M. ulcerans* isolate was recovered from a 19-year-old girl (360). These isolates were distinguished (on the basis of mycolic acid patterns) from previous *M. ulcerans* isolates and were reported to form a subcluster named *M. ulcerans* subsp. *shinshuense* (*M. ulcerans* ATCC 33728) (360). *M. ulcerans* subsp. *shinshuense* was confirmed to be the etiologic agent of Buruli ulcer in Japan (361). The first cases of Buruli ulcer in Malaysia in Southeast Asia were described in 1958 (362) and in 1983 in Kiribati, which is located in the Central Pacific region (363). Subsequently, Buruli ulcer was considered an emerging disease in Papua New Guinea (364).

In the Americas, Buruli ulcer has been diagnosed in South America, in Mexico since 1953 (352), and in French Guiana (112, 365) and Peru (366) since 1969. The first Brazilian case was reported in 2007 (367). French Guiana was qualified as the only area in the Americas where Buruli ulcer is endemic, with an average incidence of 2.09/100,000 (368). From 1969 until 2007, only 11 cases of Buruli ulcer were reported in Peru, but no countrywide survey has been conducted to evaluate its true prevalence there (366). Indeed, in Peru, Buruli ulcer is probably both infrequent and underreported and may often be misdiagnosed as leishmaniasis, which is more prevalent and better known (366).

Geography of Buruli Ulcer

Buruli ulcer is not a ubiquitous infection but is rather located in some large geographic areas scattered in 1 of the 33 countries which report cases to the WHO in Australia, Asia, Africa, and the Americas (Fig. 1). In each country where Buruli ulcer is endemic, there is a distinct geographical distribution, depending on environmental factors. In all these countries, Buruli ulcer occurs in specific discrete foci, suggesting a space-confined distribution pattern (369). To create an overview of the common characteristics of countries where Buruli ulcer is endemic, we observed that most of these countries are located within a belt limited by latitudes 10°N and 10°S (Fig. 10) and in moderate, nontropical climate areas, including Australia and Japan (233). In Australia, where the disease and the agent were first described, the incidence of Buruli ulcer has progressively risen since 1991 (18). Although Buruli ulcer is usually regarded as a disease of tropical and subtropical climates, an increasing number of cases have been recorded in temperate southeastern Australia (18, 370). Areas of endemicity include

mainly coastal Victoria, particularly the Mornington Peninsula and Bellarine Peninsula, northern Queensland near Mossman, the Capricorn Coast of Queensland near Yeppoon, and the tropical northern coast near Darwin (370). Buruli ulcer has moved as far in as Melbourne's southeast suburbs, including Bentleigh, Hampton, and Cheltenham (27), but no case has been linked to Tasmania, South Australia, or southern Western Australia. There have been cases in southern New South Wales near the border with Victoria (27). In Australia, environmental factors associated with Buruli ulcer prevalence included a low elevation with forested land cover (371). Likewise, in Côte d'Ivoire (372) and Benin (32), areas of endemicity are characterized by a high density of forest cover and low density of urban cover.

In continental Asia, the reported case of *M. ulcerans* infection in China had occurred at the highest latitudes in the Northern Hemisphere and was caused by *M. ulcerans* subspecies *shinshuense* (358). In Japan, the majority of cases are distributed in typically temperate, mountainous regions located between latitudes 34°N and 38°N, mountainous terrains at an altitude of 2,000 m in the case of the mountain ranges of Hida, Kiso, and Akaishi on Honshu, and 1,400 m in the cases of Hidaka on Hokkaido (358, 361, 373).

We reviewed the characteristics of these foci in West Africa, which occupies approximately one-fifth of the continent. The vast majority of this region is composed of plains rising to 300 m above sea level, but the northern section is composed of a semiarid terrain known as the Sahel, a transitional zone between the Sahara and the savannahs and forests of western Sudan (374) (Fig. 10). In West Africa, regions with reported cases of Buruli ulcer are all characterized by their proximity to a river, which connects the coast to mountains of >1,500 m that are less than 500 km away from the coast. In Benin, it was observed that the mean prevalences of Buruli ulcer significantly correlated inversely with elevation, from 60.7 cases/10,000 inhabitants in villages with an elevation below 50 m to 10.2/10,000 inhabitants in villages with an elevation between 50 and 100 m to 5.4/10,000 inhabitants in villages with an elevation above 100 m (144, 375). However, cases were reported at a minimum distance of 15 km from the coast of the Atlantic Ocean and a maximum distance of 18 km in a study conducted in Benin (144). In a study conducted in Benin by Portaels et al., an inverse relationship between the prevalence of the disease and the distance that a patient lived from a river was found. The prevalence gradually increased from 0.6 to 32.6/1,000 inhabitants when the distance from a river was less than 10 km (131). This observation correlates with our recent report that *M. ulcerans* tolerates a degree of salinity above 20 g/liter (186). In West Africa, where the disease is most prevalent, a dramatic increase in the incidence of Buruli ulcer has been reported by countries mostly along the Gulf of Guinea (17, 197, 334–342, 376). In all these countries, Buruli ulcer occurs in specific discrete foci, suggesting a space-confined distribution pattern (369). We reviewed the characteristics of these foci in West Africa, which occupies approximately one-fifth of the continent. The vast majority of this region is composed of plains lying at 300 m above sea level (374) (Fig. 10).

Seasonal factors may affect the epidemiology of *M. ulcerans*. In Cameroon, *M. ulcerans* dynamics are largely driven by seasonal climatic factors (188). In Ghana, the incidence of Buruli ulcer peaked at the end of the rainy season in September and October (333). It was recently shown in Ghana that the proportion of positive *M. ulcerans* samples recorded was higher during the months with higher rainfall levels (11%) than during the dry season months (3%) (210). This demonstrates that there is a seasonal pattern to the presence of *M. ulcerans* in the environment, which may be related to recent rainfall or water in the soil (210). In Cameroon, *M. ulcerans* dynamics are largely driven by seasonal climatic factors (188). In the United States (Louisiana), the environmental investigation of *M. ulcerans* DNA by IS*2404* qPCR revealed seasonal variations in the prevalences of *M. ulcerans*, with a notable decrease in prevalence in the samples collected during autumn every year in the areas between latitude 30.003537 and longitude 92.021235 (147). No cases of human Buruli ulcer have been reported in Louisiana, suggesting that the environmental distribution of *M. ulcerans* is

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not limited to areas where Buruli ulcer is endemic and that infections caused by *M. ulcerans* are not limited to humans (147).

In Central Africa, it was shown that cases of Buruli ulcer peaked in March, suggesting that the risk is highest during the rainy season (377). In Cameroon, in the Nyong River distance model, the risk of Buruli ulcer decreased when the distance to the river increased, with a dose-response relationship (378). In Japan, there is a dynamic seasonal appearance of *M. ulcerans* in the environment, which may contribute to the seasonal variation of Buruli ulcer occurrence (108).

Outbreaks of Buruli ulcer have been attributed in many cases to environmental disturbances, such as flooding, agricultural deforestation, increases in the sizes of irrigated areas for cultivation, and construction of dams or damming of rivers (31, 33, 34, 347, 370). It also was shown that the areas where Buruli ulcer is highly endemic are located most often in lowland areas (375). Environmental factors, such as climate, soil, geology, and geochemistry, may indirectly influence or contribute to *M. ulcerans* infection (379). Several screenings of *M. ulcerans* in environmental samples have been done (Table 3). In countries with a constantly high incidence of Buruli ulcer, temperature and humidity generally follow the same trends, with average temperatures ranging between 22°C and 33°C, which is the optimum temperature required for the growth and survival of *M. ulcerans* (23, 94–97). The average relative humidity is 85% in the southern areas of these countries and 71% in the north. The annual sunshine duration varies with the seasons, and the average has been estimated at 1,762 h (249, 250).

In Japan, it was proven that there is a dynamic seasonal appearance of *M. ulcerans* in the environment, which may contribute to the seasonal variation of Buruli ulcer occurrence (108). In Central Africa, it was shown that the cases of Buruli ulcer peaked in March, suggesting that the risk is at its highest during the rainy season (377). In each country of endemicity, there is a distinct geographical distribution, depending on environmental factors.

Buruli ulcer, which is rampant in foci of endemicity and scattered, but limited, in general in marshes, floodplains, and close to lakes or rivers, is an ancient disease and widespread in the world and seems to have currently reached a new level through the extension of its usual foci and impact. Man-made changes in the environment may provide new opportunities for ecological niches for *M. ulcerans* and new opportunities for contact between populations and these niches.

Descriptive Epidemiology of Buruli Ulcer

Age. *M. ulcerans* infection affects primarily children between 5 and 15 years of age (233).

Children less than 15 years of age represent approximately 42% of the overall population in West Africa. This proportion is approximately 41% in countries with a high prevalence of Buruli ulcer (Côte d'Ivoire, Ghana, Benin, Guinea, Togo, Nigeria) and 43% in countries with a lower prevalence or in which it is not endemic (Burkina Faso, Sierra Leone, Senegal, Guinea Bissau, Liberia, Mali) ($P < 0.05$). In 10 independent studies conducted in Ghana, Côte d'Ivoire, Benin, and Nigeria (23, 26, 155, 226, 235, 241, 242, 244, 253, 380), 50.7% of patients were less than 15 years old, and the median age was 18 years (Fig. 11). The peak age group in West Africa studies was 5 to 15 years, although Buruli ulcer can affect any age group (334, 340, 380). The highest detection rates were found sometimes in 75- to 79-year-old patients in West Africa, probably due to the reactivation of disease from a latent infection of *M. ulcerans* (380). It was proven in Africa that children less than 5 years old rarely develop antibody responses to the 18-kDa small heat shock protein (shsp) of *M. ulcerans* and thus seem to be considerably less exposed to the pathogen than older children (381, 382). As Buruli ulcer is not known to be an immunizing infection, this may reflect a greater exposition to sources and vectors (381, 382).

F11

Sex ratio. In West Africa, the female population represents about 49.52% of the overall population, with a sex ratio of 1.02. This proportion is approximately 49.87% in

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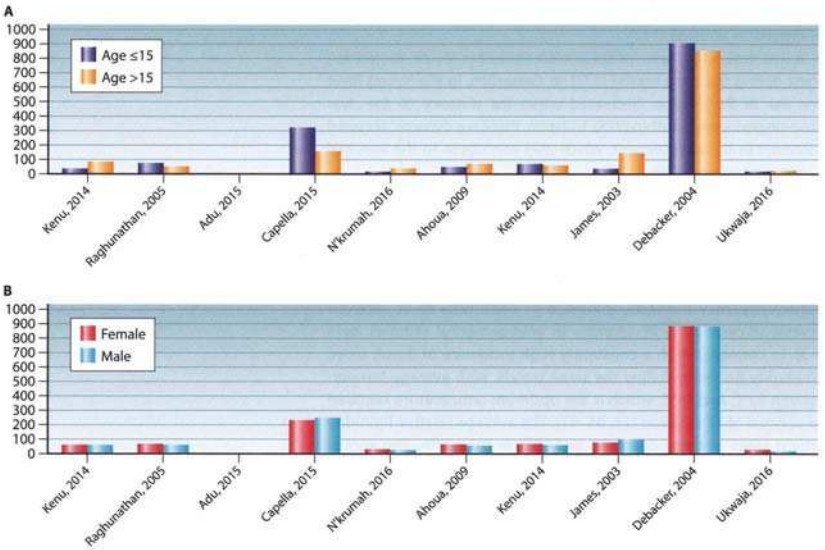

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AQ: X **FIG 11** Numbers of patients by age class (A) and sex (B) in studies conducted in West Africa (A) and the sex ratio of patients (B).

six countries with a high prevalence of Buruli ulcer (Côte d'Ivoire, Ghana, Benin, Guinea, Togo, Nigeria), while it is 50.99% in countries with a lower prevalence or in which it is not endemic (Burkina Faso, Sierra Leone, Senegal, Guinea Bissau, Liberia, Mali) ($P < 0.05$). The sex ratio is 1.01 in countries where Buruli ulcer is endemic and 0.96 in the other countries. In 10 studies conducted in the countries of endemicity of West Africa (23, 26, 155, 226, 235, 241, 242, 244, 253, 380), the global calculated sex ratio for Buruli ulcer patients (male to female) was 0.97 (0.90 to 1.04) (Fig. 11).

Farming Activities in Swampy Areas as a Risk Factor for Buruli Ulcer

In West Africa, the emergence and distribution of Buruli ulcer cases are clearly linked to aquatic ecosystems, and recent data suggest that different modes of transmission occur in specific areas and epidemiological settings (110, 150, 235, 347). Since the 1970s, some authors have formulated the hypothesis that patients may be infected through minor wounds or skin abrasions via contact with water containing *M. ulcerans* or by insect bites (99, 150, 383). Prior to recent studies, it was difficult to establish the epidemiological and ecological evidence linking the source of *M. ulcerans* to swamps and slow-flowing water (379). Past epidemiological studies have associated Buruli ulcer with human activity near, or within, slow-flowing or still bodies of water that have been created or disturbed by humans. This postulate was considered because there is strong epidemiological evidence linking the source of *M. ulcerans* to swamps and slow-flowing water or stagnant water (379). Residence near an aquatic environment has been identified as a consistent risk factor for *M. ulcerans* infection in Africa (155, 192, 334, 384). The proximity to rivers and water reservoirs has long been implicated in the emergence of Buruli ulcer in West African countries and, particularly in rural areas, especially in children less than 15 years old (63). In Côte d'Ivoire and Ghana, infections were reported to occur near rivers (198, 385, 386). The increased incidence of Buruli ulcer in Côte d'Ivoire was very much related to areas around dammed rivers and corroborates the first reported case of *M. ulcerans* infection in Côte d'Ivoire, a young

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patient living near the artificial Kossou Lake in the center of the country (386, 387). Irrigated rice and banana fields and deforested irrigation and aquaculture installations are zones for high-risk Buruli ulcer in Côte d'Ivoire (372); Buruli ulcer has also emerged in some communities (20, 153). Cases described in Nigeria were associated with the Benue River Valley in 1967 or a small artificial lake (338, 346). Similarly, in Liberia, cases were reported after dam construction following the introduction of swamp rice to replace upland rice (rice grown on dry soil) (341, 388). In Ghana, cases have clustered along the Densu River (385). The proximity of villages to rivers was a risk factor for contracting Buruli ulcer in Benin and Ghana (155, 389), and the link between a watery ecosystem and the emergence of Buruli ulcer was proven. In West Africa, Buruli ulcer afflicts primarily rural farmers in swampy environments. Also, it is thought that the use of river water for domestic purposes may contribute to the high prevalence of Buruli ulcer in settings of endemicity (389, 390). Another epidemiological study in Benin showed that foci of endemicity are organized primarily around the valley of the Ouémé and Kouffo Rivers. The communes of Lalo, Ouinhi, Bonu, Adjohoun, and Ze are the most affected (246). The first two reported patients with Buruli ulcer in Togo established a geographical continuum of the disease in all countries bordering the Gulf of Guinea (342). Cases reported in Burkina Faso (335) and Sierra Leone (391) were also related to an aquatic environment. A study conducted in Ghana suggested that swimming or activities on riverbanks were risk factors for contracting Buruli ulcer (192). Three at-risk areas for *M. ulcerans* disease were identified in Togo: the Laguna coastal area, marshy inland areas where market crops and rice are cultivated, and river valley areas (244). The foci of the disease are associated with environmental changes due to logging and mining and the creation or the extension of swampy areas, such as the construction of dams or lakes for the development of agriculture by irrigation, and are associated with exposure to river areas and sometimes with flooding (25, 155, 338).

The exposed skin of farmers and their activities in rural areas may facilitate the transmission of the pathogen (155). It has been demonstrated that there is a link between a watery ecosystem and the emergence of Buruli ulcer; preventive public health programs based on strategies that provide protected water supply systems to villages must be developed to reduce the frequency of the disease (389) (Fig. 4).

Raghunathan et al. identified wading in a river and streams in tropical climates as a risk factor for Buruli ulcer (155). A recent case-control study in Ghana showed that the risk factors for Buruli ulcer are contacts with wetland, insect bites in water, use of adhesive when injured, and bathing in the river (235). Other risk factors in Ghana were exposure to river areas, the presence of arsenic in the environment, exposed skin, use of water from rivers and ponds for drinking, and being between 2 and 14 years old (155, 191, 192). In Côte d'Ivoire, farming near the river was a risk factor (334). Another study in Côte d'Ivoire showed that regular contacts with unprotected surface water and the absence of protective equipment during agricultural activities were identified as the main factors associated with the risk of contracting Buruli ulcer (23). The contact with water was due mainly to agricultural activities (e.g., rice farming, market gardening, and fishing) and washing/bathing/swimming activities (23). The same conclusions about risk factors in Côte d'Ivoire were obtained previously by Ahoua et al. and Marston et al., and they concluded that young children and women having daily water-related activities were most at risk (253, 334). In Nigeria, the area of endemicity in Ogun state is divided into two drainage basins, the Yewa and Ogun Rivers, which are considered to be risk areas for Buruli ulcer (26). In Togo, three risk areas in swampy areas were identified: the Laguna coastal area, marshy inlands where market crops and rice are cultivated, and river valleys (244). Risk factors identified in Benin were the use of water from swamps, agricultural activities, being <15 years old or >49 years old, BCG vaccination status, and improper wound care (30, 33, 380). The greatest risk factors for acquiring Buruli ulcer included residing in an area of endemicity, close proximity to specific bodies of water, and being less than 15 years old (20) (Fig. 4). A fundamental research study conducted in Ghana with Buruli ulcer patients and control patients

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showed that a genetic polymorphism in the SLC11A1 gene played a role in the susceptibility to Buruli ulcer, with an estimated 13% population-attributable risk (392).

Protective Factors

Raghunathan et al. found that wearing a shirt while farming, sharing indoor living space with livestock, and bathing with toilet soap appeared to be protective (155). Covering limbs during farming and the use of alcohol after insect bites were also found to be protective factors against Buruli ulcer in Ghana (235). Wearing long pants was protective against *M. ulcerans* infection in Côte d'Ivoire and Australia (18, 334). N'krumah et al. found that wearing protective equipment before being in contact with surface water was a protective factor against Buruli ulcer (23) (Fig. 4). In Benin, the use of mosquito bed nets was considered to be a protective factor (30). In Australia, Quek et al. showed that immediately washing a wound received outdoors was found to decrease the odds of disease (18). In Ghana, it was proven that patients with Buruli ulcer who had received BCG vaccination had a shorter duration of the ulcer than those who were not vaccinated (333). A further study in Uganda showed that any protective effect was of short duration (393). Minimizing contact with water or soil around regions where Buruli ulcer is endemic, particularly in the presence of cuts or abrasions, had a protective effect.

Coepidemiology of Buruli Ulcer with Prevalent Infections

In an attempt to narrow the spectrum of potential reservoirs and vectors for *M. ulcerans*, we created a map of 10 infectious diseases that are prevalent in the same geographical belt as Buruli ulcer, with a focus on their vectors and reservoirs. We then focused on four infections in the tropics with significant overlap of Buruli ulcers.

Malaria

Malaria is the most important insect-transmitted human disease, and progress in its control has been slow, especially in Africa, where approximately 90% of cases occur (394, 395). Sub-Saharan Africa is home to localities with the highest global malaria transmission levels and, hence, high malarial morbidity and mortality. Human malarial protozoa are transmitted by mosquitoes of the genus *Anopheles*, including *A. arabiensis*, *A. gambiae*, *A. melas*, and *A. merus*. *Anopheles arabiensis* is considered mostly zoophilic compared to the highly anthropophilic *A. gambiae* but still plays a very important role in malaria transmission (394). The transmission of malaria in the coastal areas of West Africa is almost constant throughout the year. Further north, transmission varies from 1 month to 11 months of the year. While the role of mosquitoes in the transmission of *M. ulcerans* has not been demonstrated in West Africa, *M. ulcerans* DNA has been detected in mosquitoes (*Aedes camptorhynchus*, *Coquillettidia linealis*, *Anopheles annulipes*, *Culex australicus*, *Aedes notoscriptus*) trapped in Australia (19). Experimentally, mosquito larvae (*Aedes aegypti*, *A. albopictus*, *Ochlerotatus triseriatus*, *Culex restuans* larvae) can ingest wild-type *M. ulcerans* and *M. marinum* and remain infected throughout larval development (396). Evidence that implicates mosquitoes in the transmission of *M. ulcerans* in southeastern Australia has been established (18). The role of mosquitoes in transmission in Africa remains controversial. In particular, mosquito bites do not explain the unequal left-right distributions of lesions reported in some studies (308, 340). However, the past 15 years have seen unprecedented progress in malaria prevention and control by scaling up vector control interventions, particularly in sub-Saharan Africa (397, 398). Faced with the heavy burden of malaria, African countries decided in 2000 at the Abuja Summit to pay special attention to the fight against this disease (399). In 2005, they decided that at least 60% of the people who were most vulnerable to this disease, especially children under 5 years of age and pregnant women, should benefit from the best possible combination of personal and community protective measures, such as mosquito nets impregnated with insecticides, long-lasting insecticidal nets (LLINs), and other existing and available interventions to prevent infection and disease. This target was set at 80% for 2010 by the Organization of African

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Unity (OAU), currently replaced by the African Union (AU) (397–399). The insecticide-treated net kills or keeps away mosquitoes and other insects, such as head lice, bed bugs, and fleas. Numerous types of insecticide are used to treat the net: deltamethrin, lambda-cyhalothrin, alpha-cypermethrin, cyfluthrin, etofenprox, and permethrin (397). The WHO Global Malaria Program (WHO GMP) recommends three primary interventions for effective malaria control: the diagnosis and treatment of patients, the use of insecticide-treated nets (ITNs), and indoor residual spraying (IRS) (398). To strengthen the fight against malaria, African countries have benefited from Global Fund grants and technical support from other partners. The main aim was to contribute to the reduction of morbidity and mortality due to malaria between 2008 and 2014. Among the objectives of the application to the Global Fund is increasing the ITN utilization rate to at least 80% for people exposed to malaria, particularly pregnant women and children under 5 years of age. This mass distribution campaign has complemented LLIN distribution between 2008 and 2009 in most sub-Saharan countries. IRS is a major intervention for malaria control. There are currently 12 insecticides recommended for IRS, including dichlorodiphenyltrichloroethane, pyrethroids, and carbamates, which were used efficiently against vectors of malaria by NTP to scale up global malaria control and elimination (400). Given the incertitude as the role of mosquitoes in the transmission of *M. ulcerans* in Africa, the fact that these preventive measures caused a decrease in Buruli ulcer cases in Africa remains controversial.

AQ: J

Filariasis

In Africa, lymphatic filariasis or elephantiasis is a neglected tropical disease (401). The environmental conditions for lymphatic filariasis transmission occur around the forest and savannah regions of West Africa (401). Lymphatic filariasis is caused by the filarial worms *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori*, which are endemic in 55 countries (401–403). The transmission of lymphatic filariasis in Africa is predicted to appear across much of the coastal and savannah areas of West Africa (401), thus, in the same areas as Buruli ulcer. In Ghana, lymphatic filariasis caused by *W. bancrofti* nematodes is found in several regions where Buruli ulcer is endemic (258). Approximately 80% of the people living in areas that require preventive chemotherapy to stop the spread of infection live in the following 10 countries: Angola, Cameroon, Côte d'Ivoire, the Democratic Republic of the Congo, India, Indonesia, Mozambique, Myanmar, Nigeria, and the United Republic of Tanzania (402). *Culex* species mosquitoes are the major vectors of *W. bancrofti* (404). The major *Anopheles* vectors in West Africa are *A. gambiae sensu lato* and the *Anopheles funestus* group (405). The World Health Assembly resolution WHA50.29 (406) encourages eliminating lymphatic filariasis. In response, the WHO launched its Global Programme to Eliminate Lymphatic Filariasis (GPELF) in 2000 (401). In 2012, the WHO's neglected tropical diseases roadmap reconfirmed the target date for achieving elimination by 2020 (402). Between 2000 and 2014, 5.63 billion treatments were delivered to more than 1 billion people at least once in 63 countries, considerably reducing transmission in many places. Recent research data showed that the transmission of lymphatic filariasis in at-risk populations has dropped by 43% since the beginning of the GPELF (402). Depending on the parasite vector species, measures such as insecticide-treated nets, indoor residual spraying, and personal protection measures may help protect people from infection. Vector control has, in specific settings, contributed to the elimination of lymphatic filariasis in the absence of large-scale preventive chemotherapy (402). Between 2000 and 2009, nine of the West African countries achieved full coverage of their entire at-risk populations after the launch of the GPELF with the mass drug administration (MDA) of a single dose of diethylcarbamazine or ivermectin plus albendazole (403, 407). In West African countries, due to the fact that onchocerciasis is coendemic with lymphatic filariasis, ivermectin plus albendazole in a single dose per year was used for MDA (407). In addition to interrupting transmission, MDA provides significant collateral health benefits, such as reduced morbidity from intestinal worms and ectoparasites (291, 402, 407). Vector control to reduce mosquito populations was one of the WHO GPELF priorities for the

interruption of transmission by the recommended use of techniques such as insecticide-treated bed nets and curtains as well as residual spraying as effective vector control tools (403). Insecticide resistance among the vectors of lymphatic filariasis in Africa has been reported. The *kdr* mutation responsible for resistance to pyrethroids has been found in the M and S forms of *A. gambiae sensu stricto* (408). The GPELF is based on the MDA, but the vector control activities of the Roll Back Malaria campaign have a significant capacity to eliminate the risk of transmission of *W. bancrofti* in areas of coendemicity (408). We have formulated the following postulate, which states that the annual decrease in the number of cases of Buruli ulcer since 2010s in the world, and particularly in West Africa, is due to the associated benefit of the WHO GPELF program by the MDA (including ivermectin) to all populations at risk. This assumption is reinforced by two recent studies in which experiments demonstrated that two avermectins could inhibit the growth and kill *M. ulcerans* strains from both Africa and Australia (289, 290) (Table 5).

AQ: K

Schistosomiasis

Schistosomiasis, also known as bilharziosis, is caused by several species of parasitic platyhelminthes of the genus *Schistosoma*, which can infect the urinary tract or the intestines of hosts. Of the 207 million estimated cases of schistosomiasis worldwide, 93% occur in sub-Saharan Africa. *Schistosoma haematobium* and *Schistosoma mansoni* are endemic throughout the continent. Transmission is usually associated with poor socio-economic conditions. Compared to the other schistosomes, *S. haematobium* is responsible for approximately two-thirds of the schistosomiasis cases in sub-Saharan Africa. *S. haematobium* infection is highly endemic in many Buruli ulcer foci in West Africa, with a striking increase in transmission after river dams were constructed (409). Approximately 76% of the population lives near rivers, lakes, and other bodies of water contaminated with snail intermediate hosts (410), which are also incriminated as potential reservoirs of *M. ulcerans*. The infection has been associated with water resource development projects, such as dams and irrigation projects, and slow-flowing or stagnant water, where the snail, an intermediate host of the parasite, breeds (410, 411). The disease is essentially an infection of rural and agricultural communities, where the way of life promotes contamination of inland water with human excreta (412). Schistosomiasis and Buruli ulcer have increased rapidly in the tropical wetlands of West and Central Africa since the 1980s, particularly after irrigation and dam construction (413, 414). Whether schistosomiasis was a risk factor for Buruli ulcer by driving the host immune response toward a predominantly Th2 pattern (409) has been disputed (413, 414). The highest prevalence and intensities of human schistosomiasis occur in school-aged children, adolescents, and young adults (410), as with Buruli ulcer. The control strategies include control of the intermediate snail host, use of molluscicides, chemotherapy, and improved sanitation and health education (412). The WHO strategy for schistosomiasis control focuses on reducing the disease using periodic, targeted treatments with praziquantel through large-scale treatment of affected populations (407).

Cutaneous Leishmaniasis

Leishmaniasis in HIV-coinfected patients is a significant yet neglected public health problem in West Africa (415). It is a vector-borne parasitic disease of humans and mammals caused by cell-infecting flagellate protozoa of the genus *Leishmania*, transmitted by female phlebotomine sand flies (415, 416). In most African countries, the disease is typically caused by one of two species, *Leishmania major* or *Leishmania tropica* (410). The areas of endemicity of leishmaniasis are governed by the presence of the sand fly vector, their dietary preferences, and their ability to promote the internal development of specific *Leishmania* species (417). Sand fly species of the genera *Phlebotomus* and *Sergentomyia* are two putative vectors in the transmission of *Leishmania* in West Africa (418). In South America, *Leishmania braziliensis*, *Leishmania guyanensis*, and *Leishmania panamensis* are responsible for cutaneous ulcers (259, 419).

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One case of *M. ulcerans* and *L. braziliensis* coinfection in a European traveler in South America raised the question of possible cotransmission of the two pathogens (259).

Areas of Uncertainties and Perspectives

Sources of infection. *M. ulcerans* has been detected in soil, biofilms, aquatic insects, fish, amphibia, and wildlife, confirming the epidemiologic evidence linking Buruli ulcer to aquatic and marshy environments. This is illustrated by the clear colocalization between rice fields and regions in Côte d'Ivoire where Buruli ulcer is endemic (372). However, the exact biotopes where *M. ulcerans* resides and which constitute sources of infection remain unknown. Amoebae are natural hosts of several microbial pathogens, such as certain mycobacteria (*Mycobacterium smegmatis*, *M. marinum*, *Mycobacterium simiae*, *Mycobacterium avium*) (420). Other studies showed that *M. shottsii*, *M. pseudoshottsii*, and *M. marinum* bacilli were internalized by *A. polyphaga* trophozoites (421–424). Therefore, amoebae can be a serious niche for the investigation of environmental strains of *M. ulcerans* in settings where Buruli ulcer is endemic. In a recent high-throughput carbon substrate profile of *M. ulcerans* in our laboratory, we found a significant association between the *M. ulcerans* core biologome and bacteria, fungi, algae, and mollusks. We concluded that environmental *M. ulcerans* research should increase its focus on fungi, algae, and mollusks, because they contain the nutrients necessary for the survival of *M. ulcerans* (211).

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In addition, the route of transmission remains enigmatic. Current hypotheses regarding the role of mosquito and water bug bites are not supported by the current distribution of the disease in human populations. However, a mosquito bite might be one form of skin lesion among others giving the opportunity to *M. ulcerans* to penetrate the skin. Consequently, further laboratory studies may clarify the role of mosquitoes in the transmission of *M. ulcerans* to people from the local environment or wildlife.

Variations in the incidence of Buruli ulcer. Buruli ulcer is an infectious pathology related to ecosystems in areas of endemicity, and the incidence of Buruli ulcer is driven mainly by variations in the ecosystems, but significant variations are unpredictable. For example, it could not be anticipated that in Ghana, soil arsenic is significantly associated with the persistence of the disease in specific areas contaminated by this mineral (191).

Targeted interventions against Buruli ulcer. Early detection and treatment of the disease has been implemented by national Buruli ulcer control programs to reduce the morbidity and disability associated with the disease. Multifaceted activities at the community level are organized for the early detection of cases, with information, education, and communication campaigns in communities and schools, training of village health workers, and strengthening of community-based surveillance systems. Since the creation of national programs with WHO support in the fight against Buruli ulcer in the 2000s by health authorities of the countries concerned, valuable efforts have been made to control and fight this disease (425, 426).

CONCLUSIONS

M. ulcerans is a prototype of an opportunistic inoculated pathogen, and Buruli ulcer is a prototype for ecosystem pathology. However, the exact ecosystems in which *M. ulcerans* resides are still unknown, as are the sources of infection for the populations in areas of endemicity and the exact circumstances of transmission. Efforts must be made to unravel exact sources of infection by substituting isolation and culture of environmental specimens with an exclusive PCR-only-based approach. Active and continuous surveillance in countries at risk of Buruli ulcer is needed for mapping the areas of endemicity in order to implement targeted control actions. An effective strategy to reduce the incidence of Buruli ulcer should involve compliance with protective equipment during agricultural activities, avoidance of contact with surface water, and community capacity building through training and sensitization. It is necessary to improve the means of prevention through ongoing identification of the most at-risk *M. ulcerans* infection factors in areas of high endemicity. Preventive public health policies

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for protecting water supply systems in villages must be implemented to reduce the frequency of this infectious disease. Educational programs should especially target the population groups at risk. A better understanding of the ecology of *M. ulcerans* and its route of transmission is very important for enhanced knowledge of disease epidemiology in order to establish control and prevention strategies. Given the current decline in the incidence of Buruli ulcer since 2010, it is necessary to conduct thorough investigations to better understand the factors involved in the decreased incidence to improve Buruli ulcer control strategies for each setting where Buruli ulcer is endemic. The search for efficient, natural, and active products against *M. ulcerans* should be encouraged in resource-limited settings, because they are part of the natural heritage of these populations. They are financially affordable and can be used at the earliest stage.

In conclusion, elucidating the sources of contamination and the modes of transmission by tentative isolation of *M. ulcerans* from environmental samples is a priority for efficient guiding of the fight against this neglected "tropical" disease.

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REFERENCES

1. UBG. 1969. The Uganda Buruli group. BCG vaccination against Mycobacterium ulcerans infection (Buruli ulcer). First results of a trial in Uganda. Lancet i:111–115.
2. Clancey JK, Dodge OG, Lunn HF, Oduori ML. 1961. Mycobacterial skin ulcers in Uganda. Lancet ii:951–954.
3. Alsop DG. 1972. The Bairnsdale ulcer. Aust N Z J Surg 41:317–319.
4. Meyers WM. 1995. Mycobacterial infections of the skin. Trop Pathol (Spez Pathol Anat) 8:291–377. https://doi.org/10.1007/978-3-642-57863-2_9.
5. MacCallum P, Tolhurst JC, Buckle G, Sissons HA. 1948. A new mycobacterial infection in man. J Pathol Bacteriol 60:93–102. https://doi.org/10.1002/path.1700600111.
6. Lunn HF. 1965. Effects of climate on the localization and geographic distribution of mycobacterial skin ulcers. Dermatol Int 4:111–114. https://doi.org/10.1111/j.1365-4362.1965.tb05134.x.
7. Coloma JNN-FG, Iribe P, López-Cepeda LD. 2005. Ulcerative cutaneous mycobacteriosis. Int J Leprosy 73:5–12.
8. Kibadi K, Ajoulat I, Meyers WM, Mokassa L, Muyembe T, Portaels F. 2007. Étude des appellations et des représentations attachées à l'infection à Mycobacterium ulcerans dans différents pays endémiques d'Afrique. Med Trop 67:241–248.
9. Phanzu DM, Suykerbuyk P, Imposo DB, Lukanu PN, Minuku JB, Lehman LF, Saunderson P, de Jong BC, Lutumba PT, Portaels F, Boelaert M. 2011. Effect of a control project on clinical profiles and outcomes in Buruli ulcer: a before/after study in Bas-Congo, Democratic Republic of Congo. PLoS Negl Trop Dis 5:e1402. https://doi.org/10.1371/journal.pntd.0001402.
10. Vincent QB, Ardant M-F, Adeye A, Goundote A, Saint-André J-P, Cottin J, Kempf M, Agossadou D, Johnson C, Abel L, Marsollier L, Chauty A, Alcaïs A. 2014. Clinical epidemiology of laboratory-confirmed Buruli ulcer in Benin: a cohort study. Lancet Global Health 2:422–430. https://doi.org/10.1016/S2214-109X(14)70223-2.
11. Portaels F, Fonteyene PA, de Beenhouwer H, de Rijk P, Guedenon A, Hayman J, Meyers MW. 1996. Variability in 3′ end of 16S rRNA sequence of Mycobacterium ulcerans is related to geographic origin of isolates. J Clin Microbiol 34:962–965.
12. WHO. 2004. Buruli ulcer disease. Wkly Epidemiol Rec 79:194–199.
13. Williamson HR, Benbow ME, Nguyen KD, Beachboard DC, Kimbirauskas RK, McIntosh MD, Quaye C, Ampadu EO, Boakye D, Merritt RW, Small PL. 2008. Distribution of Mycobacterium ulcerans in buruli ulcer endemic and non-endemic aquatic sites in Ghana. PLoS Negl Trop Dis 2:e205. https://doi.org/10.1371/journal.pntd.0000205.
14. Stinear TP, Seemann T, Pidot S, Frigui W, Reysset G, Garnier T, Meurice G, Simon D, Bouchier C, Ma L, Tichit M, Porter JL, Ryan J, Johnson PD, Davies JK, Jenkin GA, Small PL, Jones LM, Tekaia F, Laval F, Daffe M, Parkhill J, Cole ST. 2007. Reductive evolution and niche adaptation inferred from the genome of Mycobacterium ulcerans, the causative agent of Buruli ulcer. Genome Res 17:192–200. https://doi.org/10.1101/gr.5942807.
15. Walsh DS, Portaels F, Meyers WM. 2011. Buruli ulcer: advances in understanding Mycobacterium ulcerans infection. Dermatol Clin 29:1–8. https://doi.org/10.1016/j.det.2010.09.006.
16. Marsollier L, Robert R, Aubry J, Saint Andre JP, Kouakou H, Legras P, Manceau AL, Mahaza C, Carbonnelle B. 2002. Aquatic insects as a vector for Mycobacterium ulcerans. Appl Environ Microbiol 68:4623–4628. https://doi.org/10.1128/AEM.68.9.4623-4628.2002.
17. Debacker M, Aguiar J, Steunou C, Zinsou C, Meyers WM, Guédénon A, Scott JT, Dramaix M, Portaels F. 2004. Mycobacterium ulcerans disease (Buruli ulcer) in rural hospital, southern Benin, 1997–2001. Emerg Infect Dis 10:1391–1398. https://doi.org/10.3201/eid1008.030886.
18. Quek TY, Athan E, Henry MJ, Pasco JA, Redden-Hoare J, Hughes A, Johnson PD. 2007. Risk factors for Mycobacterium ulcerans infection, southeastern Australia. Emerg Infect Dis 13:1661–1666. https://doi.org/10.3201/eid1311.061206.
19. Johnson PD, Azuolas J, Lavender CJ, Wishart E, Stinear TP, Hayman JA. 2007. Mycobacterium ulcerans in mosquitoes captured during outbreak of Buruli ulcer, southeastern Australia. Emerg Infect Dis 13:1653–1660. https://doi.org/10.3201/eid1311.061369.
20. Merritt RW, Walker ED, Small PLC, Wallace JR, Johnson PDR, Benbow ME, Boakye DA. 2010. Ecology and transmission of Buruli ulcer disease: a systematic review. PLoS Negl Trop Dis 4:e911. https://doi.org/10.1371/journal.pntd.0000911.
21. WHO. 2014. Buruli ulcer (Mycobacterium ulcerans infection). Fact Sheet no. 199. World Health Organization, Geneva, Switzerland.
22. Portaels F, Chemlal K, Elsen P, Johnson PD, Hayman JA, Hibble J, Kirkwood R, Meyers WM. 2001. Mycobacterium ulcerans in wild animals. Rev Sci Tech 20:252–264. https://doi.org/10.20506/rst.20.1.1270.
23. N'krumah RTAS, Koné B, Tiembre I, Cissé G, Pluschke G, Tanner M, Utzinger J. 2016. Socio-environmental factors associated with the risk of contracting Buruli ulcer in Tiassalé, South Côte d'Ivoire: a case-control study. PLoS Negl Trop Dis 10:e0004327. https://doi.org/10.1371/journal.pntd.0004327.
24. Amofah G, Bonsu F, Tetteh C, Okrah J, Asamoa K, Asiedu K, Addy J.

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2002. Buruli ulcer in Ghana: results of a national case search. Emerg Infect Dis 8:167–170. https://doi.org/10.3201/eid0802.010119.
25. Asiedu K, Sherpbier R, Raviglione M (ed). 2000. Buruli ulcer. Mycobacterium ulcerans infection. World Health Organization, Geneva, Switzerland. http://apps.who.int/iris/bitstream/10665/66164/1/WHO_CDS_CPE_GBUI_2000.1.pdf.
26. Marion E, Carolan K, Adeye A, Kempf M, Chauty A, Marsollier L. 2015. Buruli ulcer in south western Nigeria: a retrospective cohort study of patients treated in Benin. PLoS Negl Trop Dis 9:e3443. https://doi.org/10.1371/journal.pntd.0003443.
27. Booker C. 4 August 2016. Flesh-eating Buruli ulcer cases soar as disease spreads to Melbourne. The Age, Melbourne, Victoria, Australia. http://www.theage.com.au/victoria/flesheating-buruli-ulcer-cases-soar-as-disease-spreads-to-melbourne-20160804-gqksnw.html.
28. Aboagye SY, Asare P, Otchere ID, Koka E, Mensah GE, Yirenya-Tawiah D, Yeboah-Manu D. 2017. Environmental and behavioral drivers of Buruli ulcer disease in selected communities along the Densu River Basin of Ghana: a case-control study. Am J Trop Med Hyg 96:1076–1083. https://doi.org/10.4269/ajtmh.16-0749.
29. Amissah NA, Gryseels S, Tobias NJ, Ravadgar B, Suzuki M, Vandelannoote K, Durnez L, Leirs H, Stinear TP, Portaels F, Ablordey A, Eddyani M. 2014. Investigating the role of free-living amoebae as a reservoir for Mycobacterium ulcerans. PLoS Negl Trop Dis 8:e3148. https://doi.org/10.1371/journal.pntd.0003148.
30. Nackers F, Johnson RC, Glynn JR, Zinsou C, Tonglet R, Portaels F. 2007. Environmental and health-related risk factors for Mycobacterium ulcerans disease (Buruli ulcer) in Benin. Am J Trop Med Hyg 77:834–836.
31. Walsh DS, Portaels F, Meyers WM. 2008. Buruli ulcer (Mycobacterium ulcerans infection). Trans R Soc Trop Med Hyg 102:969–978. https://doi.org/10.1016/j.trstmh.2008.06.006.
32. Wagner T, Benbow ME, Brenden TO, Qi J, Johnson RC. 2008. Buruli ulcer disease prevalence in Benin, West Africa: associations with land use/cover and the identification of disease clusters. Int J Health Geogr 7:25. https://doi.org/10.1186/1476-072X-7-25.
33. Debacker M, Portaels F, Aguiar J, Steunou C, Zinsou C, Meyers W, Dramaix M. 2006. Risk factors for Buruli ulcer, Benin. Emerg Infect Dis 12:1325–1331. https://doi.org/10.3201/eid1209.050598.
34. Josse R, Guedenon A, Darie H, Anagonou S, Portaels F, Meyers WM. 1995. Mycobacterium ulcerans cutaneous infections: Buruli ulcers. Med Trop (Mars) 55:363–373. (In French.)
35. Giles-Vernick T, Owona-Ntsama J, Landier J, Eyangoh S. 2015. The puzzle of Buruli ulcer transmission, ethno-ecological history and the end of "love" in the Akonolinga district, Cameroon. Soc Sci Med 129:20–27. https://doi.org/10.1016/j.socscimed.2014.03.008.
36. Nyabadza F, Bonyah E. 2015. On the transmission dynamics of Buruli ulcer in Ghana: insights through a mathematical model. BMC Res Notes 8:656. https://doi.org/10.1186/s13104-015-1619-5.
37. Tian RD, Lepidi H, Nappez C, Drancourt M. 2016. Experimental survival of Mycobacterium ulcerans in watery soil, a potential source of Buruli ulcer. Am J Trop Med Hyg 94:89–92. https://doi.org/10.4269/ajtmh.15-0568.
38. van der Werf TS, van der Graaf WT, Tappero JW, Asiedu K. 1999. Mycobacterium ulcerans infection. Lancet 354:1013–1018. https://doi.org/10.1016/S0140-6736(99)01156-3.
39. Aboagye SY, Danso E, Ampah KA, Nakobu Z, Asare P, Otchere ID, Röltgen K, Yirenya-Tawiah D, Yeboah-Manu D. 2016. Isolation of non-tuberculous mycobacteria from the environment of Ghanian communities where Buruli ulcer is endemic. Appl Environ Microbiol 82:4320–4329. https://doi.org/10.1128/AEM.01002-16.
40. Marsollier L, Sévérin T, Aubry J, Merritt RW, Saint André J-P, Legras P, Manceau A-L, Chauty A, Carbonnelle B, Cole ST. 2004. Aquatic snails, passive hosts of Mycobacterium ulcerans. Appl Environ Microbiol 70:6296–6298. https://doi.org/10.1128/AEM.70.10.6296-6298.2004.
41. Asiedu K, Etuaful S. 1998. Socioeconomic implications of Buruli ulcer in Ghana: a three-year review. Am J Trop Med Hyg 59:1015–1022. https://doi.org/10.4269/ajtmh.1998.59.1015.
42. Henle FGJ. 1840. Von den Miasmen und Contagien, p 1–82. *In* Pathologische Untersuchungen. Friedrich Gustav Jacob Henle, Berlin, Germany.
43. Koch R. 1882. Die Aetiologie der Tuberkulose. Berl Klin Wochenschr 19:221–230.
44. Cule J. 1988. Sir William Osler and his Welsh connections. Postgrad Med J 64:568–574. https://doi.org/10.1136/pgmj.64.753.568.
45. Hayman J. 1984. Mycobacterium ulcerans: an infection from Jurassic time. Lancet ii:1015–1016.
46. Stinear TP, Jenkin GA, Johnson PDR, Davies JK. 2000. Comparative genetic analysis of Mycobacterium ulcerans and Mycobacterium marinum reveals evidence of recent divergence. J Bacteriol 182:6322–6330. https://doi.org/10.1128/JB.182.22.6322-6330.2000.
47. Pidot SJ, Asiedu K, Käser M, Fyfe JAM, Stinear TP. 2010. Mycobacterium ulcerans and other mycolactone-producing mycobacteria should be considered a single species. PLoS Negl Trop Dis 4:e663. https://doi.org/10.1371/journal.pntd.0000663.
48. Pidot SJ, Hong H, Seemann T, Porter JL, Yip MJ, Men A, Johnson M, Wilson P, Davies JK, Leadlay PF, Stinear TP. 2008. Deciphering the genetic basis for polyketide variation among mycobacteria producing mycolactones. BMC Genomics 9:462–462. https://doi.org/10.1186/1471-2164-9-462.
49. Nakanaga K, Yotsu RR, Hoshino Y, Suzuki K, Makino M, Ishii N. 2013. Buruli ulcer and mycolactone-producing mycobacteria. Jpn J Infect Dis 66:83–88. https://doi.org/10.7883/yoken.66.83.
50. Doig KD, Holt KE, Fyfe JAM, Lavender CJ, Eddyani M, Portaels F, Yeboah-Manu D, Pluschke G, Seemann T, Stinear TP. 2012. On the origin of Mycobacterium ulcerans, the causative agent of Buruli ulcer. BMC Genomics 13:258–258. https://doi.org/10.1186/1471-2164-13-258.
51. Stinear TP, Mve-Obiang A, Small PL, Frigui W, Pryor MJ, Brosch R, Jenkin GA, Johnson PD, Davies JK, Lee RE, Adusumilli S, Garnier T, Haydock SF, Leadlay PF, Cole ST. 2004. Giant plasmid-encoded polyketide synthases produce the macrolide toxin of Mycobacterium ulcerans. Proc Natl Acad Sci U S A 101:1345–1349. https://doi.org/10.1073/pnas.0305877101.
52. Singh VK, Berry L, Bernut A, Singh S, Carrere-Kremer S, Viljoen A, Alibaud L, Majlessi L, Brosch R, Chaturvedi V, Geurtsen J, Drancourt M, Kremer L. 2016. A unique PE_PGRS protein inhibiting host cell cytosolic defenses and sustaining full virulence of Mycobacterium marinum in multiple hosts. Cell Microbiol 18:1489–1507. https://doi.org/10.1111/cmi.12606.
53. Rybniker J, Kramme S, Small PL. 2006. Host range of 14 mycobacteriophages in Mycobacterium ulcerans and seven other mycobacteria including Mycobacterium tuberculosis—application for identification and susceptibility testing. J Med Microbiol 55:37–42. https://doi.org/10.1099/jmm.0.46238-0.
54. Huys G, Rigouts L, Chemlal K, Portaels F, Swings J. 2000. Evaluation of amplified fragment length polymorphism analysis for inter- and intraspecific differentiation of Mycobacterium bovis, M. tuberculosis, and M. ulcerans. J Clin Microbiol 38:3675–3680.
55. Käser M, Rondini S, Naegeli M, Stinear T, Portaels F, Certa U. 2007. Evolution of two distinct phylogenetic lineages of the emerging human pathogen Mycobacterium ulcerans. BMC Evol Biol 7:177. https://doi.org/10.1186/1471-2148-7-177.
56. Qi W, Käser M, Röltgen K, Yeboah-Manu D, Pluschke G. 2009. Genomic diversity and evolution of Mycobacterium ulcerans revealed by next-generation sequencing. PLoS Pathog 5:e1000580. https://doi.org/10.1371/journal.ppat.1000580.
57. Chemlal K, Huys G, Fonteyne PA, Vincent V, Lopez AG, Rigouts L, Swings J, Meyers WM, Portaels F. 2001. Evaluation of PCR-restriction profile analysis and IS2404 restriction fragment length polymorphism and amplified fragment length polymorphism fingerprinting for identification and typing of Mycobacterium ulcerans and M. marinum. J Clin Microbiol 39:3272–3278. https://doi.org/10.1128/JCM.39.9.3272-3278.2001.
58. Lamelas A, Ampah KA, Aboagye S, Kerber S, Danso E, Asante-Poku A, Asare P, Parkhill J, Harris SR, Pluschke G, Yeboah-Manu D, Röltgen K. 2016. Spatiotemporal co-existence of two Mycobacterium ulcerans clonal complexes in the Offin River Valley of Ghana. PLoS Negl Trop Dis 10:e0004856. https://doi.org/10.1371/journal.pntd.0004856.
59. Stragier P, Ablordey A, Bayonne LM, Lugor YL, Sindani IS, Suykerbuyk P, Wabinga H, Meyers WM, Portaels F. 2006. Heterogeneity among Mycobacterium ulcerans isolates from Africa. Emerg Infect Dis 12:844–847. https://doi.org/10.3201/eid1205.051191.
60. Bolz M, Bratschi MW, Kerber S, Minyem JC, Um Boock A, Vogel M, Bayi PF, Junghanss T, Brites D, Harris SR, Parkhill J, Pluschke G, Lamelas Cabello A. 2015. Locally confined clonal complexes of Mycobacterium ulcerans in two Buruli ulcer endemic regions of Cameroon. PLoS Negl Trop Dis 9:e0003802. https://doi.org/10.1371/journal.pntd.0003802.
61. Ablordey AS, Vandelannoote K, Frimpong IA, Ahortor EK, Amissah NA, Eddyani M, Durnez L, Portaels F, de Jong BC, Leirs H, Porter JL, Mangas KM, Lam MMC, Buultjens A, Seemann T, Tobias NJ, Stinear TP. 2015. Whole genome comparisons suggest random distribution of Mycobac-

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zcm-cmr/zcm00118/zcm2618d18z | xppws | S=6 | 11/10/17 | 21:58 | 4/Color Fig: 1,2,3,4,5,6,7,8,9,10,11 | ArtID: 00045-17 | DOI:10.1128/CMR.00045-17CE: msm

terium ulcerans genotypes in a Buruli ulcer endemic region of Ghana. PLoS Negl Trop Dis 9:e0003681. https://doi.org/10.1371/journal.pntd.0003681.
62. Vandelannoote K, Meehan CJ, Eddyani M, Affolabi D, Phanzu DM, Eyangoh S, Jordaens K, Portaels F, Mangas K, Seemann T, Marsollier L, Marion E, Chauty A, Landier J, Fontanet A, Leirs H, Stinear TP, de Jong BC. 2017. Multiple introductions and recent spread of the emerging human pathogen Mycobacterium ulcerans across Africa. Genome Biol Evol 9:414–426. https://doi.org/10.1093/gbe/evx003.
63. Narh CA, Mosi L, Quaye C, Dassi C, Konan DO, Tay SCK, de Souza DK, Boakye DA, Bonfoh B. 2015. Source tracking Mycobacterium ulcerans infections in the Ashanti Region, Ghana. PLoS Negl Trop Dis 9:e0003437. https://doi.org/10.1371/journal.pntd.0003437.
64. Dassi C, Mosi L, Akpatou B, Narh C, Quaye C, Konan D, Djaman J, Bonfoh B. 2015. Detection of Mycobacterium ulcerans in Mastomys natalensis and potential transmission in Buruli ulcer endemic areas in Côte d'Ivoire. Mycobact Dis 5:184.
65. Fyfe JA, Lavender CJ, Handasyde KA, Legione AR, O'Brien CR, Stinear TP, Pidot SJ, Seemann T, Benbow ME, Wallace JR, McCowan C, Johnson PD. 2010. A major role for mammals in the ecology of Mycobacterium ulcerans. PLoS Negl Trop Dis 4:0000791. https://doi.org/10.1371/journal.pntd.0000791.
66. George KM, Chatterjee D, Gunawardana G, Welty D, Hayman J, Lee R, Small PL. 1999. Mycolactone: a polyketide toxin from Mycobacterium ulcerans required for virulence. Science 283:854–857. https://doi.org/10.1126/science.283.5403.854.
67. Gunawardana G, Chatterjee D, George KM, Brennan P, Whittern D, Small PLC. 1999. Characterization of novel macrolide toxins, mycolactones A and B, from a human pathogen, Mycobacterium ulcerans. J Am Chem Soc 121:6092–6093. https://doi.org/10.1021/ja990017l.
68. Marion E, Prado S, Cano C, Babonneau J, Ghamrawi S, Marsollier L. 2012. Photodegradation of the Mycobacterium ulcerans toxin, mycolactones: considerations for handling and storage. PLoS One 7:e33600. https://doi.org/10.1371/journal.pone.0033600.
69. Hong H, Stinear T, Porter J, Demangel C, Leadlay PF. 2007. A novel mycolactone toxin obtained by biosynthetic engineering. Chembiochem 8:2043–2047. https://doi.org/10.1002/cbic.200700411.
70. Rhodes MW, Kator H, Kotob S, van Berkum P, Kaattari I, Vogelbein W, Floyd MM, Butler WR, Quinn FD, Ottinger C, Shotts E. 2001. A unique Mycobacterium species isolated from an epizootic of striped bass (Morone saxatilis). Emerg Infect Dis 7:896–899. https://doi.org/10.3201/eid0705.017523.
71. Mve-Obiang A, Lee RE, Portaels F, Small PL. 2003. Heterogeneity of mycolactones produced by clinical isolates of Mycobacterium ulcerans: implications for virulence. Infect Immun 71:774–783. https://doi.org/10.1128/IAI.71.2.774-783.2003.
72. Ranger BS, Mahrous EA, Mosi L, Adusumilli S, Lee RE, Colorni A, Rhodes M, Small PLC. 2006. Globally distributed mycobacterial fish pathogens produce a novel plasmid-encoded toxic macrolide, mycolactone F. Infect Immun 74:6037–6045. https://doi.org/10.1128/IAI.00970-06.
73. Tafelmeyer P, Laurent C, Lenormand P, Rousselle JC, Marsollier L, Reysset G, Zhang R, Sickmann A, Stinear TP, Namane A, Cole ST. 2008. Comprehensive proteome analysis of Mycobacterium ulcerans and quantitative comparison of mycolactone biosynthesis. Proteomics 8:3124–3138. https://doi.org/10.1002/pmic.200701018.
74. Deshayes C, Angala SK, Marion E, Brandli I, Babonneau J, Preisser L, Eyangoh S, Delneste Y, Legras P, De Chastellier C, Stinear TP, Jackson M, Marsollier L. 2013. Regulation of mycolactone, the Mycobacterium ulcerans toxin, depends on nutrient source. PLoS Negl Trop Dis 7:e2502. https://doi.org/10.1371/journal.pntd.0002502.
75. Guenin-Mace L, Veyron-Churlet R, Thoulouze MI, Romet-Lemonne G, Hong H, Leadlay PF, Danckaert A, Ruf MT, Mostowy S, Zurzolo C, Bousso P, Chretien F, Carlier MF, Demangel C. 2013. Mycolactone activation of Wiskott-Aldrich syndrome proteins underpins Buruli ulcer formation. J Clin Invest 123:1501–1512. https://doi.org/10.1172/JCI66576.
76. Sarfo FS, Phillips R, Wansbrough-Jones M, Simmonds RE. 2016. Recent advances: role of mycolactone in the pathogenesis and monitoring of Mycobacterium ulcerans infection/Buruli ulcer disease. Cell Microbiol 18:17–29. https://doi.org/10.1111/cmi.12547.
77. Phillips RO, Sarfo FS, Landier J, Oldenburg R, Frimpong M, Wansbrough-Jones M, Abass K, Thompson W, Forson M, Fontanet A, Niang F, Demangel C. 2014. Combined inflammatory and metabolic defects reflected by reduced serum protein levels in patients with Buruli ulcer disease. PLoS Negl Trop Dis 8:e2786. https://doi.org/10.1371/journal.pntd.0002786.
78. Anand U, Sinisi M, Fox M, MacQuillan A, Quick T, Korchev Y, Bountra C, McCarthy T, Anand P. 2016. Mycolactone-mediated neurite degeneration and functional effects in cultured human and rat DRG neurons: mechanisms underlying hypoalgesia in Buruli ulcer. Mol Pain 20:1–11. https://doi.org/10.1177/1744806916654144.
79. Ogbechi J, Ruf MT, Hall BS, Bodman-Smith K, Vogel M, Wu HL, Stainer A, Esmon CT, Ahnstrom J, Pluschke G, Simmonds RE. 2015. Mycolactone-dependent depletion of endothelial cell thrombomodulin is strongly associated with fibrin deposition in Buruli ulcer lesions. PLoS Pathog 11:e1005011. https://doi.org/10.1371/journal.ppat.1005011.
80. Oliveira MS, Fraga AG, Torrado E, Castro AG, Pereira JP, Filho AL, Milanezi F, Schmitt FC, Meyers WM, Portaels F, Silva MT, Pedrosa J. 2005. Infection with Mycobacterium ulcerans induces persistent inflammatory responses in mice. Infect Immun 73:6299–6310. https://doi.org/10.1128/IAI.73.10.6299-6310.2005.
81. George KM, Pascopella L, Welty DM, Small PLC. 2000. A Mycobacterium ulcerans toxin, mycolactone, causes apoptosis in guinea pig ulcers and tissue culture cells. Infect Immun 68:877–883. https://doi.org/10.1128/IAI.68.2.877-883.2000.
82. Guenin-Mace L, Baron L, Chany AC, Tresse C, Saint-Auret S, Jonsson F, Le Chevalier F, Bruhns P, Bismuth G, Hidalgo-Lucas S, Bisson JF, Blanchard N, Demangel C. 2015. Shaping mycolactone for therapeutic use against inflammatory disorders. Sci Transl Med 7:289ra85. https://doi.org/10.1126/scitranslmed.aab0458.
83. Hall BS, Hill K, McKenna M, Ogbechi J, High S, Willis AE, Simmonds RE. 2014. The pathogenic mechanism of the Mycobacterium ulcerans virulence factor, mycolactone, depends on blockade of protein translocation into the ER. PLoS Pathog 10:e1004061. https://doi.org/10.1371/journal.ppat.1004061.
84. Song OR, Marion E, Comoglio Y, Babonneau J, Guerineau N, Sandoz G, Yeramian E, Brodin P, Marsollier L. 2016. Mycolactone: the amazing analgesic mycobacterial toxin. Med Sci (Paris) 32:156–158. (In French.) https://doi.org/10.1051/medsci/20163202007.
85. En J, Goto M, Nakanaga K, Higashi M, Ishii N, Saito H, Yonezawa S, Hamada H, Small PLC. 2008. Mycolactone is responsible for the painlessness of Mycobacterium ulcerans infection (Buruli ulcer) in a murine study. Infect Immun 76:2002–2007. https://doi.org/10.1128/IAI.01588-07.
86. Sarfo FS, Phillips RO, Zhang J, Abass MK, Abotsi J, Amoako YA, Adu-Sarkodie Y, Robinson C, Wansbrough-Jones MH. 2014. Kinetics of mycolactone in human subcutaneous tissue during antibiotic therapy for Mycobacterium ulcerans disease. BMC Infect Dis 14:1471–2334. https://doi.org/10.1186/1471-2334-14-202.
87. Diaz D, Dobeli H, Yeboah-Manu D, Mensah-Quainoo E, Friedlein A, Soder N, Rondini S, Bodmer T, Pluschke G. 2006. Use of the immunodominant 18-kilodalton small heat shock protein as a serological marker for exposure to Mycobacterium ulcerans. Clin Vaccine Immunol 13:1314–1321. https://doi.org/10.1128/CVI.00254-06.
88. Hong H, Coutanceau E, Leclerc M, Caleechurn L, Leadlay PF, Demangel C. 2008. Mycolactone diffuses from Mycobacterium ulcerans-infected tissues and targets mononuclear cells in peripheral blood and lymphoid organs. PLoS Negl Trop Dis 2:e325. https://doi.org/10.1371/journal.pntd.0000325.
89. Sarfo FS, Le Chevalier F, Aka N, Phillips RO, Amoako Y, Boneca IG, Lenormand P, Dosso M, Wansbrough-Jones M, Veyron-Churlet R, Guenin-Mace L, Demangel C. 2011. Mycolactone diffuses into the peripheral blood of Buruli ulcer patients—implications for diagnosis and disease monitoring. PLoS Negl Trop Dis 5:19. https://doi.org/10.1371/journal.pntd.0001237.
90. Marsollier L, Honore N, Legras P, Manceau AL, Kouakou H, Carbonnelle B, Cole ST. 2003. Isolation of three Mycobacterium ulcerans strains resistant to rifampin after experimental chemotherapy of mice. Antimicrob Agents Chemother 47:1228–1232. https://doi.org/10.1128/AAC.47.4.1228-1232.2003.
91. Portaels F, Meyers WM, Ablordey A, Castro AG, Chemlal K, de Rijk P, Elsen P, Fissette K, Fraga AG, Lee R, Mahrous E, Small PLC, Stragier P, Torrado E, Van Aerde A, Silva MT, Pedrosa J. 2008. First cultivation and characterization of Mycobacterium ulcerans from the environment. PLoS Negl Trop Dis 2:e178. https://doi.org/10.1371/journal.pntd.0000178.
92. Bratschi MW, Ruf M-T, Andreoli A, Minyem JC, Kerber S, Wantong FG, Pritchard J, Chakwera V, Beuret C, Wittwer M, Noumen D, Schürch N, Um Book A, Pluschke G. 2014. Mycobacterium ulcerans persistence at a village water source of Buruli ulcer patients. PLoS Negl Trop Dis 8:e2756. https://doi.org/10.1371/journal.pntd.0002756.
93. Marsollier L, Stinear T, Aubry J, Saint Andre JP, Robert R, Legras P. 2004.

zcm-cmr/zcm00118/zcm2618d18z | xppws | S=6 | 11/10/17 | 21:58 | 4/Color Fig: 1,2,3,4,5,6,7,8,9,10,11 | ArtID: 00045-17 | DOI:10.1128/CMR.00045-17 CE: msm

Aquatic plants stimulate the growth of and biofilm formation by Mycobacterium ulcerans in axenic culture and harbor these bacteria in the environment. Appl Environ Microbiol 70:1097–103. https://doi.org/10.1128/AEM.70.2.1097-1103.2004.
94. Eddyani M, Debacker M, Martin A, Aguiar J, Johnson CR, Uwizeye C, Fissette K, Portaels F. 2008. Primary culture of Mycobacterium ulcerans from human tissue specimens after storage in semisolid transport medium. J Clin Microbiol 46:69–72. https://doi.org/10.1128/JCM.00301-07.
95. Portaels F (ed). 2014. Laboratory diagnosis of Buruli ulcer: a manual for health-care providers, p 117. World Health Organization, Geneva, Switzerland.
96. Palomino JC, Obiang AM, Realini L, Meyers WM, Portaels F. 1998. Effect of oxygen on growth of Mycobacterium ulcerans in the BACTEC system. J Clin Microbiol 36:3420–3422.
97. Eddyani M, Portaels F. 2007. Survival of Mycobacterium ulcerans at 37 degrees C. Clin Microbiol Infect 13:1033–1035. https://doi.org/10.1111/j.1469-0691.2007.01791.x.
98. Portaels F, Johnson P, Meyers WM (ed). 2001. Buruli ulcer. Diagnosis of Mycobacterium ulcerans disease. A manual for health care providers. World Health Organization, Geneva, Switzerland. http://whqlibdoc.who.int/hq/2001/WHO_CDS_CPE_GBUI_2001.4.pdf.
99. Tian RBD, Asmar S, Napez C, Lépidi H, Drancourt M. 2017. Effectiveness of purified methylene blue in an experimental model of Mycobacterium ulcerans infection. Int J Antimicrob Agents 49:290–295. https://doi.org/10.1016/j.ijantimicag.2016.11.012.
100. Sanhueza D, Chevillon C, Colwell R, Babonneau J, Marion E, Marsollier L, Guegan JF. 2016. Chitin promotes Mycobacterium ulcerans growth. FEMS Microbiol Ecol 92:27. https://doi.org/10.1093/femsec/fiw067.
101. Petroff SA. 1915. A new and rapid method for the isolation and cultivation of the tubercle bacillus directly from sputum and feces. J Exp Med 21:38–42. https://doi.org/10.1084/jem.21.1.38.
102. Portaels F, Agular J, Fissette K, Fonteyne PA, De Beenhouwer H, de Rijk P, Guedenon A, Lemans R, Steunou C, Zinsou C, Dumonceau JM, Meyers WM. 1997. Direct detection and identification of Mycobacterium ulcerans in clinical specimens by PCR and oligonucleotide-specific capture plate hybridization. J Clin Microbiol 35:1097–1100.
103. Collins CH. 1952. Oxalic acid and acid-iron peroxide in the routine culture of tubercle bacilli from sputum. Tubercle 33:149–151. https://doi.org/10.1016/S0041-3879(52)80084-4.
104. Yajko DM, Nassos PS, Sanders CA, Gonzalez PC, Reingold AL, Horsburgh CR, Jr, Hopewell PC, Chin DP, Hadley WK. 1993. Comparison of four decontamination methods for recovery of Mycobacterium avium complex from stools. J Clin Microbiol 31:302–306.
105. Marsollier L, Stinear T, Aubry J, Saint André JP, Robert R, Legras P, Manceau A-L, Audrain C, Bourdon S, Kouakou H, Carbonnelle B. 2004. Aquatic plants stimulate the growth of and biofilm formation by Mycobacterium ulcerans in axenic culture and harbor these bacteria in the environment. Appl Environ Microbiol 70:1097–1103. https://doi.org/10.1128/AEM.70.2.1097-1103.2004.
106. Tian RB, Niamke S, Tissot-Dupont H, Drancourt M. 2016. Detection of Mycobacterium ulcerans DNA in the environment, Ivory Coast. PLoS One 11:e0151567. https://doi.org/10.1371/journal.pone.0151567.
107. Zogo B, Djenontin A, Carolan K, Babonneau J, Guegan J-F, Eyangoh S, Marion E. 2015. A field study in Benin to investigate the role of mosquitoes and other flying insects in the ecology of Mycobacterium ulcerans. PLoS Negl Trop Dis 9:e0003941. https://doi.org/10.1371/journal.pntd.0003941.
108. Luo Y, Degang Y, Ohtsuka M, Ishido Y, Ishii N, Suzuki K. 2015. Detection of Mycobacterium ulcerans subsp. shinshuense DNA from a water channel in familial Buruli ulcer cases in Japan. Future Microbiol 10:461–469. https://doi.org/10.2217/fmb.14.152.
109. Konan KL, Doannio JM, Coulibaly NG, Ekaza E, Marion E, Asse H, Kouassi D, N'Goran KE, Dosso M, Marsollier L, Aubry J. 2015. Detection of the IS2404 insertion sequence and ketoreductase produced by Mycobacterium ulcerans in the aquatic Heteroptera in the health districts of Dabou and Tiassale in Cote d'Ivoire. Med Sante Trop 25:44–51. (In French.). https://doi.org/10.1684/mst.2014.0363.
110. Garchitorena A, Ngonghala CN, Texier G, Landier J, Eyangoh S, Bonds MH, Guégan J-F, Roche B. 2015. Environmental transmission of Mycobacterium ulcerans drives dynamics of Buruli ulcer in endemic regions of Cameroon. Sci Rep 5:18055. https://doi.org/10.1038/srep18055.
111. O'Brien CR, Handasyde KA, Hibble J, Lavender CJ, Legione AR, McCowan C, Globan M, Mitchell AT, McCracken HE, Johnson PD, Fyfe JA. 2014. Clinical, microbiological and pathological findings of Mycobacterium ulcerans infection in three Australian possum species. PLoS Negl Trop Dis 8:e2666. https://doi.org/10.1371/journal.pntd.0002666.
112. Morris A, Gozlan R, Marion E, Marsollier L, Andreou D, Sanhueza D, Ruffine R, Couppie P, Guegan JF. 2014. First detection of Mycobacterium ulcerans DNA in environmental samples from South America. PLoS Negl Trop Dis 8:e2660. https://doi.org/10.1371/journal.pntd.0002660.
113. Fyfe JAM, Lavender CJ, Johnson PDR, Globan M, Sievers A, Azuolas J, Stinear TP. 2007. Development and application of two multiplex real-time PCR assays for the detection of Mycobacterium ulcerans in clinical and environmental samples. Appl Environ Microbiol 73:4733–4740. https://doi.org/10.1128/AEM.02971-06.
114. Feldman WH, Karlson AG, Herrick JF. 1957. Mycobacterium ulcerans: pathogenesis of infection in mice, including determinations of dermal temperatures. Am J Pathol 33:1163–1179.
115. Williamson HR, Mosi L, Donnell R, Aqqad M, Merritt RW, Small PL. 2014. Mycobacterium ulcerans fails to infect through skin abrasions in a guinea pig infection model: implications for transmission. PLoS Negl Trop Dis 8:e2770. https://doi.org/10.1371/journal.pntd.0002770.
116. Schutte D, Umboock A, Pluschke G. 2009. Phagocytosis of Mycobacterium ulcerans in the course of rifampicin and streptomycin chemotherapy in Buruli ulcer lesions. Br J Dermatol 160:273–283. https://doi.org/10.1111/j.1365-2133.2008.08879.x.
117. Bozzo C, Tiberio R, Graziola F, Pertusi G, Valente G, Colombo E, Small PL, Leigheb G. 2010. A Mycobacterium ulcerans toxin, mycolactone, induces apoptosis in primary human keratinocytes and in HaCaT cells. Microbes Infect 12:1258–1263. https://doi.org/10.1016/j.micinf.2010.08.005.
118. Bieri R, Bolz M, Ruf M-T, Pluschke G. 2016. Interferon-γ is a crucial activator of early host immune defense against Mycobacterium ulcerans infection in mice. PLoS Negl Trop Dis 10:e0004450. https://doi.org/10.1371/journal.pntd.0004450.
119. Bolz M, Ruggli N, Borel N, Pluschke G, Ruf MT. 2016. Local cellular immune responses and pathogenesis of Buruli ulcer lesions in the experimental Mycobacterium ulcerans pig infection model. PLoS Negl Trop Dis 10:e0004678. https://doi.org/10.1371/journal.pntd.0004678.
120. Torrado E, Fraga AG, Castro AG, Stragier P, Meyers WM, Portaels F, Silva MT, Pedrosa J. 2007. Evidence for an intramacrophage growth phase of Mycobacterium ulcerans. Infect Immun 75:977–987. https://doi.org/10.1128/IAI.00889-06.
121. Drancourt M, Jarlier V, Raoult D. 2002. The environmental pathogen Mycobacterium ulcerans grows in amphibian cells at low temperatures. Appl Environ Microbiol 68:6403–6404. https://doi.org/10.1128/AEM.68.12.6403-6404.2002.
122. Marsollier L, Aubry J, Coutanceau E, Andre JP, Small PL, Milon G, Legras P, Guadagnini S, Carbonnelle B, Cole ST. 2005. Colonization of the salivary glands of Naucoris cimicoides by Mycobacterium ulcerans requires host plasmatocytes and a macrolide toxin, mycolactone. Cell Microbiol 7:935–943. https://doi.org/10.1111/j.1462-5822.2005.00521.x.
123. Coutanceau E, Marsollier L, Brosch R, Perret E, Goossens P, Tanguy M, Cole ST, Small PL, Demangel C. 2005. Modulation of the host immune response by a transient intracellular stage of Mycobacterium ulcerans: the contribution of endogenous mycolactone toxin. Cell Microbiol 7:1187–1196. https://doi.org/10.1111/j.1462-5822.2005.00546.x.
124. Walsh DS, Meyers WM, Portaels F, Lane JE, Mongkolsirichaikul D, Hussem K, Gosi P, Myint KSA. 2005. High rates of apoptosis in human Mycobacterium ulcerans culture-positive Buruli ulcer skin lesions. Am J Trop Med Hyg 73:410–415.
125. Torrado E, Fraga AG, Logarinho E, Martins TG, Carmona JA, Gama JB, Carvalho MA, Proença F, Castro AG, Pedrosa J. 2010. IFN-γ-dependent activation of macrophages during experimental infections by Mycobacterium ulcerans is impaired by the toxin mycolactone. J Immunol 184:947–955. https://doi.org/10.4049/jimmunol.0902717.
126. Driskell R, Jahoda CAB, Chuong C-M, Watt F, Horsley V. 2014. Defining dermal adipose tissue. Exp Dermatol 23:629–631. https://doi.org/10.1111/exd.12450.
127. Hayman J. 1993. Out of Africa: observations on the histopathology of Mycobacterium ulcerans infection. J Clin Pathol 46:5–9. https://doi.org/10.1136/jcp.46.1.5.
128. Dobos KM, Small PL, Deslauriers M, Quinn FD, King CH. 2001. Mycobacterium ulcerans cytotoxicity in an adipose cell model. Infect Immun 69:7182–7186. https://doi.org/10.1128/IAI.69.11.7182-7186.2001.
129. Bolz M, Ruggli N, Ruf M-T, Ricklin ME, Zimmer G, Pluschke G. 2014. Experimental infection of the pig with Mycobacterium ulcerans: a novel

zcm-cmr/zcm00118/zcm2618d18z | xppws | S=6 | 11/10/17 | 21:58 | 4/Color Fig: 1,2,3,4,5,6,7,8,9,10,11 | ArtID: 00045-17 | DOI:10.1128/CMR.00045-17 CE: msm

model for studying the pathogenesis of Buruli ulcer disease. PLoS Negl Trop Dis 8:e2968. https://doi.org/10.1371/journal.pntd.0002968.
130. Gryseels S, Amissah D, Durnez L, Vandelannoote K, Leirs H, De Jonckheere J, Silva MT, Portaels F, Ablordey A, Eddyani M. 2012. Amoebae as potential environmental hosts for Mycobacterium ulcerans and other mycobacteria, but doubtful actors in Buruli ulcer epidemiology. PLoS Negl Trop Dis 6:e1764. https://doi.org/10.1371/journal.pntd.0001764.
131. Portaels F, Silva MT, Meyers WM. 2009. Buruli ulcer. Clin Dermatol 27:291–305. https://doi.org/10.1016/j.clindermatol.2008.09.021.
132. Lind TK, Darre L, Domene C, Urbanczyk-Lipkowska Z, Cardenas M, Wacklin HP. 2015. Antimicrobial peptide dendrimer interacts with phosphocholine membranes in a fluidity dependent manner: a neutron reflection study combined with molecular dynamics simulations. Biochim Biophys Acta 1848:2075–2084. https://doi.org/10.1016/j.bbamem.2015.05.015.
133. Bag N, Yap DH, Wohland T. 2014. Temperature dependence of diffusion in model and live cell membranes characterized by imaging fluorescence correlation spectroscopy. Biochim Biophys Acta 1838:802–813. https://doi.org/10.1016/j.bbamem.2013.10.009.
134. Capo X, Martorell M, Sureda A, Batle JM, Tur JA, Pons A. 2016. Docosahexaenoic diet supplementation, exercise and temperature affect cytokine production by lipopolysaccharide-stimulated mononuclear cells. J Physiol Biochem 72:421–434. https://doi.org/10.1007/s13105-016-0490-8.
135. Phillips R, Sarfo FS, Guenin-Mace L, Decalf J, Wansbrough-Jones M, Albert ML, Demangel C. 2009. Immunosuppressive signature of cutaneous Mycobacterium ulcerans infection in the peripheral blood of patients with Buruli ulcer disease. J Infect Dis 200:1675–1684. https://doi.org/10.1086/646615.
136. Rolain JM, Vayssier-Taussat M, Saisongkorh W, Merhej V, Gimenez G, Robert C, Le Rhun D, Dehio C, Raoult D. 2013. Partial disruption of translational and posttranslational machinery reshapes growth rates of Bartonella birtlesii. mBio 4:e00115-13. https://doi.org/10.1128/mBio.00115-13.
137. Georgiades K, Raoult D. 2011. The rhizome of Reclinomonas americana, Homo sapiens, Pediculus humanus and Saccharomyces cerevisiae mitochondria. Biol Direct 6:55. https://doi.org/10.1186/1745-6150-6-55.
138. Gil R, Sabater-Munoz B, Latorre A, Silva FJ, Moya A. 2002. Extreme genome reduction in Buchnera spp.: toward the minimal genome needed for symbiotic life. Proc Natl Acad Sci U S A 99:4454–4458. https://doi.org/10.1073/pnas.062067299.
139. Portaels F, Elsen P, Guimaraes-Peres A, Fonteyne P-A, Meyers WM. 1999. Insects in the transmission of Mycobacterium ulcerans infection. Lancet 353:986. https://doi.org/10.1016/S0140-6736(98)05177-0.
140. Ross BC, Marino L, Oppedisano F, Edwards R, Robins-Browne RM, Johnson PD. 1997. Development of a PCR assay for rapid diagnosis of Mycobacterium ulcerans infection. J Clin Microbiol 35:1696–1700.
141. Kotlowski R, Martin A, Ablordey A, Chemlal K, Fonteyne P-A, Portaels F. 2004. One-tube cell lysis and DNA extraction procedure for PCR-based detection of Mycobacterium ulcerans in aquatic insects, molluscs and fish. J Med Microbiol 53:927–933. https://doi.org/10.1099/jmm.0.45593-0.
142. Stinear T, Ross BC, Davies JK, Marino L, Robins-Browne RM, Oppedisano F. 1999. Identification and characterization of IS*2404* and IS*2606*: two distinct repeated sequences for detection of Mycobacterium ulcerans by PCR. J Clin Microbiol 37:1018–1023.
143. Stragier P, Ablordey A, Durnez L, Portaels F. 2007. VNTR analysis differentiates Mycobacterium ulcerans and IS2404 positive mycobacteria. Syst Appl Microbiol 30:525–530. https://doi.org/10.1016/j.syapm.2007.06.001.
144. Williamson HR, Benbow ME, Campbell LP, Johnson CR, Sopoh G, Barogui Y, Merritt RW, Small PLC. 2012. Detection of Mycobacterium ulcerans in the environment predicts prevalence of Buruli ulcer in Benin. PLoS Negl Trop Dis 6:e1506. https://doi.org/10.1371/journal.pntd.0001506.
145. Vandelannoote K, Durnez L, Amissah D, Gryseels S, Dodoo A, Yeboah S, Addo P, Eddyani M, Leirs H, Ablordey A, Portaels F. 2010. Application of real-time PCR in Ghana, a Buruli ulcer-endemic country, confirms the presence of Mycobacterium ulcerans in the environment. FEMS Microbiol Lett 304:191–194. https://doi.org/10.1111/j.1574-6968.2010.01902.x.
146. Tano MB, Dassi C, Mosi L, Koussémon M, Bonfoh B. 2017. Molecular characterization of mycolactone producing mycobacteria from aquatic environments in Buruli ulcer non-endemic areas in Côte d'Ivoire. Int J Environ Res Public Health 14:178. https://doi.org/10.3390/ijerph14020178.
147. Hennigan CE, Myers L, Ferris MJ. 2013. Environmental distribution and seasonal prevalence of Mycobacterium ulcerans in southern Louisiana. Appl Environ Microbiol 79:2648–2656. https://doi.org/10.1128/AEM.03543-12.
148. Doannio JM, Konan KL, Dosso FN, Kone AB, Konan YL, Sankare Y, Ekaza E, Coulibaly ND, Odehouri KP, Dosso M, Sess ED, Marsollier L, Aubry J. 2011. Micronecta sp (Corixidae) and Diplonychus sp (Belostomatidae), two aquatic Hemiptera hosts and/or potential vectors of Mycobacterium ulcerans (pathogenic agent of Buruli ulcer) in Cote d'Ivoire. Med Trop (Mars) 71:53–57. (In French.)
149. Marion E, Deshayes C, Chauty A, Cassisa V, Tchibozo S, Cottin J, Doannio J, Marot A, Marsollier L. 2011. Detection of Mycobacterium ulcerans DNA in water bugs collected outside the aquatic environment in Benin. Med Trop (Mars) 71:169–172. (In French.)
150. Benbow ME, Williamson H, Kimbirauskas R, McIntosh MD, Kolar R, Quaye C, Akpabey F, Boakye D, Small P, Merritt RW. 2008. Aquatic invertebrates as unlikely vectors of Buruli ulcer disease. Emerg Infect Dis 14:1247–1254. https://doi.org/10.3201/eid1408.071503.
151. Willson SJ, Kaufman MG, Merritt RW, Williamson HR, Malakauskas DM, Benbow ME. 2013. Fish and amphibians as potential reservoirs of Mycobacterium ulcerans, the causative agent of Buruli ulcer disease. Infect Ecol Epidemiol 3:10.3402/iee.v3i0.19946. https://doi.org/10.3402/iee.v3i0.19946.
152. Konan KL, Fofana D, Koné AB, Konan YL, Assé H, Kouassi D, Dosso M, Marsollier L, Aubry J, N'Goran K, Doannio JMC. 2015. Inventory of aquatic heteroptera in ponds near the villages of six health districts, endemic to Buruli ulcer in Côte d'Ivoire (West Africa). Experiment 31:2012–2021. http://www.experimentjournal.com/expadmin/pdf_files/Konan%20K.L%20et%20al,%20The%20Experiment,%202015.,Vol.%2031(1),%202012-2021.pdf.
153. Garchitorena A, Roche B, Kamgang R, Ossomba J, Babonneau J, Landier J, Fontanet A, Flahault A, Eyangoh S, Guegan JF, Marsollier L. 2014. Mycobacterium ulcerans ecological dynamics and its association with freshwater ecosystems and aquatic communities: results from a 12-month environmental survey in Cameroon. PLoS Negl Trop Dis 8:e2879. https://doi.org/10.1371/journal.pntd.0002879.
154. Carolan K, Garchitorena A, García-Peña GE, Morris A, Landier J, Fontanet A, Le Gall P, Texier G, Marsollier L, Gozlan RE, Eyangoh S, Lo Seen D, Guégan J-F. 2014. Topography and land cover of watersheds predicts the distribution of the environmental pathogen Mycobacterium ulcerans in aquatic insects. PLoS Negl Trop Dis 8:e3298. https://doi.org/10.1371/journal.pntd.0003298.
155. Raghunathan PL, Whitney EAS, Asamoa K, Stienstra Y, Taylor TH, Amofah GK, Ofori-Adjei D, Dobos K, Guarner J, Martin S, Pathak S, Klutse E, Etuaful S, van der Graaf WTA, van der Werf TS, King CH, Tappero JW, Ashford DA. 2005. Risk factors for Buruli ulcer disease (Mycobacterium ulcerans infection): results from a case-control study in Ghana. Clin Infect Dis 40:1445–1453. https://doi.org/10.1086/429623.
156. Lavender CJ, Fyfe JAM, Azuolas J, Brown K, Evans RN, Ray LR, Johnson PDR. 2011. Risk of Buruli ulcer and detection of Mycobacterium ulcerans in mosquitoes in southeastern Australia. PLoS Negl Trop Dis 5:e1305. https://doi.org/10.1371/journal.pntd.0001305.
157. Eddyani M, De Jonckheere JF, Durnez L, Suykerbuyk P, Leirs H, Portaels F. 2008. Occurrence of free-living amoebae in communities of low and high endemicity for Buruli ulcer in southern Benin. Appl Environ Microbiol 74:6547–6553. https://doi.org/10.1128/AEM.01066-08.
158. McIntosh M, Williamson H, Benbow ME, Kimbirauskas R, Quaye C, Boakye D, Small P, Merritt R. 2014. Associations between Mycobacterium ulcerans and aquatic plant communities of West Africa: implications for Buruli ulcer disease. Ecohealth 11:184–196. https://doi.org/10.1007/s10393-013-0898-3.
159. Mougin B, Tian RBD, Drancourt M. 2015. Tropical plant extracts modulating the growth of Mycobacterium ulcerans. PLoS One 10:e0124626. https://doi.org/10.1371/journal.pone.0124626.
160. Heydorn A, Ersbøll BK, Hentzer M, Parsek MR, Givskov M, Molin S. 2000. Experimental reproducibility in flow-chamber biofilms. Microbiology 146:2409–2415. https://doi.org/10.1099/00221287-146-10-2409.
161. Costerton JW, Cheng KJ, Geesey GG, Ladd TI, Nickel JC, Dasgupta M, Marrie TJ. 1987. Bacterial biofilms in nature and disease. Annu Rev Microbiol 41:435–464. https://doi.org/10.1146/annurev.mi.41.100187.002251.
162. Garrett TR BM, Zhang Z. 2008. Bacterial adhesion and biofilms on surfaces. Prog Nat Sci 18:1049–1056. https://doi.org/10.1016/j.pnsc.2008.04.001.

zcm-cmr/zcm00118/zcm2618d18z | xppws | S=6 | 11/10/17 | 21:58 | 4/Color Fig: 1,2,3,4,5,6,7,8,9,10,11 | ArtID: 00045-17 | DOI:10.1128/CMR.00045-17 CE: msm

163. Gooday GW. 1990. The ecology of chitin degradation. Adv Microb Ecol 11:387–430. https://doi.org/10.1007/978-1-4684-7612-5_10.
164. Fajardo-Somera RA, Johnk B, Bayram O, Valerius O, Braus GH, Riquelme M. 2015. Dissecting the function of the different chitin synthases in vegetative growth and sexual development in Neurospora crassa. Fungal Genet Biol 75:30–45. https://doi.org/10.1016/j.fgb.2015.01.002.
165. Sarbon NM, Sandanamsamy S, Kamaruzaman SF, Ahmad F. 2015. Chitosan extracted from mud crab (Scylla olivicea) shells: physicochemical and antioxidant properties. J Food Sci Technol 52:4266–4275. https://doi.org/10.1007/s13197-014-1522-4.
166. Kaya M, Karaarslan M, Baran T, Can E, Ekemen G, Bitim B, Duman F. 2014. The quick extraction of chitin from an epizoic crustacean species (Chelonibia patula). Nat Prod Res 28:2186–2190. https://doi.org/10.1080/14786419.2014.927469.
167. Kappel L, Gaderer R, Flipphi M, Seidl-Seiboth V. 2016. The N-acetylglucosamine catabolic gene cluster in Trichoderma reesei is controlled by the Ndt80-like transcription factor RON1. Mol Microbiol 99:640–657. https://doi.org/10.1111/mmi.13256.
168. Ruocco N, Costantini S, Guariniello S, Costantini M. 2016. Polysaccharides from the marine environment with pharmacological, cosmeceutical and nutraceutical potential. Molecules 21:551. https://doi.org/10.3390/molecules21050551.
169. Fesel PH, Zuccaro A. 2016. β-Glucan: crucial component of the fungal cell wall and elusive MAMP in plants. Fungal Genet Biol 90:53–60. https://doi.org/10.1016/j.fgb.2015.12.004.
170. Klusemann J, Kleinow W, Peters W. 1990. The hard parts (trophi) of the rotifer mastax do contain chitin: evidence from studies on Brachionus plicatilis. Histochemistry 94:277–283. https://doi.org/10.1007/BF00266628.
171. Brydon LJ, Gooday GW, Chappell LH, King TP. 1987. Chitin in egg shells of Onchocerca gibsoni and Onchocerca volvulus. Mol Biochem Parasitol 25:267–272. https://doi.org/10.1016/0166-6851(87)90090-9.
172. Sromova D, Lysek H. 1990. Visualization of chitin-protein layer formation in Ascaris lumbricoides egg-shells. Folia Parasitol 37:77–80.
173. Zhu KY, Merzendorfer H, Zhang W, Zhang J, Muthukrishnan S. 2016. Biosynthesis, turnover, and functions of chitin in insects. Annu Rev Entomol 61:177–196. https://doi.org/10.1146/annurev-ento-010715-023933.
174. Tang WJ, Fernandez JG, Sohn JJ, Amemiya CT. 2015. Chitin is endogenously produced in vertebrates. Curr Biol 25:897–900. https://doi.org/10.1016/j.cub.2015.01.058.
175. Frederiksen RF, Paspaliari DK, Larsen T, Storgaard BG, Larsen MH, Ingmer H, Palcic MM, Leisner JJ. 2013. Bacterial chitinases and chitin-binding proteins as virulence factors. Microbiology 159:833–847. https://doi.org/10.1099/mic.0.051839-0.
176. Zhang YZ, Chen Q, Liu CH, Liu YB, Yi P, Niu KX, Wang YQ, Wang AQ, Yu HY, Pu ZE, Jiang QT, Wei YM, Qi PF, Zheng YL. 2016. Chitin synthase gene FgCHS8 affects virulence and fungal cell wall sensitivity to environmental stress in Fusarium graminearum. Fungal Biol 120:764–774. https://doi.org/10.1016/j.funbio.2016.02.002.
177. Wan J, Zhang X-C, Stacey G. 2008. Chitin signaling and plant disease resistance. Plant Signal Behav 3:831–833. https://doi.org/10.4161/psb.3.10.5916.
178. Morozov AA, Likhoshway YV. 2016. Evolutionary history of the chitin synthases of eukaryotes. Glycobiology 26:635–639. https://doi.org/10.1093/glycob/cww018.
179. Issola Y, Kouassi A, Dongui B, Biemi J. 2008. Caractéristiques physico-chimiques d'une lagune côtière tropicale: lagune de Fresco (Côte d'Ivoire). Afr Sci 4:368–393.
180. Kambiré O, Adingra AA, Eblin SG, Aka N, Kakou AC, Koffi-Nevry R. 2014. Characterization of waters of an estuarine lagoon of the Ivory Coast: the Aby lagoon. Larhyss J 20:95–110. (In French.)
181. Inza B, Soro M, Etchian A, Trokourey A, Bokra Y. 2009. Caractérisation physico-chimique des eaux et des sédiments de surface de la baie des milliardaires, lagune Ébrié, Cote d'Ivoire. Rev Ivoir Sci Technol 13:139–154.
182. Konan K, Kouassi K, Kouamé K, Kouassi A, Gnakri D. 2013. Hydrologie et hydrochimie des eaux dans la zone de construction du chenal du port de pêche de Grand-Lahou, Côte d'Ivoire. Int J Biol Chem Sci 7:819–831.
183. N'Guessan YA, Wango T-E, Konan KE, Adingra A, Amani EM, Monde S, Affian K, Aka K. 2015. Hydrologie et morphologie de l'estuaire du fleuve Sassandra, Basse Côte d'Ivoire. Afr Sci 11:161–172.
184. Wognin V, Monde S, Affian K, Coulibaly A, Aka K. 2007. Waters model circulation in the estuary of Bandama (Ivory Coast). Rivers flows and tide condition's incidence. Sud Sci Technol 5:12.
185. Abe J, Bakayoko S, Bamba SB, Koffi KP. 1993. Morphology and hydrodynamic in the Bandama inlet. J Ivoir Océanol Limnol 2:9–24.
186. Asmar S, Sassi M, Phelippeau M, Drancourt M. 2016. Inverse correlation between salt tolerance and host-adaptation in mycobacteria. BMC Res Notes 9:249. https://doi.org/10.1186/s13104-016-2054-y.
187. Lacharme M. 2001. Le contrôle de la salinité dans les rizières. Fascicule 9, Mémento Technique de Riziculture. Coopération Française, •••, France.
188. Garchitorena A, Guégan J-F, Léger L, Eyangoh S, Marsollier L, Roche B. 2015. Mycobacterium ulcerans dynamics in aquatic ecosystems are driven by a complex interplay of abiotic and biotic factors. Elife 4:e07616. https://doi.org/10.7554/eLife.07616.
189. Dramé KN, Manneh B, Ismail AM. 2013. Rice genetic improvement for abiotic stress tolerance in Africa, p 144–160. *In* Wopereis MCS, Johnson DE, Ahmadi N, Tollens E, Jalloh A (ed), Realizing Africa's rice promise. CABI.
190. Wansbrough-Jones M, Phillips R. 2006. Buruli ulcer: emerging from obscurity. Lancet 367:1849–1858. https://doi.org/10.1016/S0140-6736(06)68807-7.
191. Duker AA, Carranza EJM, Hale M. 2004. Spatial dependency of Buruli ulcer prevalence on arsenic-enriched domains in Amansie West District, Ghana: implications for arsenic mediation in Mycobacterium ulcerans infection. Int J Health Geogr 3:19–19. https://doi.org/10.1186/1476-072X-3-19.
192. Aiga H, Amano T, Cairncross S, Domako JA, Nanas OK, Coleman S. 2004. Assessing water-related risk factors for Buruli ulcer: a case-control study in Ghana. Am J Trop Med Hyg 71:387–392.
193. Marsollier L, Aubry J, Saint-Andre JP, Robert R, Legras P, Manceau AL, Bourdon S, Audrain C, Carbonnelle B. 2003. Ecology and transmission of Mycobacterium ulcerans. Pathol Biol 51:490–495. https://doi.org/10.1016/S0369-8114(03)00151-2.
194. Duker AA, Carranza EJ, Hale M. 2005. Arsenic geochemistry and health. Environ Int 31:631–641. https://doi.org/10.1016/j.envint.2004.10.020.
195. Garelick H, Jones H, Dybowska A, Valsami-Jones E. 2008. Arsenic pollution sources. Rev Environ Contam Toxicol 197:17–60.
196. Ampah KA, Asare P, Binnah DD-G, Maccaulley S, Opare W, Röltgen K, Pluschke G, Yeboah-Manu D. 2016. Burden and historical trend of Buruli ulcer prevalence in selected communities along the Offin River of Ghana. PLoS Negl Trop Dis 10:e0004603. https://doi.org/10.1371/journal.pntd.0004603.
197. Darie H, Le Guyadec T, Touze JE. 1993. Epidemiological and clinical aspects of Buruli ulcer in Ivory Coast. 124 recent cases. Bull Soc Pathol Exot 86:272–276.
198. Addo P, Adu-Addai B, Quartey M, Abbas M, Okang I, Owusu E, Ofori-Adjei D, Awumbila B. 2006. Clinical and histopathological presentation of Buruli ulcer in experimentally infected grasscutters (Thryonomys swinderianus). Internet J Trop Med 3:1–15.
199. Elsner L, Wayne J, O'Brien CR, McCowan C, Malik R, Hayman JA, Globan M, Lavender CJ, Fyfe JA. 2008. Localised Mycobacterium ulcerans infection in a cat in Australia. J Feline Med Surg 10:407–412. https://doi.org/10.1016/j.jfms.2008.03.003.
200. van Zyl A, Daniel J, Wayne J, McCowan C, Malik R, Jelfs P, Lavender CJ, Fyfe JA. 2010. Mycobacterium ulcerans infections in two horses in south-eastern Australia. Aust Vet J 88:101–106. https://doi.org/10.1111/j.1751-0813.2009.00544.x.
201. O'Brien CR, McMillan E, Harris O, O'Brien DP, Lavender CJ, Globan M, Legione AR, Fyfe JA. 2011. Localised Mycobacterium ulcerans infection in four dogs. Aust Vet J 89:506–510. https://doi.org/10.1111/j.1751-0813.2011.00850.x.
202. O'Brien C, Kuseff G, McMillan E, McCowan C, Lavender C, Globan M, Jerrett I, Oppedisano F, Johnson P, Fyfe J. 2013. Mycobacterium ulcerans infection in two alpacas. Aust Vet J 91:296–300. https://doi.org/10.1111/avj.12071.
203. Mitchell PJ, Jerrett IV, Slee KJ. 1984. Skin ulcers caused by Mycobacterium ulcerans in koalas near Bairnsdale, Australia. Pathology 16:256–260. https://doi.org/10.3109/00313028409068533.
204. Mitchell PJ, McOrist S, Bilney R. 1987. Epidemiology of Mycobacterium ulcerans infection in koalas (Phascolarctos cinereus) on Raymond Island, southeastern Australia. J Wildl Dis 23:386–390. https://doi.org/10.7589/0090-3558-23.3.386.
205. McOrist S, Jerrett IV, Anderson M, Hayman J. 1985. Cutaneous and respiratory tract infection with Mycobacterium ulcerans in two koalas (Phascolarctos cinereus). J Wildl Dis 21:171–173. https://doi.org/10.7589/0090-3558-21.2.171.

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206. Tobias NJ, Doig KD, Medema MH, Chen H, Haring V, Moore R, Seemann T, Stinear TP. 2013. Complete genome sequence of the frog pathogen Mycobacterium ulcerans ecovar liflandii. J Bacteriol 195:556-564. https://doi.org/10.1128/JB.02132-12.
207. Tobias NJ, Ammisah NA, Ahortor EK, Wallace JR, Ablordey A, Stinear TP. 2016. Snapshot fecal survey of domestic animals in rural Ghana for Mycobacterium ulcerans. PeerJ 4:e2065. https://doi.org/10.7717/peerj.2065.
208. Sakaguchi K, Iima H, Hirayama K, Okamoto M, Matsuda K, Miyasho T, Kasamatsu M, Hasegawa K, Taniyama H. 2011. Mycobacterium ulcerans infection in an Indian flap-shelled turtle (Lissemys punctata punctata). J Vet Med Sci 73:1217-1220. https://doi.org/10.1292/jvms.10-0386.
209. Ohtsuka M, Kikuchi N, Yamamoto T, Suzutani T, Nakanaga K, Suzuki K, Ishii N. 2014. Buruli ulcer caused by Mycobacterium ulcerans subsp shinshuense: a rare case of familial concurrent occurrence and detection of insertion sequence 2404 in Japan. JAMA Dermatol 150:64-67. https://doi.org/10.1001/jamadermatol.2013.6816.
210. Aboagye SY, Ampah KA, Ross A, Asare P, Otchere ID, Fyfe J, Yeboah-Manu D. 2017. Seasonal pattern of Mycobacterium ulcerans, the causative agent of Buruli ulcer, in the environment in Ghana. Microb Ecol 74:350-361. https://doi.org/10.1007/s00248-017-0946-6.
211. Zingue D, Bouam A, Militello M, Drancourt M. 2017. High-throughput carbon substrate profiling of Mycobacterium ulcerans suggests potential environmental reservoirs. PLoS Negl Trop Dis 11:e0005303. https://doi.org/10.1371/journal.pntd.0005303.
212. Morris AL, Guegan JF, Andreou D, Marsollier L, Carolan K, Le Croller M, Sanhueza D, Gozlan RE. 2016. Deforestation-driven food-web collapse linked to emerging tropical infectious disease, Mycobacterium ulcerans. Sci Adv 2:e1600387. https://doi.org/10.1126/sciadv.1600387.
213. Stienstra Y, van Roest MH, van Wezel MJ, Wiersma IC, Hospers IC, Dijkstra PU, Johnson RC, Ampadu EO, Gbovi J, Zinsou C, Etuaful S, Klutse EY, van der Graaf WT, van der Werf TS. 2005. Factors associated with functional limitations and subsequent employment or schooling in Buruli ulcer patients. Trop Med Int Health 10:1251-1257. https://doi.org/10.1111/j.1365-3156.2005.01519.x.
214. Lehman L, Simonet V, Saunderson P, Agbenorku P. 2006. Buruli ulcer: prevention of disability (POD). World Health Organization, Geneva, Switzerland.
215. Barogui Y, Johnson RC, van der Werf TS, Sopoh G, Dossou A, Dijkstra PU, Stienstra Y. 2009. Functional limitations after surgical or antibiotic treatment for Buruli ulcer in Benin. Am J Trop Med Hyg 81:82-87.
216. Sizaire V, Nackers F, Comte E, Portaels F. 2006. Mycobacterium ulcerans infection: control, diagnosis, and treatment. Lancet Infect Dis 6:288-296. https://doi.org/10.1016/S1473-3099(06)70464-9.
217. WHO. 2012. Treatment of Mycobacterium ulcerans disease (Buruli ulcer): guidance for health workers. World Health Organization, Geneva, Switzerland.
218. WHO. 2004. Global Buruli ulcer initiative. Provisional guidance on the role of specific antibiotics in the management of Mycobacterium ulcerans disease (Buruli ulcer). World Health Organization, Geneva, Switzerland.
219. Wallace JR, Mangas KM, Porter JL, Marcsisin R, Pidot SJ, Howden BO, Omansen TF, Zeng W, Axford JK, Johnson PDR, Stinear TP. 2017. Mycobacterium ulcerans low infectious dose and mechanical transmission support insect bites and puncturing injuries in the spread of Buruli ulcer. PLoS Negl Trop Dis 11:e0005553. https://doi.org/10.1371/journal.pntd.0005553.
220. Johnson PD, Stinear TP, Hayman JA. 1999. Mycobacterium ulcerans—a mini-review. J Med Microbiol 48:511-513. https://doi.org/10.1099/00222615-48-6-511.
221. Hayman J. 1991. Postulated epidemiology of Mycobacterium ulcerans infection. Int J Epidemiol 20:1093-1098. https://doi.org/10.1093/ije/20.4.1093.
222. Connor DH, Lunn HF. 1965. Mycobacterium ulcerans infection (with comments on pathogenesis). Int J Lepr 33:698-709.
223. Duker AA, Portaels F, Hale M. 2006. Pathways of Mycobacterium ulcerans infection: a review. Environ Int 32:567-573. https://doi.org/10.1016/j.envint.2006.01.002.
224. Pszolla N, Sarkar MR, Strecker W, Kern P, Kinzl L, Meyers WM, Portaels F. 2003. Buruli ulcer: a systemic disease. Clin Infect Dis 37:e78-e82. https://doi.org/10.1086/377170.
225. Debacker M, Zinsou C, Aguiar J, Meyers WM, Portaels F. 2003. First case of Mycobacterium ulcerans disease (Buruli ulcer) following a human bite. Clin Infect Dis 36:67-68. https://doi.org/10.1086/367660.
226. Ukwaja KN, Meka AO, Chukwuka A, Asiedu KB, Huber KL, Eddyani M, Chukwu JN, Anyim MC, Nwafor CC, Oshi DC, Madichie NO, Ekeke N, Njoku M, Ntana K. 2016. Buruli ulcer in Nigeria: results of a pilot case study in three rural districts. Infect Dis Poverty 5:39. https://doi.org/10.1186/s40249-016-0119-8.
227. Mavinga Phanzu D, Suykerbuyk P, Saunderson P, Ngwala Lukanu P, Masamba Minuku JB, Imposo DB, Mbadu Diengidi B, Kayinua M, Tamfum Muyembe JJ, Tshindele Lutumba P, de Jong BC, Portaels F, Boelaert M. 2013. Burden of Mycobacterium ulcerans disease (Buruli ulcer) and the underreporting ratio in the territory of Songololo, Democratic Republic of Congo. PLoS Negl Trop Dis 7:e2563. https://doi.org/10.1371/journal.pntd.0002563.
228. Bayonne Manou LS, Portaels F, Eddyani M, Book AU, Vandelannoote K, de Jong BC. 2013. Mycobacterium ulcerans disease (Buruli ulcer) in Gabon: 2005-2011. Med Sante Trop 23:450-457. (In French.)
229. Porten K, Sailor K, Comte E, Njikap A, Sobry A, Sihom F, Meva'a A, Eyangoh S, Myatt M, Nackers F, Grais RF. 2009. Prevalence of Buruli ulcer in Akonolinga health district, Cameroon: results of a cross sectional survey. PLoS Negl Trop Dis 3:466. https://doi.org/10.1371/journal.pntd.0000466.
230. O'Brien DP, Friedman ND, Cowan R, Pollard J, McDonald A, Callan P, Hughes A, Athan E. 2015. Mycobacterium ulcerans in the elderly: more severe disease and suboptimal outcomes. PLoS Negl Trop Dis 9:e0004253. https://doi.org/10.1371/journal.pntd.0004253.
231. Friedman ND, Athan E, Hughes AJ, Khajehnoori M, McDonald A, Callan P, Rahdon R, O'Brien DP. 2013. Mycobacterium ulcerans disease: experience with primary oral medical therapy in an Australian cohort. PLoS Negl Trop Dis 7:e2315. https://doi.org/10.1371/journal.pntd.0002315.
232. WHO. 2016. Buruli ulcer (Mycobacterium ulcerans infection). WHO, Geneva, Switzerland. http://www.who.int/mediacentre/factsheets/fs199/en/. Accessed ●●●● 2017. AQ: Q
233. Yotsu RR, Murase C, Sugawara M, Suzuki K, Nakanaga K, Ishii N, Asiedu K. 2015. Revisiting Buruli ulcer. J Dermatol 42:1033-1041. https://doi.org/10.1111/1346-8138.13049.
234. Hofer M, Hirschel B, Kirschner P, Beghetti M, Kaelin A, Siegrist CA, Suter S, Teske A, Bottger EC. 1993. Brief report: disseminated osteomyelitis from Mycobacterium ulcerans after a snakebite. N Engl J Med 328:1007-1009. https://doi.org/10.1056/NEJM199304083281405.
235. Kenu E, Nyarko KM, Seefeld L, Ganu V, Käser M, Lartey M, Calys-Tagoe BNL, Koram K, Adanu R, Razum O, Afari E, Binka FN. 2014. Risk factors for Buruli ulcer in Ghana—a case control study in the Suhum-Kraboa-Coaltar and Akuapem South Districts of the eastern region. PLoS Negl Trop Dis 8:e3279. https://doi.org/10.1371/journal.pntd.0003279.
236. Lagarrigue V, Portaels F, Meyers WM, Aguiar J. 2000. Buruli ulcer: risk of bone involvement! Apropos of 33 cases observed in Benin. Med Trop 60:262-266. (In French.)
237. Walsh DS, Dela Cruz EC, Abalos RM, Tan EV, Walsh GP, Portaels F, Meyers WM. 2007. Clinical and histologic features of skin lesions in a cynomolgus monkey experimentally infected with Mycobacterium ulcerans (Buruli ulcer) by intradermal inoculation. Am J Trop Med Hyg 76:132-134.
238. Niang F, Sarfo FS, Frimpong M, Guenin-Mace L, Wansbrough-Jones M, Stinear T, Phillips RO, Demangel C. 2015. Metabolomic profiles delineate mycolactone signature in Buruli ulcer disease. Sci Rep 5:17693. https://doi.org/10.1038/srep17693.
239. Vincent QB, Ardant M-F, Marsollier L, Chauty A, Alcaïs A. 2014. HIV infection and Buruli ulcer in Africa. Lancet Infect Dis 14:796-797. https://doi.org/10.1016/S1473-3099(14)70882-5.
240. Johnson RC, Nackers F, Glynn JR, de Biurrun Bakedano E, Zinsou C, Aguiar J, Tonglet R, Portaels F. 2008. Association of HIV infection and Mycobacterium ulcerans disease in Benin. AIDS 22:901-903. https://doi.org/10.1097/QAD.0b013e3282f7690a.
241. Adu EJK, Ampadu E. 2015. Mycobacterium ulcerans disease in the middle belt of Ghana: an eight-year review from six endemic districts. Int J Mycobacteriol 4:138-142. https://doi.org/10.1016/j.ijmyco.2015.03.006.
242. Capela C, Sopoh GE, Houezo JG, Fiodessihoué R, Dossou AD, Costa P, Fraga AG, Menino JF, Silva-Gomes R, Ouendo EM, Rodrigues F, Pedrosa J. 2015. Clinical epidemiology of Buruli ulcer from Benin (2005-2013): effect of time-delay to diagnosis on clinical forms and severe phenotypes. PLoS Negl Trop Dis 9:e0004005. https://doi.org/10.1371/journal.pntd.0004005.
243. Beissner M, Arens N, Wiedemann F, Piten E, Kobara B, Bauer M, Herbinger K-H, Badziklou K, Banla Kere A, Löscher T, Nitschke J, Bretzel G.

zcm-cmr/zcm00118/zcm2618d18z | xppws | S=6 | 11/10/17 | 21:58 | 4/Color Fig: 1,2,3,4,5,6,7,8,9,10,11 | ArtID: 00045-17 | DOI:10.1128/CMR.00045-17 CE: msm

2015. Treatment outcome of patients with Buruli ulcer disease in Togo. PLoS Negl Trop Dis 9:e0004170. https://doi.org/10.1371/journal.pntd.0004170.
244. James K, Attipou KK, James YE, Blakime M, Tignokpa N, Abete B. 2003. Buruli ulcer in Togo: a hospital study. Sante 13:43–47.
245. Berger S. 2016. Tropical skin ulcers: global status. Gideon Informatics, Los Angeles, CA.
246. Johnson RC, Sopoh GE, Barogui Y, Dossou A, Fourn L, Zohoun T. 2008. Surveillance system for Buruli ulcer in Benin: results after four years. Sante 18:9–13. (In French.)
247. Jiao J, Yao L, Li Y, Wong S, Sau-Fan Ng F, Teng Y, Guo Y. 2012. Thermal physiology and local responses of human body during exercise in hot conditions. J Fiber Bioeng Inform 5:115–124. https://doi.org/10.3993/jfbi06201201.
248. Aschoff J, Wever R. 1958. Kern und Schale im Wfumehaushalt des Menschen. Naturwissenschaften 45:477–485. https://doi.org/10.1007/BF00635546.
249. Brou G, Houndonougbo F, Aboh A, Mensah G, Fantodji A. 2012. Effet de la variation temporelle de la température ambiante journalière sur le poids des œufs de poules pondeuses ISA Brown en Côte-d'Ivoire. Int J Biol Chem Sci 6:2158–2169.
250. Miller CS, Gosling WD. 2014. Quaternary forest associations in lowland tropical West Africa. Quat Sci Rev 84:7–25. https://doi.org/10.1016/j.quascirev.2013.10.027.
251. Vogel M, Bayi PF, Ruf M-T, Bratschi MW, Bolz M, Um Boock A, Zwahlen M, Pluschke G, Junghanss T. 2016. Local heat application for the treatment of Buruli ulcer: results of a phase II open label single center non comparative clinical trial. Clin Infect Dis 62:342–350. https://doi.org/10.1093/cid/civ883.
252. Stinear TP, Seemann T, Harrison PF, Jenkin GA, Davies JK, Johnson PD, Abdellah Z, Arrowsmith C, Chillingworth T, Churcher C, Clarke K, Cronin A, Davis P, Goodhead I, Holroyd N, Jagels K, Lord A, Moule S, Mungall K, Norbertczak H, Quail MA, Rabbinowitsch E, Walker D, White B, Whitehead S, Small PL, Brosch R, Ramakrishnan L, Fischbach MA, Parkhill J, Cole ST. 2008. Insights from the complete genome sequence of Mycobacterium marinum on the evolution of Mycobacterium tuberculosis. Genome Res 18:729–741. https://doi.org/10.1101/gr.075069.107.
253. Ahoua L, Guetta A, Ekanza E, Bouzid S, N'Guessan R, Dosso M. 2009. Risk factors for Buruli ulcer in Côte d'Ivoire: results of a case-control study, August 2001. Afr J Biotechnol 8:536–546.
254. Tuffour J, Owusu-Mireku E, Ruf M-T, Aboagye S, Kpeli G, Akuoku V, Pereko J, Paintsil A, Bonney K, Ampofo W, Pluschke G, Yeboah-Manu D. 2015. Challenges associated with management of Buruli ulcer/human immunodeficiency virus coinfection in a treatment center in Ghana: a case series study. Am J Trop Med Hyg 93:216–223. https://doi.org/10.4269/ajtmh.14-0571.
255. Christinet V, Comte E, Ciaffi L, Odermatt P, Serafini M, Antierens A, Rossel L, Nomo A-B, Nkemenang P, Tsoungui A, Delhumeau C, Calmy A. 2014. Impact of human immunodeficiency virus on the severity of Buruli ulcer disease: results of a retrospective study in Cameroon. Open Forum Infect Dis 1:ofu021. https://doi.org/10.1093/ofid/ofu021.
256. Toll A, Gallardo F, Ferran M, Gilaberte M, Iglesias M, Gimeno JL, Rondini S, Pujol RM. 2005. Aggressive multifocal Buruli ulcer with associated osteomyelitis in an HIV-positive patient. Clin Exp Dermatol 30:649–651. https://doi.org/10.1111/j.1365-2230.2005.01892.x.
257. Komenan K, Elidjé EJ, Ildevert GP, Yao KI, Kanga K, Kouamé KA, Abdoulaye S, Hamdam KS, Yao YP, Jean-Marie K. 2013. Multifocal Buruli ulcer associated with secondary infection in HIV positive patient. Case Rep Med 2013:348628. https://doi.org/10.1155/2013/348628.
258. Phillips RO, Frimpong M, Sarfo FS, Kretschmer B, Beissner M, Debrah A, Ampem-Amoako Y, Abass KM, Thompson W, Duah MS, Abotsi J, Adjei O, Fleischer B, Bretzel G, Wansbrough-Jones M, Jacobsen M. 2014. Infection with Mansonella perstans nematodes in Buruli ulcer patients, Ghana. Emerg Infect Dis 20:1000–1003. https://doi.org/10.3201/eid2006.131501.
259. Mougin B, Avenel-Audran M, Hasseine L, Martin L, Cottin J, Pomares C, Delaunay P, Marty P, Ravel C, Chabasse D, Abgueguen P. 2011. A cutaneous ulcer resulting from Mycobacterium ulcerans—Leishmania braziliensis coinfection in South America. Am J Trop Med Hyg 85:897–899. https://doi.org/10.4269/ajtmh.2011.11-0126.
260. Nackers F, Tonglet R, Slachmuylder V, Johnson RC, Robert A, Zinsou C, Glynn JR, Portaels F, Gala JL. 2007. Association between haemoglobin variants S and C and Mycobacterium ulcerans disease (Buruli ulcer): a case-control study in Benin. Trop Med Int Health 12:511–518. https://doi.org/10.1111/j.1365-3156.2006.01808.x.
261. Siegmund V, Adjei O, Racz P, Berberich C, Klutse E, van Vloten F, Kruppa T, Fleischer B, Bretzel G. 2005. Dry-reagent-based PCR as a novel tool for laboratory confirmation of clinically diagnosed Mycobacterium ulcerans-associated disease in areas in the tropics where M. ulcerans is endemic. J Clin Microbiol 43:271–276.
262. Guarner J, Bartlett J, Whitney EA, Raghunathan PL, Stienstra Y, Asamoa K, Etuaful S, Klutse E, Quarshie E, van der Werf TS, van der Graaf WT, King CH, Ashford DA. 2003. Histopathologic features of Mycobacterium ulcerans infection. Emerg Infect Dis 9:651–656. https://doi.org/10.3201/eid0906.020485.
263. Tabah EN, Nsagha DS, Bissek AC, Njamnshi AK, Bratschi MW, Pluschke G, Um Boock A. 2016. Buruli ulcer in Cameroon: the development and impact of the National Control Programme. PLoS Negl Trop Dis 10:e0004224. https://doi.org/10.1371/journal.pntd.0004224.
264. Herbinger K-H, Adjei O, Awua-Boateng N-Y, Nienhuis WA, Kunaa L, Siegmund V, Nitschke J, Thompson W, Klutse E, Agbenorku P, Schipf A, Reu S, Racz P, Fleischer B, Beissner M, Fleischmann E, Helfrich K, van der Werf TS, Lüscher T, Bretzel G. 2009. Comparative study of the sensitivity of different diagnostic methods for the laboratory diagnosis of Buruli ulcer disease. Clin Infect Dis 48:1055–1064. https://doi.org/10.1086/597398.
265. WHO. 2008. Guidance on sampling techniques for laboratory-confirmation of Mycobacterium ulcerans infection (Buruli ulcer disease). WHO, Geneva, Switzerland. http://www.who.int/buruli/Guidance_sampling_techniques_MU_infection.pdf.
266. Beissner M, Herbinger KH, Bretzel G. 2010. Laboratory diagnosis of Buruli ulcer disease. Future Microbiol 5:363–370. https://doi.org/10.2217/fmb.10.3.
267. Beissner M, Phillips RO, Battke F, Bauer M, Badziklou K, Sarfo FS, Maman I, Rhomberg A, Piten E, Frimpong M, Huber KL, Symank D, Jansson M, Wiedemann FX, Banla Kere A, Herbinger KH, Loscher T, Bretzel G. 2015. Loop-mediated isothermal amplification for laboratory confirmation of Buruli ulcer disease—towards a point-of-care test. PLoS Negl Trop Dis 9:e0004219. https://doi.org/10.1371/journal.pntd.0004219.
268. Affolabi D, Bankole H, Ablordey A, Hounnouga J, Koutchakpo P, Sopoh G, Aguiar J, Dossou A, Johnson RC, Anagonou S, Portaels F. 2008. Effects of grinding surgical tissue specimens and smear staining methods on Buruli ulcer microscopic diagnosis. Trop Med Int Health 13:187–190. https://doi.org/10.1111/j.1365-3156.2007.01989.x.
269. Shinnick TM, Iademarco MF, Ridderhof JC. 2005. National plan for reliable tuberculosis laboratory services using a systems approach. Recommendations from CDC and the Association of Public Health Laboratories Task Force on Tuberculosis Laboratory Services. MMWR Recomm Rep 54:1–12.
270. Wadagni A, Frimpong M, Phanzu DM, Ablordey A, Kacou E, Gbedevi M, Marion E, Xing Y, Babu VS, Phillips RO, Wansbrough-Jones M, Kishi Y, Asiedu K. 2015. Simple, rapid Mycobacterium ulcerans disease diagnosis from clinical samples by fluorescence of mycolactone on thin layer chromatography. PLoS Negl Trop Dis 9:e0004247. https://doi.org/10.1371/journal.pntd.0004247.
271. N'Guessan K, Kouassi Y, Bouzid S, Ehuie P, Koffi K, Oniangue C, Aka N, Dosso M. 2001. Value and limits of microscopy of exudates in Mycobacterium ulcerans cutaneous infection in Cote d'Ivoire. Bull Soc Pathol Exot 94:9–10. (In French.)
272. Asiedu K, Wansbrough-Jones M. 2007. Mycobacterium ulcerans infection (Buruli or Bairnsdale ulcer): challenges in developing management strategies. Med J Aust 186:55–56.
273. Siegmund V, Adjei O, Nitschke J, Thompson W, Klutse E, Herbinger KH, Thompson R, van Vloten F, Racz P, Fleischer B, Loescher T, Bretzel G. 2007. Dry reagent-based polymerase chain reaction compared with other laboratory methods available for the diagnosis of Buruli ulcer disease. Clin Infect Dis 45:68–75. https://doi.org/10.1086/518604.
274. Babonneau J, Bernard C, Marion E, Chauty A, Kempf M, Robert R, Marsollier L. 2015. Development of a dry-reagent-based qPCR to facilitate the diagnosis of Mycobacterium ulcerans infection in endemic countries. PLoS Negl Trop Dis 9:e0003606. https://doi.org/10.1371/journal.pntd.0003606.
275. de Souza DK, Quaye C, Mosi L, Addo P, Boakye DA. 2012. A quick and cost effective method for the diagnosis of Mycobacterium ulcerans infection. BMC Infect Dis 12:1471–2334. https://doi.org/10.1186/1471-2334-12-8.
276. Njiru ZK, Yeboah-Manu D, Stinear TP, Fyfe JA. 2012. Rapid and sensitive

zcm-cmr/zcm00118/zcm2618d18z | xppws | S=6 | 11/10/17 | 21:58 | 4/Color Fig: 1,2,3,4,5,6,7,8,9,10,11 | ArtID: 00045-17 | DOI:10.1128/CMR.00045-17 CE: msm

detection of Mycobacterium ulcerans by use of a loop-mediated isothermal amplification test. J Clin Microbiol 50:1737–1741. https://doi.org/10.1128/JCM.06460-11.
277. Ablordey A, Amissah DA, Aboagye IF, Hatano B, Yamazaki T, Sata T, Ishikawa K, Katano H. 2012. Detection of Mycobacterium ulcerans by the loop mediated isothermal amplification method. PLoS Negl Trop Dis 6:e1590. https://doi.org/10.1371/journal.pntd.0001590.
278. Laval F, Laneelle MA, Deon C, Monsarrat B, Daffe M. 2001. Accurate molecular mass determination of mycolic acids by MALDI-TOF mass spectrometry. Anal Chem 73:4537–4544. https://doi.org/10.1021/ac0105181.
279. Zingue D, Flaudrops C, Drancourt M. 2016. Direct matrix-assisted laser desorption ionisation time-of-flight mass spectrometry identification of mycobacteria from colonies. Eur J Clin Microbiol Infect Dis 35:1983–1987. https://doi.org/10.1007/s10096-016-2750-5.
280. Sakyi SA, Aboagye SY, Otchere ID, Liao AM, Caltagirone IG, Yeboah-Manu D. 2016. RNA aptamer that specifically binds to mycolactone and serves as a diagnostic tool for diagnosis of Buruli ulcer. PLoS Negl Trop Dis 10:e0004950. https://doi.org/10.1371/journal.pntd.0004950.
281. Thangaraj HS, Adjei O, Allen BW, Portaels F, Evans MR, Banerjee DK, Wansbrough-Jones MH. 2000. In vitro activity of ciprofloxacin, sparfloxacin, ofloxacin, amikacin and rifampicin against Ghanaian isolates of Mycobacterium ulcerans. J Antimicrob Chemother 45:231–233. https://doi.org/10.1093/jac/45.2.231.
282. Ji B, Lefrançois S, Robert J, Chauffour A, Truffot C, Jarlier V. 2006. In vitro and in vivo activities of rifampin, streptomycin, amikacin, moxifloxacin, R207910, linezolid, and PA-824 against Mycobacterium ulcerans. Antimicrob Agents Chemother 50:1921–1926. https://doi.org/10.1128/AAC.00052-06.
283. Yeboah-Manu D, Kpeli GS, Ruf MT, Asan-Ampah K, Quenin-Fosu K, Owusu-Mireku E, Paintsil A, Lamptey I, Anku B, Kwakye-Maclean C, Newman M, Pluschke G. 2013. Secondary bacterial infections of buruli ulcer lesions before and after chemotherapy with streptomycin and rifampicin. PLoS Negl Trop Dis 7:e2191. https://doi.org/10.1371/journal.pntd.0002191.
284. Portaels F, Traore H, De Ridder K, Meyers WM. 1998. In vitro susceptibility of Mycobacterium ulcerans to clarithromycin. Antimicrob Agents Chemother 42:2070–2073.
285. Pattyn SR, van Ermengem J. 1968. DDS sensitivity of mycobacteria. Antagonistic effect of PABA for M. ulcerans and M. kansasii. Int J Lepr Other Mycobact Dis 36:427–431.
286. Portaels F, Van den Breen L, Pattyn SR. 1982. Sensitivity of mycobacteria to dapsone. Arzneimittelforschung 32:1124–1125.
287. Ji B, Chauffour A, Robert J, Lefrancois S, Jarlier V. 2007. Orally administered combined regimens for treatment of Mycobacterium ulcerans infection in mice. Antimicrob Agents Chemother 51:3737–3739. https://doi.org/10.1128/AAC.00730-07.
288. Almeida D, Converse PJ, Ahmad Z, Dooley KE, Nuermberger EL, Grosset JH. 2011. Activities of rifampin, rifapentine and clarithromycin alone and in combination against Mycobacterium ulcerans disease in mice. PLoS Negl Trop Dis 5:e933. https://doi.org/10.1371/journal.pntd.0000933.
289. Scherr N, Pluschke G, Thompson CJ, Ramón-García S. 2015. Selamectin is the avermectin with the best potential for Buruli ulcer treatment. PLoS Negl Trop Dis 9:e0003996. https://doi.org/10.1371/journal.pntd.0003996.
290. Omansen TF, Porter JL, Johnson PD, van der Werf TS, Stienstra Y, Stinear TP. 2015. In-vitro activity of avermectins against Mycobacterium ulcerans. PLoS Negl Trop Dis 9:e0003549. https://doi.org/10.1371/journal.pntd.0003549.
291. Hopla CE, Durden LA, Keirans JE. 1994. Ectoparasites and classification. Rev Sci Tech 13:985–1017. https://doi.org/10.20506/rst.13.4.815.
292. Beissner M, Awua-Boateng NY, Thompson W, Nienhuis WA, Klutse E, Agbenorku P, Nitschke J, Herbinger KH, Siegmund V, Fleischmann E, Adjei O, Fleischer B, van der Werf TS, Loscher T, Bretzel G. 2010. A genotypic approach for detection, identification, and characterization of drug resistance in Mycobacterium ulcerans in clinical samples and isolates from Ghana. Am J Trop Med Hyg 83:1059–1065. https://doi.org/10.4269/ajtmh.2010.10-0263.
293. Dega H, Bentoucha A, Robert J, Jarlier V, Grosset J. 2002. Bactericidal activity of rifampin-amikacin against Mycobacterium ulcerans in mice. Antimicrob Agents Chemother 46:3193–3196. https://doi.org/10.1128/AAC.46.10.3193-3196.2002.
294. Chauffour A, Robert J, Veziris N, Aubry A, Jarlier V. 2016. Sterilizing activity of fully oral intermittent regimens against Mycobacterium ulcerans infection in mice. PLoS Negl Trop Dis 10:e0005066. https://doi.org/10.1371/journal.pntd.0005066.
295. Friedman ND, Athan E, Walton AL, O'Brien DP. 2016. Increasing experience with primary oral medical therapy for Mycobacterium ulcerans disease in an Australian cohort. Antimicrob Agents Chemother 60:2692–2695. https://doi.org/10.1128/AAC.02853-15.
296. Cowan R, Athan E, Friedman ND, Hughes AJ, McDonald A, Callan P, Fyfe J, O'Brien DP. 2015. Mycobacterium ulcerans treatment—can antibiotic duration be reduced in selected patients? PLoS Negl Trop Dis 9:e0003503. https://doi.org/10.1371/journal.pntd.0003503.
297. Converse PJ, Tyagi S, Xing Y, Li SY, Kishi Y, Adamson J, Nuermberger EL, Grosset JH. 2015. Efficacy of rifampin plus clofazimine in a murine model of Mycobacterium ulcerans disease. PLoS Negl Trop Dis 9:e0003823. https://doi.org/10.1371/journal.pntd.0003823.
298. Junghanss T, Um Boock A, Vogel M, Schuette D, Weinlaeder H, Pluschke G. 2009. Phase change material for thermotherapy of Buruli ulcer: a prospective observational single centre proof-of-principle trial. PLoS Negl Trop Dis 3:17. https://doi.org/10.1371/journal.pntd.0000380.
299. WHO. 2012. Guidance for health workers. Treatment of Mycobacterium ulcerans disease (Buruli ulcer). World Health Organization, Geneva, Switzerland.
300. Chauty A, Ardant M-F, Adeye A, Euverte H, Guédénon A, Johnson C, Aubry J, Nuermberger E, Grosset J. 2007. Promising clinical efficacy of streptomycin-rifampin combination for treatment of Buruli ulcer (Mycobacterium ulcerans Disease). Antimicrob Agents Chemother 51:4029–4035. https://doi.org/10.1128/AAC.00175-07.
301. Teelken MA, Stienstra Y, Ellen DE, Quarshie E, Klutse E, van der Graaf WT, van der Werf TS. 2003. Buruli ulcer: differences in treatment outcome between two centres in Ghana. Acta Trop 88:51–56. https://doi.org/10.1016/S0001-706X(03)00170-0.
302. Klis S, Stienstra Y, Phillips RO, Abass KM, Tuah W, van der Werf TS. 2014. Long term streptomycin toxicity in the treatment of Buruli ulcer: follow-up of participants in the BURULICO drug trial. PLoS Negl Trop Dis 8:e2739. https://doi.org/10.1371/journal.pntd.0002739.
303. Eddyani M, Vandelannoote K, Meehan CJ, Bhuju S, Porter JL, Aguiar J, Seemann T, Jarek M, Singh M, Portaels F, Stinear TP, de Jong BC. 2015. A genomic approach to resolving relapse versus reinfection among four cases of Buruli ulcer. PLoS Negl Trop Dis 9:e0004158. https://doi.org/10.1371/journal.pntd.0004158.
304. Nienhuis WA, Stienstra Y, Abass KM, Tuah W, Thompson WA, Awuah PC, Awuah-Boateng NY, Adjei O, Bretzel G, Schouten JP, van der Werf TS. 2012. Paradoxical responses after start of antimicrobial treatment in Mycobacterium ulcerans infection. Clin Infect Dis 54:519–526. https://doi.org/10.1093/cid/cir856.
305. Sugawara M, Ishii N, Nakanaga K, Suzuki K, Umebayashi Y, Makigami K, Aihara M. 2015. Exploration of a standard treatment for Buruli ulcer through a comprehensive analysis of all cases diagnosed in Japan. J Dermatol 42:588–595. https://doi.org/10.1111/1346-8138.12851.
306. Chauty A, Ardant M-F, Marsollier L, Pluschke G, Landier J, Adeye A, Goundoté A, Cottin J, Ladikpo T, Ruf T, Ji B. 2011. Oral treatment for Mycobacterium ulcerans infection: results from a pilot study in Benin. Clin Infect Dis 52:94–96. https://doi.org/10.1093/cid/ciq072.
307. WHO. 2017. Neglected tropical diseases. Biennial meeting of the Global Buruli ulcer Initiative, Geneva, 20 to 22 March 2017. WHO, Geneva, Switzerland.
308. Nienhuis WA, Stienstra Y, Thompson WA, Awuah PC, Abass KM, Tuah W, Awua-Boateng NY, Ampadu EO, Siegmund V, Schouten JP, Adjei O, Bretzel G, van der Werf TS. 2010. Antimicrobial treatment for early, limited Mycobacterium ulcerans infection: a randomised controlled trial. Lancet 375:664–672. https://doi.org/10.1016/S0140-6736(09)61962-0.
309. Johnson PDR, Hayman JA, Quek TY, Fyfe JAM, Jenkin GA, Buntine JA, Athan E, Birrell M, Graham J, Lavender CJ on behalf of the Mycobacterium ulcerans Study Team. 2007. Consensus recommendations for the diagnosis, treatment and control of Mycobacterium ulcerans infection (Bairnsdale or Buruli ulcer) in Victoria, Australia. Med J Aust 186:64–68.
310. Braxmeier S, Hellmann M, Beck A, Umboock A, Pluschke G, Junghanss T, Weinlaeder H. 2009. Phase change material for thermotherapy of Buruli ulcer: modelling as an aid to implementation. J Med Eng. Technol 33:559–566. https://doi.org/10.1080/03091900903067457.
311. Rathnayake D, Sinclair R. 2014. Tropical and exotic dermatoses and ulcers. Aust Fam Physician 43:604–609.

zcm-cmr/zcm00118/zcm2618d18z | xppws | S=6 | 11/10/17 | 21:58 | 4/Color Fig: 1,2,3,4,5,6,7,8,9,10,11 | ArtID: 00045-17 | DOI:10.1128/CMR.00045-17 CE: msm

312. Amissah NA, Chlebowicz MA, Ablordey A, Tetteh CS, Prah I, van der Werf TS, Friedrich AW, van Dijl JM, Stienstra Y, Rossen JW. 2017. Virulence potential of Staphylococcus aureus isolates from Buruli ulcer patients. Int J Med Microbiol 307:223–232. https://doi.org/10.1016/j.ijmm.2017.04.002.
313. Yemoa A, Gbenou J, Affolabi D, Moudachirou M, Bigot A, Anagonou S, Portaels F, Quetin-Leclercq J, Martin A. 2011. Buruli ulcer: a review of in vitro tests to screen natural products for activity against Mycobacterium ulcerans. Planta Med 77:641–646. https://doi.org/10.1055/s-0030-1250642.
314. Johnson RC, Makoutode M, Hougnihin R, Guedenon A, Ifebe D, Boko M, Portaels F. 2004. Traditional treatment for Buruli ulcer in Benin. Med Trop 64:145–150. (In French.)
315. Webb BJ, Hauck FR, Houp E, Portaels F. 2009. Buruli ulcer in West Africa: strategies for early detection and treatment in the antibiotic era. East Afr J Public Health 6:144–147.
316. Kibadi K, Boelaert M, Kayinua M, Minuku JB, Muyembe-Tamfum JJ, Portaels F, Lefevre P. 2009. Therapeutic itineraries of patients with ulcerated forms of Mycobacterium ulcerans (Buruli ulcer) disease in a rural health zone in the Democratic Republic of Congo. Trop Med Int Health 14:1110–1116. https://doi.org/10.1111/j.1365-3156.2009.02324.x.
317. Aujoulat I, Johnson C, Zinsou C, Guedenon A, Portaels F. 2003. Psychosocial aspects of health seeking behaviours of patients with Buruli ulcer in southern Benin. Trop Med Int Health 8:750–759. https://doi.org/10.1046/j.1365-3156.2003.01089.x.
318. Yemoa A, Gbenou J, Affolabi D, Moudachirou M, Bigot A, Anagonou S, Portaels F, Martin A, Quetin-Leclercq J. 2015. Beninese medicinal plants as a source of antimycobacterial agents: bioguided fractionation and in vitro activity of alkaloids isolated from Holarrhena floribunda used in traditional treatment of Buruli ulcer. Biomed Res Int 2015:835767. https://doi.org/10.1155/2015/835767.
319. Tsouh Fokou PV, Nyarko AK, Appiah-Opong R, Tchokouaha Yamthe LR, Addo P, Asante IK, Boyom FF. 2015. Ethnopharmacological reports on anti-Buruli ulcer medicinal plants in three West African countries. J Ethnopharmacol 172:297–311. https://doi.org/10.1016/j.jep.2015.06.024.
320. Tsouh Fokou PV, Kissi-Twum AA, Yeboah-Manu D, Appiah-Opong R, Addo P, Tchokouaha Yamthe LR, Ngoutane Mfopa A, Fekam Boyom F, Nyarko AK. 2016. In vitro activity of selected West African medicinal plants against Mycobacterium ulcerans disease. Molecules 21:445. https://doi.org/10.3390/molecules21040445.
321. O'Brien DP, Jenkin G, Buntine J, Steffen CM, McDonald A, Horne S, Friedman ND, Athan E, Hughes A, Callan PP, Johnson PD. 2014. Treatment and prevention of Mycobacterium ulcerans infection (Buruli ulcer) in Australia: guideline update. Med J Aust 200:267–270. https://doi.org/10.5694/mja13.11331.
322. WHO. 2012. Treatment of Mycobacterium ulcerans disease (Buruli ulcer): guidance for health workers, p 73. World Health Organization, Geneva, Switzerland.
323. Hart BE, Hale LP, Lee S. 2015. Recombinant BCG expressing Mycobacterium ulcerans Ag85A imparts enhanced protection against experimental Buruli ulcer. PLoS Negl Trop Dis 9:e0004046. https://doi.org/10.1371/journal.pntd.0004046.
324. Ahorlu CK, Koka E, Yeboah-Manu D, Lamptey I, Ampadu E. 2013. Enhancing Buruli ulcer control in Ghana through social interventions: a case study from the Obom sub-district. BMC Public Health 13:59. https://doi.org/10.1186/1471-2458-13-59.
325. Roupie V, Pidot SJ, Einarsdottir T, Van Den Poel C, Jurion F, Stinear TP, Huygen K. 2014. Analysis of the vaccine potential of plasmid DNA encoding nine mycolactone polyketide synthase domains in Mycobacterium ulcerans infected mice. PLoS Negl Trop Dis 8:e2604. https://doi.org/10.1371/journal.pntd.0002604.
326. Tanghe A, Content J, Van Vooren JP, Portaels F, Huygen K. 2001. Protective efficacy of a DNA vaccine encoding antigen 85A from Mycobacterium bovis BCG against Buruli ulcer. Infect Immun 69:5403–5411. https://doi.org/10.1128/IAI.69.9.5403-5411.2001.
327. Tanghe A, Dangy JP, Pluschke G, Huygen K. 2008. Improved protective efficacy of a species-specific DNA vaccine encoding mycolyl-transferase Ag85A from Mycobacterium ulcerans by homologous protein boosting. PLoS Negl Trop Dis 2:199. https://doi.org/10.1371/journal.pntd.0000199.
328. Tanghe A, Adnet PY, Gartner T, Huygen K. 2007. A booster vaccination with Mycobacterium bovis BCG does not increase the protective effect of the vaccine against experimental Mycobacterium ulcerans infection in mice. Infect Immun 75:2642–2644. https://doi.org/10.1128/IAI.01622-06.
329. Watanabe M, Nakamura H, Nabekura R, Shinoda N, Suzuki E, Saito H. 2015. Protective effect of a dewaxed whole-cell vaccine against Mycobacterium ulcerans infection in mice. Vaccine 33:2232–2239. https://doi.org/10.1016/j.vaccine.2015.03.046.
330. Bolz M, Kerber S, Zimmer G, Pluschke G. 2015. Use of recombinant virus replicon particles for vaccination against Mycobacterium ulcerans disease. PLoS Negl Trop Dis 9:e0004011. https://doi.org/10.1371/journal.pntd.0004011.
331. van der Werf TS, Stienstra Y, Johnson RC, Phillips R, Adjei O, Fleischer B, Wansbrough-Jones MH, Johnson PD, Portaels F, van der Graaf WT, Asiedu K. 2005. Mycobacterium ulcerans disease. Bull World Health Organ 83:785–791.
332. Aguiar J, Domingo M, Guédénon A, Meyers W, Steunou C, Portaels F. 1997. L'ulcère de Buruli, une maladie mycobactérienne importante et en recrudescence au Bénin. Bull Seances Acad R Sci Outre Mer 3:325–356.
333. Amofah GK, Sagoe-Moses C, Adjei-Acquah C, Frimpong EH. 1993. Epidemiology of Buruli ulcer in Amansie West district, Ghana. Trans R Soc Trop Med Hyg 87:644–645. https://doi.org/10.1016/0035-9203(93)90272-R.
334. Marston BJ, Diallo MO, Horsburgh CR, Diomande I, Saki MZ, Kanga J-M, Patrice GB, Lipman HB, Ostroff SM, Good RC. 1995. Emergence of Buruli ulcer disease in the Daloa region of Cote D'Ivoire. Am J Trop Med Hyg 52:219–224. https://doi.org/10.4269/ajtmh.1995.52.219.
335. Ouoba K, Sano D, Traore A, Ouedraogo R, Sakande B, Sanou A. 1998. Buruli ulcers in Burkina Faso: a propos of 6 cases. Tunis Med 76:46–50.
336. Coulibaly-N'Golo GM, Ekaza E, Coulibaly B, Aka N, N'Guessan RK, Thiberge JM, Caro V, Brisse S, Bretin-Dosso M. 2011. Multilocus VNTR analysis of Mycobacterium ulcerans strains isolated in Cote d'Ivoire. J Infect Dev Ctries 5:59–63.
337. Muelder K. 1988. Buruli ulcer in Benin. Trop Doct 18:53. https://doi.org/10.1177/004947558801800204.
338. Oluwasanmi JO, Solanke TF, Olurin EO, Itayemi SO, Alabi GO, Lucas AO. 1976. Mycobacterium ulcerans (Buruli) skin ulceration in Nigeria. Am J Trop Med Hyg 25:122–128. https://doi.org/10.4269/ajtmh.1976.25.122.
339. Bayley AC. 1971. Buruli ulcer in Ghana. Br Med J 2:401–402. https://doi.org/10.1136/bmj.2.5758.401-c.
340. van der Werf TS, van der Graaf WT, Groothuis DG, Knell AJ. 1989. Mycobacterium ulcerans infection in Ashanti region, Ghana. Trans R Soc Trop Med Hyg 83:410–413. https://doi.org/10.1016/0035-9203(89)90521-X.
341. Monson MH, Gibson DW, Connor DH, Kappes R, Hienz HA. 1984. Mycobacterium ulcerans in Liberia: a clinicopathologic study of 6 patients with Buruli ulcer. Acta Trop 41:165–172.
342. Meyers WM, Tignokpa N, Priuli GB, Portaels F. 1996. Mycobacterium ulcerans infection (Buruli ulcer): first reported patients in Togo. Brit J Dermatol 134:1116–1121. https://doi.org/10.1046/j.1365-2133.1996.d01-914.x.
343. Ratmanov P, Mediannikov O, Raoult D. 2013. Vectorborne diseases in West Africa: geographic distribution and geospatial characteristics. Trans R Soc Trop Med Hyg 107:273–284. https://doi.org/10.1093/trstmh/trt020.
344. WHO. 2015. World distribution of Buruli ulcer, 2014. Abstr WHO Annu Meet Buruli Ulcer. AQ: R
345. Bessis D, Kempf M, Marsollier L. 2015. Mycobacterium ulcerans disease (Buruli ulcer) in Mali: a new potential African endemic country. Acta Dermatol Venereol 95:489–490. https://doi.org/10.2340/00015555-1942.
346. Gray HH, Kingma S, Kok SH. 1967. Mycobacterial skin ulcers in Nigeria. Trans R Soc Trop Med Hyg 61:712–714. https://doi.org/10.1016/0035-9203(67)90139-3.
347. Johnson PDR, Stinear T, Small PLC, Pluschke G, Merritt RW, Portaels F, Huygen K, Hayman JA, Asiedu K. 2005. Buruli ulcer (M. ulcerans infection): new insights, new hope for disease control. PLoS Med 2:e108. https://doi.org/10.1371/journal.pmed.0020108.
348. Minime-Lingoupou F, Beyam N, Zandanga G, Manirakiza A, N'Domackrah A, Njuimo S, Eyangoh S, Cottin J, Marsollier L, Marion E, Portaels F, Le Faou A, Bercion R. 2010. Buruli ulcer, Central African Republic. Emerg Infect Dis 16:746–748. https://doi.org/10.3201/eid1604.090195.
349. Kibadi K, Panda M, Tamfum J-JM, Fraga AG, Filho AL, Anyo G, Pedrosa J, Nakazawa Y, Suykerbuyk P, Meyers WM, Portaels F. 2008. New foci of Buruli ulcer, Angola and Democratic Republic of Congo. Emerg Infect Dis 14:1790–1792. https://doi.org/10.3201/eid1411.071649.

zcm-cmr/zcm00118/zcm2618d18z | xppws | S=6 | 11/10/17 | 21:58 | 4/Color Fig: 1,2,3,4,5,6,7,8,9,10,11 | ArtID: 00045-17 | DOI:10.1128/CMR.00045-17 CE: msm

350. Marion E, Obvala D, Babonneau J, Kempf M, Asiedu KB, Marsollier L. 2014. Buruli ulcer disease in Republic of the Congo. Emerg Infect Dis 20:1070–1072. https://doi.org/10.3201/eid2006.131498.
351. Ravisse P. 1977. L'ulcere cutane a Mycobacterium ulcerans au Cameroun. I. Etude clinique, epidemiologique et histologique. Bull Soc Pathol Exot 70:109–124.
352. Janssens PG PS, Meyers WM, Portaels F. 2005. Buruli ulcer: an historical overview with updating to 2005. Bull Seances Acad R Sci Outre Mer 51:165–199.
353. Bar W, Rusch-Gerdes S, Richter E, Marquez de Bar G, Dittmer C, Papsdorf H, Stosiek P, de Rijk PB, Meyers WM, Portaels F. 1998. Mycobacterium ulcerans infection in a child from Angola: diagnosis by direct detection and culture. Trop Med Int Health 3:189–196. https://doi.org/10.1046/j.1365-3156.1998.00225.x.
354. Walsh DS, Eyase F, Onyango D, Odindo A, Otieno W, Waitumbi JN, Bulimo WD, Schnabel DC, Meyers WM, Portaels F. 2009. Clinical and molecular evidence for a case of Buruli ulcer (Mycobacterium ulcerans infection) in Kenya. Am J Trop Med Hyg 81:1110–1113. https://doi.org/10.4269/ajtmh.2009.09-0313.
355. Komolafe OO. 2001. Buruli ulcer in Malawi—a first report. Malawi Med J 13:37–38. https://doi.org/10.4314/mmj.v13i2.10829.
356. Stienstra Y, van der Graaf WT, Asamoa K, van der Werf TS. 2002. Beliefs and attitudes toward Buruli ulcer in Ghana. Am J Trop Med Hyg 67:207–213. https://doi.org/10.4269/ajtmh.2002.67.207.
357. O'Brien DP, Robson M, Friedman ND, Walton A, McDonald A, Callan P, Hughes A, Rahdon R, Athan E. 2013. Incidence, clinical spectrum, diagnostic features, treatment and predictors of paradoxical reactions during antibiotic treatment of Mycobacterium ulcerans infections. BMC Infect Dis 13:416–416. https://doi.org/10.1186/1471-2334-13-416.
358. Faber WR, Arias-Bouda LM, Zeegelaar JE, Kolk AH, Fonteyne PA, Toonstra J, Portaels F. 2000. First reported case of Mycobacterium ulcerans infection in a patient from China. Trans R Soc Trop Med Hyg 94:277–279. https://doi.org/10.1016/S0035-9203(00)90320-1.
359. Mikoshiba H, Shindo Y, Matsumoto H, Mochizuki M, Tsukamura M. 1982. A case of typical mycobacteriosis due to Mycobacterium ulcerans-like organism. Nihon Hifuka Gakkai Zasshi 92:557–565. (In Japanese.)
360. Tsukamura M, Kaneda K, Imaeda T, Mikoshiba H. 1989. A taxonomic study on a mycobacterium which caused a skin ulcer in a Japanese girl and resembled Mycobacterium ulcerans. Kekkaku 64:691–697. (In Japanese.)
361. Nakanaga K, Hoshino Y, Yotsu RR, Makino M, Ishii N. 2011. Nineteen cases of Buruli ulcer diagnosed in Japan from 1980 to 2010. J Clin Microbiol 49:3829–3836. https://doi.org/10.1128/JCM.00783-11.
362. Pettit JH, Marchette NJ, Rees RJ. 1966. Mycobacterium ulcerans infection. Clinical and bacteriological study of the first cases recognized in South East Asia. Br J Dermatol 78:187–197.
363. Christie M. 1987. Suspected Mycobacterium ulcerans disease in Kiribati. Med J Aust 146:600–604.
364. Jacquemart Y, Josse R. 2002. Papua New Guinea. Med Trop 62:583–588.
365. Sambourg E, Dufour J, Edouard S, Morris A, Mosnier E, Reynaud Y, Sainte-Marie D, Nacher M, Guegan JF, Couppie P. 2014. Paradoxical reactions and responses during antibiotic treatment for Mycobacterium ulcerans infection (Buruli ulcer). Four cases from French Guiana. Ann Dermatol Venereol 141:413–418. (In French.) https://doi.org/10.1016/j.annder.2014.01.010.
366. Guerra H, Palomino JC, Falconi E, Bravo F, Donaires N, Van Marck E, Portaels F. 2008. Mycobacterium ulcerans disease, Peru. Emerg Infect Dis 14:373–377. https://doi.org/10.3201/eid1403.070904.
367. dos Santos VM, Noronha FL, Vicentina EC, Lima CC. 2007. Mycobacterium ulcerans infection in Brazil. Med J Aust 187:63–64.
368. Reynaud Y, Millet J, Couvin D, Rastogi N, Brown C, Couppié P, Legrand E. 2015. Heterogeneity among Mycobacterium ulcerans from French Guiana revealed by multilocus variable number tandem repeat analysis (MLVA). PLoS One 10:e0118597. https://doi.org/10.1371/journal.pone.0118597.
369. Osei FB, Duker AA. 2015. Analysis of Buruli ulcer prevalence in Amansie West District: a geostatistical approach. Austin Biom Biostat 2:1011.
370. Quek TYJ, Henry MJ, Pasco JA, O'Brien DP, Johnson PDR, Hughes A, Cheng AC, Redden-Hoare J, Athan E. 2007. Mycobacterium ulcerans infection: factors influencing diagnostic delay. Med J Aust 187:561–563.
371. van Ravensway J, Benbow ME, Tsonis AA, Pierce SJ, Campbell LP, Fyfe JA, Hayman JA, Johnson PD, Wallace JR, Qi J. 2012. Climate and landscape factors associated with Buruli ulcer incidence in Victoria, Australia. PLoS One 7:e51074. https://doi.org/10.1371/journal.pone.0051074.
372. Brou T, Broutin H, Elguero E, Asse H, Guegan J-F. 2008. Landscape diversity related to Buruli ulcer disease in Côte d'Ivoire. PLoS Negl Trop Dis 2:e271. https://doi.org/10.1371/journal.pntd.0000271.
373. Yoshikawa T, Kaizuka S, Ōta Y. 1981. Landforms of Japan. University of Tokyo Press, Tokyo, Japan.
374. Herman JS, Chiodini PL. 2011. West Africa, p 128–138. *In* Petersen E, Chen LH, Schlagenhauf P (ed), West Africa, infectious diseases: a geographic guide. Wiley-Blackwell, Oxford, United Kingdom.
375. Sopoh GE, Johnson RC, Anagonou SY, Barogui YT, Dossou AD, Houézo JG, Phanzu DM, Tente BH, Meyers WM, Portaels F. 2011. Buruli ulcer prevalence and altitude, Benin. Emerg Infect Dis 17:153–154. https://doi.org/10.3201/eid1701.100644.
376. Muelder K, Nourou A. 1990. Buruli ulcer in Benin. Lancet 336:1109–1111. https://doi.org/10.1016/0140-6736(90)92581-2.
377. Landier J, Constantin de Magny G, Garchitorena A, Guegan JF, Gaudart J, Marsollier L, Le Gall P, Giles-Vernick T, Eyangoh S, Fontanet A, Texier G. 2015. Seasonal patterns of Buruli ulcer incidence, Central Africa, 2002–2012. Emerg Infect Dis 21:1414–1417. https://doi.org/10.3201/eid2108.141336.
378. Landier J, Gaudart J, Carolan K, Lo Seen D, Guégan J-F, Eyangoh S, Fontanet A, Texier G. 2014. Spatio-temporal patterns and landscape-associated risk of Buruli ulcer in Akonolinga, Cameroon. PLoS Negl Trop Dis 8:e3123. https://doi.org/10.1371/journal.pntd.0003123.
379. Portaels F. 1995. Epidemiology of mycobacterial diseases. Clin Dermatol 13:207–222. https://doi.org/10.1016/0738-081X(95)00004-Y.
380. Debacker M, Aguiar J, Steunou C, Zinsou C, Meyers WM, Scott JT, Dramaix M, Portaels F. 2004. Mycobacterium ulcerans disease: role of age and gender in incidence and morbidity. Trop Med Int Health 9:1297–1304. https://doi.org/10.1111/j.1365-3156.2004.01339.x.
381. Roltgen K, Bratschi MW, Ross A, Aboagye SY, Ampah KA, Bolz M, Andreoli A, Pritchard J, Minyem JC, Noumen D, Koka E, Um Boock A, Yeboah-Manu D, Pluschke G. 2014. Late onset of the serological response against the 18 kDa small heat shock protein of Mycobacterium ulcerans in children. PLoS Negl Trop Dis 8:e2904. https://doi.org/10.1371/journal.pntd.0002904.
382. Ampah KA, Nickel B, Asare P, Ross A, De-Graft D, Kerber S, Spallek R, Singh M, Pluschke G, Yeboah-Manu D, Roltgen K. 2016. A sero-epidemiological approach to explore transmission of Mycobacterium ulcerans. PLoS Negl Trop Dis 10:e0004387. https://doi.org/10.1371/journal.pntd.0004387.
383. UBG. 1971. The Uganda Buruli Group. Epidemiology of Mycobacterium ulcerans infection (Buruli ulcer) at Kinyara, Uganda. Trans R Soc Trop Med Hyg 65:763–775.
384. Sopoh GE, Barogui YT, Johnson RC, Dossou AD, Makoutodé M, Anagonou SY, Kestens L, Portaels F. 2010. Family relationship, water contact and occurrence of Buruli ulcer in Benin. PLoS Negl Trop Dis 4:e746. https://doi.org/10.1371/journal.pntd.0000746.
385. Mensah-Quainoo EK. 1998. A study of the magnitude and determinants of Buruli ulcer disease in the Ga District of Ghana. Abstr Int Conf Buruli Ulcer Contr Res, Yamoussoukro, Cote d'Ivoire, 6 to 8 July 1998.
386. Aujoulat I, Huguet-Ribas M-P, Koita Y. 1996. L'ulcère de Buruli: un problème de santé publique méconnu appellant une mobilization internationale. Développement et santé. Rev Int Perfect Méd Sanit 125:22–30.
387. Peraudin ML, Herrault A, Desbois JC. 1980. Ulcère cutanée à Mycobacterium ulcerans (ulcère de Buruli). Ann Pediatr (Paris) 27:687–692.
388. Ziefer AM, Connor DH, Gibson DW. 1981. Mycobacterium ulcerans. Infection of two patients in Liberia. Int J Dermatol 20:362–367. https://doi.org/10.1111/j.1365-4362.1981.tb00822.x.
389. Johnson RC, Makoutodé M, Sopoh GE, Elsen P, Gbovi J, Pouteau LH, Meyers WM, Boko M, Portaels F. 2005. Buruli ulcer distribution in Benin. Emerg Infect Dis 11:500–501. https://doi.org/10.3201/eid1103.040597.
390. Stoffel V, Barthelme B, Chague F. 2005. Tropical ecopathology: up hill and down dale Buruli ulcer. Sante Publique 17:191–197. https://doi.org/10.3917/spub.052.0191.
391. Gibson J. 1975. Buruli ulcers in Bo. Bull Sierra Leone Med Dental Assoc 2:64–66.
392. Stienstra Y, van der Werf TS, Oosterom E, Nolte IM, van der Graaf WT, Etuaful S, Raghunathan PL, Whitney EA, Ampadu EO, Asamoa K, Klutse EY, te Meerman GJ, Tappero JW, Ashford DA, van der Steege G. 2006. Susceptibility to Buruli ulcer is associated with the SLC11A1 (NRAMP1)

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D543N polymorphism. Genes Immun 7:185–189. https://doi.org/10.1038/sj.gene.6364281.
393. Smith PG, Revill WD, Lukwago E, Rykushin YP. 1976. The protective effect of BCG against Mycobacterium ulcerans disease: a controlled trial in an endemic area of Uganda. Trans R Soc Trop Med Hyg 70:449–457. https://doi.org/10.1016/0035-9203(76)90128-0.
394. Collins FH, Paskewitz SM. 1995. Malaria: current and future prospects for control. Annu Rev Entomol 40:195–219. https://doi.org/10.1146/annurev.en.40.010195.001211.
395. WHO. 2003. Climate change and human health: risks and responses. WHO, Geneva, Switzerland.
396. Wallace JR, Gordon MC, Hartsell L, Mosi L, Benbow ME, Merritt RW, Small PLC. 2010. Interaction of Mycobacterium ulcerans with mosquito species: implications for transmission and trophic relationships. Appl Environ Microbiol 76:6215–6222. https://doi.org/10.1128/AEM.00340-10.
397. WHO. 2002. Instructions for treatment and use of insecticide-treated mosquito nets. Use insecticide-treated mosquito nets to sleep in peace—and protect your health. WHO, Geneva, Switzerland.
398. WHO/GMP. 2009. Global Malaria Programme. Insecticide-treated mosquito nets: a WHO position statement. WHO, Geneva, Switzerland. http://files.givewell.org/files/DWDA%202009/Interventions/Nets/itnspospaperfinal.pdf.
399. OAU. 4 June 2000. African Summit on Roll Back Malaria. The Abuja Declaration on Roll Back Malaria in Africa by the African Heads of State and Government. USAID, Washington, DC. https://www.usaid.gov/sites/default/files/documents/1864/abuja.pdf.
400. WHO. 2011. The use of DDT in malaria vector control: World Health Organization position statement. WHO, Geneva, Switzerland.
401. Cano J, Rebollo MP, Golding N, Pullan RL, Crellen T, Soler A, Hope LAK, Lindsay SW, Hay SI, Bockarie MJ, Brooker SJ. 2014. The global distribution and transmission limits of lymphatic filariasis: past and present. Parasit Vectors 7:466. https://doi.org/10.1186/s13071-014-0466-x.
402. WHO. 2016. Lymphatic filariasis. Fact Sheet 102. WHO, Geneva, Switzerland.
403. WHO. 2010. Global programme to eliminate lymphatic filariasis. WHO GPELF Progress Report 2000–2009 and strategic plan 2010–2020. WHO, Geneva, Switzerland.
404. Abdullah S, Adazu K, Masanja H, Diallo D, Hodgson A, Ilboudo-Sanogo E, Nhacolo A, Owusu-Agyei S, Thompson R, Smith T, Binka FN. 2007. Patterns of age-specific mortality in children in endemic areas of Sub-Saharan Africa. Am J Trop Med Hyg 77:99–105. https://doi.org/10.1016/0035-9203(83)90028-7.
405. Boakye DA, Wilson MD, Appawu MA, Gyapong J. 2004. Vector competence, for Wuchereria bancrofti, of the Anopheles populations in the Bongo district of Ghana. Ann Trop Med Parasitol 98:501–508. https://doi.org/10.1179/000349804225003514.
406. World Health Assembly. 1997. Elimination of lymphatic filariasis as a public health problem. World Health Assembly Resolution and Decision WHA50.29. WHO, Geneva, Switzerland. http://www.who.int/neglected_diseases/mediacentre/WHA_50.29_Eng.pdf.
407. WHO. 2006. Preventive chemotherapy in human helminthiasis. Coordinated use of anthelminthic drugs in control interventions: a manual for health professionals and programme managers. WHO, Geneva, Switzerland.
408. de Souza DK, Koudou B, Kelly-Hope LA, Wilson MD, Bockarie MJ, Boakye DA. 2012. Diversity and transmission competence in lymphatic filariasis vectors in West Africa, and the implications for accelerated elimination of Anopheles-transmitted filariasis. Parasit Vectors 5:259. https://doi.org/10.1186/1756-3305-5-259.
409. Stienstra Y, van der Graaf WT, te Meerman GJ, The TH, de Leij LF, van der Werf TS. 2001. Susceptibility to development of Mycobacterium ulcerans disease: review of possible risk factors. Trop Med Int Health 6:554–562. https://doi.org/10.1046/j.1365-3156.2001.00746.x.
410. Hotez PJ, Kamath A. 2009. Neglected tropical diseases in sub-Saharan Africa: review of their prevalence, distribution, and disease burden. PLoS Negl Trop Dis 3:e412. https://doi.org/10.1371/journal.pntd.0000412.
411. Imevbore A, Ofoezie I, Obot E. 1988. Vector-borne disease problems of small scale water resources development projects in Kano State: snail vectors of schistosomiasis. Afrancet 1:17–23.
412. Akinwale OP, Ajayi MB, Akande DO, Gyang PV, Adeleke MA, Adeneye AK, Adebayo MO, Dike AA. 2010. Urinary schistosomiasis around Oyan Reservoir, Nigeria: twenty years after the first outbreak. Iranian J Public Health 39:92–95.
413. Scott JT, Johnson RC, Aguiar J, Debacker M, Kestens L, Guedenon A, Gryseels B, Portaels F. 2004. Schistosoma haematobium Infection and Buruli ulcer. Emerg Infect Dis 10:551–552. https://doi.org/10.3201/eid1003.020514.
414. Stienstra Y, van der Werf TS, van der Graaf WT, Secor WE, Kihlstrom SL, Dobos KM, Asamoa K, Quarshi E, Etuaful SN, Klutse EY, King CH. 2004. Buruli ulcer and schistosomiasis: no association found. Am J Trop Med Hyg 71:318–321.
415. Kimutai A, Ngure P, Tonui W, Gicheru M, Nyamwamu L. 2009. Leishmaniasis in Northern and Western Africa: a review. Afr J Infect Dis 3:14–25.
416. Nzelu CO, Kato H, Puplampu N, Desewu K, Odoom S, Wilson MD, Sakurai T, Katakura K, Boakye DA. 2014. First detection of Leishmania tropica DNA and Trypanosoma species in Sergentomyia sand flies (Diptera: Psychodidae) from an outbreak area of cutaneous leishmaniasis in Ghana. PLoS Negl Trop Dis 8:e2630. https://doi.org/10.1371/journal.pntd.0002630.
417. Kweku MA, Odoom S, Puplampu N, Desewu K, Nuako GK, Gyan B, Raczniak G, Kronmann KC, Koram K, Botero S, Boakye D, Akuffo H. 2011. An outbreak of suspected cutaneous leishmaniasis in Ghana: lessons learnt and preparation for future outbreaks. Global Health Action 2011:4. https://doi.org/10.3402/gha.v4i0.5527.
418. Romero GAS, Boelaert M. 2010. Control of visceral leishmaniasis in Latin America—a systematic review. PLoS Negl Trop Dis 4:e584. https://doi.org/10.1371/journal.pntd.0000584.
419. Veland N, Valencia BM, Alba M, Adaui V, Llanos-Cuentas A, Arevalo J, Boggild AK. 2013. Simultaneous infection with Leishmania (Viannia) braziliensis and L. (V.) lainsoni in a Peruvian patient with cutaneous leishmaniasis. Am J Trop Med Hyg 88:774–777. https://doi.org/10.4269/ajtmh.12-0594.
420. Adekambi T, Ben Salah S, Khlif M, Raoult D, Drancourt M. 2006. Survival of environmental mycobacteria in Acanthamoeba polyphaga. Appl Environ Microbiol 72:5974–5981. https://doi.org/10.1128/AEM.03075-05.
421. Gupta T, Fine-Coulson K, Karls R, Gauthier D, Quinn F. 2013. Internalization of Mycobacterium shottsii and Mycobacterium pseudoshottsii by Acanthamoeba polyphaga. Can J Microbiol 59:570–576. https://doi.org/10.1139/cjm-2013-0079.
422. Steinert M, Birkness K, White E, Fields B, Quinn F. 1998. Mycobacterium avium bacilli grow saprozoically in coculture with Acanthamoeba polyphaga and survive within cyst walls. Appl Environ Microbiol 64:2256–2261.
423. Lamrabet O, Mba Medie F, Drancourt M. 2012. Acanthamoeba polyphaga-enhanced growth of Mycobacterium smegmatis. PLoS One 7:11. https://doi.org/10.1371/journal.pone.0029833.
424. Wilson MD, Boakye DA, Mosi L, Asiedu K. 2011. In the case of transmission of Mycobacterium ulcerans in Buruli ulcer disease, Acanthamoeba species stand accused. Ghana Med J 45:31–34. https://doi.org/10.4314/gmj.v45i1.68920.
425. Aka P. 2002. Buruli ulcer, an emerging disease in public health in Cote D'Ivoire (Ivory Coast). Forum Nord Derm Ven 7:22–26.
426. Affolabi D, Tanimomo-Kledjo B, Anyo G, Johnson RC, Anagonou SY, Portaels F. 2008. Setting up a national reference laboratory for Buruli ulcer: the case of Benin. Trop Med Int Health 13:365–368. https://doi.org/10.1111/j.1365-3156.2008.02011.x.
427. Gullan PJ, Cranston PS. 2014. The insects: an outline of entomology, 5th ed, p 190–226. Wiley-Blackwell, Oxford, United Kingdom.
428. Eddyani M, Ofori-Adjei D, Teugels G, De Weirdt D, Boakye D, Meyers WM, Portaels F. 2004. Potential role for fish in transmission of Mycobacterium ulcerans disease (Buruli ulcer): an environmental study. Appl Environ Microbiol 70:5679–5681. https://doi.org/10.1128/AEM.70.9.5679-5681.2004.

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Buruli Ulcer, a Prototype for Ecosystem-Related Infection, Caused by *Mycobacterium ulcerans*

Dezemon Zingue, Amar Bouam, Roger B. D. Tian, Michel Drancourt

Summary: Buruli ulcer is a noncontagious disabling cutaneous and subcutaneous mycobacteriosis reported by 33 countries in Africa, Asia, Oceania, and South America. The causative agent, *Mycobacterium ulcerans*, derives from *Mycobacterium marinum* by genomic reduction and acquisition of a plasmid-borne, nonribosomal cytotoxin mycolactone, the major virulence factor. *M. ulcerans*-specific sequences have been readily detected in aquatic environments in food chains involving small mammals. Skin contamination combined with any type of puncture, including insect bites, is the most plausible route of transmission, and skin temperature of <30°C significantly correlates with the topography of lesions. After 30 years of emergence and increasing prevalence between 1970 and 2010, mainly in Africa, factors related to ongoing decreasing prevalence in the same countries remain unexplained. Rapid diagnosis, including laboratory confirmation at the point of care, is mandatory in order to reduce delays in effective treatment. Parenteral and potentially toxic streptomycin-rifampin is to be replaced by oral clarithromycin or fluoroquinolone combined with rifampin. In the absence of proven effective primary prevention, avoiding skin contamination by means of clothing can be implemented in areas of endemicity. Buruli ulcer is a prototype of ecosystem pathology, illustrating the impact of human activities on the environment as a source for emerging tropical infectious diseases.

AUTHOR QUERIES

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AQE—I am unsure how the "detection of related mycolactone-producing mycobacteria" in the caption of Table 2 relates to the table. Clarification needed?

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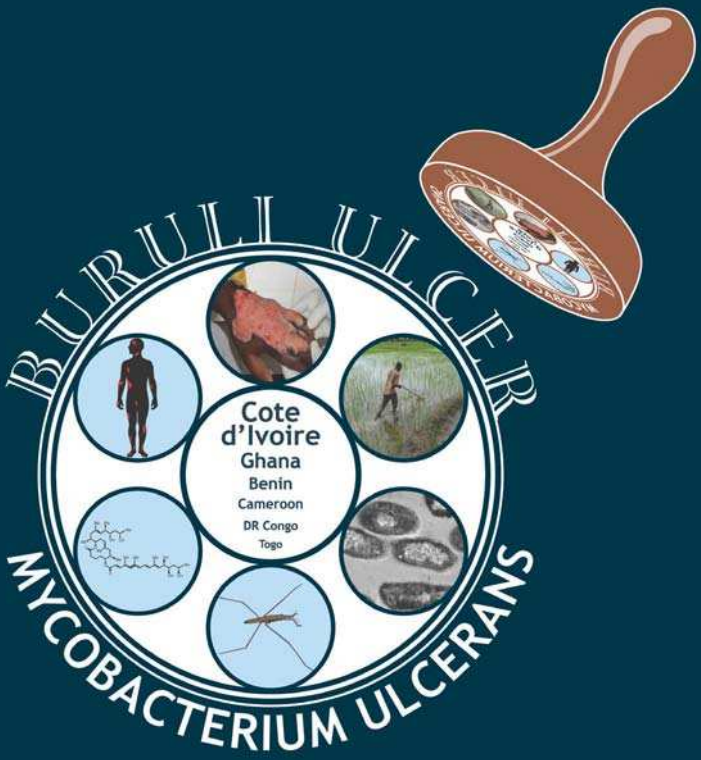

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Article 2: Climate change, global warming and incidence of Buruli ulcer in Africa

American Journal of Tropical Medicine & Hygiene

(submitted)

From our review, it emerged that since the year 2010, a regular decrease in the incidence of cases of Buruli ulcer was noted; this was particularly true in Africa that pays the heaviest tribe for this disabling disease. Until now, the causes of this regular decline are unknown. We have asked ourselves what factors may be responsible for this decrease. A number of factors have been identified, including the mass administration of ivermectin in Africa against filariasis, improvements in the activities of the various Buruli ulcer control programs, and climate change. The last factor was all the more attractive because there was some paralleling between the temperature anomalies and the incidence of Buruli ulcer. Based on WHO data for Buruli ulcer cases reported each year for two decades, as well as temperature anomaly data, we found that there was an inverse correlation between the two types of data.

Global warming could be a significant cause of the decline in the incidence of Buruli ulcer and this is all the more plausible as the growth of *M. ulcerans* is not optimal from 37 °C and more.

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Climate change, global warming and incidence of Buruli ulcer in Africa

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Abstract

Mycobacterium ulcerans is an environmental mycobacterium responsible of Buruli ulcer, a World Health Organization-notifiable chronic infection which is mainly reported in tropical areas in West Africa, Central Africa and certain regions of Australia and Japan. *M. ulcerans* lives in aquatic environments and we hypothesized that global warming may affect environmental *M. ulcerans* ecological niches and thus the epidemiology of Buruli ulcer. We recovered WHO data of Buruli ulcer incidence at http://apps.who.int/gho/data/node.main.A1631?lang=en and temperature trend from 2002 to 2015 in countries where Buruli ulcer is endemic at https://www.ncdc.noaa.gov/sotc/. Correlations between annual temperature and the incidence of Buruli ulcer were analyzed by inverse regression. Decrease in the worldwide incidence of Buruli ulcer began in 2010, from an average of 5,092 cases in 2007-2009 down to 4,043 cases in 2010-2012 and an on-going trend of decreasing incidence curve. Then, the incidence of Buruli ulcer showed an inverse correlation with temperature trend. This inverse correlation was of 0.30; 0.75 without the 2010 values and of 0.9 without the 2009-2010 values. Warming may affect the temperature-sensitive survival of *M. ulcerans* in its reservoirs and vectors, the distribution of these reservoirs and vectors and the exposure of populations to them. These environmental factors have to be taken into consideration for the accurate evaluation of public health interventions in endemic countries.

43 INTRODUCTION

44 *Mycobacterium ulcerans* is a non-tuberculous mycobacterium causing Buruli ulcer (BU), a
45 tropical disease classified as a neglected one by the World Health Organization in 1997 during
46 the Yamoussoukro Conference.[1] BU is notified to the WHO by a total of 33 countries in Africa,
47 Oceania, South-East Asia, South America and Western Pacific Regions.[2-6] It occurs in tropical
48 and sub-tropical areas near stagnant or slowly flowing water and marshlands [2, 7] where infection
49 develops after inoculation of a four colony-forming units mycobacterial inoculum through the
50 skin and subcutaneous tissues leading to ulcers on the legs and arms, which can further extend to
51 other skin regions and soft tissues.[8-10] Human-to-human transmission seems rare, suggesting
52 transmission of *M. ulcerans* from environmental sources.[11] However, both environmental
53 sources and vectors remain elusive.

54 BU has emerged dramatically since the 1980's,[12] reportedly coupled with rapid
55 environmental changes in the landscape including deforestation, eutrophication, dam
56 construction, irrigation, farming, mining and habitat fragmentation.[13-17] Deforestation of the
57 riverbanks was almost complete in the 1980s, following the rising pressure for agricultural land
58 and drought-associated fires.[18]

59 During the period from 2003 to 2010, the prevalence of BU increased especially in West
60 and Central Africa [19] as illustrated by the outbreak of BU in Côte d'Ivoire (Figure 1).

61 This outbreak also has been reported to be related to man-made changes in the natural
62 environment.[12, 20] The disease has been reported in over 33 countries[3, 12, 21, 22] where 5,000 to
63 6,000 cases have been notified every year by the 15 most endemic countries, predominantly from
64 rural regions across West and Central Africa, until 2009.[19, 23] Since 2010, a regular decrease in
65 new cases of Buruli ulcer has been observed in endemic countries of Africa. In 2014, 12 of these

15 most endemic countries reported 2,251 new cases and 2,037 new cases in 2015, which is less than half the number of reported cases in 2009;[19] and the same trend is now observed worldwide.[19] The exact cause of this decline in incidence is unknown.[22]

Here, we observed a significant correlation between the decrease in BU incidence and the parallel increase in mean temperature in BU endemic regions, questioning the mechanisms of global warming in the progressive decrease of BU.

METHODS

Decade trends in BU incidence. In order to describe the decade trends in the incidence of BU, we recovered the incidence data of the WHO at http://apps.who.int/gho/data/node.main.A1631.[19] We then classified the data by year from 2002 to 2015 and expressed them in the form of a trend figure of BU cases listed in the world and in Africa.

Decade trends in global warming and temperature anomaly. In order to describe the decade trends in environmental temperature in the geographic areas where BU have been reported, we recovered the anomaly temperature trend from 2002 to 2015 at https://www.ncdc.noaa.gov/sotc/,[24] https://data.giss.nasa.gov/gistemp/ [25] and expressed them in the form of a trend figure in the world and in Africa.

Correlation between trends. The land annual temperature and the annual Buruli ulcer cases data table were analyzed by inverse regression and the chart was drawn. Values of Buruli ulcer cases in Africa and annual land temperature in Africa were used to estimate the inverse correlation following the formula: $y = A + \frac{B}{x}$ where “x” refers to Buruli ulcer annual cases and

"y" to the land annual anomaly temperature. The guidelines used for interpreting the correlation coefficient r were: $0.7 < |r| \leqq 1$ indicates a strong correlation, $0.4 < |r| < 0.7$ a moderate correlation, $0.2 < |r| < 0.4$ a weak correlation and $0 \leqq |r| < 0.2$ no significant correlation.

RESULTS

Decade trends in BU incidence. Decade trends were assessed by the use of BU annual cases notified to the WHO by endemic countries. The notified cases between 2002 and 2015 were considered to establish the total cases in the word and total cases in Africa endemic countries. The correlation between BU cases in the word and BU cases in Africa was of 0.99. We noted that 97.78% of BU cases notified to the WHO during this period, were notified by fifteen African endemic countries[19] (Figure 2, Figure 3)

Decade trends in global warming and temperature anomaly. Inland temperatures are measured by the network of weather stations spread across the world but also by satellites.[26] In meteorology and climatology, seasonal average is calculated over periods of 30 years to smooth the values and avoid variations due to recurrent anomalies.[26] The temperature deviation (anomaly) is established depending on the average temperature over the reference period 1951-1980. "*Best estimate for absolute global mean for 1951-1980 is 14.0 deg-C or 57.2 deg-F, so add that to the temperature change if you want to use an absolute scale"*.[27] The ten warmest years in the 134-year record all have occurred since 1997. The year 2015 ranks as the warmest, closely followed by 2014 and 2010. A one-degree global change is significant because it takes a vast amount of heat to warm all the oceans, atmosphere, and land.[28] The rate of change in climate is faster now than in any other period in the past thousand years.[29] The African continent observed its second warmest year, only behind 2010. The year 2010 was marked by several

notable extreme temperature events. The global land surface temperature for 2010 tied with 2005 as the second warmest on record, with 0.96°C (1.73°F) above the 20th century average. The global ocean surface temperature for 2010 tied with 2005 as the third warmest on record, with 0.49°C (0.88°F) above the 20th century average. The temperature of tropical waters has increased by 1.2 °C over the twentieth century, causing coral reef bleaching [25]. Also, global precipitation in 2010 was well above the 1961–1990 average, ranking as the wettest on record since 1900 [30]. Overall, the global annual temperature has increased at an average rate of 0.07°C (0.13°F) per decade since 1880 and at an average rate of 0.17°C (0.31°F) per decade since 1970.[24] The temperature trend by decade is shown on Figure 2, and Figure 3.

Correlation between trends. Analyzing temperature anomaly and the BU incidence trend showed an inverse correlation (Figure 2, Figure 3). More precisely, the inverse correlation between all the trends values was of 0.30 (week correlation), it was of 0.75 (strong correlation) without the 2010 values and of 0.9 (strong correlation) without the 2009 and 2010 values (Figure 4). This analysis indicated an obvious inverse correlation between land temperature and BU cases in Africa (Figure 2, Figure 3 and Figure 4).

DISCUSSION

We observed a significant inverse correlation between environmental warming and the incidence of BU in geographic areas of African endemic countries which have declared BU cases to the WHO for the last decade. More precisely, we observed that an increase in environmental temperature correlated with a decrease in the incidence of BU in affected countries.

Climate changes marked by increasing temperatures across the world are occurring as a result of the warming of the earth's atmosphere, partially due to human activity generating excessive amounts of greenhouse gases,[28, 31] a phenomena called global warming.[32] Changes in global climate over the past decades are well documented. The average global surface temperature, for example, has risen by approximately 0.75°C during the past 100 years (1906–2005)[33] due to the El Niño/Southern Oscillation (ENSO) phenomenon. The ENSO phenomenon, which originates in the Tropical Pacific and affects global climate, is the strongest internal climate mode on inter-annual timescales.[27] The ENSO is characterized by strong variations of the eastern and central equatorial Pacific sea surface temperature that reoccur on average every four years. The El Niño record of 1997/1998 strongly contributed to the global average record of surface temperature in 1998.[27, 34] Since the mid-19th century, human activities have increased the greenhouse gases production such as carbon dioxide, methane and nitrous oxide in the Earth's atmosphere, which resulted in an increased average temperature.[29]

Whether our observation of a decrease in BU incidence correlating with warming in Africa is resulting from chance or is translating one or several temperature-dependant phenomenon implied in the epidemiology of BU remains to be established. We do favor the latter hypothesis as the correlation we discovered was statistically significant and temperature is already known to influence some parameters potentially implicated in the epidemiology of BU.

At first, the causative agent of BU, *M. ulcerans,* is highly susceptible to temperature.[35] In particular, its optimal temperature for growth is comprised in a narrow range between 28°C and 33°C.[8, 36] Any slight increase in environmental temperature may slow down the growth of *M. ulcerans* in its poorly characterized aquatic reservoirs in Africa. Moreover, it has been suggested that increased temperatures caused microbes to undergo physiological changes that resulted in

reduced carbon use efficiency. Indeed, we previously reported that *M. ulcerans* was using a narrow spectrum of carbon sources.[37, 38] *M. ulcerans* is also highly susceptible to ultraviolet light and sunlight.[3, 39, 40] This susceptibility is partly driven by the loss of genes encoding for pigments otherwise present in its parent *Mycobacterium marinum*, another aquatic environmental non-tuberculous mycobacterium.[3, 39, 41] We noticed that climate reports indicated a 0.2°C increase in mean temperature in BU endemic areas from the same period resulting in an average temperature of 0.15°C and 0.3°C per decade for 1990 to 2005.[42] The average global temperature across land surfaces was of 1.29°C higher than the 20th century average of 12.0°C [24]. Therefore, elevation in temperature and the solar exposition would indeed be harmful to *M. ulcerans*. At last, exposure to ultraviolet or sunlight results in the rapid photo-degradation of the *M. ulcerans* plasmid-encoded mycolactone, which is the main virulence factor of *M. ulcerans* responsible for skin lesions characteristic of BU.[43]

Secondly, the precise reservoirs of *M. ulcerans* remain doubtful, yet all epidemiological and microbiological studies point towards stagnant water environments as probable reservoirs in African countries.[44] Deforestation and either agricultural or urban intrusion led to a decline in local trophic niche width, resulting in decreased regional mean vulnerability of taxa, coupled with a decrease in generality.[45] Host taxa, which on average carried a high level of *M. ulcerans*, were most abundant at sites where there was a very low level of vulnerability and a midlevel of generality.[45] A consequence of the rapid increase in human populations, settlements, and encroachments is the concomitant rapid decline in biological diversity, with significant shifts in species community composition and severe disruption to established food webs, which are directly linked to land-use changes and deforestation.[45, 46] Estimates suggest that 10,000 to 20,000 freshwater species are under severe threat of extinction.[45] A severe change in a trophic

network may have a significant impact on an emerging infectious disease, with promotion or decline of certain host species in the community network.[45] It is conceivable that the continuous warming tends to dry some stagnant water sources in newly conquered fields in affected countries, leading to a decreased exposure of the populations to waterborne *M. ulcerans*. This is endorsed by the work of Morris et al. who stated that, as the diversity of basal organisms begins to decrease, the abundance of *M. ulcerans* hosts and the *M. ulcerans* load then start to increase, until the number of basal organisms becomes too low, leading again to a decrease in *M. ulcerans* abundance.[45] Homogenization is the outcome of three interacting processes: introduction of non-native species, extirpation of native species and habitat alterations that facilitate these two processes.[46] The homogenization process has generally increased biodiversity in most freshwater faunas, as the establishment of new species has outpaced the extinction of native species.[46]

Thirdly *M. ulcerans* may be passively or actively transmitted by the bite of insects.[5, 10, 47] The global warming may affect species abundance and distribution, as well as temperature-dependent morphometric traits[48] and thus the biting habits and capabilities of vectors; or the subtle interplay between vectors and *M. ulcerans*. Indeed, climate changes have an impact on microbial flora and the animate and inanimate ecology in general. Pathogens transmitted by vectors are particularly susceptible to climate change because they spend most of their life cycle in a cold-blooded invertebrate host whose temperature is similar to the environment.[29]

Climate change is a current global concern and, despite continuing controversy about the extent and importance of its causes and effects, it seems likely that it will affect the incidence and prevalence of infections.[34, 49, 50, 51] It was shown in several studies that, there is consistent evidence that foodborne pathogens infection with bacterial pathogens is positively correlated with ambient temperature, as warmer temperatures enable more rapid replication.[34, 49, 50]

According to the IPCC (2007) report, climate change will alter patterns of infectious disease outbreaks in humans and animals.[33] However, the effects of global warming on infections is probably unpredictable, especially for infections for which all the ecological and epidemiological parameters are not yet understood, such as BU. We argue that, besides targeted interventions against BU[52, 53] and other interventions,[54-59] global warming also participates to BU decline in Africa.

CONCLUSION

The impact of the increasing global temperature in African regions is evident. Efforts are made at the national and international level to combat global warming. The 2015 Paris Agreement aims at limiting global warming to 2°C and pursues efforts to even limit it to 1.5°C, relative to pre-industrial levels.[60] A warming of 0.5°C leads to significant increases in temperature and extreme rainfall in most regions.[60]

Socio-cultural determinants, rainfall and temperature frequently underlie overall tropical infectious disease prevalence, particularly for vector-borne diseases.[51, 61] These tropical diseases have an increased prevalence in tropical as compared to temperate regions.[61] As for BU however, unexpectedly, global warming may act in the same way as the targeted interventions in public health in Africa , contributing to the drastic decrease in BU incidence. The findings indicate, however, that higher climate change are important and reinforce the need for further research.

Figure legends:

Figure 1: Trend in Buruli ulcer cases notification between 1978 and 2015 in Ivory Coast

Figure 2: Inverse correlation between cases of Buruli ulcer and temperature anomaly in the world and Africa (2002-2015). The trend in the global number of Buruli ulcer cases was similar to that in Africa with a correlation of 0.99. Line plot of annual mean global surface temperature anomaly between 2002 and 2015, calculated in relation to the 1951-1980 average temperatures.

Figure 3: Inverse correlation between cases of Buruli ulcer and temperature anomaly in Africa (2002-2015). Line plot of annual mean global surface temperature anomaly between 2002 and 2015, calculated in relation to the 1951-1980 average temperatures.

Figure 4: Inverse correlation between the trend of Buruli ulcer annual cases and annual anomaly temperature in Africa.

References

1. Asiedu K, Sherpbier R, Raviglione M, 2000. Buruli Ulcer Mycobacterium ulcerans infection.Global Buruli Ulcer initiative. Geneva, Switzerland.
2. Williamson HR, Benbow ME, Nguyen KD, Beachboard DC, Kimbirauskas RK, McIntosh MD, Quaye C, Ampadu EO, Boakye D, Merritt RW, Small PL, 2008. Distribution of Mycobacterium ulcerans in buruli ulcer endemic and non-endemic aquatic sites in Ghana. *PLoS Negl Trop Dis 2:* e205.
3. Stinear TP, Seemann T, Pidot S, Frigui W, Reysset G, Garnier T, Meurice G, Simon D, Bouchier C, Ma L, Tichit M, Porter JL, Ryan J, Johnson PD, Davies JK, Jenkin GA, Small PL, Jones LM, Tekaia F, Laval F, Daffe M, Parkhill J, Cole ST, 2007. Reductive evolution and niche adaptation inferred from the genome of Mycobacterium ulcerans, the causative agent of Buruli ulcer. *Genome Res 17:* 192-200.
4. Walsh DS, Portaels F, Meyers WM, 2011. Buruli ulcer: Advances in understanding Mycobacterium ulcerans infection. *Dermatol Clin 29:* 1-8.
5. Marsollier L, Robert R, Aubry J, Saint Andre JP, Kouakou H, Legras P, Manceau AL, Mahaza C, Carbonnelle B, 2002. Aquatic insects as a vector for Mycobacterium ulcerans. *Appl Environ Microbiol 68:* 4623-4628.
6. Debacker M, Aguiar J, Steunou C, Zinsou C, Meyers WM, Guédénon A, Scott JT, Dramaix M, Portaels F, 2004. Mycobacterium ulcerans Disease (Buruli Ulcer) in Rural Hospital, Southern Benin, 1997–2001. *Emerg Infect Dis 10:* 1391-1398.
7. Sopoh GE, Barogui YT, Johnson RC, Dossou AD, Makoutodé M, Anagonou SY, Kestens L, Portaels F, 2010. Family Relationship, Water Contact and Occurrence of Buruli Ulcer in Benin. *PLoS Negl Trop Dis 4:* e746.

8. Portaels F, 2014. Laboratory diagnosis of Buruli ulcer: A manual for health-care providers. World Health Organization. Geneva, 117.

9. Williamson HR, Mosi L, Donnell R, Aqqad M, Merritt RW, Small PL, 2014. Mycobacterium ulcerans fails to infect through skin abrasions in a guinea pig infection model: implications for transmission. *PLoS Negl Trop Dis 8*.

10. Wallace JR, Mangas KM, Porter JL, Marcsisin R, Pidot SJ, Howden BO, Omansen TF, Zeng W, Axford JK, Johnson PDR, Stinear TP, 2017. Mycobacterium ulcerans low infectious dose and atypical mechanical transmission support insect bites and puncturing injuries in the spread of Buruli ulcer. *PLoS Negl Trop Dis 11:* e0005553.

11. Nackers F, Johnson RC, Glynn JR, Zinsou C, Tonglet R, Portaels F, 2007. Environmental and Health-Related Risk Factors for Mycobacterium ulcerans Disease (Buruli Ulcer) in Benin. A*m J Trop Med Hyg 77:* 834-836.

12. Merritt RW, Walker ED, Small PLC, Wallace JR, Johnson PDR, Benbow ME, Boakye DA, 2010. Ecology and Transmission of Buruli Ulcer Disease: A Systematic Review. *PLoS Negl Trop Dis 4:* e911.

13. Walsh DS, Portaels F, Meyers WM, 2008. Buruli ulcer (Mycobacterium ulcerans infection). *Trans R Soc Trop Med Hyg 102:* 969-978.

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

15. Wansbrough-Jones M, Phillips R, 2006. Buruli ulcer: emerging from obscurity. *Lancet 367:* 1849-1858.

16. Johnson PDR, Stinear T, Small PLC, Pluschke G, Merritt RW, Portaels F, Huygen K, Hayman JA, Asiedu K, 2005. Buruli Ulcer (M. ulcerans Infection): New Insights, New Hope for Disease Control. *PLoS Medicine 2:* e108.

17. Janssens PG PS, Meyers WM, Portaels F, 2005. Buruli ulcer: an historical overview with updating to 2005. *Bull Seances Acad R Sci Outre Mer 51:* 165-199.

18. Giles-Vernick T, Owona-Ntsama J, Landier J, Eyangoh S, 2015. The puzzle of Buruli ulcer transmission, ethno-ecological history and the end of "love" in the Akonolinga district, Cameroon. *Soc Sci Med 129:* 20-27.

19. WHO, 2016. Global Health Observatory data repository.Number of new reported cases, Data by country.

20. Josse R, Guedenon A, Darie H, Anagonou S, Portaels F, Meyers WM, 1995. [Mycobacterium ulcerans cutaneous infections: Buruli ulcers]. *Med Trop 55:* 363-373.

21. Johnson PD, Azuolas J, Lavender CJ, Wishart E, Stinear TP, Hayman JA, Brown L, Jenkin GA, Fyfe JA, 2007. Mycobacterium ulcerans in mosquitoes captured during outbreak of Buruli ulcer, southeastern Australia. *Emerg Infect Dis 13:* 1653-1660.

22. WHO, 2016. Buruli ulcer (Mycobacterium ulcerans infection). Available at: http://www.who.int/mediacentre/factsheets/fs199/en/: World Health Organization.

23. WHO, 2014. Buruli ulcer (Mycobacterium ulcerans infection).Fact Sheet. N°199. World Health Organization.

24. NOAA, 2016. National Centers for Environmental Information, State of the Climate: Global Analysis for September 2016, published online October 2016, retrieved on November 28, 2016

25. NASA, 2016. http://data.giss.nasa.gov/. Accessed 25/05/2016.

26. NOAA, 2011. National Centers for Environmental Information, State of the Climate: Global Analysis for Annual 2010 Available at: http://www.ncdc.noaa.gov/sotc/global/201013.

27. Tisdale B, 2014. The 2014/15 El Niño – Part 20 Available at: https://bobtisdale.wordpress.com/2014/11/09/on-the-elusive-absolute-global-mean-surface-temperature-a-model-data-comparison/.

28. Carlowicz M, 2016. "Global Temperatures."Retrieved 28/05/2016, 2016, from http://earthobservatory.nasa.gov/Features/WorldOfChange/decadaltemp.php.

29. Rossati A, 2017. Global Warming and Its Health Impact. *Int J Occup Environ Med 8:* 7-20.

30. NOAA, 2010. Global Climate Report - November 2010.

31. Shuman EK, 2011. Global climate change and infectious diseases. *Int J Occup Environ Med 2:* 11-9.

32. NASA., 2010. NOAA National Climatic Data Center (n.d.). Global Warming Frequently Asked Questions. Available at: http://earthobservatory.nasa.gov/Features/GlobalWarming/. Accessed 28/05/2016.

33. IPCC, 2007. Climate Change 2007: Synthesis Report. Contribution of Working Groups I, II and III to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change [Core Writing Team, Pachauri, R.K and Reisinger, A. (eds.)]. IPCC, Geneva, Switzerland, , 104 pp.

34. Bezirtzoglou C, Dekas K, Charvalos E, 2011. Climate changes, environment and infection: facts, scenarios and growing awareness from the public health community within Europe. *Anaerobe 17:* 337-340.

35. Riad EM, Amany ND, Dalia MM, 2015. The uncommon Mycobaterium ulcerans infection and its public health importance. *Egypt J Chem Environ Health 1:* 1058-1066.

36. Eddyani M, Debacker M, Martin A, Aguiar J, Johnson CR, Uwizeye C, Fissette K, Portaels F, 2008. Primary Culture of Mycobacterium ulcerans from Human Tissue Specimens after Storage in Semisolid Transport Medium. *J Clin Microbiol 46:* 69-72.

37. Allison SD, Wallenstein, M.D., Bradford, M.A, 2010. Soil-carbon response to warming dependent on microbial physiology. *Nature Geoscience 3:* 336-340.

38. Zingue D, Bouam A, Militello M, Drancourt M, 2017. High-Throughput Carbon Substrate Profiling of Mycobacterium ulcerans Suggests Potential Environmental Reservoirs. *PLOS Negl Trop Dis 11:* e0005303.

39. Amissah NA, Gryseels S, Tobias NJ, Ravadgar B, Suzuki M, Vandelannoote K, Durnez L, Leirs H, Stinear TP, Portaels F, Ablordey A, Eddyani M, 2014. Investigating the Role of Free-living Amoebae as a Reservoir for Mycobacterium ulcerans. *PLoS Negl Trop Dis 8:* e3148.

40. Tian RD, Lepidi H, Nappez C, Drancourt M, 2016. Experimental Survival of Mycobacterium ulcerans in Watery Soil, a Potential Source of Buruli Ulcer. *Am J Trop Med Hyg 94:* 89-92.

41. Stinear TP, Jenkin GA, Johnson PDR, Davies JK, 2000. Comparative Genetic Analysis of Mycobacterium ulcerans and Mycobacterium marinum Reveals Evidence of Recent Divergence. *J Bacteriol 182:* 6322-6330.

42. IPCC, 2007. Climate Change 2007. Working Group I: The Physical Science Basis. Projections of Future Changes in Climate. IPCC Fourth Assessment Report. Geneva, Switzerland: World Meteorological Organization.

43. Marion E, Prado S, Cano C, Babonneau J, Ghamrawi S, Marsollier L, 2012. Photodegradation of the Mycobacterium ulcerans Toxin, Mycolactones: Considerations for Handling and Storage. *PLoS ONE 7:* e33600.

44. Portaels F, 1995. Epidemiology of mycobacterial diseases. *Clin Dermatol 13:* 207-222.

45. Morris AL, Guegan JF, Andreou D, Marsollier L, Carolan K, Le Croller M, Sanhueza D, Gozlan RE, 2016. Deforestation-driven food-web collapse linked to emerging tropical infectious disease, Mycobacterium ulcerans. *Sci Adv 2:* e1600387.

46. Rahel FJ, 2002. Homogenization of Freshwater Faunas. *Ann Rev Ecol Syst 33:* 291-315.

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

48. Schuldiner-Harpaz T, Coll M, 2013. Effects of Global Warming on Predatory Bugs Supported by Data Across Geographic and Seasonal Climatic Gradients. *PLoS ONE 8:* e66622.

49. Kovats S, 2003. Climate change, temperature and foodborne disease. *Euro Surveill 7:* pii=2339. Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=2339

50. Kendrovski V, Gjorgjev D, 2012. Climate Change: Implication for Food-Borne Diseases (Salmonella and Food Poisoning Among Humans in R. Macedonia). Eissa AA, ed. Structure and Function of Food Engineering. Rijeka: InTech, Ch. 07.

51. Wu X, Lu Y, Zhou S, Chen L, Xu B, 2016. Impact of climate change on human infectious diseases: Empirical evidence and human adaptation. *Environ Int 86:* 14-23.

52. Aka P, 2002. Buruli Ulcer, an Emerging Disease in Public Health in Cote D'Ivoire (Ivory Coast). *Forum for Nord Derm Ven 7:* 22-26.

53. Affolabi D, Tanimomo-Kledjo B, Anyo G, Johnson RC, Anagonou SY, Portaels F, 2008. Setting up a national reference laboratory for Buruli ulcer: the case of Benin. *Trop Med Int Health 13:* 365-368.

54. WHO, 2002. Instructions for treatment and use of insecticide-treated mosquito nets. Use insecticide-treated mosquito nets to sleep in peace – and protect your health. WHO/CDS/WHOPES/GCDPP/2002.4, ed.

55. WHO/GMP, 2009. GLOBAL MALARIA PROGRAMME. INSECTICIDE-TREATED MOSQUITO NETS: a WHO Position Statement

56. OAU, 2000. AFRICAN SUMMIT ON ROLL BACK MALARIA .The Abuja Declaration on Roll Back Malaria in Africa. Available at: https://www.usaid.gov/sites/default/files/documents/1864/abuja.pdf.

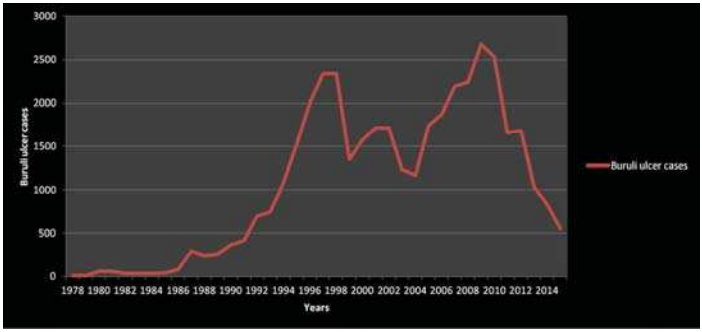
57. WHO, 2011. The use of DDT in malaria vector control: World Health Organzation position statement

58. WHO, 2010. Global programme to eliminate lymphatic filariasis. WHO GPELF progress report 2000–2009 and strategic plan 2010–2020. WHO/HTM/NTD/PCT/2010.6

59. WHO, 2006. Preventive chemotherapy in human helminthiasis.Coordinated use of anthelminthic drugs in control interventions: a manual for health professionals and programme managers.

60. Wang Z, Lin L, Zhang X, Zhang H, Liu L, Xu Y, 2017. Scenario dependence of future changes in climate extremes under 1.5 degrees C and 2 degrees C global warming. *Sci Rep 7:* 46432.

61. Beckley CS, Shaban S, Palmer GH, Hudak AT, Noh SM, Futse JE, 2016. Disaggregating Tropical Disease Prevalence by Climatic and Vegetative Zones within Tropical West Africa. *PLoS One 11:* e0152560.



Trend in Buruli ulcer cases notification between 1978 and 2015 in Ivory Coast

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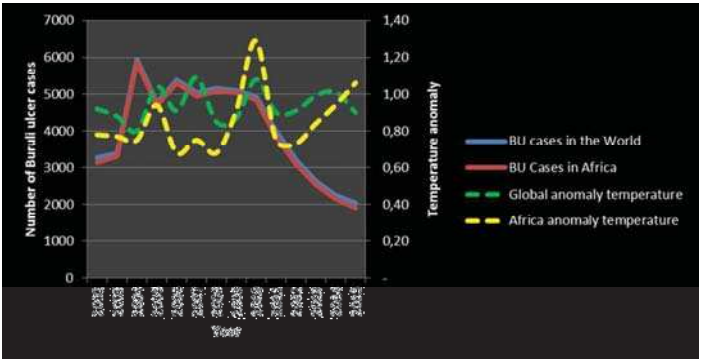
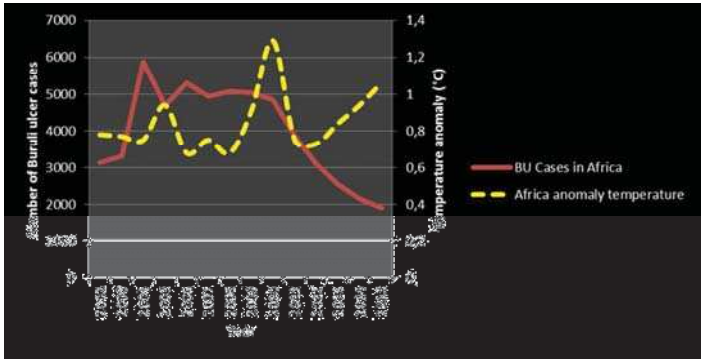


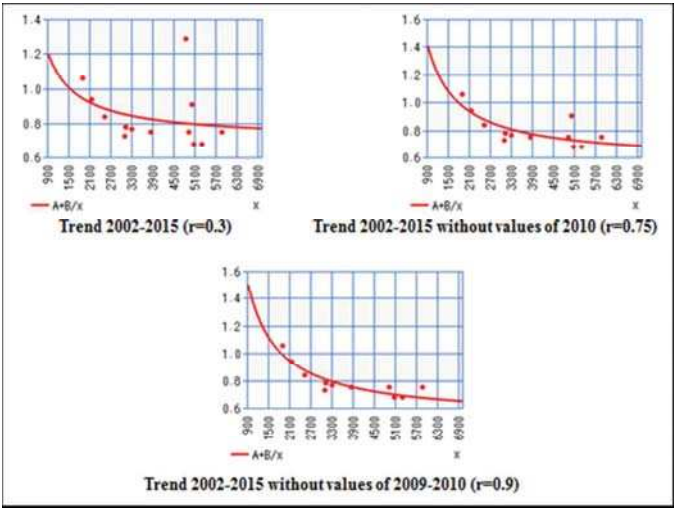
Inverse correlation between cases of Buruli ulcer and temperature anomaly in the world and Africa (2002-2015). The trend in the global number of Buruli ulcer cases was similar to that in Africa with a correlation of 0.99. Line plot of annual mean global surface temperature anomaly between 2002 and 2015, calculated in relation to the 1951-1980 average temperatures.

235x120mm (106 x 106 DPI)



Inverse correlation between cases of Buruli ulcer and temperature anomaly in Africa (2002-2015). Line plot of annual mean global surface temperature anomaly between 2002 and 2015, calculated in relation to the 1951-1980 average temperatures.

235x120mm (106 x 106 DPI)



Inverse correlation between the trend of Buruli ulcer annual cases and annual anomaly temperature in Africa.

200x150mm (61 x 61 DPI)

Chapter 2: Phenotype MicroArray (Biolog Inc.) for the high-throughput carbon substrate profiling of *Mycobacterium ulcerans*

Identification at species level can be performed by using the Biolog microplate system (Biolog, Hayward, CA, USA) which was originally developed for the rapid identification of bacteria by sole-carbon source utilization. The principle is based on the redox reaction of tetrazolium violet of substrates in a 96-well microtiter plate. Each well contains a redox dye, tetrazolium violet that permits colorimetric determination of the increased respiration that occurs when cells are oxidizing a carbon source. The micro-plates are incubated between four and 72 h following inoculation with a pre-grown isolate according to manufacturer's specification. Individual species may be identified by the specific pattern of color change on the plate, providing an identifiable metabolic fingerprint.The micro-plate are read with the Biolog MicroStation TM system and compared to the Biolog database enabling the rapid identification of 1,449 aerobic/anaerobic bacterial yeast and fungi species/taxa. For the first time, the Biolog system was used

for high-throughput phenotyping of *M. ulcerans* and *M. marinum* strains. We have highlighted in this study the nutrients used by *M. ulcerans* strains for their metabolism and growth. We have learned that there are nutrients that are used by all *M. ulcerans* strains regardless of their geographic origin, whereas the use of certain nutrients depends on the genotypic family of *M. ulcerans*. These interesting results have allowed us to locate in the environment the sources of these nutrients which by extrapolation provide reservoir and host research paths of *M. ulcerans* whose knowledge is an invaluable asset in the fight against the spread of Buruli ulcer. This validated high-throughput phenotyping protocol of *M. ulcerans* opens the way to the study of other clinical or environmental mycobacteria.

Article 3: High-Throughput Carbon Substrate Profiling of *Mycobacterium ulcerans* Suggests Potential Environmental Reservoirs

Zingue D, Bouam A, Militello M, Drancourt M, 2017. High-Throughput Carbon Substrate Profiling of *Mycobacterium ulcerans* Suggests Potential Environmental Reservoirs. PLOS Neglected Tropical Diseases 11: e0005303.

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

High-Throughput Carbon Substrate Profiling of *Mycobacterium ulcerans* Suggests Potential Environmental Reservoirs

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Abstract

Background

Mycobacterium ulcerans is a close derivative of *Mycobacterium marinum* and the agent of Buruli ulcer in some tropical countries. Epidemiological and environmental studies pointed towards stagnant water ecosystems as potential sources of *M. ulcerans*, yet the ultimate reservoirs remain elusive. We hypothesized that carbon substrate determination may help elucidating the spectrum of potential reservoirs.

Methodology/Principal findings

In a first step, high-throughput phenotype microarray Biolog was used to profile carbon substrates in one *M. marinum* and five *M. ulcerans* strains. A total of 131/190 (69%) carbon substrates were metabolized by at least one *M. ulcerans* strain, including 28/190 (15%) carbon substrates metabolized by all five *M. ulcerans* strains of which 21 substrates were also metabolized by *M. marinum*. In a second step, 131 carbon substrates were investigated, through a bibliographical search, for their known environmental sources including plants, fruits and vegetables, bacteria, algae, fungi, nematodes, mollusks, mammals, insects and the inanimate environment. This analysis yielded significant association of *M. ulcerans* with bacteria ($p = 0.000$), fungi ($p = 0.001$), algae ($p = 0.003$) and mollusks ($p = 0.007$). In a third step, the Medline database was cross-searched for bacteria, fungi, mollusks and algae as potential sources of carbon substrates metabolized by all tested *M. ulcerans*; it indicated that 57% of *M. ulcerans* substrates were associated with bacteria, 18% with alga, 11% with mollusks and 7% with fungi.

Conclusions

This first report of high-throughput carbon substrate utilization by *M. ulcerans* would help designing media to isolate and grow this pathogen. Furthermore, the presented data suggest that potential *M. ulcerans* environmental reservoirs might be related to micro-habitats where bacteria, fungi, algae and mollusks are abundant. This should be followed by targeted investigations in Buruli ulcer endemic regions.

Author Summary

Buruli ulcer is a neglected tropical disease which has been reported in over 33 countries, mainly located in tropical and subtropical regions. It is caused by *Mycobacterium ulcerans*, an environmental pathogen associated to slow-moving water. The sources and reservoirs of *M. ulcerans* remain elusive and are still to be discovered. In a first attempt to address this issue we used high-throughput carbon substrate profiling of *M. ulcerans*. The reported results show that some nutrients, naturally available in organisms present in *M. ulcerans*' environment, are metabolized by this microorganism. This carbon substrate determination should help improve the culture of *M. ulcerans* as well as suggest potential environmental reservoirs in Buruli ulcer endemic regions.

Introduction

Mycobacterium ulcerans is the etiologic agent of Buruli ulcer, a disabling infection of the cutaneous and subcutaneous tissues [1–3]. *M. ulcerans* has been discovered in Bairnsdale, Australia, where Buruli ulcer was initially described [4,5]. Buruli ulcer is a World Health Organization notifiable infection and has been reported at least once by 33 countries located in the rural tropical regions of Africa and South America, in addition to Australia and Japan [6,7]. Over the past ten years, 83.6% (80.89–86.30) of cases were declared by eight West African countries [8]. In these highly endemic regions, the exact reservoirs of *M. ulcerans* remain elusive [6, 9–11]. However, epidemiological studies conducted in West African countries all indicated a significant association between the prevalence of Buruli ulcer and the contact of populations with stagnant water sources [12–17] through routine activities such as washing, swimming, fishing and farming [18,19]. A significant progress was recently made by narrowing the possible sources down to contacts with rice fields in Côte d'Ivoire which are sources of stagnant water [16,18,20,21]. Parallel environmental investigations of stagnant water [20,22], water insects [23–25], fishes [26,27] and aquatic mammals [12] showed the presence of PCR-amplified *M. ulcerans* insertion sequences (IS) IS*2404*, IS*2606* and KR-B gene. Furthermore, *M. ulcerans* partial DNA coding sequences were also recovered from the soil in the vicinity of stagnant water [20,22,26,28,29]. This finding was strengthened by an experimental study confirming a four-month survival of *M. ulcerans* in soil [30]. *M. ulcerans* DNA has been also detected in water plants [28,31] and in *Thryonhuomys swinderianus* (agouti), a small mammal causing damages to rice fields and in close contacts with rural populations in West Africa [20].

Moreover, this compelling amount of information concerning the presence of *M. ulcerans* DNA-related sequences found in the environment has been strengthened by the isolation of five wild strains from those sources [3,32,33].

Here, we propose that a characterization of the metabolic profile of *M. ulcerans* may give clues to better define its natural environment including its environmental reservoirs. In this perspective, we used the Biolog Phenotype MicroArray (Biolog Inc., Hayward, CA) for high-throughput carbon substrate profiling of *M. ulcerans*. Indeed, Biolog Phenotype MicroArray was previously used to classify and characterize heterotrophic microbial communities from different natural habitats according to their sole-carbon-source utilization profiles [34]. Accordingly, this approach previously unraveled the phenotypic patterns of some *Mycobacterium tuberculosis* complex mycobacteria [35] and *Mycobacterium avium* subsp. *paratuberculosis* [36]. It is used here in the context of unique carbon metabolisms such as chitinase exhibited by *M. ulcerans* [37].

Materials and Methods

M. ulcerans strains

This experimental study investigated *M. ulcerans* strain CU001 (a gift from Pr V. Jarlier, Paris, France), a clinical isolate representative of the West African epidemic, *M. ulcerans* ATCC 19423 isolated in Australia, *M. ulcerans* ATCC 33728 isolated in Japan, *M. ulcerans* ATCC 25900 isolated in the USA and *Mycobacterium buruli* ATCC 25894 isolated in Uganda [38]. These strains were manipulated into a BLS3 laboratory and a clinical isolate of *Mycobacterium marinum* was isolated in our laboratory [39]. All strains were cultured at 30˚C in Middlebrook 7H10 agar medium supplemented with 10% (v/v) oleic acid/albumin/dextrose/catalase (OADC) (Becton Dickinson, Sparks, MD, USA) and 0.5% (v/v) glycerol in a microaerophilic atmosphere for one week for *M. marinum* and four weeks for *M. ulcerans*.

Biolog Phenotype microarray

The Biolog Phenotype MicroArray (Biolog Inc.), which consists of 96-well microtiter plates containing each a defined medium that incorporates a unique carbon source (plates PM1 and PM2A for 190 different carbon sources) plus a dye indicator of cell respiration was used, according to the previously reported standard Biolog Inc. protocol [40,41]. *M. ulcerans* and *M. marinum* colonies were removed from Middlebrook 7H10 medium using a cotton swab previously dipped in 0.1% Tween 80 (WGK Germany, Sigma Aldrich). Mycobacteria were taken with the wet swab off the agar plate culture by gently sweeping on the surface of the culture and then rubbed against the wall of a dry glass tube containing glass beads. The cells were then suspended in GN/GP-IF-0a (Biolog inoculating fluid n˚133), the suspension was vigorously vortexed, passed three times through a 29-gauge needle in order to separate aggregates and adjusted to 81% transmittance using a turbidimeter (Biolog Inc). The PM-additive solutions for each plate were prepared according to Table 1. The inoculating fluid (Table 2) consisted of 20 mL of IF-0a GN/GP (1.2 x), 0.24 mL of dye mix G (100x) and 2.0 mL of PM additive (12x) added to the *M. ulcerans* or *M. marinum* suspension in IF-0a GN/GP (1.76 mL). Each PM plate was then inoculated in duplicate with 100 μL of inoculating fluid. The PM plates were incubated in the OmniLog PM System (Biolog Inc.) which measures the growth of mycobacteria every fifteen minutes for eight days at 30˚C. In each well the substrate was reduced to a purple color which was directly proportional to the growth of the mycobacteria. The intensity of the purple color was recorded as dye reduction value, which was then plotted as area under the curve (AUC) by Biolog's parametric software. Negative control wells containing non-inoculated additive solutions in each PM1 and PM2 plates were run at the same time as a quality control element. The threshold separating the wells which exhibited a positive reaction from those with a negative reaction was set for each plate according to the value of the area under the curve (AUC) of the negative control Well (NCW). We defined moderately positive growing wells (MPW) and highly positive growing wells (HPW) as follows: MPW is when the AUC

Table 1. Composition and preparation of 12 x PM additive solutions.

Ingredient	Final Conc.	120x Conc.	Formula Weight	Grams/ 100 ml	PM 1	PM 2
$MgCl_2$, $6H_2O$ Ca Cl_2, 2 H_2O	2mM 1mM	240mM 120mM	203.3 147.0	4.88 1.76	10 mL	10 mL
Tween 80	0.01%	1.2%	-	1.2	10 mL	10 mL
D-glucose	5mM	600mM	180.2	10.8	-	-
Sterile water					80 mL	80 mL
Total					100 mL	100 mL

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Table 2. Recipe for 1x PM inoculating fluids from stock solutions.

PM Stock Solution	PM1	PM2
IF-0a GN/GP (1.2x)	20 mL	20
PM additive (12x)	2 mL	2
Dye mix G (100x)	0.24 mL	0.24
cells (13.64x)	1.76 mL	1.76
Total	24 mL	24

doi:10.1371/journal.pntd.0005303.t002

value of the well is equal to or lower than 1.25 times the AUC value of the negative control well, and HPW is when the AUC value of the well is equal to or higher than 1.50 times the AUC value of the negative control. PM plates were further examined visually at the end of each incubation period to ensure an independent verification of the results.

Environmental sources of substrates metabolized by all tested *M. ulcerans* strains

In order to find the potential environmental origin of the carbon substrates metabolized by *M. ulcerans*, we used the PubMed database to obtain information on the environmental sources for each of the 190 carbon substrates present in the PM1and PM2 plates. The environmental sources were organized in 10 categories (plants, fruits and vegetables, bacteria, algae, fungi, nematodes, mollusks, mammals, insects and the inanimate environment). The Chi-square test was used to compare the proportion of each category for substrates not metabolized by *M. ulcerans* versus substrates metabolized by all tested *M. ulcerans* strains; a P value < 0.05 was used as the criterion for statistical significance. We then used the PubMed database to match each substrate, used as a key-word, with all environmental sources significantly associated with substrates metabolized by all tested *M. ulcerans* strains, used as the second key-word (e.g., D-glucosamine and fungi). We calculated the number of hits obtained in this research and compared it to the number of hits obtained by searching only for the key word corresponding to the environmental sources (e.g., fungi).

Results

Carbone substrate profiling in *M. marinum* and *M. ulcerans*

The negative control wells remained negative in all the PMs plates, and results obtained with the five *M. ulcerans* strains and the *M. marinum* strain were duplicated. A total of 131/190 (69%) carbon substrates were metabolized by at least one of the five *M. ulcerans* strains, including 28/190 (15%) carbon substrates common to the five *M. ulcerans* strains and 16/190 (8%) carbon substrates metabolized by only one *M. ulcerans* strain (Table 3). A total of 21/28 (75%) substrates metabolized by all tested *M. ulcerans* strains were also metabolized by *M. marinum* (Table 3). In detail, 17/95 (18%) carbon sources in PM1 plates were metabolized by all *M. ulcerans* strains and comprised D-glucose-6-phosphate, D-ribose, L-asparagine, uridine, D-fructose-6-phosphate, adenosine, inosine, acetoacetic acid, methyl pyruvate, L-malic acid, D-psicose, L-lyxose, glucuronamide, pyruvic acid, L-galactonic acid-g-lactone, D-galacturonic acid and phenylethylamine. Six of these substrates exhibited a strong positive reaction (D-ribose, L-malic acid, L-lyxose, glucuronamide, pyruvic acid and D-galacturonic acid). Then, 11/95 (11.5%) carbon sources in PM2 plates metabolized by all *M. ulcerans* strains comprised D-raffinose, butyric acid, D-glucosamine, α-keto-valeric acid, 5-keto-D-gluconic acid, oxalo-malic acid, sorbic acid, L-isoleucine, L-lysine, putrescine and dihydroxyacetone. Five of these

Table 3. Carbone substrates metabolized by at least one of the five tested *M. ulcerans* strains compared with carbon substrates metabolized by *Mycobacterium marinum* on Biolog PM1 & PM2 plates.

Substrates	CU001	ATCC 25900	ATCC 33728	ATCC 19423	ATCC 25894	*M. marinum*
D-Ribose						
L-Malicacid						
L-Lyxose						
Glucuronamide						
Pyruvic acid						
D-Galacturonicacid						X
D-Glucosamine						
5-Keto-D-Gluconic acid						
Oxalomalic acid						
Sorbic acid						
Dihydroxyacetone						
Inosine						
L-Galactonic acid-g-Lactone						
D-Raffinose						
Butyric acid						
Putrescine						X
Phenylethylamine						
D-Glucose-6-Phosphate						
Adenosine						
L-Asparagine						
D-Fructose-6-Phosphate						
Acetoacetic acid						
D-Psicose						
α-Keto-Valeric acid						X
L-Isoleucine						X
L-Lysine						X
Methylpyruvate						X
Uridine						X
Fumaricacid						
Tricarballylicacid						
L-Serine						
L-Threonine						
L-Alanine						
L-Alanine-Glycine						
N-Acetyl-β-D-Mannosamine						
Glycyl-L-Proline						
2-Aminoethanol						
3-Methylglucose						
β-Methyl-D-Xyloside						
N-Acetyl-D-Glucosaminitol						
Citramalicacid						
Malonicacid						
Succinamicacid						
3-Hydroxy-2-butanone						
D-Tartaricacid						
L-Tartaricacid						

(*Continued*)

Table 3. (*Continued*)

Substrates	CU001	ATCC 25900	ATCC 33728	ATCC 19423	ATCC 25894	*M. marinum*
Acetamide						
L-Arginine						
Glycine						
L-Histidine						
L-Homoserine						
Hydroxy-L-Proline						
L-Leucine						
L-Methionine						
L-Ornithine						
L-Phenylalanine						
L-Pyroglutamicacid						
L-Valine						
D,L-Carnitine						
sec-Butylamine						
D,L-Octopamine						
2,3-Butanediol						
2,3-Butanedione						
Itaconicacid						
D-Lactic acid Methyl Ester						
Melibionicacid						
Oxalicacid						
Quinicacid						
D-Ribono-1,4-Lactone						
Sebacicacid						
Salicin						
Sedoheptulosan						
L-Sorbose						
Stachyose						
D-Tagatose						
Turanose						
Xylitol						
γ-Amino-N-Butyric acid						
δ-Amino Valeric acid						
Capricacid						
Caproicacid						
4-Hydroxybenzoic acid						
β-Hydroxybutyricacid						
γ-Hydroxybutyricacid						
Pectin						
N-Acetyl-D-Galactosamine						
N-Acetyl-Neuraminicacid						
β-D-Allose						
D-Arabinose						
2-Deoxy-D-Ribose						
3-O-β-D-Galactopyranosyl-D-Arabinose						
Gentiobiose						
L-Glucose						

(*Continued*)

Table 3. (*Continued*)

Substrates	CU001	ATCC 25900	ATCC 33728	ATCC 19423	ATCC 25894	*M. marinum*
D-Lactitol						
D-Melezitose						
Maltitol						
α-Methyl-D-Glucoside						
2-Deoxyadenosine						
Glycyl-L-Aspartic acid						
Citricacid						
Bromosuccinicacid						
Propionicacid						
Mucicacid						
Glycolicacid						
Glyoxylicacid						
D-Cellobiose						
Glycyl-L-Glutamic acid						
Mono-Methylsuccinate						
D-Malicacid						
Tyramine						
D-Asparticacid						
1,2-Propanediol						
Tween 40						
α-Ketoglutaricacid						
α-Ketobutyricacid						
L-Glutamine						
Tween 80						
α-Hydroxybutyric acid						
β-Methyl-D-Glucoside						
Adonitol						
Maltotriose						
Dulcitol						
D-Serine						
D-Galactonic acid-γ-Lactone						
DL-Malicacid						
Tween 20						
L-Rhamnose						
D-Fructose						
Aceticacid						
α-D-Glucose						
Thymidine						

	Carbone substrates metabolized by at least one of the five tested *M. ulcerans* strains.
	carbon substrates metabolized by only one of the five tested *M. ulcerans* strains.
	carbon substrates metabolized by all tested*M. ulcerans* strains.
	Moderately positive wells
	Highly positive wells
X	Carbon substrates which are not metabolized by *M. marinum* and metabolized by all tested *M. ulcerans* strains.

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substrates exhibited a strong positive reaction (D-glucosamine, 5-keto-D-gluconic acid, oxalomalic acid, sorbic acid and dihydroxyacetone). A total of 21/28 carbon substrates were also metabolized by *M. marinum* leaving D-galacturonic acid, uridine, methyl pyruvate, α-ketovaleric acid, L-isoleucine, L-lysine and putrescine as the only substrates specific to *M. ulcerans* (Table 3).

Environmental sources for substrates metabolized by all tested *M. ulcerans* strains

Comparing the potential environmental sources in search of substrates metabolized by all tested *M. ulcerans* strains versus non-metabolized substrates, we found a significant association between *M. ulcerans* metabolized substrates and bacteria ($p = 0.000$), fungi ($p = 0.001$), algae ($p = 0.003$) and mollusks ($p = 0.007$). The differences were not significant for plants ($p = 0.535$), fruits and vegetables ($p = 0.870$), mammals ($p = 0.064$), insects ($p = 0.234$) and the inanimate environment ($p = 0.477$). No carbon source was found to be associated with nematodes. Further MedLine research incorporating bacteria, fungi, algae and mollusks as keywords disclosed that 16/28 (57%) metabolized substrates were associated with bacteria, 5/28 (18%) were associated with alga, 3/28 (11%) were associated with mollusks and 2/28 with fungi. Discarding bacteria because of a potential bias since Biolog was designed for the study of bacterial metabolism, 15/28 (54%) metabolized substrates were associated with fungi whereas 6/28 (21%) were associated with the algae and 6/28 (21%) with mollusks (Table 4).

Discussion

We determined that five different strains of *M. ulcerans* could use 28 different substrates as sources of carbon. These results were authenticated by the negativity of the negative controls introduced in every plate and the reproduction of data over two replicates. Moreover, stringent criteria were used to ensure the predictive value of the positive results. However, only seven of these 28 substrates were found to be specifically used by *M. ulcerans* and not by the phylogenetically closest species *M. marinum*. Three of these seven carbon sources indeed contain indispensable amino-acids.

The carbon sources here determined for *M. ulcerans* may by incorporated in culture media in the perspective of enhancing the isolation and culture of this pathogen. Indeed, *M. ulcerans* is a slow-growing mycobacterium and the availability of an improved method for its culture would improve the diagnosis of Buruli ulcer patients and the quest for environmental reservoirs [32]. As an example, it has been shown that the incorporation of chitin into the Middlebrook 7H9 broth enhances the growth of *M. ulcerans* [37]. Accordingly, our study points towards a possible association of *M. ulcerans* with fungi as a potential source of chitin, a polysaccharide possibly degraded by *M. ulcerans*' genome-encoded chitinase [42]. Likewise, the other carbon sources here disclosed should be tested for their potential to increase the cultivation of *M. ulcerans*.

Moreover, our analyses suggested that *M. ulcerans* may have found some sources of carbon in microbial communities including alive and dead bacteria, fungi and algae. As for bacteria, it has been previously reported that *M. ulcerans* was isolated in environments where 17 other mycobacteria species were also isolated, including *M. fortuitum* as a constant co-inhabitant [3, 32, 33]. These results suggest cross-feeding between various bacterial complexes including mycobacteria, for the acquisition of carbon. Likewise, green algae extracts have been shown to halve the *in vitro* doubling time of *M. ulcerans* and promote the formation of biofilm [31]. We observed that *M. ulcerans* metabolizes D-galacturonic acid, the main component of pectin contained in the primary cell walls of terrestrial plants, and putrescine, a foul-smelling

Table 4. Cross-search of the Medline database (May, 2016) for fungi, mollusks and algae as potential sources of carbon substrates; and substrates metabolized by all tested *M. ulcerans* strains. The total number of hits for fungi, mollusks and algae is indicated into brackets. Each cell contains the number of cross-hits and green cells indicate the higher relative hit for each carbon source.

	Algae (19292)	fungi (1392904)	Molluscs (52885)	Bacteria (1934745)
D-ribose	24/19292	2571/1392904	114/52885	5133/1934745
Glucuronamide	0/19292	4/1392904	0/52885	7/1934745
D-Galacturonicacid	0/19292	79/1392904	3/52885	218/1934745
D-Glucosamine	8/19292	498//1392904	28/52885	4499/1934745
Oxalomalic acid	0/19292	0/1392904	0/52885	0/1934745
Sorbic acid	0/19292	357/1392904	2/52885	411/1934745
Dihydroxyacetone	7/19292	294/1392904	3/52885	504/1934745
L-Galactonic acid-g-Lactone	0/19292	0/1392904	0/52885	0/1934745
D-Raffinose	3/19292	646/1392904	4/52885	728/1934745
Butyric acid	26/19292	3089/1392904	266/52885	5689/1934745
Putrescine	17/19292	1174/1392904	27/52885	2226/1934745
Phenylethylamine	10/19292	683/1392904	225/52885	908/1934745
D-Psicose	1/19292	14/1392904	0/52885	77/1934745
L-Malicacid	1/19292	107/1392904	0/52885	301/1934745
L-Lyxose	0/19292	4/1392904	0/52885	28/1934745
Pyruvic acid	19/19292	686/1392904	19/52885	1484/1934745
5-Keto-D-Gluconic acid	0/19292	5/1392904	0/52885	21/1934745
Inosine	2/19292	999/1392904	37/52885	1540/1934745
D-Glucose-6-Phosphate	1/19292	57/1392904	2/52885	94/1934745
Adenosine	182/19292	14109/1392904	1029/52885	27257/1934745
L-Asparagine	17/19292	1980/1392904	37/52885	3428/1934745
D-Fructose-6-Phosphate	2/19292	7/1392904	0/52885	92/1934745
Acetoacetic acid	0/19292	20/1392904	1/52885	62/1934745
a-Keto-Valeric acid	0/19292	2/1392904	0/52885	10/1934745
L-Isoleucine	4/19292	1400/1392904	40/52885	3296/1934745
L-Lysine	65/19292	6989/1392904	254/52885	11894/1934745
Methyl pyruvate	7/19292	4/1392904	0/52885	7/1934745
Uridine	30/19292	3408/1392904	112/52885	6435/1934745

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chemical derived from the decomposition of dead plants, which indicates that *M. ulcerans* may live in assemblages of dead aquatic plants. This finding is reinforced by the observation that *M. ulcerans's* genome encodes five putative cutinases. Cutinases are mainly produced by phytopathogenic fungi to hydrolyze cutin (a main component of the cuticle which covers the aerial surfaces of plants) during plant colonization process [43].

Green algae are among the main food of freshwater mollusks pointed out in our study; mollusks are herbivores like other species of the freshwater snail family [44]. The principal genera of mollusks met in freshwater in West Africa are *Bulinus*, *Planorbis*, *Pila*, *Lanistes*, *Melania*, *Bithynia*, *Lymnaea*, *Biomphalaria*, *Mutela*, *Aspatharia* and *Sphaerium* [23,45]. Previous molecular investigations reported the detection of specific *M. ulcerans* DNA sequences in *Bulinus* spp. [23,46], in *Planorbis* spp. [23] and in mollusks of different Gastropoda order, Bivalvia order and Basommatophora order [26]. Furthermore, the experimental infection of *Pomacea canaliculata* (Ampullariidae) and *Planorbis planorbis* (*Planorbidae*) by plants contaminated by *M. ulcerans*- showed through optic microscopy digestive tract observation that snails remained infected by viable mycobacteria up to 25 days [23]. Small mollusks are also known to be a prey for water bugs which are involved in the transmission of *M. ulcerans* in Buruli ulcer endemic

regions [3]. In West Africa, approximately 76% of the population lives next to rivers, lakes, and other water bodies contaminated with intermediate hosts such as snails [47].

In conclusion, our study is suggesting paths to improve culture media for the enhanced isolation of *M. ulcerans* by mimicking the natural ecosystem of *M. ulcerans* which is probably living in microbial communities with other bacteria, fungi and algae. These data support the recent hypothesis that mollusks could be part of a larger food chain including several hosts giving appropriate shelters to *M. ulcerans*, as recently reported [48]. Small mollusks should be further investigated using culture-based appropriate methods in the search for *M. ulcerans*.

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Author Contributions

Conceptualization: MD.

Methodology: DZ AB.

Software: MM.

Validation: DZ AB MM.

Writing – original draft: DZ AB MD.

References

1. Doig KD, Holt KE, Fyfe JAM, Lavender CJ, Eddyani M, Portaels F, et al. On the origin of Mycobacterium ulcerans, the causative agent of Buruli ulcer. BMC Genomics 2012; 13, 258. doi: 10.1186/1471-2164-13-258 PMID: 22712622
2. Josse R, Guedenon A, Darie H, Anagonou S, Portaels F, Meyers WM. [Mycobacterium ulcerans cutaneous infections: Buruli ulcers]. Medecine tropicale: revue du Corps de sante colonial 1995; 55(4), 363–373.
3. Portaels F, Meyers WM, Ablordey A, Castro AG, Chemlal K, de Rijk P, et al. First Cultivation and Characterization of Mycobacterium ulcerans from the Environment. PLoS Negl Trop Dis. 2008; 2(3), e178. doi: 10.1371/journal.pntd.0000178 PMID: 18365032
4. Portaels F, Fonteyene PA, de Beenhouwer H, de Rijk P, Guedenon A, Hayman J, et al. Variability in 3' end of 16S rRNA sequence of Mycobacterium ulcerans is related to geographic origin of isolates. J Clin Microbiol. 1996; 34(4), 962–965. PMID: 8815117
5. Maccallum P, Tolhurst JC, Buckle G, Sissons HA A new mycobacterial infection in man. J Pathol Bacteriol. 1948; 60(1), 93–122.
6. Johnson PD, Azuolas J, Lavender CJ, Wishart E, Stinear TP, Hayman JA, et al. Mycobacterium ulcerans in mosquitoes captured during outbreak of Buruli ulcer, southeastern Australia. Emerg Infect Dis. 2007; 13(11), 1653–1660. doi: 10.3201/eid1311.061369 PMID: 18217547
7. Stinear TP, Seemann T, Pidot S, Frigui W, Reysset G, Garnier T, et al. Reductive evolution and niche adaptation inferred from the genome of Mycobacterium ulcerans, the causative agent of Buruli ulcer. Genome Res. 2007; 17(2), 192–200. doi: 10.1101/gr.5942807 PMID: 17210928
8. WHO. World: Distribution of buruli ulcer, 2014 WHO Annual meeting on Buruli ulcer 2015.
9. Benbow ME, Williamson H, Kimbirauskas R, McIntosh MD, Kolar R, Quaye C, et al. Aquatic invertebrates as unlikely vectors of Buruli ulcer disease. Emerg Infect Dis. 2008; 14(8), 1247–1254. doi: 10.3201/eid1408.071503 PMID: 18680648
10. Garchitorena A, Ngonghala CN, Texier G, Landier J, Eyangoh S, Bonds MH, et al. Environmental transmission of Mycobacterium ulcerans drives dynamics of Buruli ulcer in endemic regions of Cameroon. Sci Rep. 2015; 5, 18055. doi: 10.1038/srep18055 PMID: 26658922
11. Kenu E, Nyarko KM, Seefeld L, Ganu V, Käser M, Lartey M, et al. Risk Factors for Buruli Ulcer in Ghana—A Case Control Study in the Suhum-Kraboa-Coaltar and Akuapem South Districts of the Eastern Region. PLoS Negl Trop Dis. 2014; 8(11), e3279. doi: 10.1371/journal.pntd.0003279 PMID: 25411974

12. Narh CA, Mosi L, Quaye C, Dassi C, Konan DO, Tay SCK, et al. Source Tracking Mycobacterium ulcerans Infections in the Ashanti Region, Ghana. PLoS Negl Trop Dis. 2015; 9, e0003437. doi: 10.1371/journal.pntd.0003437 PMID: 25612300

13. Addo P, Adu-Addai B, Quartey M, Abbas M, Okang I, Owusu E, et al. Clinical and Histopathological Presentation of Buruli Ulcer in Experimentally Infected Grasscutters (Thryonomys swinderianus). Intern J Trop Med. 2006; 3(2).

14. Mensah-Quainoo EK. A study of the magnitude and determinants of Buruli ulcer disease in the Ga District of Ghana. International Conference on Buruli ulcer Control and Research; 6–8 July 1998; Yamoussoukro, Cote d'Ivoire 1998.

15. Peraudin ML, Herrault A, Desbois JC Ulcère cutanée à Mycobacterium ulcerans (ulcère de Buruli). Annales de Pédiatrie 1980; 27(10), 687–692. PMID: 7212557

16. Brou T, Broutin H, Elguero E, Asse H, Guegan JF Landscape diversity related to Buruli ulcer disease in Cote d'Ivoire. PLoS Negl Trop Dis. 2008; 2(7), 0000271.

17. Johnson RC, Makoutodé M, Sopoh GE, Elsen P, Gbovi J, Pouteau LH, et al. Buruli Ulcer Distribution in Benin. Emerg Infect Dis. 2005; 11(3), 500–501. doi: 10.3201/eid1103.040597 PMID: 15789490

18. N'krumah RTAS, Koné B, Tiembre I, Cissé G, Pluschke G, Tanner M, et al. Socio-Environmental Factors Associated with the Risk of Contracting Buruli Ulcer in Tiassalé, South Côte d'Ivoire: A Case-Control Study. PLoS Negl Trop Dis. 2016; 10(1), e0004327. doi: 10.1371/journal.pntd.0004327 PMID: 26745723

19. Aiga H, Amano T, Cairncross S, Domako JA, Nanas OK, Coleman S Assessing water-related risk factors for Buruli ulcer: a case-control study in Ghana. Am J Trop Med Hyg. 2004; 71(4), 387–392. PMID: 15516631

20. Tian RB, Niamke S, Tissot-Dupont H, Drancourt M. Detection of Mycobacterium ulcerans DNA in the Environment, Ivory Coast. PLoS One 2016; 11(3): e0151567 doi: 10.1371/journal.pone.0151567 PMID: 26982581

21. Ahoua L, Guetta A, Ekanza E, Bouzid S, N'Guessan R, Dosso M Risk factors for Buruli ulcer in Côte d'Ivoire: Results of a cas-control study, August 2001. Afr J Biotechnol. 2009; 8, 536–546.

22. Vandelannoote K, Durnez L, Amissah D, Gryseels S, Dodoo A, Yeboah S, et al. Application of real-time PCR in Ghana, a Buruli ulcer-endemic country, confirms the presence of Mycobacterium ulcerans in the environment. FEMS Microbiol Lett. 2010; 304(2), 191–194. doi: 10.1111/j.1574-6968.2010.01902.x PMID: 20146745

23. Marsollier L, Severin T, Aubry J, Merritt RW, Saint Andre JP, Legras P, et al. Aquatic snails, passive hosts of Mycobacterium ulcerans. Appl Environ Microbiol. 2004; 70(10), 6296–6298. doi: 10.1128/AEM.70.10.6296-6298.2004 PMID: 15466578

24. Marsollier L, Robert R, Aubry J, Saint Andre JP, Kouakou H, Legras P, et al. Aquatic insects as a vector for Mycobacterium ulcerans. Appl Environ Microbiol. 2002; 68(9), 4623–4628. doi: 10.1128/AEM.68.9.4623-4628.2002 PMID: 12200321

25. Konan KL, Doannio JM, Coulibaly NG, Ekaza E, Marion E, Asse H, et al. [Detection of the IS2404 insertion sequence and ketoreductase produced by Mycobacterium ulcerans in the aquatic Heteroptera in the health districts of Dabou and Tiassale in Cote d'Ivoire]. Med Sante Trop. 2015; 25(1), 44–51. doi: 10.1684/mst.2014.0363 PMID: 25499000

26. Williamson HR, Benbow ME, Nguyen KD, Beachboard DC, Kimbirauskas RK, McIntosh MD, et al. Distribution of Mycobacterium ulcerans in buruli ulcer endemic and non-endemic aquatic sites in Ghana. PLoS Negl Trop Dis. 2008; 2(3), e205. doi: 10.1371/journal.pntd.0000205 PMID: 18365034

27. Zogo B, Djenontin A, Carolan K, Babonneau J, Guegan J-F, Eyangoh S, et al. A Field Study in Benin to Investigate the Role of Mosquitoes and Other Flying Insects in the Ecology of Mycobacterium ulcerans. PLoS Negl Trop Dis. 2015; 9(7), e0003941. doi: 10.1371/journal.pntd.0003941 PMID: 26196901

28. Amissah NA, Gryseels S, Tobias NJ, Ravadgar B, Suzuki M, Vandelannoote K, et al. Investigating the Role of Free-living Amoebae as a Reservoir for Mycobacterium ulcerans. PLoS Negl Trop Dis. 2014; 8(9), e3148. doi: 10.1371/journal.pntd.0003148 PMID: 25188535

29. Williamson HR, Benbow ME, Campbell LP, Johnson CR, Sopoh G, Barogui Y, et al. Detection of Mycobacterium ulcerans in the Environment Predicts Prevalence of Buruli Ulcer in Benin. PLoS Negl Trop Dis. 2012; 6(1), e1506. doi: 10.1371/journal.pntd.0001506 PMID: 22303498

30. Tian RD, Lepidi H, Nappez C, Drancourt M Experimental Survival of Mycobacterium ulcerans in Watery Soil, a Potential Source of Buruli Ulcer. Am J Trop Med Hyg. 2016; 94(1), 89–92. doi: 10.4269/ajtmh.15-0568 PMID: 26526927

31. Marsollier L, Stinear T, Aubry J, Saint André JP, Robert R, Legras P, et al. Aquatic Plants Stimulate the Growth of and Biofilm Formation by Mycobacterium ulcerans in Axenic Culture and Harbor These Bacteria in the Environment. App Environm Microbiol. 2004; 70(2), 1097–1103.

32. Aboagye SY, Danso E, Ampah KA, Nakobu Z, Asare P, Otchere ID, et al. Isolation of Nontuberculous Mycobacteria from the Environment of Buruli Ulcer Endemic Communities in Ghana. App Environ Microbiol. 2016; 6, 2016.

33. Marsollier L, Aubry J, Saint-andré J, Robert R, Legras P, Manceau A. Ecology and transmission of Mycobacterium ulcerans. 2003; 51:490–5.

34. Garland JAYL, Mills AL. Classification and Characterization of Heterotrophic Microbial Communities on the Basis of Patterns of Community-Level Sole-Carbon-Source Utilization. 1991; 57(8):2351–9.

35. Khatri B, Fielder M, Jones G, Newell W, Abu-Oun M, Wheeler PR High Throughput Phenotypic Analysis of Mycobacterium tuberculosis and Mycobacterium bovis Strains' Metabolism Using Biolog Phenotype Microarrays. PLoS ONE 2013; 8(1), e52673. doi: 10.1371/journal.pone.0052673 PMID: 23326347

36. Chen J-W, Scaria J, Chang Y-F Phenotypic and Transcriptomic Response of Auxotrophic Mycobacterium avium subsp. paratuberculosis leuD Mutant under Environmental Stress. PLoS ONE 2012; 7(6), e37884. doi: 10.1371/journal.pone.0037884 PMID: 22675497

37. Sanhueza D, Chevillon C, Colwell R, Marion E, Marsollier L. Chitin promotes Mycobacterium ulcerans growth. J er. 2016; 2010(October 2015):1–6.

38. Schoröder K. H. Investigation into the relatioship of M. ulcerans to M. burili and other mycobacteria. American review of resperatory disease 1975; 111, 559–562.

39. Bouricha M, Castan B, Duchene-Parisi E, Drancourt M Mycobacterium marinum infection following contact with reptiles: vivarium granuloma. Int J Infect Dis. 2014; 21, 17–18. doi: 10.1016/j.ijid.2013.11.020 PMID: 24530276

40. Bochner BR Global phenotypic characterization of bacteria. Fems Microbiol Rev. 2009; 33(1), 191–205. doi: 10.1111/j.1574-6976.2008.00149.x PMID: 19054113

41. Bochner BR, Gadzinski P, Panomitros E Phenotype MicroArrays for High-Throughput Phenotypic Testing and Assay of Gene Function. Genome Res. 2001; 11(7), 1246–1255. doi: 10.1101/gr.186501 PMID: 11435407

42. Stinear TP, Seemann T, Pidot S, Frigui W, Reysset G, Garnier T, et al. Reductive evolution and niche adaptation inferred from the genome of Mycobacterium ulcerans, the causative agent of Buruli ulcer. Genome Res. 2007; 17(2):192–200. doi: 10.1101/gr.5942807 PMID: 17210928

43. Côtes K, Bakala N'Goma JC, Dhouib R, Douchet I, Maurin D, Carrière F, et al. Lipolytic enzymes in Mycobacterium tuberculosis. Appl Microbiol Biotechnol. 2008; 78(5):741–9. doi: 10.1007/s00253-008-1397-2 PMID: 18309478

44. El-Assal FM, Shanab SMM, Abou-El-Hassan AA, Mahmoud KMA. Effect of some algal species on the snail intermediate hosts of schistosomiasis in Egypt II. Growth, infection and mortality rates. Egypt J Phycol. 2005; 6(1), 93–110.

45. Thiam N, Diallo A Intégration de la biodiversité d'eau douce dans le processus de développement en Afrique: Module de formation des formateurs sur Le suivi des Mollusques d'eau douce. Wetlands International Afrique [serial on the Internet]. 2010. https://cmsdata.iucn.org/downloads/module_mollusques_fr.pdf.

46. Kotlowski R, Martin A, Ablordey A, Chemlal K, Fonteyne P-A, Portaels F One-tube cell lysis and DNA extraction procedure for PCR-based detection of Mycobacterium ulcerans in aquatic insects, molluscs and fish. J Med Microbiol. 2004; 53(9), 927–933.

47. Hotez PJ, Kamath A Neglected Tropical Diseases in Sub-Saharan Africa: Review of Their Prevalence, Distribution, and Disease Burden. PLoS Negl Trop Dis. 2009, 3(8), e412. doi: 10.1371/journal.pntd.0000412 PMID: 19707588

48. Morris AL, Guégan J, Andreou D, Marsollier L, Carolan K, Le Croller M, et al. Deforestation-driven food-web collapse linked to emerging tropical infectious disease, Mycobacterium ulcerans. 2016;1–7.

Chapter3: Improving culture-based detection of *Mycobacterium ulcerans*from environnemental sources

M. ulcerans is a slow-growing mycobacterium. Its cultivation from clinical specimens is very tedious. And it takes about nine months to decide the negative result of a bacterial culture. The main limitation is the culture of this pathogen from environmental sources. The culture of *M. ulcerans* from environmental samples is even more tedious and the attempts of their culture have generally led to failures despite the detection of their DNA in these same samples. Nowadays, only one environmental *M. ulcerans* isolate has been firmly confirmed after two years of hard work by Françoise Portaels and colleagues. The development of improve methods of environmental samples decontamination and innovate culture media is of major interest for research on Buruli ulcer disease, This study is timely and very important to go further with the understanding of *M. ulcerans* ecology, evolution and transmission, which is still a puzzling question today.

This paper describes new decontamination and culture methods in order to improve the growth of *M. ulcerans* clinical strains but also to allow the isolation of this pathogen from environmental sources (i.e. water, plant

debris, agouti faeces). The approach for the composition of the innovative medium is based on the results of high throughput phenotyping of *M. ulcerans* strains by Biolog System as presented in Chapter 2 combined with data issued from published genome sequences. This work is the first one to really develop a new experimental protocol for the specific isolation and culture of *M. ulcerans* environmentally-persistent strains. We shown that the combination of 1% chlorhexidine-decontaminated samples followed by their culture on DZ medium enhanced the growth of clinical strains compared to the classic Middlebrook 7h10 media and allowed the isolation of a unique colony from faeces of a single agouti. Whilst the developed protocol constitutes a first step in the culture of *M. ulcerans* from the environment samples, the sub-culture of this colony failed and future studies will thus have to focus on this limitation.

The possible role of wild animals in the chain of transmission of Buruli ulcer in Africa should be study on a large scale based on isolation and culture in complement to molecular detection.

Article 4: "A protocol for culturing environmental strains of the Buruli ulcer agent, *Mycobacterium ulcerans*"

Scientific Reports

(submitted)

REVISED VERSION

A protocol for culturing environmental strains of the Buruli ulcer agent, *Mycobacterium ulcerans*

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ABSTRACT

Contaminations and fastidiousness of *M. ulcerans* may have both hamper isolation of strains from environmental sources. We aimed to optimize decontamination and culture of environmental samples to circumvent both limitations. Three strains of *M. ulcerans* cultured onto Middlebrook 7H10 at 30°C for 20 days yielded a significantly higher number of colonies in micro-aerophilic atmosphere compared to ambient atmosphere, 5% CO_2 and anaerobic atmosphere. In a second step, we observed that *M. ulcerans* genome uniquely encoded chitinase, fucosidase and A-D-GlcNAc-diphosphoryl polyprenol A-3-L-rhamnosyl transferase giving *M. ulcerans* the potential to metabolize chitine, fucose and N-acetyl galactosamine (NAG), respectively. A significant growth-promoting effect of 0.2 mg/mL chitin ($p < 0.05$), 0.01 mg/mL N-acetyl galactosamine ($p < 0.05$), 0.01 mg/mL fucose ($p < 0.05$) was observed with *M. ulcerans*; leading to the present design of the DZ-medium (Middlebrook 7H10 medium containing chitin (0.2%), NAG (0.01%) and fucose (0.01%)) . Finally, the protocol combining 1% chlorhexidine decontamination with micro-aerophilic incubation on the DZ-medium and auto-fluorescence detection of colonies allowed for the isolation of one firmly identified *M. ulcerans* strain from *Thryonomys swinderianus* (aulacode) feces specimens collected near the Kossou Dam, Côte d'Ivoire. We propose that incubation of chlorhexidine-decontaminated environmental specimens on the DZ-medium under micro-aerophilic atmosphere at 30°C may be used for the tentative isolation of *M. ulcerans* strains from potential environmental sources.

Introduction

Buruli ulcer is a World Health Organization (WHO)-notifiable, yet neglected infection of the cutaneous and subcutaneous tissues caused by the nontuberculous *Mycobacterium ulcerans* [1]. This pathogen emerged from a common ancestor with the environmental *Mycobacterium marinum,* after genomic reduction and the acquisition of a 174-kb pMUM001 plasmid encoding a macrolide mycolactone toxin, the major virulence factor for *M. ulcerans* [2-4]. *M. ulcerans* was initially isolated from sub-cutaneous lesions in patients in Bairnsdale, Australia, where Buruli ulcer (Bairnsdale ulcer) was initially described [5]. For more than 70 years, Buruli ulcer cases have been notified in patients residing in 33 countries mainly in the rural and tropical regions of Africa, significantly less in South America, in addition to Australia and Japan [1]. For an example, in 2014, 2,200 new cases were notified by 12 countries and most of the patients were children under 15 years [1]. The laboratory diagnostic of Buruli ulcer is made by microscopy, histopathology and PCR-based detection of *M. ulcerans*-specific sequences, including the IS*2404*, IS*2606* and ketoreductase-B domain of the mycolactone polyketide synthase genes [1,6-8]. Successful isolation and culture of *M. ulcerans* from clinical lesions depends on several parameters, including the exact sampled site (most bacilli are in the deepest areas of the skin), or the type of decontamination method or culture medium and culture conditions used [8-11]. Whilst *M. ulcerans* grows on similar culture media as *Mycobacterium tuberculosis*, i.e. on Löwenstein-Jensen medium, Brown and Buckle or Ogawa medium, microaerophilic atmosphere and optimal temperature of 28-33°C are required for this pathogen [8-11]. In a clinical diagnostic laboratory, primary cultures are usually positive within a 6-12-week incubation, but a much longer incubation period of up to nine months may be necessary to obtain isolates, illustrating the fastidiousness of this microbe [11,12].

The fact that thousands *M. ulcerans* isolates have been made from clinical sources sharply contrasts with the fact that several attempts to culture *M. ulcerans* from many

specimens of flora and fauna remained unsuccessful [13,14]. However, numerous PCR-based investigations indicated that potential reservoirs or host carriers were localized in aquatic environments where *M. ulcerans* may be able to colonize different ecological niches eventually scattered along a food chain [15-19]. Culture of *M. ulcerans* from environmental samples is tedious and understanding the ecology of *M. ulcerans* has been severely hampered by the extreme difficulty of culturing the organism directly from the environment[14,20,21]. Cultures of collected diverse samples (water, soil, fish, rodents, biting flies, reptiles) from Buruli ulcer endemic areas failed to yield *M. ulcerans* a long time ago [13], though testing of samples by molecular biology found *M. ulcerans* DNA [7,22-30]. Finally, only one environmental *M. ulcerans* (*M. ulcerans* 00-1441 from a Buruli ulcer endemic area in Benin, West Africa) isolate has been firmly confirmed on Löwenstein-Jensen medium after 15-day of incubation in BACTEC 12b broth and three successive passages in mouse footpad P1, P2 and P3 for nine months, six months and 12 months, followed by culture on Löwenstein-Jensen for two months [21]. Three additional reported strains included two IS*2404*-PCR positive strains from two samples of aquatic plants and two wild aquatic insects collected in a Buruli ulcer endemic area of Côte d'Ivoire [31,32] and two *M. ulcerans* strains from moss and soil in Ghana [33]. None of these strains have been deposited in public collection.

The fact that only a few environmental isolates have been made after such a long experiment, suggests that contamination by fast-growing bacteria or mycobacteria and fungi of the environmental samples along with poorly appropriate culture media limited the isolation of *M. ulcerans* from environmental sources. Also when contaminants are present in the sample, they limit and inhibit the growth of *M. ulcerans*. Contaminants not eliminated by the decontamination method eventually rot or transferring the culture medium rendering impossible the incubation of cultures on a long period necessary for the isolation of *M. ulcerans*. There is thus a need to develop new protocols and innovative

media to improve the recovery of *M. ulcerans* in primary culture from environmental sources in order to assess the viability of the pathogen in these sources [34]. The availability of the complete genome sequence of *M. ulcerans* is a boon to better known metabolic activities which could support the development of innovative culture media, as previously reported for some other fastidious pathogens [35].

In the perspective of achieving a culture-based field investigation of *M. ulcerans*, we aimed at improving the decontamination of samples along with the composition of culture media in order to optimize the chance of recovering additional environmental *M. ulcerans* isolates.

RESULTS

Effect of atmosphere in culturing *M. ulcerans* onto Middlebrook 7H10. We observed an enhanced growth of *M. ulcerans* under micro-aerophilic atmosphere at day 20 post-incubation on Middlebrook 7H10 medium at 30°C. The number of colonies of *M. ulcerans* CU001 was significantly higher in micro-aerophilic atmosphere (133 ± 6 CFUs) than under ambient atmosphere (65 ± 14 CFUs; p=0.002) or 5% CO_2 atmosphere (36 ±14 CFUs; p=0.002) or anaerobic atmosphere (10 ± 5 CFUs; p<0.001); likewise, *M. ulcerans* ATCC25900 yielded 125 ± 18 CFUs in micro-aerophilic atmosphere compared to 49 ± 1.7 CFUs (p=0.002) under ambient atmosphere, 45 ± 11 CFUs (p=0.003) under 5% CO_2 atmosphere and (44 ± 7 CFUs; p=0.002) in anaerobic atmosphere; and *M. ulcerans* ATCC33728 yielded 195 ± 13 CFUs under micro-aerophilic atmosphere compared to 53 ± 6 CFUs (p=0.0001) in ambient atmosphere , 64 ± 32 CFUs (p=0.003) under 5% CO2 atmosphere and 99 ± 11 CFUs; p=0.001 in anaerobic atmosphere (Figure 1).

Cultures at 37 ° C. remained negative during the same period of incubation.

Effect of growth promoters on culturing *M. ulcerans* strains. We incorporated chitin, fucose and N-acetylgalactosamine into Middlebrook 7H10 medium in order to experimentally test their growth-promoting effect on *M. ulcerans*. The experimental data were authenticated by the negativity of the negative controls used in every experiment and the reproducibility of data over three different *M. ulcerans* strains and three independent experiments. Incorporation of 0.1 mg/mL fucose or 0.1 mg/mL N-acetyl galactosamine into Middlebrook 7H10 base yielded no significant difference in the growth of *M. ulcerans* until day 15. However, from day 15 to day 40, the number of colonies was significantly higher on Middlebrook 7H10 medium enriched with 0.1 mg/mL N-acetyl galactosamine (1,485 ± 275 CFUs for *M. ulcerans* CU001, 340 ± 28 CFUs for *M. ulcerans* ATCC25900, 788 ± 125 CFUs for *M. ulcerans* ATCC33728) or Middlebrook 7H10 enriched with 0.1 mg/mL fucose (1,770 ± 241 CFUs for *M. ulcerans* CU001, 770 ± 9 CFUs for *M. ulcerans* ATCC25900, 516 ± 11 CFUs for *M. ulcerans* ATCC33728) than in standard Middlebrook 7H10 medium (359 ± 16 CFUs for *M. ulcerans* CU001, 129 ± 8 CFUs for *M. ulcerans* ATCC25900, 278 ± 9 CFUs for *M. ulcerans* ATCC33728) ($p<0.05$) (Figure 2).

Furthermore, the number of colonies was significantly higher on Middlebrook 7H10 medium enriched with 0.01 mg/mL N-acetyl galactosamine (1,054 ± 84 CFUs for *M. ulcerans* CU001, 920 ± 7 CFUs for *M. ulcerans* ATCC25900, 967 ± 40 CFUs for *M. ulcerans* ATCC33728) or Middlebrook 7H10 enriched with 0.01 mg/mL fucose (871 ± 102 CFUs for *M. ulcerans* CU001, 784 ± 53 CFUs for *M. ulcerans* ATCC25900, 821 ± 56 CFUs for *M. ulcerans* ATCC33728) or Middlebrook 7H10 enriched with 0.2 mg/mL chitin (802 ± 82 CFUs for *M. ulcerans* CU001, 741 ± 42 CFUs for *M. ulcerans* ATCC25900, 655 ± 25 CFUs for *M. ulcerans* ATCC33728) than in standard Middlebrook 7H10 medium (67 ± 1 CFUs for *M. ulcerans* CU001, 97 ± 2 CFUs for *M. ulcerans* ATCC25900, 80 ± 2 CFUs for *M. ulcerans* ATCC33728) ($p <0.05$) (Figure 3).

The number of colonies was significantly higher on Middlebrook 7H10 medium enriched with a mix of the three growth promoters called DZ medium (0.01 mg/mL N-acetyl galactosamine, 0.01 mg/mL fucose and 0.2 mg/mL chitin) (936 ± 21 CFUs for *M. ulcerans* CU001, 927 ± 57 CFUs for *M. ulcerans* ATCC25900, 1087 ± 82 CFUs for *M. ulcerans* ATCC33728) than in standard Middlebrook 7H10 medium (67 ± 1 CFUs for *M. ulcerans* CU001, 97 ± 2 CFUs for *M. ulcerans* ATCC25900, 80 ± 2 CFUs for *M. ulcerans* ATCC33728) ($p < 0.05$) (Figure 3). The doubling time of *M. ulcerans* was measured at 2.95 ± 0.98 days with chitin, 1.3 ± 0.23 days with NAG, 1.31 ± 0.28 days for fucose, 2.37 ±.0.41 days with chitin/NAG/Fucose and 5.96 ± 2.99 days with the control Middlebrook 7H10 medium.

Chlorhexidine decontamination of river freshwater artificially inoculated with *M. ulcerans* strains. Direct seeding without decontamination of river freshwater sample onto 5 % sheep-blood Columbia agar combined with MALDI-TOF-MS identification of colonies yielded *Acinetobacter baumannii, Acinetobacter baylyi, Acinetobacter junii, Acinetobacter nosocomialis, Aeromonas caviae, Aeromonas ichthiosmia, Aeromonas hydrophila, Aeromonas veronii, Brevibacterium luteolum, Brevibacterium paucivorans, Escherichia coli, Klebsiella pneumoniae* and *Serratia marcescens* within 48 hours of incubation.

After 1% chlorhexidine decontamination of freshwater sample, the culture onto sheep-blood Columbia agar remained sterile without contamination. The same freshwater sample mocked-inoculated with *M. ulcerans* strain (CU001 and ATCC33728 separately) and chlorhexidine-decontaminated yielded autofluorescent colonies of *M. ulcerans*, starting at day 15 after inoculation onto the chitin, fucose and NAG growth promoters media. Colony counting was done on day 30 (Figure 4). Several colonies obtained during this step were confirmed by RT-PCR targeting specific genomic regions of *M. ulcerans* (IS*2404*, IS*2606*, KR-B) in order to

confirm *M. ulcerans* colonies . RT-PCR results where positive for all IS*2404*, IS*2606* and KR-B targets.

Detecting *M. ulcerans* DNA in environment samples, Côte d'Ivoire. Mycolactone-producing mycobacteria (MPM) DNAs were detected by RT-PCR in the environmental samples collected in Côte d'Ivoire, while the negative controls remained negative and all the samples were free of PCR inhibition. Five of the 12 water without plant debris (41.66%) were positive for KR-B gene, insertion sequence IS*2404* and IS*2606*; five (41.66%) were positive for KR-B and IS*2404*, ten (83.33%) were positive for KR-B gene and IS*2606*. Among the eight water with plants debris, two (25%) were positive for KR-B and IS*2404*. No *Thryonomys swinderianus's* aulacode feces were definitely found positive according to our criteria while two of 11 feces samples (18.18%) were positive for IS*2404* (Table 1). Further, the calculated values for Δ*Ct* (IS*2606*-IS*2404*) from MPM-PCR positive environmental samples were ≤3.32 (95% CI=0.43– 1.70), suggesting that all the sequences detected were attributable to *M. ulcerans* which typically give higher Δ*Ct* values than the other MPM, as previously reported[7].

Culturing *M. ulcerans* micro-colony in environment samples, Côte d'Ivoire. The culture of the 31 samples decontaminated with 1% chlorexidine yielded one auto-fluorescent micro-colony isolated from one IS*2404*-positive aulacode feces sample after 45-day incubation on the DZ medium at 30°C under a micro-aerophilic atmosphere condition. This unique micro-colony yielded Ziehl-Neelsen -positive bacilli identified as a MPM after the RT-PCR positivity for KR-B, IS*2404* and IS*2606* in the presence of negative controls (Figure 5). Further, calculated value for Δ*Ct* (IS*2606*-IS*2404*) of 2. 97 (Δ*Ct* ≤3.32) indicated that it was more likely a colony of *M. ulcerans* according to previously described criteria[7].

Sub-culturing onto the DZ medium of the micro-colony material remaining after identification failed.

DISCUSSION

We here report on a culture protocol for the recovery of viable *M. ulcerans* mycobacteria from environmental sources and we successfully applied it to the first isolation of micro-colonies from aulacode feces collected in Côte d'Ivoire.

Temperature of incubation is a crucial point for the culture of mycobacteria as previously reported [36]. Indeed, *M. ulcerans* strains have optimal growth between 28- 32°C; they are very sensitive to higher temperatures, a temperature of 41°C over a period of 24 hours kills more than 90 % of the bacilli [13]. This observation gives indications for the storage of environmental samples for *M. ulcerans* culture in endemic countries where the ambient temperature may reach values in-between 37°C-45°C, suggesting that samples should be stored at lower temperatures into any appropriate transport medium before inoculation. We then observed that growth of the *M. ulcerans* strains here investigated was significantly more rapid in micro-aerophilic atmosphere than in ambient atmosphere and in a 5% CO_2-enriched atmosphere. It was previously suggested that *M. ulcerans* is capable of growth under aerobic but not anaerobic conditions[3] and to survive anaerobic conditions [37]. During our experiment we observed a significant growth of the three strains of *M. ulcerans* cultured under anaerobic conditions after day 20 of incubation. Indeed, breaking anaerobic atmosphere may occur during which a suitable atmospheric condition for the growth of mycobacteria was created during colonies count and / or pocket replacement every five days. Further, a possible activity of the *cydA* locus in *M. ulcerans* may sustain the ability of this strains to survive under low-oxygen conditions [37].

To develop an innovative culture medium for improve isolation and growth of *M. ulcerans*, we thought that comparative genome analysis reveal unique metabolic features and clues to enrich a Middlebrook 7H10 medium. Indeed, this approach has been successfully used to design a new culture medium for the fasditious pathogen *Tropheryma whipplei*, another Actinobacterium [35]. Accordingly, we incorporated chitin, fucose and N-acetylgalactosamine into a Middlebrook 7H10 medium in order to experimentally test their effect on the growth of *M. ulcerans*. The experimental data were authenticated by the negativity of the negative controls used in every experiment and the reproducibility of data over three different *M. ulcerans* strains culture and triplicate experiments. Chitin is the second most abundant organic and renewable source in nature, after cellulose [38]. This linear homopolymer can be hydrolyzed at β-1,4-linkages by the enzymatic action of glycoside hydrolase enzymes, the chitinases (E.C. 3.2.1.14) and the N-acetylglucosaminidases (E.C. 3.2.1.52) [39,40].Chitin is found in the structure of fungi, crustaceans (crabs, lobsters), insects, mollusks, cephalopods, fishes such as zebrafish (*Danio rerio*) and amphibians [41-43]. The derivatives of chitin play a crucial role in the interaction between higher plants and symbiotic bacteria; suggesting that chitin synthesis may serve roles other than the production of skeletal material [42]. N-acetyl galactosamine has been recognized as a minor sugar component covalently-bound amino sugar component of the cell wall of some slow-growing mycobacteria and orthologs of polyprenyl-phospho-N-acetyl-galactosaminyl synthase (ppgS), which are found in the genomes of slowly-growing mycobacteria including *M. bovis*, *M. bovis* BCG, *M. leprae*, *M. marinum* and *M. avium* subsp. *paratuberculosis*, as well as in *M. abscessus*; but not in the genomes of other rapidly growing *Mycobacterium* species such as *Mycobacterium smegmatis* [44,45].

Chitinases are chitin-degrading enzymes belonging to the glycoside hydrolase family 18 (GH18) and 19 (GH19) [39] (www.cazy.org). They act in a synergistic to perform the complete enzymatic hydrolysis of chitin to N-acetylglucosamine [46,47]. The GH18 family is widely

distributed in all kingdoms, including viruses, bacteria, plants, fungi and animals [38]. Bacterial chitinases and chitin-binding proteins (CBPs) play a fundamental role in the degradation of the ubiquitous biopolymer chitin, and the degradation products serve as an important nutrient source for marine- and soil-dwelling bacteria [48-51]. *M. ulcerans* genomes encode for a GH18 compatible with a putative chitinase activity [3]. N-acetylglucosaminidases belong to glycoside hydrolase family 20 (GH20) [39]. α-l-Fucosidases are enzymes involved in metabolism of α-l-fucosylated molecules , compounds with a fundamental role in different life essential processes including development [52]. These enzymes play a fundamental role in the degradation of the ubiquitous biopolymer [39,46,50]. The degradation products serve as an important nutrient source for bacteria in the nature [51]. *M. ulcerans* may obtain energy and carbon from the degradation of plant saccharides which were demonstrated to stimulate *M. ulcerans* growth *in vitro* [14,53]. It was recently shown that chitin promoted growth of *M. ulcerans* [43]. Fucose was here tested after we observed that *M. ulcerans* contained an alpha-L-fucosidase cytoplasmic protein involved in carbohydrate transport and metabolism[39].

It was proved that some green algae extracts stimulate the growth of *M. ulcerans*[14]. In a later study however, the growth of *M. ulcerans* was indirectly observed by using quantitative PCR kinetics, instead of the simple observation of growing colonies as reported here[43].

The doubling time obtained with the culture of *M. ulcerans* strains onto each growth promoter was less than two days and was in agreement with previously reported values. The doubling time was estimated to be of approximately 36 h [54], 1-2 days [55] and 3.3 ± 0.56 days [14]. In the Dubos medium (a liquid medium) , the doubling time was less than 48 hours at 33°C [56] and 44 hours in the Dubos medium without serum [57]. A doubling time estimated to be between three and four days has been reported [58]. In mice, the doubling time was approximately 3.5 days [59-61]. The discrepancy may be first due to the strain of *M. ulcerans*,

secondly to the calculation method [57] or the type of culture medium. In contrast, *M. marinum* has a doubling time of 6-11 h [3].

Culture onto 5 % sheep-blood Columbia agar of freshwater sample after chlorhexidine decontamination remained sterile so, we deduced that the chlorhexidine decontamination method has been effective. Culture onto growth promoters media of chlorhexidine decontaminated freshwater sample mocked- inoculated with *M. ulcerans* strains allowed to isolate *M. ulcerans* colonies confirmed by RT-PCR.

M. ulcerans DNA were detected by RT-PCR in water, plants debris and aulacodes feces, all collected in Côte d'Ivoire. *M. ulcerans's* DNA has been detected previously in two feces from the aulacode collected in Côte d'Ivoire [22] and in small mammal (*Mastomys*) in Ivory Coast [27] suggesting that these animals may shelter and vehicle *M. ulcerans*. In our study, several strains yielded IS*2404* region but not KR-B region detection. It may suggest that such environmental *M. ulcerans* strains did not produce mycolactone or were mycolactone-deficient strain of *M. ulcerans* or other mycobacteria such as *M. marinum*. Other several strains had KR-B but not IS*2404*. It may suggest that these strains were not *M. ulcerans*, but had mycolactone-producing gene.

Growth promoters allow the isolation of micro-colony which was positive for IS*2404*, IS*2606* and KR-B. However, direct sub-culture onto the growth-promoters for more biological material failed. This failure could be explained by the very low inoculum remaining for the culture after we realized Zielh-Neelsen staining and the RT-PCR for the identification of the micro-colony. Our samples were collected in a Buruli ulcer endemic region in the centre of Côte d'Ivoire, closely related but different from a site where we previously PCR-amplified *M. ulcerans* DNA in the feces of aulacodes [22]. However, the isolation of one *M. ulcerans* micro-colony from one feces sample does not prove that

295 aulacodees constitute one reservoir for *M. ulcerans* in Côte d'Ivoire. Indeed, only one of 11
296 tested aulacode feces samples produced only one single viable micro-colony of *M. ulcerans*,
297 which could have arose by chance or natural contamination in the environment. Moreover, the
298 aulacode could have fed upon some infected organic materials, thus having *M. ulcerans* into
299 its digestive tract, acting thus as host spreaders, instead of being the natural host reservoirs.
300 Therefore, this interesting yet limited observation warrants further field studies for
301 confirmation.
302

303 **Conclusions**
304 A 1% chlorhexidine decontamination and addition of appropriate concentration of chitin, N-
305 acetyl galactosamine and fucose to the standard Middlebrook 7H10 culture medium (an
306 innovative medium here reported as DZ medium named after its inventor) promoted the
307 growth of *M. ulcerans* under microaerophilic atmosphere at 30°C. This protocol allowed for
308 the discovery of the first *M. ulcerans* isolate from the aulacode feces collected in a Buruli
309 ulcer endemic region in Côte d'Ivoire. This protocol is proposed as a first-line protocol for the
310 tentative isolation of additional *M. ulcerans* strains during field campaigns in Buruli ulcer
311 endemic areas.
312

313 **METHODS**
314 **Ethics statement.** The study has been conducted with collection references strains of *M.*
315 *ulcerans* and no experiment or test has been performed on patients or/and animals.
316 ***M. ulcerans* strains**. Three strains of *M. ulcerans* isolated from different geographic origins
317 were used throughout the study. *M. ulcerans* strain Cu001 (a gift from Prof Vincent Jarlier,
318 Centre National de Référence des Mycobactéries, Paris France) was from Côte d'Ivoire [62], *M.*
319 *ulcerans* ATCC 33728 isolated from Japan and *M. ulcerans* ATCC 25900 belongs to the

Borstel collection (Schröder 5392) and was probably isolated in Africa. The identification of these three strains were ensured by *rpoB* gene sequence analysis prior to experiments [63]. *M. ulcerans* strains were sub-cultured at 30°C onto Middlebrook 7H10 agar medium supplemented with 10% (v/v) oleic acid/albumin/dextrose/catalase (OADC) (Becton Dickinson, Sparks, MD, USA) and 0.5% (v/v) glycerol (Sigma-Aldrich, Lyon, France) in a microaerophilic atmosphere until we have enough colonies to prepare inoculum. Then, a mycobacterial suspension was prepared by placing a loopful of colonies in a tube containing sterile phosphate buffered saline (PBS, pH 6.5) and sterile glass beads. The tube was vigorously vortexed in order to separate any bacterial aggregates and adjusted with PBS in setting the mycobacterial concentration of inoculum to 0.5 McFarland standards in order to obtain a final suspension containing 10^7 acid-fast bacteria (AFB)/mL using a turbidimeter (Biolog Inc., Hayward, U.S.A). This suspension was shown to be free of clumps by microscopic examination after Ziehl-Neelsen staining. Then, a 10^6 AFB/mL working suspension was prepared.

In all further experiments, colonies were observed and count by using a MZ-FLIII fluorescence microscope (Leica, Nanterre, France) equipped with a GFP filter and an ICA digital camera (Leica) to detect mycobacterial auto-fluorescence as previously described [64]. Counting of fluorescent colonies was performed using the Leica Application Suite software (Leica). The identification of colonies was confirmed by matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS) (Bruker Daltonics, Bremen, Germany) as previously described [65]

Testing atmosphere conditions for culturing *M. ulcerans*.

The first step consisted in the culture of *M. ulcerans* under different conditions. For each *M. ulcerans* strain, a 100 µL-volume of a 10^5 AFB/mL suspension corresponding to 10^4 AFB

was cultured in triplicate onto Middlebrook 7H10 medium supplemented with 10% (v/v) OADC and 0.5% (v/v) glycerol then, incubated at 30°C under four different atmospheric conditions. The atmospheric growing conditions were artificially created by the use of sealed plastic pouch that can hold ten Middlebrook 7H10 medium poured in 55-mm diameter Petri dishes. The conditions of culture to produce standard, microaerophilic (5% oxygen), anaerobic (total absence of free oxygen and 8%-14% CO_2) and 5% CO_2 rich atmosphere conditions with a 15% final concentration of oxygen, all in closes pouch were made respectively by simple culture method, microaerophilic condition using CampyGen Compact (OXOID Ltd, Basingstoke, Hampshire, England), Anaerobie Poche System/AnaeroGen Compact (OXOID Ltd, Basingstoke, Hants RG24 8PW, UK) and CO_2 Rich atmosphere using CO_2 Gen Compact (OXOID Ltd). All inoculated cultures and negative control inoculated with sterile PBS were incubated at 30°C. In parallel, we cultured onto Middlebrook 7H10 medium supplemented with 10% (v/v) OADC and 0.5% (v/v) glycerol, the three strains of *M. ulcerans* (10^4 AFB per Petrish dish), incubated at 37°C . Cultures were all examined at day 5, day 10, day 15, day 20 and day 30 by a MZ-FLIII fluorescence microscope. Counting of fluorescent colonies was performed using the Leica Application Suite software.

Growth-promoters. In a second step, we searched for genes encoding chitinase, N-acetyl galactosaminase (NAG) and fucosidase in completely sequenced *Mycobacterium* genomes (NCBI Gene Bank, last accessed in February 2016). To estimate the copy number of these genes, we considered protein functional description of the respective strains as documented in the gff files of NCBI Gene Bank bacterial genome repository. In each completely sequenced *Mycobacterium* genome, we counted the number of genes using the key terms “chitinase”, “cellulase”, “fucosidase” in their protein functional annotation column.

A total of 109 *Mycobacterium* genomes were screened in this analysis. The presence of one of these three genes was detected in 70 genomes and the presence of the three genes at once was detected only in the *M. ulcerans* genome.

Therefore, these three substances were tested as growth promoters for *M. ulcerans*. N-acetylgalactosamine (Sigma-Aldrich) and fucose (Sigma-Aldrich) were dissolved in sterile distilled water (Sigma-Aldrich) at a concentration of 1 mg/mL. They were then prepared on Middlebrook 7H10 agar medium supplemented with 10% (v/v) OADC and 0.5% (v/v) glycerol (Sigma) at a concentration of 0.1 mg/mL in the first step followed by a preparation with a final concentration of 0.01 mg/mL in a second step. These media were poured into 55-mm Petri dishes (Gosselin, Borre, France). Solubilization of chitin (Sigma-Aldrich) was achieved as previously described with few modifications by slowly dissolving chitin in 37% concentrated hydrochloric acid "HCl" (Sigma-Aldrich) [66] (Supplementary material). The obtained colloidal chitin was then dissolved into Middlebrook 7H10 medium supplemented with 10% (v/v) OADC and 0.5% (v/v) glycerol at a concentration of 0.2 mg/mL and this medium was poured into 55-mm Petri dishes. The purity of chitin, fucose and NAG delivered by Sigma-Aldrich was controlled by MALDI-TOF-MS before incorporation into the enriched culture media. For each of the three reagents, one microliter of the supernatant was spotted per spot onto the polished-steel MSP 96 target plate (Bruker Daltonics) and allowed to dry at room temperature. Each dry spot was then overlaid with 1µL of matrix solution (saturated α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) (Bruker Daltonics). The plate was air-dried for 5 minutes and loaded for manual processing into the MALDI-TOF mass spectrometer (delay: 170 ns; ion source 1 (IS1) voltage: 20 kV; ion source 2 (IS2) voltage: 16.65 kV; lens voltage: 7.20 kV; mass range: 0 kDa to 1 kDa) taking into account the known molar mass of reagents. Molar mass was identified by the m/z (mass/charge) parameter on spectra. The specific peaks were obtained for chitin (molar mass

394 of 627.59 g/mol), N-acetyl galactosamine (molar mass of 221. 20 g/mol) and fucose (molar
395 mass of 164.15 g/mol) confirming the presence and purity of the reagents.
396
397 **Testing growth-promoters for *M. ulcerans* culture.** For each one of the *M. ulcerans* strains
398 under study, a 100 µL-volume of a 10^5 AFB/mL suspension and PBS as negative control were
399 cultured in parallel in triplicate onto each of the three enriched media and onto Middlebrooks
400 7H10 reference medium supplemented with 10% (v/v) OADC and 0.5% (v/v) glycerol. We
401 seeded an inoculum of 10^4 AFB by Petri dish rather that a greater concentration of AFB in
402 order to minimize bias of counting because in bacterium kinetic, population growth strongly
403 depends on initial conditions. The 55-mm plates were incubated at 30°C in microaerophilic
404 atmosphere. Five days, ten days, fifteen days, twenty days and thirty days after inoculation,
405 colonies were observed by a MZ-FLIII fluorescence microscope (Leica, Nanterre, France)
406 equipped with a GFP filter and an ICA digital camera (Leica) to detect mycobacterial
407 autofluorescence [64]. Counting of fluorescent colonies was performed using the Leica
408 Application Suite software (Leica) [64]. Identification of colonies was confirmed by MALDI-
409 TOF-MS as previously described [65,67] and by *rpoB* gene sequence analysis [63].
410
411 **Decontamination of river freshwater** experimentally inoculated **with *M. ulcerans* strains.**
412 Freshwater obtained from the Huveaune River, a small river of southern France was used. In
413 first time, we analyzed without decontamination formerly direct cultured samples of the river
414 freshwater on 5 % sheep-blood Columbia agar (COS, bioMérieux, La Balme-Les-Grottes,
415 France) and after two days of culture we identified by MALDI-TOF-MS the growth bacterial
416 colonies. The direct culture aimed to list bacteria and fungi contained in the native
417 freshwater for the differential appreciation of germs between direct culture and culture after
418 chlorhexidine decontamination. Secondarily, we decontaminated the freshwater of Huveaune

River with 1% chlorhexidine and culture the pellet onto 5 % sheep-blood Columbia agar. The decontamination was processed as previously described [68].

The next step consisted of on the chlorhexidine decontamination of freshwater experimentally inoculated with *M. ulcerans* suspension. Briefly, in 50mL Corning Falcon conical centrifuge tubes (Becton Dickinson) containing 5 mL of river freshwater experimentally inoculated with one milliliter of 10^7 AFB/mL of each *M. ulcerans* Cu001 and *M. ulcerans* ATCC25900, the chlorhexidine decontamination was done as previously described [68]. Then, a 100-µL volume of pellet was inoculated in parallel on 5 % sheep-blood Columbia agar, in triplicate on each of the growth-promoting media (chitin, N-acetyl galactosamine, fucose) and on the standard Middlebrook 7H10 medium. The negative control was consisted of seeding 100 µL of sterile PBS onto 5 % sheep-blood Columbia agar, standard Middlebrook 7H10 medium and onto each of the growth-promoting media. Cultures and negative controls were examined at day 3, day 7 to appreciate the quality of the decontamination method and quality of the cultures. Colonies were counted at day 15 and day 30 using the Leica Application Suite software as described above.

Isolation of *M. ulcerans* from environmental samples, Côte d'Ivoire. Tentative isolation of *M. ulcerans* was done on 12 water samples without plant debris (40-45mL), 8 water samples with plant debris (40-45 mL) and 11 aulacode (*Thryonomys swinderianus*) feces collected around the Kossou dam near the village of Kongouanou, an endemic area of Buruli ulcer in the district of Yamoussoukro located in the centre of Côte d'Ivoire. The feces of aulacodes which are easy to recognize have been collected with the support of professional hunters around Kossou dam. All the samples were collected onto 50mL Corning Falcon conical centrifuge tubes.

For the treatment of samples for seeding, water samples without and with plants debris were concentrated by centrifugation at 1,700 g for 15 min and the supernatants were removed. The resulting pellets from water without plant debris were suspended in PBS before decontamination. The resulting pellets from water with plant debris were suspended in PBS and mechanically disrupted with silica beads (0.5 mm diameter) and the obtained suspensions were transferred into 50mL Corning Falcon conical centrifuge tubes before decontamination . 500 mg aulacode feces added into 50mL Corning Falcon conical centrifuge tubes containing 5 mL of PBS were mechanically disrupted with silica beads (0.5 mm diameter) and the obtained suspensions were transferred into another 50mL Corning Falcon conical centrifuge tubes before decontamination. Then, each sample was decontaminated using 1% chlorhexidine as previously described [68] . 100 µL of decontaminated pellet were seeded onto the chitin medium, NAG medium, fucose medium and DZ medium (Middlebrook 7H10 medium containing chitin (0.2%), NAG (0.01%) and fucose (0.01%)) and incubated for 24 weeks at 30°C under a micro-aerophilic atmosphere condition using CampyGen Compact. 100µL of sterile PBS was seeded in each media as negative controls and incubated in the same conditions. Colonies were screened with auto-fluorescence three times a month and the obtained colonies were identified by Real-Time PCR (RT-PCR) incorporating three independent gene targets, IS*2404*, IS*2606* and KR-B, within the *M. ulcerans* genome [7,22,69,70] and internal positive control to determine the level of inhibition as previously described [7]. The presence of *M. ulcerans* DNA in environmental samples was analyzed by RT-PCR. We used the three primers (KR-B, IS*2404* and IS*2606*) to improve the specificity of the *M. ulcerans* DNA detection in the environmental samples [71,72]. Primers and probes from Applied Biosystems that were selected from regions of the sequences for IS*2404*, IS*2606* and KR present on the plasmid pMUM001 were used [7]. Probes IS*2404*TP and KR-BTP were labelled with the fluorescent dye 6-carboxyfluorescein (FAM) at the 5′ end and a non-

fluorescent quencher at the 3′ end. Probe IS*2606*TP was labelled with the fluorescent dye VIC at the 5′ end and a non-fluorescent quencher at the 3′ end [7] (Table 2). Total DNA was extracted from feces using the QIAmp® DNA Stool kit according to the manufacturer's instructions (Qiagen, Stochach, Germany); total DNA was extracted from plant debris and sediment water using the NucleoSpin Tissue Kit (Macherey-Nagel, Hoerdt, France). *M. ulcerans* Cu001 DNA was extracted using a commercial Nucleospin Tissue kit (Macherey-Nagel, Hoerdt, France) which was used as a positive control in PCR-based identification of micro-colonies while distilled water was used as a negative control. The PCR inhibition was assessed by adding 10 µL of internal control into 190 µL of sample, as previously described [73]. Each IS*2404, IS2606 and KR* real-time PCR mixtures contained 5 µl of DNA or negative control, 20 µM of each primer, 5 µM of probe, 3.5 µL of sterile water and 10 µL of mastermix (Eurogentec) in a total volume of 20 µl. The RT-PCR program comprised one cycle at 50°C for two minutes and 40 cycles at 95°C for 15 seconds and 60°C for one minute [22], amplification was done in a CFX 96™real time PCR thermocycler and detection system (BIO-Rad, Marnes-la-Coquette, France). Two negative controls were incorporated into each PCR run. All samples were tested in triplicate. A specimen was considered as positive for the detection of *M. ulcerans* when both the insertion sequences IS*2606* and/or IS*2404* and the KR-B detection were positive (Ct ≤ 40 cycles) in ≥ 2/3 replicates. The Ct cut-off value was chosen in order to increase the sensitivity of the detection as previously described [22].

Statistics

The results of growth kinetics were expressed as mean value ± standard error of the mean (SEM) of counted colonies. The Student's T–test was used to compare the number of colonies growing in the media with growth promoters versus standard media. P values lower than 0.05

493 ($p < 0.05$) were considered significant. The doubling time was determined by calculating the

494 average slope of the mycobacterial replication curve during the early phase of culture.

Table 1. Real-time PCR (RT-PCR) results of *M. ulcerans* DNA detection in aulacode feces (FAG), water (EAU) and water containing plant debris (VEG) by using detection of KR-B gene and insertion sequences IS*2404* and IS*2606*.

The green boxes are the positive results for the corresponding primer and CT values.

Samples	KR-B	IS2404	IS2606
KON-FAG 1			+29.65
KON-FAG 2			
KON-FAG 3			
KON-FAG 4		+33.04	+31.58
KON-FAG 5			
KON-FAG 6			
KON-FAG 7			
KON-FAG 8		+36.30	
KON-FAG 9			
KON-FAG 10			
KON-FAG 11			
KON-EAU1	+33.11	+30.97	+29.85
KON-EAU 2	+32.14	+32.00	+30.77
KON-EAU 3			
KON-EAU 4	+31.56		+29.94
KON-EAU 5	+31.36		+29.64
KON-EAU 6	+32.74		+30.64
KON-EAU 7	+30.67		+29.18
KON-EAU 8	+30.15		+27.99
KON-EAU 9		+30.15	+30.08
KON-EAU 10	+32.75	+32.55	+31.75
KON-EAU 11	+32.22	+31.58	+30.51
KON-EAU 12	+32.37	+32.12	+29.85
KON-VEG 1			+30.97
KON-VEG 2		+32.85	
KON-VEG 3			

KON-VEG 4	+34.70		+30.09
KON-VEG 5			
KON-VEG 6		+32.34	+31.64
KON-VEG 7			
KON-VEG 8	+32.23		+29.07

499

500

501

502 Table 2. Primers and probes designed for real-time PCR detection of *M. ulcerans* by targeting

503 IS*2404*, IS*2606*, and KR-B gene.

Target sequence	Prime or Probe [a]	N° of bases	Amplicon size	Sequences (5'-3')	Nucleotide positions [b]	No. of copies of amplicon per plasmid/chromosome
IS*2404*	IS*2404* TF	19	59	AAAGCACCACGCAGCATCT	27746-27762	4/201
IS*2404*	IS*2404* TR	18		AGCGACCCCAGTGGATTG	27787-27804	
IS*2404*	IS*2404* TP			6FAM-CGTCCAACGCGATC-MGBNFQ	27768-27781	
IS*2606*	IS*2606* TF	21	58	CCGTCACAGACCAGGAAGAAG	28912-28932	8/82
IS*2606*	IS*2606* TR	21		TGCTGACGGAGTTGAAAAACC	28947-28969	
IS*2606*	IS*2606* TP			VIC-TGTCGGCCACGCCG-MGBNFQ	28933-28946	
KRB	KR-BTF	18	65	TCACGGCCTGCGATATCA	3178-3195	15/0
KR-B	KR-BTR	21		TTGTGTGGGCACTGAATTGAC	3222-3242	
KR-B	KR-BTP			6FAM-ACCCCGAAGCACTG-MGBNFQ	3199-3212	

504

505

Figure legends.

Figure 1. Triplicate culture of 10^4 bacilli of *M. ulcerans* CU001 (A), *M. ulcerans* ATCC25900 (B) and *M. ulcerans* ATCC33728 (C) in Middlebrook 7H10 under different atmospheres (ambient atmosphere, and microaerophilic, anaerobic and 5% CO_2-enriched atmosphere). Microaerophilic atmosphere yielded the optimal growth of *M. ulcerans* (red line).

Figure 2. Growth of *M. ulcerans* (10^4 bacilli) onto Middlebrook 7H10 enriched with high concentration of N-acethyl galactosamine (0.1 mg/mL, 0.5 mg/mL) and fucose (0.1 mg/mL, 0.5 mg/mL) versus growth onto standard Middlebrook 7H10 ($p<0.05$ after Day 15 of incubation). **A. *M. ulcerans* CU001; B. *M. ulcerans* ATCC25900; C. *M. ulcerans* ATCC33728**

Figure 3. Improved growth of *M. ulcerans* strains (10^4 bacilli) by N-acetyl galactosamine (NAG) (0.01 mg/mL), fucose (0.01 mg/mL), chitin (0,2mg/mL) and DZ medium (NAG (0.01 mg/mL), fucose (0.01 mg/mL), chitin (0,2mg/mL)) versus growth onto Middlebrook 7H10 ($p<0.05$). **A. *M. ulcerans* CU001; B. *M. ulcerans* ATCC25900; C. *M. ulcerans* ATCC33728**

Figure 4. Growth count at day 30 of 10^4 bacilli of *M. ulcerans* CU001 and *M. ulcerans* ATCC33728 colonies after 1% chlorhexidine decontamination of river freshwater artificially inoculated with *M. ulcerans* strains and cultured onto growth promoters and Middlebrook 7H10. Blue column: *Mycobacterium ulcerans* CU001; Red column: *Mycobacterium ulcerans* ATCC33728

Figure 5. Culturing one aulacode feces sample collected in Côte d'Ivoire on Middlebrook 7H10 enriched with growth promoters yielded one micro-colony observed by autofluorescence (arrow) (left panel). Right panel exhibits Ziehl-Neelsen staining of the micro colony further identified as *M. ulcerans* by positive RT-qPCR for KR-B gene, IS*2404* and IS*2606*.

Author Contributions

DZ: performed the experiments and drafted the manuscript

AP: performed genome analyses and drafted the manuscript

MD: designed the experiments, interpreted data and drafted the manuscript.

All the three authors corrected and approved the final version of the manuscript.

Competing Interests

The authors declare that they have no competing interests.

REFERENCES

1 WHO. Buruli ulcer (Mycobacterium ulcerans infection). Available at: http://www.who.int/mediacentre/factsheets/fs199/en/. (2016).

2 Stinear, T. P. *et al.* Insights from the complete genome sequence of Mycobacterium marinum on the evolution of Mycobacterium tuberculosis. *Genome Res* **18**, 729-741 (2008).

3 Stinear, T. P. *et al.* Reductive evolution and niche adaptation inferred from the genome of Mycobacterium ulcerans, the causative agent of Buruli ulcer. *Genome Res* **17**, 192-200, doi:10.1101/gr.5942807 (2007).

4 Stinear, T. P., Pryor, M. J., Porter, J. L. & Cole, S. T. Functional analysis and annotation of the virulence plasmid pMUM001 from Mycobacterium ulcerans. *Microbiology* **151**, 683-692, doi:doi:10.1099/mic.0.27674-0 (2005).

5 Mac Callum, P., Tolhurst, J. C. & et al. A new mycobacterial infection in man. *J Pathol Bacteriol* **60**, 93-122 (1948).

6 Walsh, D. S., Portaels, F. & Meyers, W. M. Buruli ulcer: Advances in understanding Mycobacterium ulcerans infection. *Dermatol Clin* **29**, 1-8 (2011).

7 Fyfe, J. A. M. *et al.* Development and Application of Two Multiplex Real-Time PCR Assays for the Detection of Mycobacterium ulcerans in Clinical and Environmental Samples. *Applied and environmental microbiology* **73**, 4733-4740, doi:10.1128/aem.02971-06 (2007).

8 Portaels, F. in *World Health Organization* 117 (Geneva, 2014).

9 Eddyani, M. *et al.* Primary Culture of Mycobacterium ulcerans from Human Tissue Specimens after Storage in Semisolid Transport Medium. *Journal of Clinical Microbiology* **46**, 69-72, doi:10.1128/jcm.00301-07 (2008).

10 Palomino, J. C., Obiang, A. M., Realini, L., Meyers, W. M. & Portaels, F. Effect of Oxygen on Growth of Mycobacterium ulcerans in the BACTEC System. *Journal of Clinical Microbiology* **36**, 3420-3422 (1998).

11 WHO. *Portaels F, Johnson P, Meyers WM. Buruli ulcer. Diagnosis of Mycobacterium ulcerans disease. A manual for health care providers.* *http://whqlibdoc.who.int/hq/2001/WHO_CDS_CPE_GBUI_2001.4.pdf*. (World Health Organization, 2001).

12 Josse, R. *et al.* [Mycobacterium ulcerans cutaneous infections: Buruli ulcers]. *Med Trop* **55**, 363-373 (1995).

13 Portaels, F. *et al.* Mycobacterium ulcerans in wild animals. *Rev Sci Tech* **20**, 252-264 (2001).

14 Marsollier, L. *et al.* Aquatic Plants Stimulate the Growth of and Biofilm Formation by Mycobacterium ulcerans in Axenic Culture and Harbor These Bacteria in the Environment. *Applied and Environmental Microbiology* **70**, 1097-1103, doi:10.1128/aem.70.2.1097-1103.2004 (2004).

15 Portaels, F. [Mycobacteria and tropical regions: from the environment to man]. *Med Trop* **64**, 219-220 (2004).

16 UBG. The Uganda Buruli Group. Epidemiology of Mycobacterium ulcerans infection (Buruli ulcer) at Kinyara, Uganda. *Trans R Soc Trop Med Hyg* **65**, 763-775 (1971).

17 Morris, A. L. *et al.* Deforestation-driven food-web collapse linked to emerging tropical infectious disease, Mycobacterium ulcerans. *Sci Adv* **2**, e1600387 (2016).

18 Roche, B. *et al.* Identifying the Achilles' heel of multi-host pathogens: The concept of keystone "host" species illustrated by Mycobacterium ulcerans transmission. *Environmental research letters : ERL [Web site]* **8**, 045009, doi:10.1088/1748-9326/8/4/045009 (2013).

19 Meyin, A. E. S. *et al.* Ecology and Feeding Habits Drive Infection of Water Bugs with Mycobacterium ulcerans. *EcoHealth* **14**, 329-341, doi:10.1007/s10393-017-1228-y (2017).

20 Portaels, F. Epidemiology of mycobacterial diseases. *Clinics in Dermatology* **13**, 207-222, doi:http://dx.doi.org/10.1016/0738-081X(95)00004-Y (1995).

21 Portaels, F. *et al.* First Cultivation and Characterization of Mycobacterium ulcerans from the Environment. *PLoS neglected tropical diseases* **2**, e178, doi:10.1371/journal.pntd.0000178 (2008).

22 Tian, R. B., Niamke, S., Tissot-Dupont, H. & Drancourt, M. Detection of Mycobacterium ulcerans DNA in the Environment, Ivory Coast. *PLoS One* **11**, e0151567 (2016).

23 Zogo, B. *et al.* A Field Study in Benin to Investigate the Role of Mosquitoes and Other Flying Insects in the Ecology of Mycobacterium ulcerans. *PLoS neglected tropical diseases* **9**, e0003941, doi:10.1371/journal.pntd.0003941 (2015).

24 Luo, Y. *et al.* Detection of Mycobacterium ulcerans subsp. shinshuense DNA from a water channel in familial Buruli ulcer cases in Japan. *Future microbiology* **10**, 461-469 (2015).

25 Konan, K. L. *et al.* [Detection of the IS2404 insertion sequence and ketoreductase produced by Mycobacterium ulcerans in the aquatic Heteroptera in the health districts of Dabou and Tiassale in Cote d'Ivoire]. *Med Sante Trop* **25**, 44-51 (2015).

26 Garchitorena, A. *et al.* Environmental transmission of Mycobacterium ulcerans drives dynamics of Buruli ulcer in endemic regions of Cameroon. *Scientific reports* **5**, 18055, doi:10.1038/srep18055

http://www.nature.com/articles/srep18055#supplementary-information (2015).

27 Dassi, C. *et al.* Detection of Mycobacterium ulcerans in Mastomys natalensis and Potential Transmission in Buruli ulcer Endemic Areas in Côte d'Ivoire. *Mycobact Dis* **5**, 184 (2015).

28 O'Brien, C. R. *et al.* Clinical, microbiological and pathological findings of Mycobacterium ulcerans infection in three Australian Possum species. *PLoS neglected tropical diseases* **8**, e2666 (2014).

29 Morris, A. *et al.* First detection of Mycobacterium ulcerans DNA in environmental samples from South America. *PLoS neglected tropical diseases* **8**, e2660 (2014).

30 Johnson, P. D. *et al.* Mycobacterium ulcerans in mosquitoes captured during outbreak of Buruli ulcer, southeastern Australia. *Emerg Infect Dis* **13**, 1653-1660, doi:10.3201/eid1311.061369 (2007).

31 Marsollier, L. *et al.* Aquatic Snails, Passive Hosts of Mycobacterium ulcerans. *Applied and environmental microbiology* **70**, 6296-6298, doi:10.1128/aem.70.10.6296-6298.2004 (2004).

32 Marsollier, L. *et al.* Aquatic insects as a vector for Mycobacterium ulcerans. *Applied and environmental microbiology* **68**, 4623-4628 (2002).

33 Aboagye, S. Y. *et al.* Isolation of Nontuberculous Mycobacteria from the Environment of Ghanian Communities Where Buruli Ulcer Is Endemic. *Appl Environ Microbiol* **82**, 4320-4329., doi:10.1128/aem.01002-16 (2016).

34 Palomino, J. C. & Portaels, F. Effects of Decontamination Methods and Culture Conditions on Viability of Mycobacterium ulcerans in the BACTEC System. *Journal of Clinical Microbiology* **36**, 402-408 (1998).

35 Renesto, P. *et al.* Genome-based design of a cell-free culture medium for Tropheryma whipplei. *Lancet (London, England)* **362**, 447-449 (2003).

36 Eddyani, M. & Portaels, F. Survival of Mycobacterium ulcerans at 37 degrees C. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases* **13**, 1033-1035, doi:10.1111/j.1469-0691.2007.01791.x (2007).

37 Tobias, N. J. *et al.* Complete Genome Sequence of the Frog Pathogen Mycobacterium ulcerans Ecovar Liflandii. *Journal of bacteriology* **195**, 556-564, doi:10.1128/jb.02132-12 (2013).

38 Duo-Chuan, L. Review of fungal chitinases. *Mycopathologia* **161**, 345-360, doi:10.1007/s11046-006-0024-y (2006).

39 Cantarel, B. L. *et al.* The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics. *Nucleic acids research* **37**, D233-238, doi:10.1093/nar/gkn663 (2009).

40 Gooday, G. W. in *Advances in Microbial Ecology* (ed K. C. Marshall) 387-430 (Springer US, 1990).

41 Tang, W. J., Fernandez, Javier G., Sohn, Joel J. & Amemiya, Chris T. Chitin Is Endogenously Produced in Vertebrates. *Current Biology* **25**, 897-900, doi:10.1016/j.cub.2015.01.058 (2015).

42 Wagner, G. P. in *Molecular Ecology and Evolution: Approaches and Applications* (eds B. Schierwater, B. Streit, G. P. Wagner, & R. DeSalle) 559-577 (Birkhäuser Basel, 1994).

43 Sanhueza, D. *et al.* Chitin promotes Mycobacterium ulcerans growth. *FEMS Microbiol Ecol* **92**, 27 (2016).

44 Škovierová, H. *et al.* Biosynthetic Origin of the Galactosamine Substituent of Arabinogalactan in Mycobacterium tuberculosis. *Journal of Biological Chemistry* **285**, 41348-41355, doi:10.1074/jbc.M110.188110 (2010).

45 Wheat, W. H. *et al.* The presence of a galactosamine substituent on the arabinogalactan of Mycobacterium tuberculosis abrogates full maturation of human peripheral blood monocyte-derived dendritic cells and increases secretion of IL-10. *Tuberculosis* **95**, 476-489 (2015).

46 Jollès, P. & Muzzarelli, R. A. A. Vol. 43 (ed Chitin and Chitinases) 340 (Birkhäuser, Basel, 1999).

47 Patil, R. S., Ghormade, V. V. & Deshpande, M. V. Chitinolytic enzymes: an exploration. *Enzyme and microbial technology* **26**, 473-483 (2000).

48 Xiao, X. *et al.* Chitinase genes in lake sediments of Ardley Island, Antarctica. *Applied and environmental microbiology* **71**, 7904-7909 (2005).

49 Wan, J., Zhang, X.-C. & Stacey, G. Chitin signaling and plant disease resistance. *Plant Signaling & Behavior* **3**, 831-833 (2008).

50 Frederiksen, R. F. *et al.* Bacterial chitinases and chitin-binding proteins as virulence factors. *Microbiology* **159**, 833-847, doi:doi:10.1099/mic.0.051839-0 (2013).

51 Beier, S. & Bertilsson, S. Bacterial chitin degradation—mechanisms and ecophysiological strategies. *Frontiers in Microbiology* **4**, 149, doi:10.3389/fmicb.2013.00149 (2013).

52 Benešová, E. *et al.* Alpha-l-Fucosidase Isoenzyme iso2 from Paenibacillus thiaminolyticus. *BMC Biotechnology* **15**, 36, doi:10.1186/s12896-015-0160-x (2015).

53 Zingue, D., Bouam, A., Militello, M. & Drancourt, M. High-Throughput Carbon Substrate Profiling of Mycobacterium ulcerans Suggests Potential Environmental Reservoirs. *PLoS neglected tropical diseases* **11**, e0005303, doi:10.1371/journal.pntd.0005303 (2017).

54 Pattyn, S. R. Bactériologie et pathologie humaine et expérimentale des ulcères à Mycobacterium ulcerans. *Ann.Soc. Belge Me¨d. Trop.* **45** (1965).

55 Cadapan, L. D. *et al.* Suspension cultivation of Mycobacterium ulcerans for the production of mycolactones. *FEMS Microbiol Lett* **205**, 385-389 (2001).

56 Leach, R. H. & Fenner, F. Studies on Mycobacterium ulcerans and Mycobacterium balnei. III. Growth in the semi-synthetic culture media of Dubos and drug sensitivity in vitro and in vivo. *Aust J Exp Biol Med Sci* **32**, 835-852 (1954).

57 Yaoi, H., Takei, M. & Tsuji, Y. Biological studies on Mycobacterium ulcerans (Maccallum). III. The rate of growth in culture media. *Jpn J Microbiol* **1**, 177-182 (1957).

58 Dega, H., Bentoucha, A., Robert, J., Jarlier, V. & Grosset, J. Bactericidal activity of rifampin-amikacin against Mycobacterium ulcerans in mice. *Antimicrobial agents and chemotherapy* **46**, 3193-3196 (2002).

59 Marsollier, L. *et al.* Colonization of the salivary glands of Naucoris cimicoides by Mycobacterium ulcerans requires host plasmatocytes and a macrolide toxin, mycolactone. *Cellular microbiology* **7**, 935-943 (2005).

60 Marsollier, L. *et al.* Susceptibility of Mycobacterium ulcerans to a combination of amikacin/rifampicin. *International Journal of Antimicrobial Agents* **22**, 562-566, doi:10.1016/s0924-8579(03)00240-1 (2003).

61 Dega, H., Robert, J., Bonnafous, P., Jarlier, V. & Grosset, J. Activities of several antimicrobials against Mycobacterium ulcerans infection in mice. *Antimicrobial agents and chemotherapy* **44**, 2367-2372 (2000).

62 Dega, H., Bentoucha, A., Robert, J., Jarlier, V. & Grosset, J. Bactericidal Activity of Rifampin-Amikacin against Mycobacterium ulcerans in Mice. *Antimicrobial agents and chemotherapy* **46**, 3193-3196, doi:10.1128/aac.46.10.3193-3196.2002 (2002).

63 Adékambi, T., Colson, P. & Drancourt, M. rpoB-Based Identification of Nonpigmented and Late-Pigmenting Rapidly Growing Mycobacteria. *Journal of clinical microbiology* **41**, 5699-5708, doi:10.1128/jcm.41.12.5699-5708.2003 (2003).

64 Mougin, B., Tian, R. B. D. & Drancourt, M. Tropical Plant Extracts Modulating the Growth of Mycobacterium ulcerans. *PLoS ONE* **10**, e0124626, doi:10.1371/journal.pone.0124626 (2015).

65 Zingue, D., Flaudrops, C. & Drancourt, M. Direct matrix-assisted laser desorption ionisation time-of-flight mass spectrometry identification of mycobacteria from colonies. *European Journal of Clinical Microbiology & Infectious Diseases*, 1-5, doi:10.1007/s10096-016-2750-5 (2016).

66 N Murthy, N. & Bleakley, B. Simplified Method of Preparing Colloidal Chitin Used For Screening of Chitinase- Producing Microorganisms. *The Internet Journal of Microbiology* **10**, 7 (2012).

67 Balazova, T. *et al.* The influence of culture conditions on the identification of Mycobacterium species by MALDI-TOF MS profiling. *FEMS microbiology letters* **353**, 77-84, doi:10.1111/1574-6968.12408 (2014).

68 Asmar, S. & Drancourt, M. Chlorhexidine decontamination of sputum for culturing Mycobacterium tuberculosis. *BMC Microbiology* **15**, 1-6, doi:10.1186/s12866-015-0479-4 (2015).

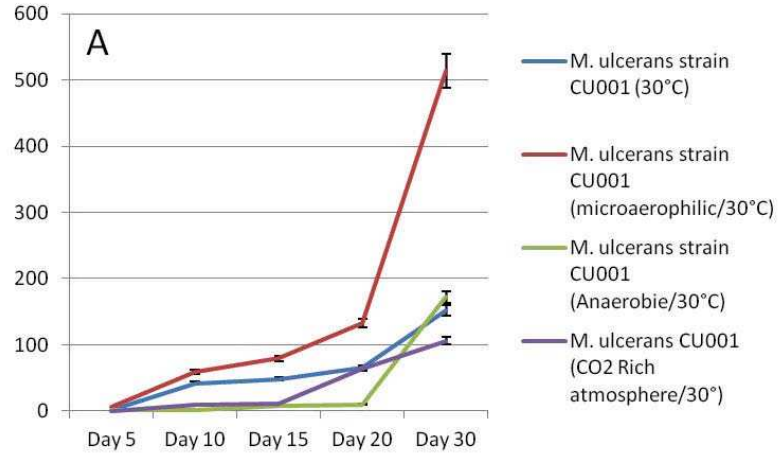
69 Tian, R. B. D., Asmar, S., Napez, C., Lépidi, H. & Drancourt, M. Effectiveness of purified methylene blue in an experimental model of Mycobacterium ulcerans infection. *Int J Antimicrob Agents* **49**, 290-295, doi:10.1016/j.ijantimicag.2016.11.012 (2016).

70 Gryseels, S. *et al.* Amoebae as Potential Environmental Hosts for Mycobacterium ulcerans and Other Mycobacteria, but Doubtful Actors in Buruli Ulcer Epidemiology. *PLoS neglected tropical diseases* **6**, e1764, doi:10.1371/journal.pntd.0001764 (2012).

71 Mve-Obiang, A. *et al.* A newly discovered mycobacterial pathogen isolated from laboratory colonies of Xenopus species with lethal infections produces a novel form of mycolactone, the Mycobacterium ulcerans macrolide toxin. *Infection and immunity* **73**, 3307-3312, doi:10.1128/iai.73.6.3307-3312.2005 (2005).

72 Afeke, I. *et al.* Cultivation of two IS2404 positive Mycobacterium spp from the environment of Asante Akim district of Ghana. *BMJ Global Health* **2**, A58-A59, doi:10.1136/bmjgh-2016-000260.156 (2017).

73 Ninove, L. *et al.* RNA and DNA bacteriophages as molecular diagnosis controls in clinical virology: a comprehensive study of more than 45,000 routine PCR tests. *PLoS One* **6**, 0016142 (2011).


A
600
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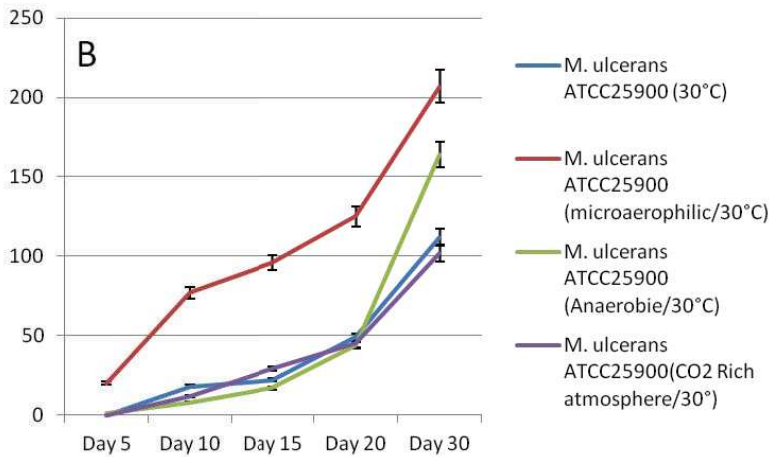
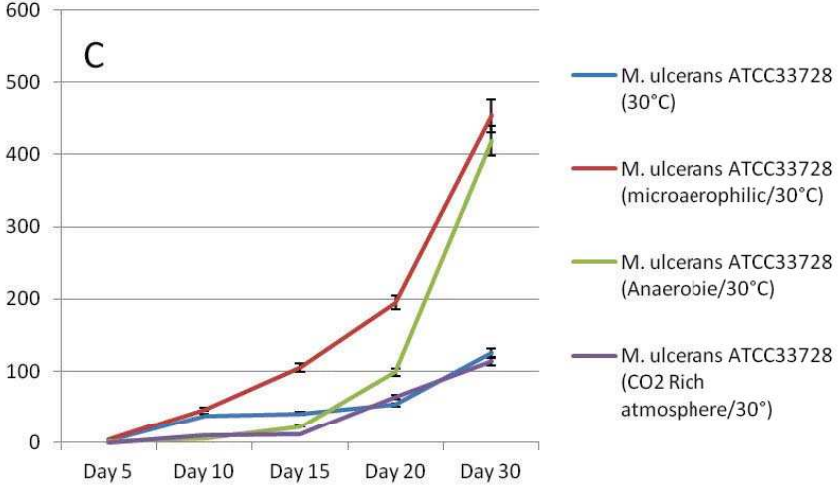
Day 5
Day 10
Day 15
Day 20
Day 30
M. ulcerans strain CU001 (30°C)
M. ulcerans strain CU001 (microaerophilic/30°C)
M. ulcerans strain CU001 (Anaerobie/30°C)
M. ulcerans CU001 (CO2 Rich atmosphere/30°)


B
250
200
150
100
50
0
Day 5
Day 10
Day 15
Day 20
Day 30
M. ulcerans ATCC25900 (30°C)
M. ulcerans ATCC25900 (microaerophilic/30°C)
M. ulcerans ATCC25900 (Anaerobie/30°C)
M. ulcerans ATCC25900(CO2 Rich atmosphere/30°)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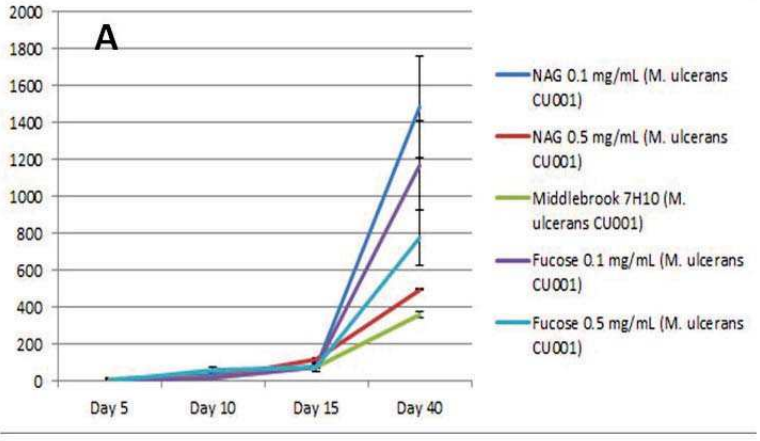
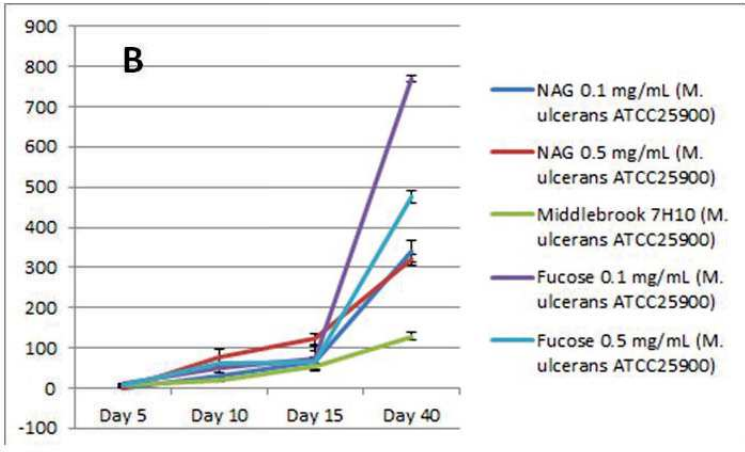
100
0
Day 5
Day 10
Day 15
Day 20
Day 30
M. ulcerans ATCC33728 (30°C)
M. ulcerans ATCC33728 (microaerophilic/30°C)
M. ulcerans ATCC33728 (Anaerobie/30°C)
M. ulcerans ATCC33728 (CO2 Rich atmosphere/30°)


A
2000
1800
1600
1400
1200
1000
800
600
400
200
0
Day 5
Day 10
Day 15
Day 40
NAG 0.1 mg/mL (M. ulcerans CU001)
NAG 0.5 mg/mL (M. ulcerans CU001)
Middlebrook 7H10 (M. ulcerans CU001)
Fucose 0.1 mg/mL (M. ulcerans CU001)
Fucose 0.5 mg/mL (M. ulcerans CU001)


B
900
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400
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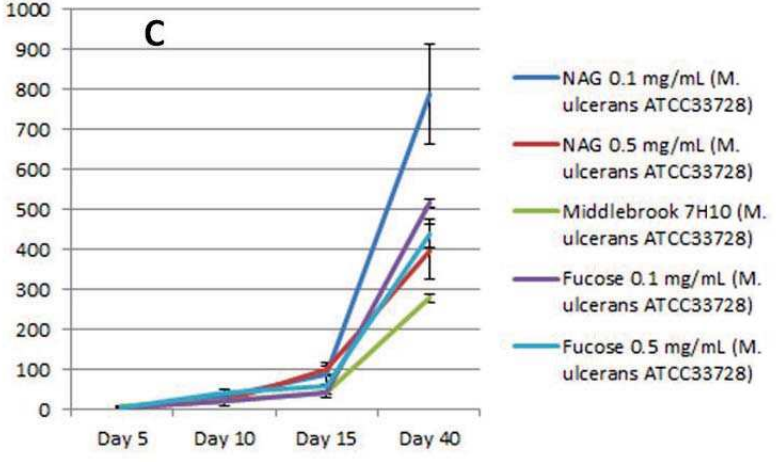
200
100
0
-100
Day 5
Day 10
Day 15
Day 40
NAG 0.1 mg/mL (M. ulcerans ATCC25900)
NAG 0.5 mg/mL (M. ulcerans ATCC25900)
Middlebrook 7H10 (M. ulcerans ATCC25900)
Fucose 0.1 mg/mL (M. ulcerans ATCC25900)
Fucose 0.5 mg/mL (M. ulcerans ATCC25900)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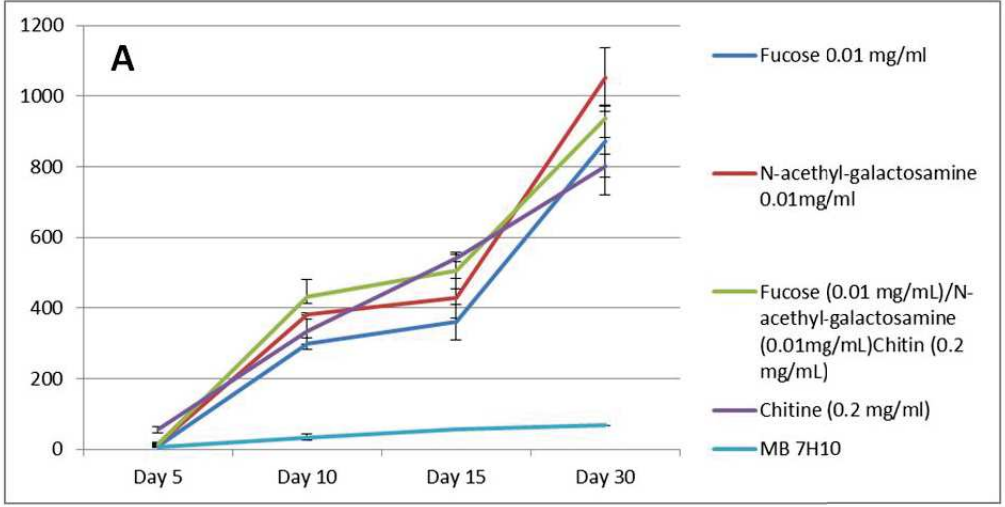
0
Day 5
Day 10
Day 15
Day 40
NAG 0.1 mg/mL (M. ulcerans ATCC33728)
NAG 0.5 mg/mL (M. ulcerans ATCC33728)
Middlebrook 7H10 (M. ulcerans ATCC33728)
Fucose 0.1 mg/mL (M. ulcerans ATCC33728)
Fucose 0.5 mg/mL (M. ulcerans ATCC33728)


A
1200
1000
800
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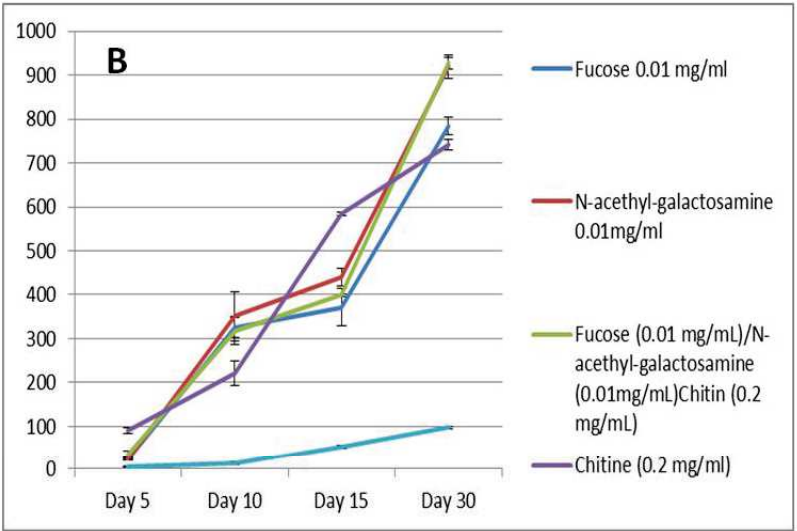
400
200
0
Day 5
Day 10
Day 15
Day 30
Fucose 0.01 mg/ml
N-acethyl-galactosamine 0.01mg/ml
Fucose (0.01 mg/mL)/N-acethyl-galactosamine (0.01mg/mL)Chitin (0.2 mg/mL)
Chitine (0.2 mg/ml)
MB 7H10


B
1000
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700
600
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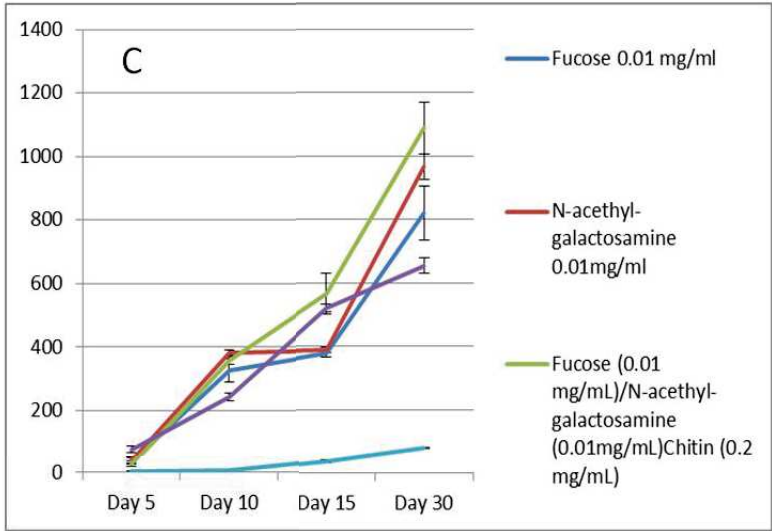
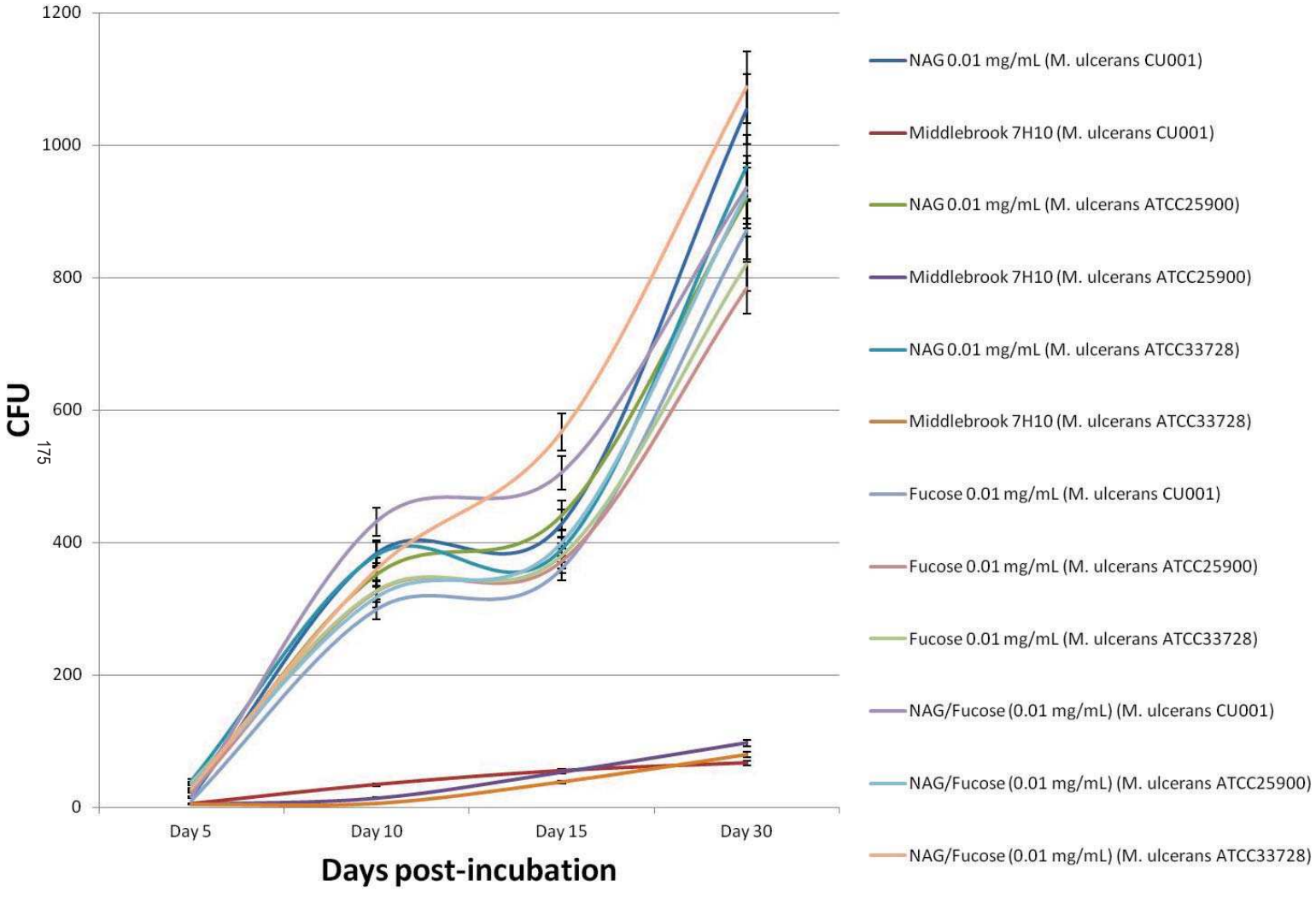
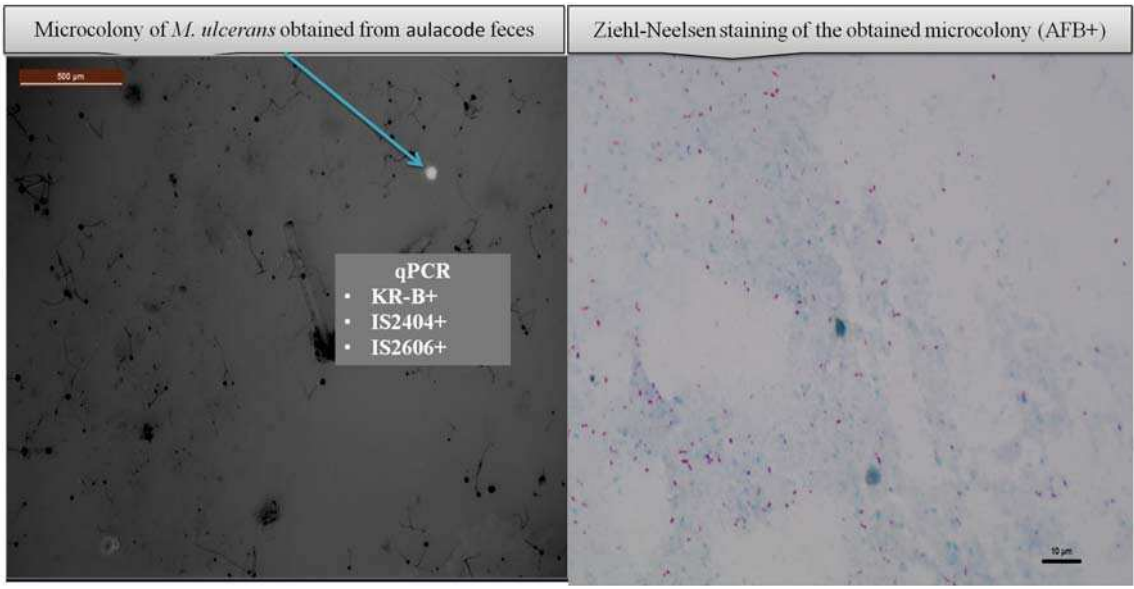
300
200
100
0
Day 5
Day 10
Day 15
Day 30
Fucose 0.01 mg/ml
N-acethyl-galactosamine 0.01mg/ml
Fucose (0.01 mg/mL)/N-acethyl-galactosamine (0.01mg/mL)Chitin (0.2 mg/mL)
Chitine (0.2 mg/ml)


C
1400
1200
1000
800
600
400
200
0
Day 5
Day 10
Day 15
Day 30
Fucose 0.01 mg/ml
N-acethyl-galactosamine 0.01mg/ml
Fucose (0.01 mg/mL)/N-acethyl-galactosamine (0.01mg/mL)Chitin (0.2 mg/mL)


1200
1000
800
600
400
200
0
CFU
175
Day 5
Day 10
Day 15
Day 30
Days post-incubation
NAG 0.01 mg/mL (M. ulcerans CU001)
Middlebrook 7H10 (M. ulcerans CU001)
NAG 0.01 mg/mL (M. ulcerans ATCC25900)
Middlebrook 7H10 (M. ulcerans ATCC25900)
NAG 0.01 mg/mL (M. ulcerans ATCC33728)
Middlebrook 7H10 (M. ulcerans ATCC33728)
Fucose 0.01 mg/mL (M. ulcerans CU001)
Fucose 0.01 mg/mL (M. ulcerans ATCC25900)
Fucose 0.01 mg/mL (M. ulcerans ATCC33728)
NAG/Fucose (0.01 mg/mL) (M. ulcerans CU001)
NAG/Fucose (0.01 mg/mL) (M. ulcerans ATCC25900)
NAG/Fucose (0.01 mg/mL) (M. ulcerans ATCC33728)


Microcolony of *M. ulcerans* obtained from aulacode feces
Ziehl-Neelsen staining of the obtained microcolony (AFB+)
500 µm
qPCR
• KR-B+
• IS2404+
• IS2606+
10 µm

A protocol for culturing environmental strains of the Buruli ulcer agent, *Mycobacterium ulcerans*

Dezemon Zingue[1], Arup Panda[1, 2], Michel Drancourt[1*]

Supplement 1: Supplementary information

Chitin dissolution protocol

Ten grams of chitin were slowly dissolved in 400 ml of 37% concentrated HCl in a 1000 ml beaker. The HCl was added slowly with continuous stirring with the use of a glass pipette for 5 minutes, followed by stirring for 1 minute at an interval of every 5 minutes for 60 minutes in a chemical fume hood at room temperature (25 ° C). The mixture was incubated in a water bath at 37°C until viscosity decreased. The chitin- HCl mixture was then passed through 8 layers of cheesecloth to remove large chitin chunks. The clear filtrate obtained (100 ml) was then treated with 2 liters of ice cold sterile distilled water to allow precipitation of colloidal chitin. This was incubated overnight under static conditions at 4°C to facilitate better precipitation of colloidal chitin. This was later passed through two layers of coffee filter paper, housed in a Buchner funnel (130 mm) seated in a vacuum filtration flask under vacuum. Approximately 3 liters of tap water (pH of ~8.0) were passed through the colloidal chitin cake using this filter assembly, until the pH of the filtrate had risen to 7.0 (estimated by pH paper). The colloidal chitin obtained was pressed between coffee filter papers (to remove additional moisture), and then placed in a 100 ml glass beaker covered with two layers of aluminum foil and sterilized by autoclaving at standard temperature and pressure (STP) (15 psi, 20 minutes, 121 ° C). The autoclaved colloidal chitin was stored at 4°C until further use. The colloidal chitin we obtained had a soft cake-like texture.

Chapter 4: MALDI-TOF-MS for the identification of cultured mycobacteria

There are more than 170 recognized species and subspecies of mycobacteria. Some of them are clinically relevant and cause disease in humans, animals while others are environmental microorganisms. Several diagnostic methods, such as biochemical, sequencing, and molecular methods, are used for mycobacterial identification. The current gold standard for the identification of mycobacteria is DNA sequencing with several targets including the 16S rRNA gene, *rpoB*, *secA*, and *hsp65*. In the last few years, matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) has been shown to be a reliable and rapid technique for the identification of tuberculous and nontuberculous mycobacteria through the recognition of specific proteins using specifique database as Mycobacteria Library version. MALDI-TOF-MS is a famous device which has a low operating cost with speed and reliability quality of identification for bacteria and it is based upon the detection of highly abundant proteins in a mass range between 2 and 20 kDa by computing their mass (m) to charge (z), m/z values. However, the identification of

mycobacteria by MALDI-TOF-MS is done after a long trifluoroacetic acid and acetonitrile protein extraction procedure. In this study, we developed a rapid method for the identification of mycobacteria by MALDI-TOF-MS directly from colonies obtained on solid medium, without a protein extraction step (article 5). Currently this protocol is used routinely in our Clinical Microbiology laboratory for the identification of mycobacteria.

Article 5: “Direct matrix-assisted laser desorption ionisation time-of-flight mass spectrometry identification of mycobacteria from colonies “

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ORIGINAL ARTICLE

Direct matrix-assisted laser desorption ionisation time-of-flight mass spectrometry identification of mycobacteria from colonies

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Abstract Matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) identification of mycobacteria requires a standard acetonitrile/formic acid pre-MALDI-TOF-MS. We prospectively compared this standard protocol with direct deposit with matrix for the identification of mycobacteria cultured on solid media. We first verified that *Mycobacterium tuberculosis* was killed after it was mixed with matrix. Then, 111 *Mycobacterium* isolates previously identified by partial *rpo*B gene sequencing were tested in parallel by the two protocols. An identification score >1.7 was obtained in 86/111 (77.5 %) isolates after protein extraction versus 97/111 (87.4 %) isolates after direct deposit ($p = 0.039$, Chi-squared test). In a third step, we determined that direct deposit achieved identification for as few as 2.10^4 *M. tuberculosis* organisms. In a fourth step, we evaluated direct deposit of one colony for 116 solid medium-cultured clinical isolates finally identified as representative of 12 species (63.8 % *M. tuberculosis*). For 114/116 (98.3 %) isolates with an identification score >1.2, the MALDI-TOF-MS identification was in complete agreement with the reference *rpo*B gene sequencing identification. One isolate with a MALDI-TOF-MS identification score of 1.22 for *M. fortuitum* was identified as *M. avium* by partial *rpo*B gene sequencing. One other isolate with a MALDI-TOF-MS identification score of 1.22 for *M. tuberculosis* was identified as *M. tuberculosis* by genotyping. All the original MALDI-TOF-MS spectra reported here have been deposited in a public database. Direct deposit of one colony on a MALDI-TOF-MS plate allows for an accurate identification of mycobacteria for an identification score >1.3.

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Introduction

Mycobacterial infections, including tuberculosis and non-tuberculous mycobacterioses, are a major public health concern, despite the efforts of health authorities at national and international levels [1–4]. *Mycobacterium* strains are routinely identified by traditional microbiological identification methods as well as by various molecular biology methods [5, 6]. These methods are expensive, time consuming and require trained laboratory personnel [7].

During the last decade, the development of matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) has vastly increased the possibilities for the rapid identification of bacteria in clinical laboratories [8]. Indeed, MALDI-TOF-MS has emerged as an effective, inexpensive, innovative and rapid method for the identification of bacteria and fungi in the clinical laboratory [9]. For mycobacteria, progress has been slower, and several protocols have been specifically designed for the MALDI-TOF-MS identification of tuberculous and non-tuberculous mycobacteria [1]. All of these protocols include pre-MALDI-TOF-MS processing of the isolate [1, 6, 10–12]. Processing includes suspension in water and alcohol for partial inactivation, heat inactivation, mechanical disruption with silica beads followed by acetonitrile/formic acid for mycobacterial protein

extraction, spotting suspension onto a MALDI-TOF target and cover with matrix solution (saturated α-cyano-4-hydroxycinnamic acid, 50 % acetonitrile, 2.5 % trifluoroacetic acid) [1, 6, 10–12]. This procedure is mainly justified by the toughness of the *Mycobacterium* cell wall [13, 14]. This is in contrast with the identification of non-mycobacterial bacteria, which is easily achieved after a simple deposit of solid culture medium-grown colonies on the MALDI-TOF-MS target [15]. It is noteworthy that all these protocols have been designed for mycobacteria isolates in broth cultures, such as those incorporated in automated broth cultures [1, 16–18].

In the meantime, we have renewed interest in the isolation and culture of mycobacteria grown in solid culture media with improved qualities compared to broth culture [7, 19, 20]. We, therefore, investigated whether the same simple protocol could be used for the rapid MALDI-TOF-MS identification of solid medium-grown mycobacteria.

We here report that the simple deposit of one colony on a MALDI-TOF-MS plate is sufficient for its rapid and accurate identification, using an updated database and scores.

Materials and methods

Mycobacterial strains

The following mycobacterial strains, kept in our URMITE laboratory collection at Aix-Marseille University, France, were used in this study. This collection of mycobacterial isolates was composed of one strain each of *Mycobacterium xenopi*, *Mycobacterium setense*, *Mycobacterium marinum* [21], *Mycobacterium neworleansense* and *Mycobacterium kansasii*; two strains each of *Mycobacterium mageritense*, *Mycobacterium boenickei*, *Mycobacterium chelonae* and *Mycobacterium ulcerans*; three strains each of *Mycobacterium bolletii*, *Mycobacterium bovis*, *Mycobacterium canettii*, *Mycobacterium intracellulare* and *Mycobacterium senegalense*; four strains each of *Mycobacterium simiae* and *Mycobacterium smegmatis*; six strains of *Mycobacterium fortuitum*, eight strains of *Mycobacterium avium*, ten strains of *Mycobacterium porcinum*, 11 strains of *Mycobacterium abscessus* and 40 strains of *Mycobacterium tuberculosis*, including the *M. tuberculosis* H37Rv reference strain. The identification of all mycobacteria isolates was previously confirmed by partial *rpo*B gene sequencing, as previously described [22]. All the isolates were sub-cultured onto a previously described solid medium MOD9 [7] or on a Middlebrook 7H10 medium (Becton Dickinson, Franklin Lakes, NJ, USA) at 37 °C. The *M. marinum* and *M. ulcerans* strains were grown in Middlebrook 7H10 medium at 30 °C in an atmosphere enriched in 5 % CO_2.

MALDI-TOF-MS preparation

Reference proteins extraction protocol (protocol 1)

We used a previously published protocol incorporating protein extraction by acetonitrile and formic acid, with a few modifications, as a reference protocol [23]. A loopful of mycobacterium biomass was collected into a 1.5-mL screw-top microcentrifuge tube (VWR International, Radnor, PA, USA) containing 300 μL of HPLC-grade water (Sigma-Aldrich, St. Louis, MO, USA). Then, 900 μL of 70 % ethanol was added, vortexed and incubated for 10 min at room temperature. The suspension was then centrifuged for 2 min at 13,000 × g and the supernatant was discarded. The pellet was suspended in 500 μL of HPLC-grade water and centrifuged for 2 min at 13,000 × g. The supernatant was discarded and the pellet was suspended in 50 μL of HPLC-grade water, heat-inactivated for 30 min at 95 °C and then cooled for 2 min. A 1200-μL volume of absolute ethanol pre-cooled at −20 °C was added and vortexed for a few seconds and then centrifuged for 2 min at 13,000 × g. The supernatant was discarded and the pellet was allowed to dry for 5 min at room temperature. Silica beads (0.5 mm in diameter) and 25 μL of pure acetonitrile (Sigma-Aldrich) were added and vortexed for 1 min. The same volume of 70 % formic acid (Sigma-Aldrich) was added, the mixture was vortexed thoroughly for 1 min and then centrifuged for 2 min at 13,000 × g. The supernatant was used for MALDI-TOF-MS. One microlitre of the supernatant was spotted per spot onto the polished-steel MSP 96 target plate (Bruker Daltonics, Bremen, Germany) and allowed to dry at room temperature. Each dry spot was then overlaid with 1 μL of matrix solution (saturated α-cyano-4-hydroxycinnamic acid in 50 % acetonitrile and 2.5 % trifluoroacetic acid) (Bruker Daltonics). The plate was air-dried for 5 min and loaded for processing into the MALDI-TOF mass spectrometer as described below. The overall procedure took 70 min for one sample to be completed.

Direct deposit protocol (protocol 2)

The direct deposit protocol consisted in picking a portion of a mycobacterial colony grown from solid media by using the tip of a sterile 200-μL tip (Sigma Aldrich), and the collected biomass was applied directly on a ground-steel MALDI target plate using a circular motion at each spot in order to obtain a translucent layer. One microlitre of a matrix solution (saturated α-cyano-4-hydroxycinnamic acid in 50 % acetonitrile and 2.5 % trifluoroacetic acid) (Bruker Daltonics) was used to overlay the sample and allowed to co-crystallise at room

temperature for 5 min. The plate was then loaded for processing into the MALDI-TOF mass spectrometer, as described below. The overall procedure took 10 min to complete.

MALDI-TOF-MS analysis

Measurements were performed with a Microflex LT (Bruker Daltonics) mass spectrometer laser. Spectra were recorded in the positive linear mode (delay: 170 ns; ion source 1 (IS1) voltage: 20 kV; ion source 2 (IS2) voltage: 16.65 kV; lens voltage: 7.20 kV; mass range: 2 kDa to 20 kDa). Each spectrum was obtained after 6 × 40 shots (240 shots) in automatic mode at a variable laser power, and the acquisition time ranged from 60 to 120 s per spot. All signals with resolution ≥400 were automatically acquired using AutoXecute acquisition control in flexControl software version 3.0 and the identifications were obtained by MALDI Biotyper software version 3.0 with the Mycobacteria Library v2.0 (2014) database, which contains 313 main spectrum profiles representing 128 species. An identification score was given using Biotyper software version 3.1 (Bruker Daltonics).

Evaluation of the sensitivity

To estimate the minimal number of *Mycobacterium* organisms required to achieve a valid identification, colonies of *M. tuberculosis* H37Rv strain were taken off the solid medium and added to the test tube (20 mm/150 mm) containing phosphate-buffered saline (PBS) and sterile 0.5-mm glass beads. The tube was vortexed and the suspension was passed once through a 29-gauge needle in order to separate aggregates. This suspension was calibrated in McFarland (MF) units by measuring its turbidity (Biolog, Inc., Hayward, CA, USA) in the presence of a negative control tube containing PBS. Suspensions of mycobacteria were prepared and adjusted with PBS at MF 0.5. In order to confirm the equivalence between MF and the actual concentration of mycobacteria, serial 1:10 dilutions were prepared in PBS starting from an MF 0.5 suspension (equivalent to 10^7 bacteria/mL) to obtain 5.10^6, 4.10^6, 3.10^6, 2.10^6 and 1.10^6 bacteria/mL. Then, 100 μL of each suspension was stained by Ziehl–Neelsen staining and microscopically examined to count the number of bacilli per field (1 bacillus per field corresponds to 10^4 mycobacteria/mL, 10 mycobacteria per field corresponds to 10^5 mycobacteria/mL). In parallel, the suspensions were used to perform the MALDI-TOF-MS identification by using protocol 1, as reported above.

Killing of M. tuberculosis by matrix

In order to assess that matrix incorporating 2.5 % trifluoroacetic acid killed *M. tuberculosis*, 10^4 to 10^6 *M. tuberculosis* H37Rv were mixed with matrix for 10 min and spread on Middlebrook 7H10 for a 21-day incubation at 37 °C in a 5 % CO_2 atmosphere. A non-treated suspension was manipulated in parallel. Plates were inspected by naked eye for the presence of colonies.

Routine identification of mycobacterial isolates

From January 2015 to April 2016, all clinical *Mycobacterium* strains prospectively isolated in our Mycobacteria Reference Laboratory, Institut Hospitalier Universitaire Méditerranée Infection, Marseilles, were analysed using 'protocol 2' as presented above for the MALDI-TOF-MS identification, using the default settings and MALDI Biotyper software version 3.0 with the Mycobacteria Library v2.0 (2014) database. Identification of these isolates was confirmed by either real-time polymerase chain reaction (PCR) GeneXpert MTB/RIF (Cepheid, Sunnyvale, CA, USA) [24] as for *M. tuberculosis* or partial *rpo*B gene sequencing [22] as for non-tuberculous isolates.

Statistical analysis

The χ^2 test with Yates' correction was used to analyse qualitative variables. The alpha level was set at 0.05, in which $p < 0.05$ was considered statistically significant.

Results and discussion

We evaluated the rapid identification of solid medium-grown *Mycobacterium* isolates by using direct deposit, without any processing of the colony on a MALDI-TOF-MS plate.

In a first step, we compared this basic protocol 2 with a standard, reference protocol 1, on a 111-strain collection representative of 20 *Mycobacterium* species. By using a conventional log score of 1.7 for identification, 86 isolates (77.5 %) were identified after protein extraction (protocol 1) and 97 isolates (87.4 %) were identified after direct deposit onto the MALDI target (protocol 2) ($p = 0.039$) (Table 1). The MALDI-TOF-MS spectra for these 111 strains have been deposited into our freely available Mediterranée Infection Institute website database (http://www.mediterranee-infection.com/article.php?laref=256&titre=urmsdatabase) to assist other scientists with their identification work.

In a second step, we measured that it was possible to achieve an identification score >1.7 for the *M. tuberculosis* H37Rv strain after protein extraction of a minimal number of 2.10^6 organisms, while direct deposit of 2.10^4 organisms allowed for an accurate identification with a score >1.7 (Fig. 1). This result indicated that direct deposit of mycobacterial biomass on the MALDI-TOF plate was a hundred times more sensitive than the extraction protocol for the MALDI-

Table 1 Comparative matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) identification of clinical isolates of mycobacteria by two colony processing methods

	Identification after protein extraction (method 1)		Direct identification (method 2)	
	Score ≥1.7	Score ≥2.0	Score ≥1.7	Score ≥2.0
Number (percentage) of identified isolates	86 (77.5 %)	34 (30.63 %)	97 (87.4 %)	42 (37.84 %)
Chi-squared of global result	3.111			
p-Value	0.039 (<0.05)			

TOF-MS identification of *M. tuberculosis*. Moreover, we ensured that 10^4 to 10^6 *M. tuberculosis* H37Rv mixed with matrix were dead. This observation allowed performing MALDI-TOF-MS identification of mycobacteria in a BLS2 laboratory.

In a third step, we evaluated the routine use of 'protocol 2' over a 16-month period. For this, 116 clinical isolates cultured on MOD9 solid medium were prospectively identified by depositing one colony on the MALDI-TOF-MS plate. The final identification, as confirmed by appropriate molecular analysis, was 74 *M. tuberculosis* isolates (63.8 %), 12 *M. avium* (10 %), seven *M. simiae* (6 %), six *M. abscessus* (5 %), six *M. intracellulare* (5 %), two *M. chelonae* (2 %), two *M. chimaera* (2 %), two *M. mageritense* (2 %), two *M. xenopi* (2 %), one *M. colombiense* (1 %), one *M. fortuitum* (1 %) and one *M. kansasii* (1 %). For these 116 isolates, the MALDI-TOF-MS identification score using protocol 2 was 1.22 to 2.41. For the 114/116 (98.3 %) isolates with an identification score ≥1.3, MALDI-TOF-MS identification was in complete agreement with that of molecular identification. One *M. tuberculosis* was correctly identified with an identification score of 1.22, and one isolate with a MALDI-TOF-MS identification score of 1.22 for *M. fortuitum* was identified as *M. avium* by partial *rpo*B gene sequencing. The MALDI-TOF-MS spectra for the 115 clinical isolates with an exact MALDI-TOF-MS identification have been deposited into our freely available Mediterranée Infection Institute website database (http://www.mediterranee-infection.com/article.php?laref=256&titre=urmsdatabase).

Conclusions

The data here reported indicate that the routine matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) identification of mycobacteria of medical interest can be done by the direct deposit of one piece of colony on the MALDI-TOF-MS target. The positive predictive value of the identification is 100 % for an identification score ≥1.3. Recently, the identification of colonies of *Mycobacterium fortuitum* directly deposited on a MALDI-TOF-MS plate after this isolate had been misidentified as *Corynebacterium jeikeium* by phenotypic tests was reported [25]. Indeed, mycobacteria do not behave differently from the other organisms routinely isolated in the clinical microbiology laboratory in respect to their MALDI-TOF-MS identification. The simplicity and rapidity of the protocol further argues for isolating mycobacteria of medical interest on a solid medium such as the MOD9 reported here rather than using broth. We are now using protocol 2 described in this paper for the routine MALDI-TOF-MS identification of mycobacterial colonies grown on solid medium.

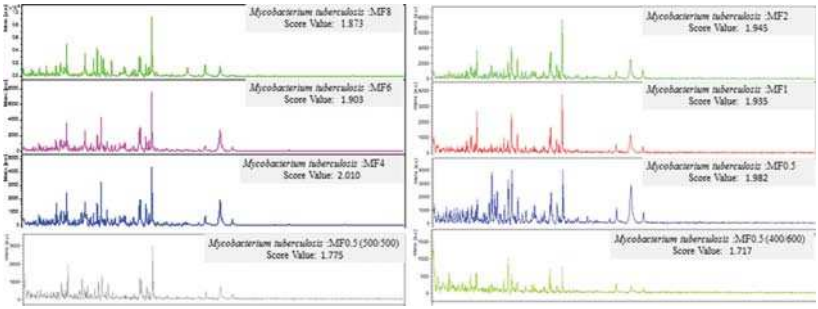

Fig. 1 *Mycobacterium tuberculosis* spectra and identification scores obtained at different McFarland (MF) values

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Compliance with ethical standards

Funding This study was supported by IHU Méditerranée Infection, Marseille, France.

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures in this study involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethic standards.

Informed consent Not applicable.

References

1. El Khéchine A, Couderc C, Flaudrops C, Raoult D, Drancourt M (2011) Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry identification of mycobacteria in routine clinical practice. PLoS One 6:e24720
2. Johnson MM, Odell JA (2014) Nontuberculous mycobacterial pulmonary infections. J Thorac Dis 6:210–220
3. Brown-Elliott BA, Nash KA, Wallace RJ Jr (2012) Antimicrobial susceptibility testing, drug resistance mechanisms, and therapy of infections with nontuberculous mycobacteria. Clin Microbiol Rev 25: 545–582
4. Escobar-Escamilla N, Ramírez-González JE, González-Villa M, Torres-Mazadiego P, Mandujano-Martínez A, Barrón-Rivera C, Bäcker CE, Fragoso-Fonseca DE, Olivera-Díaz H, Alcántara-Pérez P, Hernández-Solís A, Cícero-Sabido R, Cortés-Ortíz IA (2014) Hsp65 phylogenetic assay for molecular diagnosis of nontuberculous mycobacteria isolated in Mexico. Arch Med Res 45:90–97
5. Butcher PD, Hutchinson NA, Doran TJ, Dale JW (1996) The application of molecular techniques to the diagnosis and epidemiology of mycobacterial diseases. Soc Appl Bacteriol Symp Ser 25:53S–71S
6. Dunne WM Jr, Doing K, Miller E, Miller E, Moreno E, Baghli M, Mailler S, Girard V, van Belkum A, Deol P (2014) Rapid inactivation of Mycobacterium and nocardia species before identification using matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 52:3654–3659
7. Asmar S, Chatellier S, Mirande C, van Belkum A, Canard I, Raoult D, Drancourt M (2015) A novel solid medium for culturing Mycobacterium tuberculosis isolates from clinical specimens. J Clin Microbiol 53:2566–2569
8. Seng P, Rolain J-M, Fournier PE, La Scola B, Drancourt M, Raoult D (2010) MALDI-TOF-mass spectrometry applications in clinical microbiology. Future Microbiol 5:1733–1754
9. Tran A, Alby K, Kerr A, Jones M, Gilligan PH (2015) Cost savings realized by implementation of routine microbiological identification by matrix-assisted laser desorption ionization–time of flight mass spectrometry. J Clin Microbiol 53:2473–2479
10. Saleeb PG, Drake SK, Murray PR, Zelazny AM (2011) Identification of mycobacteria in solid-culture media by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 49(5):1790–1794
11. Mather CA, Rivera SF, Butler-Wu SM (2014) Comparison of the Bruker Biotyper and Vitek MS matrix-assisted laser desorption ionization-time of flight mass spectrometry systems for identification of mycobacteria using simplified protein extraction protocols. J Clin Microbiol 52(1):130–138
12. Machen A, Kobayashi M, Connelly MR, Wang YF (2013) Comparison of heat inactivation and cell disruption protocols for identification of mycobacteria from solid culture media by use of vitek matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 51:4226–4229
13. Djelouagji Z, Drancourt M (2006) Inactivation of cultured Mycobacterium tuberculosis organisms prior to DNA extraction. J Clin Microbiol 44:1594–1595
14. Somerville W, Thibert L, Schwartzman K, Behr MA (2005) Extraction of Mycobacterium tuberculosis DNA: a question of containment. J Clin Microbiol 43:2996–2997
15. Seng P, Drancourt M, Gouriet F, La Scola B, Fournier PE, Rolain JM, Raoult D (2009) Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clin Infect Dis 49(4):543–551
16. Lotz A, Ferroni A, Beretti J-L, Dauphin B, Carbonnelle E, Guet-Revillet H, Veziris N, Heym B, Jarlier V, Gaillard J-L, Pierre-Audigier C, Frapy E, Berche P, Nassif X, Bille E (2010) Rapid identification of mycobacterial whole cells in solid and liquid culture media by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 48:4481–4486
17. Mareković I, Bošnjak Z, Jakopović M, Boras Z, Janković M, Popović-Grle S (2016) Evaluation of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry in identification of nontuberculous mycobacteria. Chemotherapy 61:167–170
18. Kehrmann J, Wessel S, Murali R, Hampel A, Bange F-C, Buer J, Mosel F (2016) Principal component analysis of MALDI TOF MS mass spectra separates M. abscessus (sensu stricto) from M. massiliense isolates. BMC Microbiol 16:24
19. Asmar S, Sassi M, Phelippeau M, Drancourt M (2016) Inverse correlation between salt tolerance and host-adaptation in mycobacteria. BMC Res Notes 9:249
20. Asmar S, Chatellier S, Mirande C, van Belkum A, Canard I, Raoult D, Drancourt M (2016) A chlorhexidine-agar plate culture medium protocol to complement standard broth culture of Mycobacterium tuberculosis. Front Microbiol 7:30
21. Bouricha M, Castan B, Duchene-Parisi E, Drancourt M (2014) Mycobacterium marinum infection following contact with reptiles: vivarium granuloma. Int J Infect Dis 21:17–18
22. Adékambi T, Colson P, Drancourt M (2003) rpoB-based identification of nonpigmented and late-pigmenting rapidly growing mycobacteria. J Clin Microbiol 41:5699–5708
23. Balážová T, Makovcová J, Šedo O, Slaný M, Faldyna M, Zdráhal Z (2014) The influence of culture conditions on the identification of Mycobacterium species by MALDI-TOF MS profiling. FEMS Microbiol Lett 353:77–84
24. Pai M, Minion J, Sohn H, Zwerling A, Perkins MD (2009) Novel and improved technologies for tuberculosis diagnosis: progress and challenges. Clin Chest Med 30:701–716
25. Visconti V, La Martire G, Brunetti G, Ghezzi MC, Venditti M, Raponi G (2016) Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry assay solves misidentification of rapidly growing mycobacteria. Am J Infect Control 44:614–616

Article 6: Emerging of Matrix-assisted laser desorption ionization-time of flight mass spectrometry for identification of mycobacteria in a clinical microbiological laboratory

This mini-review provides a brief history of the development of MALDI-TOF-MS until it is used in research and diagnostic laboratories for the identification of mycobacteria strains. The various protocols for extracting the proteins of mycobacteria developed for identification by MALDI-TOF-MS have been described in this review. MALDI-TOF-MS has a great ability to identify mycobacteria and would be a great asset for the diagnosis of mycobacterial diseases in resource-limited countries where it is not always easy to make a differential diagnosis between different strains of mycobacteria

Emerging of Matrix-assisted laser desorption ionization-time of flight mass spectrometry for identification of mycobacteria in a clinical microbiological laboratory

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Abstract:

The diagnosis of mycobacterium infections is routinely based on microscopy, culture, phenotypic assays and molecular biology assays. Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF-MS) has emerged as an alternative method to microbiological identification and several studies showed that this technology was fast, efficient and less expensive for its use in diagnostic laboratories for the identification of microorganisms. In this review, we describe and discuss the use of this technology for identification and typing mycobacterium strains. Herein, we discuss also the advantages and disadvantages of this method.

Keywords: *Mycobacterium tuberculosis*-Non tuberculosis mycobacterium-protein profile-MALDI-TOF-MS

INTRODUCTION

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* complex (MTBC), remains a major problem of public health worldwide (1). TB ranks as the second leading cause of death from an infectious disease after human immunodeficiency virus (HIV) infection (2). It's actually high incidence is strongly associated with the emergence of M. tuberculosis Beijing lineage associated with the increase of drug resistance worldwide (2-6). During the recent two decades, it has been observed the emergence of pulmonary infections and other mycobacterial infections due to nontuberculous mycobacteria (NTM) (7-10). The NTM are ubiquitous organisms, highly prevalent in the environment then and recognized as human pathogens in 1950s (11). The incidence of diseases caused by NTM has gradually increased (12). Over 150 species of NTM have been described (7), more than 172 mycobacterial species/subspecies are listed in the Genus Mycobacterium database http://www.bacterio.cict.fr/m/mycobacterium .html; accessed on 13 August 2015). Recognition of the

potential pathogenic of NTM has lagged behind recognition of the potential pathogenic of *M. tuberculosis* (13, 14). The correct identification of mycobacteria by conventional methods is not always conclusive (15). Currently, the conventional smear microscopy with the Ziehl-Neelsen stain is the main method used in microbiology laboratory for the detection of acid-fast bacilli. The problem is that this method can't distinguish *M. tuberculosis* complex and NTM (16). In the best equipped laboratories, the conventional biochemical tests and molecular tools are used but, they are time-consuming and expensive (9, 12, 17, 18). Rapid and accurate diagnostic tools for the identification of mycobacterial species are essential for optimal management of mycobacterial infections. So, there is an urgent need to develop alternative, cost-effective methods for mycobacteria infections diagnosis. In 1998, the whole genome of *M. tuberculosis* H37 Rv was sequenced, bringing enough information and knowledge in the fields of “omics” (19). Many studies in genomics areas have shown that TB is caused by several genotypic families of *M. tuberculosis*

according to the presence or absence of specific genetic markers (20-22). The recent advances in proteomics have resulted in the ability to separate and identify individual proteins or peptides from complex biological samples, provide an opportunity for the identification of *M. tuberculosi*s complex and NTM (23). The field of proteomics has made significant advances using a variety of techniques for identification and quantification of proteins (24). Identification of complex protein mixtures have been facilitated by mass spectrometry-based quantitative proteomic techniques (24, 25). Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF-MS) instrument emerged as novel, cost-effective, rapid and reliable proteomic tool of microorganisms identification by analysis of protein profiles (26). MALDI-TOF MS showed promising results for reducing the time of identification of mycobacteria compared to conventional methods (27). Until recently, MALDI-TOF MS was unappropriated for the identification of whole mybacteria. However, it can be used

from either disrupted cells or intact bacterial cells (26). The application of MALDI-TOF MS for bacterial identification is a major advance in the field of medical bacteriology. It has been successfully applied as an identification method in clinical microbiology and has been widely used in routine laboratory practice during the last decade. This review provides an overview of the literature of the applications of MALDI-TOF MS for mycobacteria identification in microbiological diagnostics facility. It also explores the progress on the area of MALDI-TOF-MS development for the identification of mycobacterium strains

Study selection and search strategy

We conducted a review of published literature in English concerning identification of mycobacteria by MALDI-TOF-MS. A paper was included if it provided protein profile results of the identification of mycobacteria by MALDI-TOF and the software used for the analyses.

Literature was identified from electronic databases. Only PUBMED/MEDLINE and google scholar databases were

searched for articles based on MALDI-TOF-MS studies of mycobacterial. The following search terms were used: "Mycobacterium", and "MALDI-TOF-MS". In addition to this search procedure, we used the reference lists of the identified publications to find further relevant articles. In addition, we searched the reference lists of some primary studies and several previously published reviews on proteomics of mycobacterium proteins characterization by MALDI-TOF-MS.

- **Mycobacteria cell envelope**

Mycobacteria are aerobic and non-motile bacteria that are characteristically acid fast bacilli. The cell envelope of mycobacteria is a thick, complex structure. Its components include a plasma membrane, a cell wall core built of peptidoglycan, arabinogalactan and mycolic acids and a polysaccharide capsule-like material. Surrounding this core is a capsule-like outer structure of non-covalently linked glycan, lipids and proteins (5, 28). The unique composition of the mycobacterial cell wall allows the

bacterium to adapt to its intracellular environment and promote its survival and pathogenicity within an infected host cell. The cell function is directly regulated through proteins but not through genes. Analysis of the proteins coded for by these genes has traditionally been performed on a single protein at a time (29). The mycobacterial cell wall is compound of an inner layer and an outer layer that surround the plasma membrane (30). The cell envelope of *mycobacteria* is a thick, complex structure (31, 32). These bacteria are generally classified as high G + C positive Gram, but their cell envelope present characteristics of both Gram-positive and Gram-negative bacteria (31). The cell wall of mycobacteria is comprised of three covalently linked macromolecules which are peptidoglycan, arabinogalactan and mycolic acids (33). The cell wall of mycobacteria contains lipids and glycolipids that contribute to extreme hydrophobicity to the outer surface. These lipids which include mycolic acids, phosphatidyl inositol mannosides, phthiocerol dimycocerosates and lipoglycans such as lipomannan and lipoarabinomannan

play important roles in maintaining integrity of the cell envelope (34, 35). Its components include a plasma membrane, a cell wall core built of peptidoglycan covalently attached *via* a linker unit to a linear galactofuran in turn attached to several strands of a highly branched arabinofuran, in turn attached to mycolic acids (30, 34). Surrounding this core is a capsule-like outer structure of non-covalently linked glycan, lipids and proteins (5, 28).

The *M. tuberculosis* membrane is rich in antigens that are potential targets for diagnostics and the development of new vaccines (36). Proteome research permit to have information about specific proteins present in the different steps of TB infection, TB post infection and the actual physiological status of the bacilli in the host and sets the basis for development of novel diagnostic targets and drug designs (5, 28). Research on the cell membrane of pathogen mycobacteria is encouraged because it contains ingredients of diagnostic and therapeutic protein targets which need to be screened (37). The low permeability of the mycobacterial cell wall, with its unusual structure, is known to be a major

factor in this resistance (32). The mycobacterial cell wall is a rigid structure that enables the bacilli to survive under unfavorable intracellular environment (34). The highly complex cell wall of mycobacteria is implicated for its resistance to various anti-mycobacterial drugs and environmental stressors (32).

Proteomics of Mycobacterium

Evaluation of protein abundance in both qualitative and quantitative terms offers important information on different aspects of cell physiology and biology with implications in medicine, infectious diseases and cell development (38, 39). Proteins are the main components of these models and the analysis of proteomes of diverse biological organisms represents one of the challenges in the post-genome era and is a rich source of biological information because they carry out most of the fundamental processes in the cell (39, 40). In 1996, the term “proteome” was defined as the protein composition of a cell, organism, organelle, tissue, or body fluid at a given time (41). The proteome describes the

protein compliment expressed by the genome. It is the entire set of proteins encoded by the genome and proteomics is the study of this global set of proteins and their expression, function and structure.(42). Proteomics is defined as the large-scale study of proteins within a system. Proteomics, the global analysis of the proteins expressed in a cell or tissue, provides a very promising approach for the large scale identification of proteins, their complexes, and their functions(37). Proteomics encompasses a group of technologies that attempt to separate, identify and characterize a global set of proteins. It provides information about abundance, location, chemical modification and protein-protein interactions that is not available from genomic technologies (29). Proteomic technologies are improving and developing rapidly. These techniques will be valuable tools to develop markers for disease, identify and evaluate proteins as drug targets and understand renal physiology at the protein level. The US Food and Drug Administration (FDA) defines a biomarker as, “A characteristic that is objectively measured and evaluated as

an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention". Quantification of changes in biological systems in response to certain treatments is an important but challenging task in proteomics. The proteomic analysis of *M. tuberculosis* strains is critical for an understanding of the molecular basis of its virulence and pathogenicity(37). Herein, we described MALDI-TOF-MS use to identify mycobacterium.

Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry

Thomson built mass spectrometry prototype, an analytical technique in which chemical compounds are ionized into charged molecules to measure mass/charge (m/z) ratio of electron and it awarded Nobel Prize in 1906 (43). The first use of mass spectrometry in microbiology began in the 1970s (44). The evolution of ionization techniques such as Matrix-assisted laser desorption ionization (MALDI) and electrospray ionization developed in the late 1980s made

possible analysis by MS of large biomolecules such as intact proteins (45). The “MALDI” term was first used in 1985 by Franz Hillenkamp and Michael Karas which used the mass spectrometry to ionize high molecular weight compounds, then it was used for the first trial in microbiology (45-52). Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF-MS) was developed in the 1988 by Franz Hillemkamp and a group of his assistants at the University of Munster at Germany (45, 47-52). MALDI-TOF-MS has been widely used in research settings since 1990s (53, 54), then in diagnostic microbiological laboratories and recently it was implemented for the identification of mycobacteria strains by protein profiling (9, 50, 52, 55). MALDI-TOF-MS instrument emerged recently as novel, cheap, rapid and reliable proteomic tool of microorganisms identification by analysis of protein profiles and characterization based upon the detection of proteins in a mass of molecules range (56). The method can be used from either disrupted cells or intact bacterial cells and represents a cornerstone of proteomic (26). It is a new

approach used for identification and typing of microorganisms, rapid screening of pathogenic strains and species, detection of unique proteins and biomarkers(57). Obtaining a spectrum characteristic or "fingerprint spectral "of particular species is the basis for the use of mass spectrometry in microbial identification. It is a real revolution in clinical microbiology laboratories (44, 58). MALDI-TOF-MS has been widely used in diagnostic microbiological laboratories. It was implemented for the identification of mycobacteria strains by protein profiling after its first used to characterize protein profile in 1996 then, in 2006. by the use of intact germs (9, 50, 52, 55, 59, 60). Unlike other bacteria, the identification of mycobacteria by MALDI-TOF-MS had to go through a critical step of inactivation and extracting their protein, due to the fact of the toughness of their membrane. El Khéchine et al. developed an original protocol for the identification of mycobacteria by MALDI-TOF-MS after extraction by bead method of the mycobacterium protein (9) and then, others modified and simple procedures were developed (51, 61-

63). Actually, MALDI-TOF-MS is currently used in routine for diagnosis of bacteria, fungi, inactivated clinical isolates of *M. tuberculosis* and NTM (9, 50, 51, 55). The protocol using heat inactivation with sonication and cell disruption with glass beads result permit the characterization of mycobacterial isolates at species and genus level with MALDI-TOF-MS (64). As for mycobacteria, progresses were slower and several protocols have been specifically designed for the MALDI-TOF-MS identification of tuberculous and non-tuberculous mycobacteria (65). The introduction of MALDI-TOF-MS in mycobacteriology laboratories is improving the diagnosis of infections caused by mycobacterial strains. With continued research in mass spectrometry, MALDI-TOF could be used henceforth for identification of the different genotypes of mycobacterium strains as well as cases of resistant *M. tuberculosis* strains by the development of a new adequate database (66). All these protocols include pre-MALDI-TOF-MS processing of isolate (65, 67-70). Processing included suspension in water and alcohol for partial inactivation, heat inactivation,

mechanically disruption with silica beads followed by acetonitrile/formic acid for mycobacterial protein extraction, spotting suspension onto MALDI-TOF target and cover with matrix solution (saturated α cyano-4-hydroxycinnamic acid, 50% acetonitrile, 2.5% TFA) (65, 67-70). This procedure has mainly justified by the toughness of the *Mycobacterium* cell wall (71, 72). It is in contrast with the identification of non-mycobacterial bacteria easily achieved after a simple deposit of solid culture medium-grown colonies on the MALDI-TOF-MS target (73). Noteworthy, all these protocols have been designed for mycobacteria isolates in broth cultures such us the ones incorporated in automates
(65, 74-76).

However, the identification of mycobacterium strains by MALDI-TOF-MS can only be done after an adequate protein extraction method due to the toughness of their cell membrane and the use of the corresponding database (77). Bruker in vitro diagnostics (IVD) matrix-assisted laser desorption/ionization (MALDI) Biotyper and the VITEK

MS from bioMérieux are MALDI-time of flight (ToF)-mass spectrometry based platform for the identification of bacterial and yeast which obtained the US Food and Drug Adminsitration (FDA) clearance in 2013 (44). These two MALDI-TOF-MS methods and other mass spectrometry techniques were widely used into clinic laboratories for microorganisms identification. During the last decade, the development of matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) vastly increased the possibilities for the rapid identification of bacteria in clinical laboratories (78). As for mycobacteria, progresses were slower and several protocols have been specifically designed for the MALDI-TOF-MS identification of tuberculous and non-tuberculous mycobacteria (65). All these protocols include pre-MALDI-TOF-MS processing of isolate (65, 67-70). Processing included suspension in water and alcohol for partial inactivation, heat inactivation, mechanically disruption with silica beads followed by acetonitrile/formic acid for mycobacterial protein extraction, spotting suspension onto

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Today, MALDI-TOF-MS is currently used in routine for diagnosis of bacteria, yeast, arthropod, inactivated clinical isolates of *Mycobacterium tuberculosis* and Non-tuberculosis mycobacteria (9, 50, 51, 55, 79, 80). The identification of bacteria other than mycobacteria is directly done from the deposit of colonies onto the MALDI-TOF target and covered by the matrix. This is not the case with mycobacteria which must be extracted beforehand proteins to their deposition on the target (68-70). Since 2010s, it was developed original protocol for the identification of

mycobacteria by MALDI-TOF-MS after extraction of mycobacteria protein by the bead method (9).In MALDI analysis, samples are prepared by mixing the samples with a matrix which results in the crystallization of the sample within the matrix. The main used matrice in mycobacteriology laboratories is α-cyano- 4-hydroxycinnamic acid (CHCA). In mycobacteriology, the protocol using heat inactivation with sonication and cell disruption with glass beads result permit the characterization of mycobacterial isolates at species and genus level with MALDI-TOF-MS (64). The introduction of MALDI-TOF-MS in mycobacteriology laboratories is improving the diagnosis of infections caused by mycobacterial strains. With continued research in mass spectrometry, MALDI-TOF could be used henceforth for identification of *M. tuberculosis* genotype Beijing as well as cases of resistant *M. tuberculosis* strains by the development of a new adequate database (66).

Principle of MALDI-TOF-MS

The development of devices, miniaturization as well as the emergence of new ionization techniques, have made this method of chemical analysis which has the largest scope.

Mass spectrometer is composed of three main units which are the ion source to ionize and transfer sample molecules ions into a gas phase, the mass analyser that separate ions according to their mass-to-charge ratio (m/z), and the detection device to monitor separated ions.

Several ionization methods have been developed including chemical ionization (CI), atmospheric pressure CI, electrospray (ESI), and MALDI. The method of ionization is determined according to the nature of the sample and the goal of the MS analysis, but ESI and MALDI are soft ionization techniques that allow ionization and vaporization of large nonvolatile biomolecules such as intact proteins (81).

MALDI-TOF uses a laser ionization source assisted by a matrix and a time of flight analyzer (TOF). Co-crystallized sample with a donor template electron will be bombarded by

a laser. Being desorbed from the inert plate, the ionized particles are then accelerated in an electric field by printing their time of flight in a pipe where a high vacuum is maintained. The adsorbed molecule is projected onto a detector and flight time will be proportional to the mass ratio (m) of charge (z) (m/z). Proteins and peptides are separated by increasing mass. Particles are detected at the top of the TOF analyzer This flight time obtained from whole bacteria is measured and allows obtaining a mass spectrum by a generation of a pattern of characteristic peaks "spectrum".
Each controller comes with control software, a database and an expert system for the identification. The identification of microorganism based on the analysis of the spectrum generated by the mass spectrometer. Dedicated software analyzes mass spectra against library of stored spectra.

- **Identification of mycobacterium by MALDI-TOF-MS**

The discovery of suitable matrices, analyzing of microorganisms in the mass range (m/z) of 2,000 to

20,000Da, followed by the availability of the dedicated databases for germs identification has made MALDI-TOF MS an alternative method for microorganisms identification in microbiology laboratories. For successful identification of mycobacteria strains by MALDI-TOF-MS , certain conditions must be met for optimal results : work with fresh mycobacterial cultures(82), using a validate protein extraction protocol for obtaining high quality spectra, spot the freshly obtained protein extracts on the MALDI-TOF-MS target using the validated matrix for mycobacteria, use the dedicated database to mycobacteria identification. Different mycobacterial culture media (solid media and liquid media) were used in several studies to implement the identification of mycobacterium by MALDI- TOF-MS. The solid media as Lowenstein-Jensen medium (LJ), Middlebrook 7H11 (Remel, Lenexa, KS, USA).

Middlebrook 7H10/7H11 plates (Becton, Dickinson Microbiology Systems, Cockeysville, MD) and 5% sheep blood-agar (BioMérieux, La Balme-les-Grottes, France) were successful used in different studies with

MALDI-TOF-MS (9, 12, 17, 18, 62, 82-84) as soon as the use of liquid media as liquid media mycobacterium growth indicator tube (MGIT) (Becton Dickinson Microbiology Systems, Cockeysville, MD), MBBacT ALERT 3D bottles (9, 12, 18, 83). Some studies showed that there is no significant differences between MALDI-TOF-MS results obtained from solid and liquid medi a(12, 83). Unlike previous authors, some authors have found smaller identification score with the use of liquid media (17, 18, 77). It has been also shown that when mycobacterial cultures are old , the more the identification scores are low (61). But this should not impact negatively on the care of patients because the diagnosis must be made as soon as possible in order to provide the best care. In our lab, the directly identification of mycobacterium biomass without extraction procedure was done (Result not publish) with successful identification results by MALDI-TOF mass spectrometer (Brucker Daltonik GmbH, Bremen, Germany). The method consist to peak mycobacterium colony and smear on target slide then, overlaid by CHCA matrix and load to the MALDI-

TOF-MS system. The directly applied of mycobacterium biomass (MTBC, NTM) for MALDI-TOF-MS identification was found to be an accurate, rapid, and cost-effective system for identification of mycobacteria species than the identification after protein extraction. Obtaining encouraging preliminary results could be explained by the improvement of Bruker database.

To date, there are few studies in the field of mycobacterium resistance for diagnosing resistant forms of mycobacterium. But increasingly , researchers are interested in the development of MALDI-TOF-MS for the diagnosis of resistance of mycobacterium strains and their demonstrated that MALDI-TOF MS is a relevant tool for the detection of antibiotic resistance (85). But, it's necessary to release several studies to standardize the method for accuracy results.

MALDI-TOF-MS and Library of stored spectra developed for mycobacterium strains

Since the use of MALDI-TOF-MS, multiple versions of software containing mycobacteria reference spectra which are termed “Main Spectra Projection” (abbreviated as MSPs) have been developed taking into account the maximum of tuberculosis or atypical mycobacterium and named "Mycobacterial Library". In parallel, dedicated software were developed to analyze mass spectra against library of stored spectra of mycobacterium. Prior to the development of a specific database to mycobacteria belonging to the first spectra forty mycobacteria were contained only in the general database containing the reference spectra of several microorganisms and then, study was permit to include others *Mycobacterium* species in the MALDI-TOF database to improve it with home-made databases (9). In the case of the Bruker Microflex LT MALDI-TOF MS (Bruker), the first independent version of mycobacteria database was the library v3.0.2.0, which contained 18 species of mycobacterium comprising 18

strains(12). Then, there was the improvement of the Mycobacterial database by the addition of new spectra of other mycobacterium to give the version of Mycobacterial database named Mycobacteria Library v1.0. (Bruker Daltonik, Bremen, Germany) which contains 173 mycobacterial main spectrum profiles representing 94 species (12, 86) was released in 2012. The second version of the database is the Mycobacteria Library v 2.0 (Bruker Daltonik, Bremen, Germany) which contained 313 main spectrum profiles from 128 species of mycobacterium was released in 2014. The last version is the Mycobacterium library V3.0 (Bruker Daltonik, Bremen, Germany) which contained 853 main spectrum profile from 149 species of mycobacterium was released in 2015 (figure 1). With this version, *Mycobacterium africanum* could be identified as soon as *M. salmoniphilum* and others atypical mycobacterium. So, MALDI-TOF MS has been used successfully for mycobacteria typing and identification at the subspecies level, demonstrating that MALDI-TOF-MS for taxonomical classification.

Although the latest version of the database takes into account a greater number of mycobacteria strains, work remains to consent to the Bruker enricher base further. The third version as the second does not allow for a differential diagnosis of all species of the *Mycobacterium* tuberculosis complex except *M. tuberculosis*, *M. bovis* and *M. africanum*. The ability to identify *M. africanum* lineages (*M. africanum* West African 1 and *M. africanum* West African 2) by MALDIT -TOF. However, it is a clear advance and a great contribution to the management of tuberculosis in West Africa, where the strain is ubiquitous in TB cases (87). Further studies are still required to enable the development of the database for the diagnosis of other species of *M. tuberculosis* complex (ie. *M. pinnipedii*, *M. microti*, *M. caprae* and *M. canettii*) and genetic families of *M. tuberculosis* us Beijing genotype due to its worldwide expanding, LAM, T, Haarlem, Ural, Manu2 (88, 89). The whole contained of the two last versions of mycobacterium database are summarized in the figure 1. Each mycobacteriology can regularly augment the commercial

database with an in-house database containing others clinical Mycobacterium strains. Database upgrades and sample enrichment are essential elements to refine the MALDI-TOF MS technique, allowing the method to increase its power. However, it is essential to conduct multicenter evaluations whenever a new database is created in order to better assess their diagnostic performance.

The brief history of MALDI-TOF-MS development is summarized on figure 1.

Evolution of mycobacterium protein extraction and standardization of protocol

Mycobacteriums are rigid microorganisms and their identification by MALDI-TOF-MS need a special protein extraction step of their protein to obtain high quality spectra and valid identification results. The total microbial proteins are analyzed to identify mycobacterium. Since the main study by El Khéchine et al (9) which develop different inactivation and protein extraction protocols of mycobacterium strains to retain in fine the first original protocol, many extraction methods have been developed without the mycobacteria aggregate dissociation step to

simplify the previous during the implementation of MALDI-TOF-MS in the clinical microbiology laboratories(1, 9, 51, 61-64, 77, 84). The identification of mycobacterium outside biocontainment facilities requires that the organisms first be rendered inactive (71, 90) For the majority of the extraction protocols, strains inactivation and vortexing with silica beads to optimize the quality of the extracts for the obtain of bests spectra were used. All the simplified protein extraction protocols described in this review are easy to use for identifying commonly encountered Mycobacterium species. The mains steps of the successful mycobacterium sample preparation are summarized on biomass inactivation, cell disruption by aid of small beads and protein extraction with acetonitrile and formic acid. Actually, these protocols are standardized by the MALDI-TOF-MS manufactures after a synthesis of results of the different studies. The time to release the different steps to the identification of mycobacterium is approximately 1 to 2 hours. Some mycobacteria protein extraction protocols are summarized in the table 1.

Advantages and limitations of MALDI-TOF-MS for the identification of mycobacterium

Several studies have proved that MALDI-TOF MS is a promising tool for the rapid identification of mycobacterium isolates. The main advantage of this technique in mycobacteriology consists in a short-time analysis of fresh culture and the high discriminatory power of the MALDI-TOF-MS for disitinguishing among mycobacterium genus on the basis of the protein profiles of the severals species of mycobacterium. Although, With the introduction of more protein spectra of mycobacterium strains into the mycobacterium database, this device can now be used for the rapid identification of several strains of mycobacterium in a clinical microbiology laboratories as a routine activity. The new library update, Mycobacterium library V3.0 (Bruker Daltonik, Bremen, Germany) is able to identify 149 species of mycobacterial isolates. The database can also be continiously enhanced by the testing laboratory by adding new spectra when additional Mycobacterium species are encountered for which there is no identified good match.

MALDI-TOF-MS is now a boon to clinical microbiology laboratories with low levels of laboratory infrastructure and trained personnel to slightly raise the level of support infectious diseases within their coverage area. The cost of identification by MALDI-TOF MS is significantly less compared to other methods. Also, it generates less waste than other methods that are based on molecular and biochemical tests that use many disposable materials (12). The transfer of this technology in resource-limited countries where health insurance is still at an embryonic stage would significantly raise the quality of care for patients and at lower cost. Excluding the purchase cost of MALDI-TOF-MS device, the cost of identification is significantly less compared to other methods, including genomic sequencing and biochemical techniques (57). Actually, MALDI-TOF-MS is successful used in combination with genomic and conventional phenotyping (Culture and biochemical characteristics) to describe and characterize new species of mycobacterium in research laboratories(91-93). MALDI-TOF MS remains, however, limited by the previous time-

consuming culture steps, and is not suited for strain typing in epidemic contexts (81). The main advantages and disadvantages of the use of MALDI-TOF-MS are summarized in table 3.

CONCLUSION

Research of mycobacterial biomarker is the cornerstone and one the of the most challenging in the development of new diagnostic tools, drug targets and new TB vaccines. The actual multiple tools used in proteomics area permitted the discover of several intesting biomarkers. This review summarized the use of MALDI-TOF in the diagnosis of mycobacteria, and showed us, that this tools is regularly used to improve the detection of the mycobacteria. For successful identification of mycobacteria , it is important to use the same method of inactivation and protein extraction that was used to create the spectra library of the databse used by the MALDI-TOF-MS system . Always work with fresh cultures of mycobacteria for better identification and optimal management of patients. The short turn-around time

and expandability of the database demonstrate that this is a suitable first-line test for the identification of yeasts in the routine clinical microbiology laboratory.

Actually, MALDI-TOF-MS is currently used in routine for diagnosis of bacteria, fungi, inactivated clinical isolates of *Mycobacterium tuberculosis* and Non-tuberculosis mycobacteria (9, 50, 51, 55).

CONFLICT OF INTERESTS

The author declares that there is no conflict of interests regarding the publication of this paper.

REFERENCES

Albrethsen, J. (2013). "Proteomic profiling of the Mycobacterium tuberculosis identifies nutrient starvation responsive toxin-antitoxin systems." Molecular and Cellular Proteomics **doi: /10.1074/mcp.M112.018846**.

Amlerova, J., V. Studentova, et al. (2014). "[Identification of Mycobacterium spp. isolates using matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS)]." Epidemiol Mikrobiol Imunol **63**(3): 196-199.

Anhalt, J. P. and C. Fenselau (1975). "Identification of bacteria using mass spectrometry." Analytical Chemistry **47**(2): 219-225.

Arthur, J. M. (2003). "Proteomics." Curr Opin Nephrol Hypertens **12**(4): 423-430.

Balada-Llasat, J. M., K. Kamboj, et al. (2013). "Identification of mycobacteria from solid and liquid media by matrix-assisted laser desorption ionization-time of flight mass spectrometry in the clinical laboratory." J Clin Microbiol **51**(9): 2875-2879.

Balazova, T., J. Makovcova, et al. (2014). "The influence of culture conditions on the identification of Mycobacterium species by MALDI-TOF MS profiling." FEMS Microbiol Lett **353**(1): 77-84.

Bantscheff, M., M. Schirle, et al. (2007). "Quantitative mass spectrometry in proteomics: a critical review." Anal Bioanal Chem **389**(4): 1017-1031.

Bizzini, A. and G. Greub (2010). "Matrix-assisted laser desorption ionization time-of-flight mass spectrometry, a revolution in clinical microbial identification." Clin Microbiol Infect **16**(11): 1614-1619.

Bownds, S. E., T. A. Kurzynski, et al. (1996). "Rapid susceptibility testing for nontuberculosis mycobacteria using flow cytometry." J Clin Microbiol **34**(6): 1386-1390.

Brennan, P. J. "Structure, function, and biogenesis of the cell wall of Mycobacterium tuberculosis." Tuberculosis **83**(1): 91-97.

Briones, C. (2011). Proteome, Proteomics. Encyclopedia of Astrobiology. M. Gargaud, R. Amils, J. C. Quintanillaet al. Berlin, Heidelberg, Springer Berlin Heidelberg**:** 1351-1351.

Brown-Elliott, B. A., K. A. Nash, et al. (2012). "Antimicrobial Susceptibility Testing, Drug Resistance Mechanisms, and Therapy of Infections with Nontuberculous Mycobacteria." Clinical Microbiology Reviews **25**(3): 545-582.

Buchan, B. W., K. M. Riebe, et al. (2014). "Comparison of MALDI-TOF MS With HPLC and Nucleic Acid Sequencing for the Identification of Mycobacterium Species in Cultures Using Solid Medium and Broth." American Journal of Clinical Pathology **141**(1): 25-34.

Cain, T. C., D. M. Lubman, et al. (1994). "Differentiation of bacteria using protein profiles from matrix-assisted laser desorption/ionization time-of-flight mass spectrometry." Rapid Communications in Mass Spectrometry **8**(12): 1026-1030.

Chen, J. H. K., W.-C. Yam, et al. (2013). "Advantages of Using Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry as a Rapid Diagnostic Tool for Identification of Yeasts and Mycobacteria in the Clinical Microbiological Laboratory." Journal of Clinical Microbiology **51**(12): 3981-3987.

Claydon, M. A., S. N. Davey, et al. (1996). "The rapid identification of intact microorganisms using mass spectrometry." Nat Biotechnol **14**(11): 1584-1586.

Claydon, M. A., S. N. Davey, et al. (1996). "The rapid identification of intact microorganisms using mass spectrometry." Nat Biotech **14**(11): 1584-1586.

Cobo, F. (2013). "Application of MALDI-TOF Mass Spectrometry in Clinical Virology: A Review." The Open Virology Journal **7**: 84-90.

Comas, I., M. Coscolla, et al. (2013). "Out-of-Africa migration and Neolithic coexpansion of Mycobacterium tuberculosis with modern humans." Nat Genet **45**(10): 1176-1182.

Couvin, D. and N. Rastogi (2015). "Tuberculosis - A global emergency: Tools and methods to monitor, understand, and control the epidemic with specific example of the Beijing lineage." Tuberculosis (Edinb) **95 Suppl 1**: S177-189.

de Jong, B. C., M. Antonio, et al. (2010). "Mycobacterium africanum--review of an important cause of human tuberculosis in West Africa." PLoS Negl Trop Dis **4**(9): e744.

de Souza, G. A., S. Fortuin, et al. (2010). "Using a Label-free Proteomics Method to Identify Differentially Abundant Proteins in Closely

Related Hypo- and Hypervirulent Clinical Mycobacterium tuberculosis Beijing Isolates." Molecular & Cellular Proteomics **9**(11): 2414-2423.

Djelouadji, Z., M. Henry, et al. (2009). "Pyrosequencing identification of Mycobacterium tuberculosis W-Beijing." BMC Research Notes **2**: 239-239.

Djelouagji, Z. and M. Drancourt (2006). "Inactivation of Cultured Mycobacterium tuberculosis Organisms Prior to DNA Extraction." J Clin Microbiol **44**(4): 1594-1595.

Donnan, F. G. (1923). "Rays of positive electricity and their application to chemical analyses. By Sir J. J. Thomson, O. M. F.R.S. Second edition. Pp. x + 237. London: Longmans, Green and Co., 1921. Price 16s." Journal of the Society of Chemical Industry **42**(36): 861-861.

Dunne, W. M., K. Doing, et al. (2014). "Rapid Inactivation of Mycobacterium and Nocardia Species before Identification Using Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry." Journal of Clinical Microbiology **52**(10): 3654-3659.

Dunne, W. M., Jr., K. Doing, et al. (2014). "Rapid inactivation of Mycobacterium and nocardia species before identification using matrix-assisted laser desorption ionization-time of flight mass spectrometry." J Clin Microbiol **52**(10): 3654-3659.

Dunne, W. M., Jr., K. Doing, et al. (2014). "Rapid inactivation of Mycobacterium and nocardia species before identification using matrix-assisted laser desorption ionization-time of flight mass spectrometry." J Clin Microbiol **52**(10): 3654-3659.

El Khechine, A., C. Couderc, et al. (2011). "Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry identification of mycobacteria in routine clinical practice." PLoS One **6**(9): 13.

El Khéchine, A., C. Couderc, et al. (2011). "Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry Identification of Mycobacteria in Routine Clinical Practice." PLoS ONE **6**(9): e24720.

Emonet, S., H. N. Shah, et al. (2010). "Application and use of various mass spectrometry methods in clinical microbiology." Clin Microbiol Infect **16**(11): 1604-1613.

Escobar-Escamilla, N., J. E. Ramirez-Gonzalez, et al. (2014). "Hsp65 phylogenetic assay for molecular diagnosis of nontuberculous mycobacteria isolated in Mexico." Arch Med Res **45**(1): 90-97.

Falkinham, J. O., 3rd (1996). "Epidemiology of infection by nontuberculous mycobacteria." Clin Microbiol Rev **9**(2): 177-215.

Fang, J. and P. C. Dorrestein (2014). "Emerging mass spectrometry techniques for the direct analysis of microbial colonies." Current opinion in microbiology **0**: 120-129.

Fournier, P.-E., M. Drancourt, et al. (2013). "Modern clinical microbiology: new challenges and solutions." Nat Rev Micro **11**(8): 574-585.

Fournier, P. E., M. Drancourt, et al. (2013). "Modern clinical microbiology: new challenges and solutions." Nat Rev Microbiol **11**(8): 574-585.

Gagneux, S. (2012). "Host–pathogen coevolution in human tuberculosis." Philosophical Transactions of the Royal Society B: Biological Sciences **367**(1590): 850-859.

Gagneux, S., K. DeRiemer, et al. (2006). "Variable host–pathogen compatibility in Mycobacterium tuberculosis." Proceedings of the National Academy of Sciences of the United States of America **103**(8): 2869-2873.

Gu, S., J. Chen, et al. (2003). "Comprehensive Proteomic Profiling of the Membrane Constituents of a Mycobacterium tuberculosis Strain." Molecular & Cellular Proteomics **2**(12): 1284-1296.

Gunawardena, H. P., M. E. Feltcher, et al. (2013). "Comparison of the membrane proteome of virulent Mycobacterium tuberculosis and the attenuated Mycobacterium bovis BCG vaccine strain by label-free quantitative proteomics." J Proteome Res **12**(12): 5463-5474.

Holland, R. D., J. G. Wilkes, et al. (1996). "Rapid identification of intact whole bacteria based on spectral patterns using matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry." Rapid Commun Mass Spectrom **10**(10): 1227-1232.

Ishihama, Y., T. Schmidt, et al. (2008). "Protein abundance profiling of the Escherichia coli cytosol." BMC Genomics **9**(1): 102.

Jang, M.-A., W.-J. Koh, et al. (2014). "Distribution of Nontuberculous Mycobacteria by Multigene Sequence-Based Typing and Clinical Significance of Isolated Strains." Journal of Clinical Microbiology **52**(4): 1207-1212.

Jarlier, V. and H. Nikaido (1994). "Mycobacterial cell wall: Structure and role in natural resistance to antibiotics." FEMS Microbiol Lett **123**(1-2): 11.

Jiang, X., W. Zhang, et al. (2006). "Comparison of the proteome of isoniazid-resistant and -susceptible strains of Mycobacterium tuberculosis." Microb Drug Resist **12**(4): 231-238.

Johnson, M. M. and J. A. Odell (2014). "Nontuberculous mycobacterial pulmonary infections." Journal of Thoracic Disease **6**(3): 210-220.

Karas, M., D. Bachmann, et al. (1985). "Influence of the wavelength in high-irradiance ultraviolet laser desorption mass spectrometry of organic molecules." Analytical Chemistry **57**(14): 2935-2939.

Karas, M. B., D.; Hillenkamp, F. (1985). "Influence of the Wavelength in High-Irradiance Ultraviolet Laser Desorption Mass Spectrometry of Organic Molecules." Anal. Chem **78**: 53-68.

Kehrmann, J., S. Wessel, et al. (2016). "Principal component analysis of MALDI TOF MS mass spectra separates M. abscessus (sensu stricto) from M. massiliense isolates." BMC Microbiology **16**(1): 1-7.

Kim, B. J., S. H. Hong, et al. (2014). "Mycobacterium paragordonae sp. nov., a slowly growing, scotochromogenic species closely related to Mycobacterium gordonae." Int J Syst Evol Microbiol **64**(Pt 1): 39-45.

Kim, B. J., J. M. Kim, et al. (2015). "Mycobacterium anyangense sp. nov., a rapidly growing species isolated from blood of Korean native cattle, Hanwoo (Bos taurus coreanae)." Int J Syst Evol Microbiol **65**(7): 2277-2285.

Kim, B. J., R. K. Math, et al. (2013). "Mycobacterium yongonense sp. nov., a slow-growing non-chromogenic species closely related to Mycobacterium intracellulare." Int J Syst Evol Microbiol **63**(Pt 1): 192-199.

Kruh, N. A., J. Troudt, et al. (2010). "Portrait of a pathogen: the Mycobacterium tuberculosis proteome in vivo." PLoS One **5**(11): e13938.

Lotz, A., A. Ferroni, et al. (2010). "Rapid Identification of Mycobacterial Whole Cells in Solid and Liquid Culture Media by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry." J Clin Microbiol **48**(12): 4481-4486.

Lotz, A., A. Ferroni, et al. (2010). "Rapid identification of mycobacterial whole cells in solid and liquid culture media by matrix-assisted laser

desorption ionization-time of flight mass spectrometry." J Clin Microbiol **48**(12): 4481-4486.

Machen, A., M. Kobayashi, et al. (2013). "Comparison of heat inactivation and cell disruption protocols for identification of mycobacteria from solid culture media by use of vitek matrix-assisted laser desorption ionization-time of flight mass spectrometry." J Clin Microbiol **51**(12): 4226-4229.

Machen, A., M. Kobayashi, et al. (2013). "Comparison of Heat Inactivation and Cell Disruption Protocols for Identification of Mycobacteria from Solid Culture Media by Use of Vitek Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry." Journal of Clinical Microbiology **51**(12): 4226-4229.

Mareković, I., Z. Bošnjak, et al. (2015). "Evaluation of Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry in Identification of Nontuberculous Mycobacteria." Chemotherapy **61**(4): 167-170.

Mather, C. A., S. F. Rivera, et al. (2014). "Comparison of the Bruker Biotyper and Vitek MS matrix-assisted laser desorption ionization-time of flight mass spectrometry systems for identification of mycobacteria using simplified protein extraction protocols." J Clin Microbiol **52**.

Mather, C. A., S. F. Rivera, et al. (2014). "Comparison of the Bruker Biotyper and Vitek MS Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry Systems for Identification of Mycobacteria Using Simplified Protein Extraction Protocols." Journal of Clinical Microbiology **52**(1): 130-138.

Mdluli, K. and M. Spigelman (2006). "Novel targets for tuberculosis drug discovery." Curr Opin Pharmacol **6**(5): 459-467.

Mediavilla-Gradolph, M. C., I. De Toro-Peinado, et al. (2015). "Use of MALDI-TOF MS for Identification of Nontuberculous Mycobacterium Species Isolated from Clinical Specimens." BioMed Research International **2015**: 854078.

Mehaffy, C., A. Hess, et al. (2010). "Descriptive proteomic analysis shows protein variability between closely related clinical isolates of Mycobacterium tuberculosis." Proteomics **10**(10): 1966-1984.

Mehaffy, C., A. Hess, et al. (2010). "Descriptive proteomic analysis shows protein variability between closely related clinical isolates of Mycobacterium tuberculosis." Proteomics **10**(10): 1966-1984.

Mehaffy, M. C., N. A. Kruh-Garcia, et al. (2011). "Prospective on Mycobacterium tuberculosis Proteomics." Journal of Proteome Research **11**(1): 17-25.

Mokrousov, I., A. Vyazovaya, et al. (2012). "Mycobacterium tuberculosis Population in Northwestern Russia: An Update from Russian-EU/Latvian Border Region." PLoS ONE **7**(7): e41318.

Neyrolles, O. and C. Guilhot "Recent advances in deciphering the contribution of Mycobacterium tuberculosis lipids to pathogenesis." Tuberculosis **91**(3): 187-195.

Panda, A., S. Kurapati, et al. (2013). "Rapid identification of clinical mycobacterial isolates by protein profiling using matrix assisted laser desorption ionization-time of flight mass spectrometry." Indian J Med Microbiol **31**(2): 117-122.

Pignone, M., K. M. Greth, et al. (2006). "Identification of mycobacteria by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry." J Clin Microbiol **44**(6): 1963-1970.

Pleissner, K. P., T. Eifert, et al. (2004). "Web-accessible proteome databases for microbial research." Proteomics **4**(5): 1305-1313.

Quinlan, P., E. Phelan, et al. (2015). "Matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry (MS) for the identification of mycobacteria from MBBacT ALERT 3D liquid cultures and Lowenstein-Jensen (LJ) solid cultures." J Clin Pathol **68**(3): 229-235.

Saleeb, P. G., S. K. Drake, et al. (2011). "Identification of mycobacteria in solid-culture media by matrix-assisted laser desorption ionization-time of flight mass spectrometry." J Clin Microbiol **49**(5): 1790-1794.

Saleeb, P. G., S. K. Drake, et al. (2011). "Identification of mycobacteria in solid-culture media by matrix-assisted laser desorption ionization-time of flight mass spectrometry." J Clin Microbiol **49**.

Saleeb, P. G., S. K. Drake, et al. (2011). "Identification of Mycobacteria in Solid-Culture Media by Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry." Journal of Clinical Microbiology **49**(5): 1790-1794.

Schmidt, F., S. Donahoe, et al. (2004). "Complementary analysis of the Mycobacterium tuberculosis proteome by two-dimensional

electrophoresis and isotope-coded affinity tag technology." Mol Cell Proteomics **3**(1): 24-42.

Scott, J. S., S. A. Sterling, et al. (2016). "Diagnostic performance of matrix-assisted laser desorption ionisation time-of-flight mass spectrometry in blood bacterial infections: a systematic review and meta-analysis." Infect Dis **48**(7): 530-536.

Seng, P., M. Drancourt, et al. (2009). "Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry." Clin Infect Dis. **49**.

Seng, P., J.-M. Rolain, et al. (2010). "MALDI-TOF-mass spectrometry applications in clinical microbiology." Future Microbiology **5**(11): 1733-1754.

Silhavy, T. J., D. Kahne, et al. (2010). "The Bacterial Cell Envelope." Cold Spring Harbor Perspectives in Biology **2**(5): a000414.

Singh, P., R. N. Rao, et al. (2016). "PE11, a PE/PPE family protein of Mycobacterium tuberculosis is involved in cell wall remodeling and virulence." Sci Rep **6**(21624).

Singhal, N., M. Kumar, et al. (2015). "MALDI-TOF mass spectrometry: an emerging technology for microbial identification and diagnosis." Frontiers in Microbiology **6**: 791.

Singhal, N., M. Kumar, et al. (2015). "MALDI-TOF mass spectrometry: an emerging technology for microbial identification and diagnosis." Front Microbiol **6**: 791.

Somerville, W., L. Thibert, et al. (2005). "Extraction of Mycobacterium tuberculosis DNA: a Question of Containment." J Clin Microbiol **43**(6): 2996-2997.

Tanaka, K., H. Waki, et al. (1988). "Protein and polymer analyses up to m/z 100 000 by laser ionization time-of-flight mass spectrometry." Rapid Communications in Mass Spectrometry **2**(8): 151-153.

Thiede, B., C. J. Koehler, et al. (2013). "High Resolution Quantitative Proteomics of HeLa Cells Protein Species Using Stable Isotope Labeling with Amino Acids in Cell Culture(SILAC), Two-Dimensional Gel Electrophoresis(2DE) and Nano-Liquid Chromatograpohy Coupled to an LTQ-OrbitrapMass Spectrometer." Molecular & Cellular Proteomics **12**(2): 529-538.

Tudo, G., M. R. Monte, et al. (2015). "Implementation of MALDI-TOF MS technology for the identification of clinical isolates of Mycobacterium spp. in mycobacterial diagnosis." Eur J Clin Microbiol Infect Dis **34**(8): 1527-1532.

Wania, R. (2013). "Tuberculosis 2: Pathophysiology and microbiology of pulmonary tuberculosis." SSMJ **6**: 10-12.

WHO (2014). Global tuberculosis report 2014. Geneva, World Health Organization.http://www.who.int/tb/publications/global_report/en/ (accessed Dec 12, 2014).

WHO (2014). Global Tuberculosis Report 2014. WHO/HTM/TB/2014.08**:** 171.

Wilkins, M. R., E. Gasteiger, et al. (1998). "Protein identification with N and C-terminal sequence tags in proteome projects." J Mol Biol **278**(3): 599-608.

Wu, X., J. Zhang, et al. (2007). "Comparison of Three Methods for Rapid Identification of Mycobacterial Clinical Isolates to the Species Level." Journal of Clinical Microbiology **45**(6): 1898-1903.

Xu, D. D., D. F. Deng, et al. (2014). "Discovery and identification of serum potential biomarkers for pulmonary tuberculosis using iTRAQ-coupled two-dimensional LC-MS/MS." Proteomics **14**(2-3): 322-331.

Yssouf, A., L. Almeras, et al. (2016). "Emerging tools for identification of arthropod vectors." Future Microbiol **11**(4): 549-566.

Yssouf, A., C. Socolovschi, et al. (2013). "Matrix-assisted laser desorption ionization--time of flight mass spectrometry: an emerging tool for the rapid identification of mosquito vectors." PLoS ONE **8**(8).

Zingue, D., C. Flaudrops, et al. (2016). "Direct Matrix Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry identification of mycobacteria from colonies." European Journal of Clinical Microbiology & Infectious Diseases: Ms. No. EJCM-D-16-00565R00561.

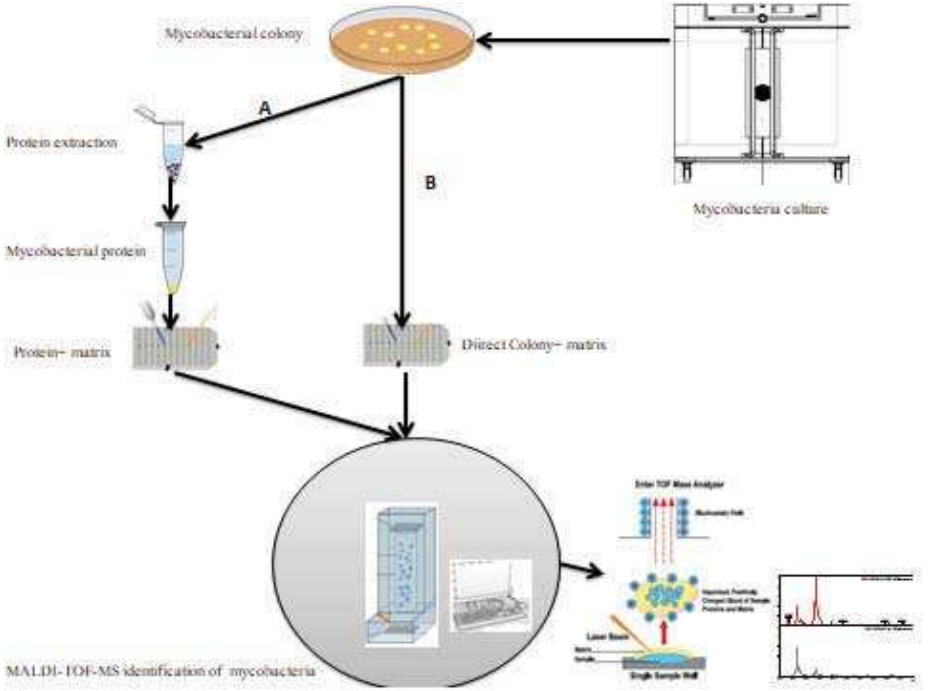

Figure 1: Schematic diagram showing the work-flow in a MALDI-TOF MS. (A) MALDI-TOF-MS identification of mycobacteria after protein extraction, (B) Direct MALDI-TOF-MS identification of mycobacteria from colony

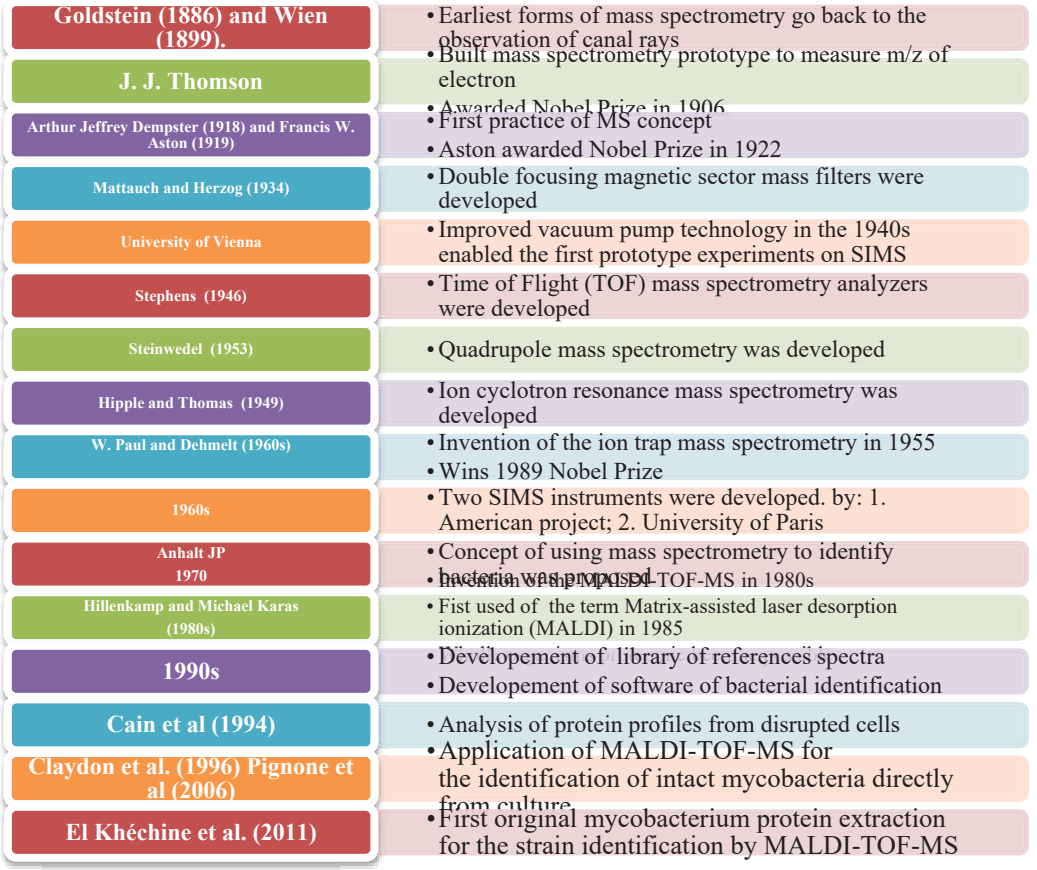

Figure 2:Chronology of major discoveries in mass spectrometry development to the MALDI-TOF-MS and its first use in microbiology and mycobacterium identification

Figure 3: Contained of Mycobacteria Library v2.0 and Mycobacteria Library v3.0 of Brucker database

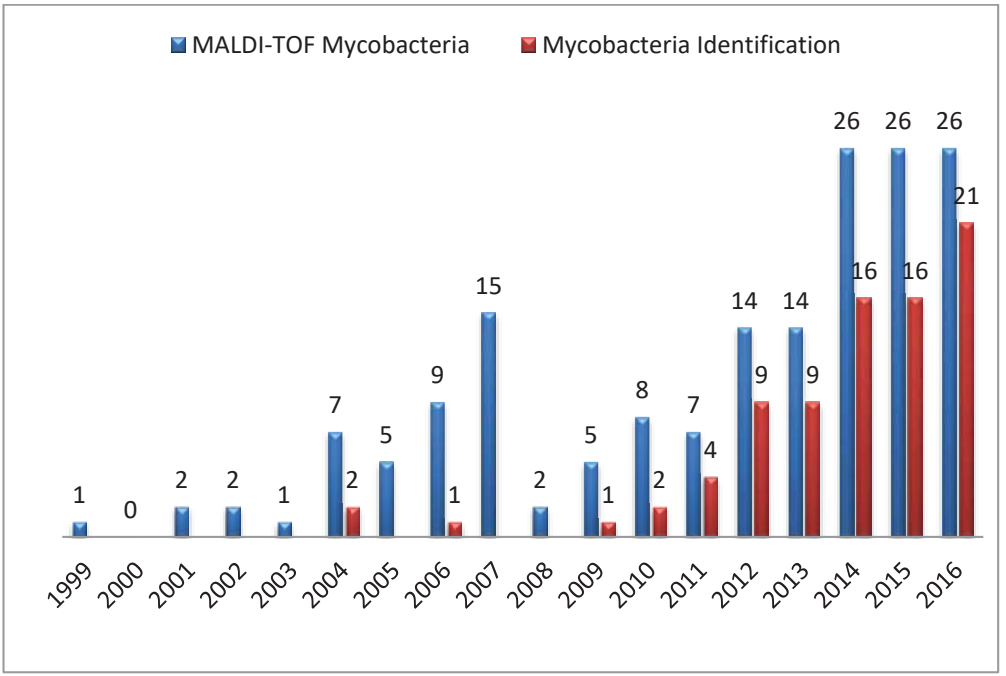

Figure 4: Annual evolution of the number of publications on the use of MALDI-TOF-MS for the characterization and identification of mycobacteria

Tableau 1:Mycobacteria inactivation and protein extraction protocols used prior to identification by MALDI-TOF MS

Steps	**Protocol 1** (first original extraction protocol)	**Protocol 2**	**Protocol 3**	**Protocole 4**	**Protocol 5**	**Protocol 6**	**Protocol 7**	**Protocol 8**	Protocol 9	Protocol 10
Ref.	(9)	(82)	(62)	(84)	(64)	(63)	(63)	(12, 77)	(61)	(94)
1.	Collected biomass or pellet from liquid media culture (obtained after centrifugation)									Colony
2.	In a screw-cap Eppendorf tube containing 500 µl of HPLC-grade water and 0.5% Tween-20	transfer in Eppendorf tube with 300 µL of water (HPLC grade)	Resuspended in 500 µl of 70% ethanol into a sterile screw-cap 2-ml microcentrifuge tube containing 0.2 ml of sterile 0.5-mm glass beads	Add 300 µl HPLC-grade H2O	Suspend organisms in 500 µl of distilled water in a 1.5-ml screw-cap tube	Resuspend in 500 µl of 70% ethanol.	In screw-cap containing 500 ml of 70% ethanol and 200 ml of 0.5-mm glass beads	Into a 1.5-ml screw-cap microcentrifug e tube containing 300 µl of distilled wate	In screw-top microcentrifu ge tube containing 300 µl of water and 200 µl of 1-mm silica beads	
3.	Heat at 95°C/1H	Heat at 95°C/ 30min	10-min exposure to 70% EtOH at room temperature	Add 900 µl of absolute ethanol	Heat to 95°C/30 min	Heat 30 min at 95°C ± 5°C	Vortex for 15 min	Add 900µl of 100% ethanol	Heat at 95°C/30 min	
243	Wash twice the suspensions with 500 µL of HPLC-grade water and centrifugation at 13,000×g/10 min.	Centrifuge at 13.000-15.000 rpm/2 min	mechanically disruption at 2,500 oscillations/min for 5 min	Vortex	Vortex briefly	Centrifuge at 18,000 × g/2 min	Incubate at room temperature for 10 min	Vortex	Add 900µl of absolute ethanol	
	Remove supernatant	Remove supernatant	centrifuge	10 min incubation at room temperature	Centrifuge at 13,000 rpm/2 min	Discard supernatant	Vortex for 5 to 10s	Centrifuge at 13,000 rpm/2 min	Vortex for 10 min	
	Add 500 µL of HPLC-grade water and 0.3 g acid-washed glass beads	Add 300 µL of water (HPLC grade)		Centrifuge at 13,000 rpm/ 2min	Remove supernatant	Wash pellet, disperse with water	Transfer suspension to an empty microcentrifuge tube with care to avoid the transfer of any bead	Discard supernatant	Transfer the liquid to a new 1.5-ml snap-top microcentrifu ge tube	
	Vortex in a FastPrep at full speed/3 min.	Mix the sediment carefully	removal of the ethanol supernatant	Remove the supernatant	Wash pellet with 300 µl of distilled water	Centrifuge at 18,000 × g/2 min	Discard the supernatant	Resuspend the pellet in 500 µl of distilled water	Centrifuge at 13,000 × g for 2 min	
	Centrifuge at 13,000×g/10 min	Add 900 µL of 100% ethanol	Reconstitute the pellet with 10µl of 70%	Wash the pellet	Centrifuged at 13,000	Discard supernatant	Resuspend the pellet in 10 µl of	Vortex	Remove supernatant	

			formic and 10µl of acetonitrile		rpm for 2 min		70% formic acid			
	Resuspend the pellet in 5-50 µl 70% formic acid and and 5-50 µl acetonitrile	Vortex	Centrifuge	suspended the pellet into 500 µl H2O	Remove supernatant	Wash pellet, disperse with water	Incubate for 2 to 5 min at room temperature	Centrifuge at 13,000 rpm/2 min	Allow pellet to air dry for 10 min.	
	Centrifuge at 11,000×g/1 min	Centrifuge 13,000–15,000 rpm/2 min	Use the supernatant for analysis	Centrifuge (13,000 rpm/ 2 min)	Suspend pellet in 1.2 ml of 70% ethanol	Sonication 15 min	Add 10µl of acetonitrile	Discard the supernatant	Resuspend the pellet in 10 µl of 70% formic acid	
		Remove supernatant		Remove supernatant	vortex briefly	Centrifuge at 18,000 × g/10 min	Centrifuge at 10,000 × g/ 2 min	Resuspend the pellet in 50 µl of water	Add 10 µl of 98% acetonitrile	
		Resuspend the pellet in 500 µL of water (HPLC grade)		Resuspend pellet into 50 µl H2O	Centrifuge at 13,000 rpm for 2 min	Remove the supernatant		heat inactivate at 100°C/30 min	Vortex for 20 s	
4.		Centrifuge 13,000–15,000 rpm/2 min		Heat inactivation 95°C/30 min	Discard supernatant	Resuspend the pellet was in 5µl of 85% formic acid		Allow to cool at room temperature/2 min	Centrifuge at 10,000×g/1min	
5.		Remove the supernatant		Cool samples at room temperature	Brief spun the pellet in the centrifuge	Centrifuge at 15,000 × g/1 min		Add 1.2 ml of precooled 100% ethanol		
6.		Resuspend the pellet in 50 µL of water (HPLC grade)		Add 1.2 ml precooled absolute ethanol	Add 50µl of 70% formic acid &100 to 200 µl of 0.1-mm-diameter glass beads	Add 5µl of acetonitrile		Vortex		
7.		Heat it for 10 min at 95°C		Centrifuge at 13,000 rpm/2 min	Vortex for 10 min	Centrifuge at 15,000 × g/1 min		Centrifuge at 13,000 rpm/2 min		
8.		Cool at room temperature	Vitek MS system (bioMérieux, Marcy l'Etoile, France)	Remove supernatant	Add 50 µl of 100% acetonitrile			Discard the supernatant		
9.		Add 1200 µL of absolute alcohol		air dry pellet for 10 min	Vortex for 10 min			Allow the pellet to dry at room		

		previously cooled (−18°C/−20°C)						temperature/2-3 min		
10.		Centrifuge 13,000–15,000 rpm/2 min		Add 0.5mm silica bead and 10 to 50 µl acetonitrile	Centrifuge for 2 min at 13,000 rpm			Suspend the pellet in 10-50 µl of acetonitrile and 50-100 0.5-mm-diameter glass beads		
11.		Remove supernatant		Vortex vigorously at maximum speed for 2 to 5 min.	Bruker Microflex LT bioMérieux Vitek MS IVD system.			Vortex 1min		
12.		Leave the pellet to dry for 5 minutes		Add 10 to 50 µl 70% formic acid				Add 70% formic acid		
13.		Add silica beads (0.5 mm beads) and 20 µL of pure acetonitrile		Vortex for 5 s				Vortex 1min		
14.		Bruker Microflex LT Biotyper system (Bruker Daltonics, Bremen, Germany)		Centrifuge (13,000 rpm/2 min)				Centrifuge at 13,000 rpm/2 min		
15.		Mix well for one minute								
16.		Add 20 µL of 70% formic acid								
17.		Centrifuge								
18.		Spot 1 to 1.5µl of supernatant on target slides and allow to dry								Spot colony
19.		Overlaid with 1 to 1.5 µl of matrix solution HCCA (saturated α cyano-4-hydroxycinnamic acid, 50% acetonitrile, 2.5% TFA)								
20.		MALDI-TOF-MS analysis								

Chapter 5: Improve automated microscopy for mycobacteria detection

Lung disease and suspected mycobacterial infections are diagnosed by the detection of acid-fast bacilli in sputum smears or in related samples by the Ziehl-Neelsen method, which is commonly used in laboratories around the world due to its simplicity and low cost. Microscopy Ziehl-Neelsen method has a low sensitivity (20-53%) and a low specificity because it cannotdistinguish tuberculosis mycobacteria from non-tuberculosis ones. Since Ziehl-Neelsen method was first described in the 1800s by the bacteriologist Franz Ziehl and the pathologist Friedrich Neelsen, several modifications have been attempted to improve its sensitivity, even attempts to automate the method have been done. Recently, the Slide Scanner ZEISS Axio Scan.Z1automate was introduced on the market for automatic reading of slides but to date, there are no publications about its performance for the diagnosis of mycobacteria infections after Ziehl-Neelsein staining. Slide Scanner ZEISS Axio Scan.Z1 is a highly automated and simple to operate and we developped protocol for its use of Ziehl-Neelsen staining slide for routine diagnosis of mycobacteria.

In our study performed on a large number of samples, the Slide Scanner ZEISS Axio Scan.Z1 showed a sensitivity of 97.06 % [84.67%- 99.93%].

Article 7: Automatic detection of Ziehl-Neelsen-stained mycobacteria in sputum specimens: a proof-of-concept

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Automatic microscopic numeration of mycobacteria in sputum: a proof-of-concept

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ABSTRACT

The laboratory diagnosis of lung mycobacterioses including tuberculosis comprises microscopic examination of sputum smear after appropriate staining such as Ziehl-Neelsen staining to observe acid-fast bacilli. This standard procedure is operator-dependant and its sensitivity depends on the duration of observation. We developed and evaluated an operator-independent microscopic examination of sputum smears for the automated detection and enumeration of acid-fast bacilli using a ZEISS Axio Scan.Z1 microscope. The sensitivity, specificity, positive predictive value, negative predictive values and accuracy were calculated using standard formulations by comparison with standard microscopic examination. After in-house parameterization of the automatic microscope and the counting software, the limit of detection evaluated by seeding negative sputa with *Mycobacterium bovis* BCG or *Mycobacterium tuberculosis* H37Rv (10^0-10^5 bacilli/mL) was 10^2 bacilli/mL of sputum with a 100% positivity rate. Then, the evaluation of 93 sputum specimens including 34 smear-positive and 59 smear-negative specimens yielded a sensitivity of 97.06 %

[84.67%- 99.93%], a specificity of 86.44 % [73.01%-92.78%], a positive predictive value of 80.49% [65.99%-89.76%] and a negative predictive value of 98.04% [87.85%-99.71%]. Up to 100 smear slides could be stocked for reading in the microscope hotel and results were exportable into the laboratory information system. Based on these preliminary results, we are implanting this automatic protocol in the routine workflow so that only smears detected positive are confirmed by standard microscopic examination.

Keywords : Mycobacterioses, tuberculosis, diagnosis, microscopic examination, Ziehl Neelsen staining.

INTRODUCTION

Life-threatening lung tuberculosis is of public health concern in several regions in the world after the World Health Organization reported 10.4 million new cases and 1.4 million deaths in 2016 worldwide (95). In all affected countries, the laboratory diagnosis of lung tuberculosis is one important component of the fight against lung tuberculosis, by assisting the medical management of patients including the isolation of contagious patients (96). For that purpose, microscopic observation of tubercle bacilli in sputum smears, invented more than 100 years ago (95, 97) remains the corner-stone of the laboratory diagnosis of lung tuberculosis, infirming or confirming the diagnosis and contributing to assess the contagiousness of the patient (98, 99). In some remote areas across low- and middle income countries, microscopic examination of sputum smear is the only tool available for the laboratory diagnosis of lung tuberculosis (100-102). Microscopic examination is performed after appropriate staining of mycobacteria and the Ziehl-Neelsen staining is used worldwide for that purpose, staining in purple red acid-fast bacilli (103).

However, it is an operator-dependant technique of diagnosis, requiring specifically trained personnel (96). Indeed, it is a time-consuming technique as the observation of at least 100 microscopic fields for at least 15 minutes is recommended (104). Following this recommendation results in the fact that one laboratory personal can examine only a limited number of slides per one day (104); and routine observation may not be optimum resulting in variable sensitivity of 60% to 70% compared to culture (105, 106). Moreover, results of the observation are manually reported, exposing to the risk of miss-reporting (107). In countries with a high-prevalence of lung tuberculosis, it has been noted that the demands on technicians lead to overload and fatigue resulting in reduced quality of microscopy (108).

Therefore, developing an operator-independent, automated reading of stained sputum spears to ensure the reproducibility of the observations may be of value (109). Accordingly, previous studies have been conducted to automatize the microscopic detection of mycobacteria in Ziehl-Neelsen-stained sputum smears or fluorescent auramine stained smears (96, 98, 104, 110-115). Aims of

automation were to speed-up the screening process to cope efficiently with large numbers of smears (98, 110), to improve sensitivity and to reduce reliance on technicians (98). However, none of these automated techniques readily replaced the standard microscopic examination in routine (116).

In France where our laboratory is operating, the prevalence of lung tuberculosis is low at 5.1 cases per 10^5 inhabitants (117, 118). We thought that in this specific context, a first step of automated microscopic detection of acid-fast bacilli would complement standard microscopic detection by sorting negative smears with a high predictive value, so to spare valuable technical work time. We here present a proof-of-concept of this approach which may assist other laboratories in their decision to implement automated smear reading in the routine workflow.

MATERIALS AND METHODS

Clinical specimens. Sputum specimens collected as part of the routine diagnosis activity of our clinical microbiology laboratory (Institut Hospitalier Universitaire- Méditerranée Infection, Marseille, France) were prospectively included in this investigation. This study using anonymous, left-over routine specimens which have not been collected specifically for this study, received the agreement of the Institut Hospitalier Universitaire Méditerranée Infection, Marseille, France (CE 2016-025). Sputum specimens were collected into a sterile, dry container and processed 24-72 hours after collection. Microscopic detection of AFB was done as detailed below and chlorexidine-decontaminated sputum specimens were inoculated onto MOD9 culture medium as previously reported (119, 120). Colonies were detected by combining naked-eye detection and scanning detection as previously described (121). Colonies were identified as *Mycobacterium tuberculosis* by using matrix-assisted laser desorption ionisation time-of-flight mass spectrometry as previously described (122).

Microscopic examinations. As for microscopy, sputum were used to prepare duplicate smears per sample which were air dried, heat fixed and stained using a commercially-available kit featuring Ziehl-Neelsen staining (kit Cold ZN, RAL, Toulouse, France). Standard microscopic observation was routinely done by the laboratory technicians using an Olympus BX40 light microscope (New York Microscope Co., USA) under oil immersion at x100 magnitude. Quantification of detected AFB was done according to international laboratory guidelines (107). Automated microscopic detection was done using a Zeiss Axio Scan.Z1 Digital Slide Scanner (Carl Zeiss Microscopy, Marly-le-Roi, France). This automated microscope is featuring a 100-slide hotel, a LED light source, a Hitachi HV-F202SCL color camera with tri CCD 1,800 x 1,200 pixels, a plan apochromat 20X/0.8 objective comprising a 18,000 X 12,000-pixel microscopic field. Axio Scan.Z1 employs fast filter wheels and can change channel in less than 50 milliseconds. Using 3-band and 4-band filter sets with Colibri.2 as a fluorescence light source, Axio Scan.Z1 makes millisecond-fast switches. The wavelength range is

400 nm to 700 nm and a resolution of 10X (0.44 µm/pixel), 20X (0.22 µm/pixel), 40X (0.11 µm/pixel).

Axio Scan.Z1 allows to scan the samples and to create virtual slide allowing retrieving a record of all virtual microscopy operations at the click of a button (Figure 1). The software module ZEN slidescan is capturing high volume quantitative image of 100 slides for 28 mm x 48 mm slides and the Scan time is four minutes by slide. Each slide can be equipped with a barcode for recognition and archiving of the digitalized slides. Acquired images are saved in Jpeg or Tiff format and data could be accessed anywhere by documenting and storing specimens as virtual slides and view them on iPad with the free ZEN browser app. The results of reading and counting the number of acid-fast bacilli found in a smear by the two methods of microscopy were graded according to the WHO and recommendation IUATLD (103).

Parameterization of the Zeiss Slide Scanner and software.

The particular application of detecting AFB required a specific parameterization of the Zeiss Slide Scanner. MetaMorph® Microscopy Automation & Image Analysis Software (Molecular Devices Sunnyvale, Californie, USA) (https://www.moleculardevices.com/systems/metamorph-research-imaging/metamorph-microscopy-automation-and-image-analysis-software) were used for the treatment of image and AFB counting. The procedure is described in supplement1. Five fields were acquired per slide (Figure 1).

Limit of detection by automated microscopy.

In order to determine the limit of detection of AFB by using the Zeiss Slide Scanner, two AFB-negative sputum specimens were inoculated with either *Mycobacterium bovis* BCG strain Pasteur (Collection de l'Institut Pasteur, Paris, France) or with *Mycobacterium tuberculosis* H37Rv (Collection de l'Institut Pasteur) at inoculum concentration of 0, 10^0, 10^1,10^2, 10^3, 10^4, 10^5 mycobacteria/mL; then stained as described above and read by the Zeiss Slide Scanner without immersion oil.

Performances of automated microscopy.

The performances of automated microscopy were established by prospectively analyzing 34 AFB positive from 15 patients and 59 AFB negative sputum specimens collected as part of the routine diagnosis activity of the laboratory. The sensitivity (Se), specificity (Sp), positive predictive value (PPV) and negative predictive values (NPV) were calculated by comparison with the results of standard microscopic examination of the same sputum specimens used as the reference method.

Statistical analysis.

The statistical software used was MedCalc Statistical Software Version 17.6 (MedCalc Software bvba, Ostend, Belgium). Performance of the method was estimated by the intrinsic and extrinsic characteristics: sensibility, specificity, positive / negative predictive values, prevalence and likelihood ratio. The confidence intervals of the percentage calculations were calculated at the risk α at 0.05 of the binomial distribution and the performance results were expressed with IC95%.

Results

Limit of the detection by automated microscopy.

The acquisition / analysis combination detects AFB in sputum inoculation at a concentration of 10^2/mL and the detection is even more tangible with an inoculum of 10^3/mL. For an inoculum of 10^1/mL, there is no detection of AFB by the ZEISS Axio Scan.Z1 Digital Slide Scanner. Each of the five fields analyzed contains approximately 11,000 x 7,500 points.

Performances of automated microscopy.

Among 93 sputum smear slides read by standard microscopy, 34/93 (36.56%) were positive by standard microscopy examination; automated microscopy detected 97% (33/34) of these slides as positive in addition to eight slides which had not been detected by standard microscopy. The resulting set includes 295 critical negative images from 59 healthy subjects without lung disease caused by mycobacteria and 170 positive images from 34 positive sputum specimens. These data yielded calculated values for four above mentioned performance parameters of Zeiss

automated method were 97.06 % [84.67%- 99.93%], 86.44 % [73.01%-92.78%], 80.49% [65.99%-89.76%] and 98.04% [87.85%-99.71%] respectively for the sensitivity (Se), specificity (Sp), positive predictive value (PPV) and negative predictive value (NPV). The accuracy was 90.32. The Youden Index was 0.82, the Yule coefficient was 0.99, the X^2 (Chi square) value was 58.28 ($p < 0.001$) and area under the ROC curve (AUC) was 0.91 [0.83-0.96].

As for the 33 slides detected positive in common by standard and automated microscopy, numeration of AFB grading according WHO and IUATLD recommendation yielded the same scale grade in 23/33 (68.6%) of slides. One positive slide with standard microscope was negative with automate microscope.

The types of images obtained from positive smears by the two methods are illustrated in figure 2 (Figure 1). All the 34 slides detected positive by standard microscopy were culture positive including 27 *M. tuberculosis* isolates, four *Mycobacterium intracellulare/ chimeara* isolates and three *Mycobacterium simiae* complex isolates. Eight sputum specimens detected negative by standard microscopy and

positive by automated microscopy remained sterile in culture. Further microscopic observation indicated that false-positivity was due to clusters of red dyes left on the slides after washing (Figure 3).

Discussion

We developed an original protocol for the automated microscopic detection of AFB in sputum specimens with the aim of sorting negative smears and confirming only positive smears by the standard microscopic examination. We compared the two procedures of microscopy as the very same sputum specimens were prospectively examined in parallel by the routine standard microscopic examination and the challenger automated microscopy; with both analyses performed by independent operators blind one to each other.

Attempts to automate the reading of the slides after ZN staining were carried out with little success for their routine use for the detection of mycobacteria (96, 98, 104, 110-115). Indeed, these studies were limited by the small number of samples used resulting in preliminary results. The present

study of microscopy automation is the first of its kind to be successfully conducted on a large number of samples. We proceeded step by step to allow us to determine the minimum threshold of AFB in a sample required to have a positive result, the focusing in a second step on achieving a negative predictive value as high as possible in order to fulfill our aim.

The automated microscopic detection here reported required a specific parameterization of the automated microscope and software in order to overcome unanticipated problems. In particular, we encountered problems when analyzing slides that had some black debris; and thick smears which retained dye clumps and staining artifacts; all initially read as AFB which yielded false positive results. These obstacles were overcome by focusing on the quality of smear preparation and careful washing of the slide; and repeated adjustments of the controller parameters to avoid debris counting. However, the algorithm used with Zeiss axio analyzes all the pixels in equal parts and makes it possible to detect all the bacilli present colored by ZN and to quantify them. ZEISS Axio Scan.Z1 Digital Slide

Scanner, in addition to giving qualitative values for the diagnosis also allows quantifying the AFB which is a parameter of monitoring of the effectiveness of the treatment. The detection of mycobacteria by the new algorithm proved to be an effective test with a Youden index close to 1.

False positive results were not annoying in our study as we have decided that all the smears automatically detected as positive, had to be confirmed by standard microscopic examination. More interestingly, this proof-of-concept study of automated microscopy yielded a negative predictive value of 98.04%, indicating that one slide that was read negative by automated microscopy had not to be controlled by standard microscopy.

Potential benefits of automated screening for mycobacterial lung disease are rapid and accurate, diagnosis, increased screening of the population, and reduced health risk to staff processing slides (123). When screening AFBs under an optical microscope at a convenient speed, a human observer may fail to observe bacilli, especially when the sample is paucibacillary. One of the main advantages of the automate

zeiss axio is the ability to read more than five fields per slide to increase its sensitivity. However, the playback time increases with increasing the number of fields.

Conclusions

In the specific context of a low prevalence of lung tuberculosis, automated microscopic detection of AFB using a Zeiss Axio Slide Scanner and home-adapted protocol for microscopy and software analyses, achieved a high predictive negative value. This performance allowed us to the on-going implementation of automated microscopic detection of AFB in the routine workflow of the laboratory to sort negative slides and control positive slides by the standard microscopy. Additional advantages are a 100-slide hotel and digitalization of data for direct exporting in the laboratory information system, further optimizing workflow. We therefore propose that automated microscopic detection of AFB could already be used as a first-line microscopy diagnosis of lung tuberculosis in low prevalence countries. Further improvements in the specificity of the detection are required in order to

progressively implement automated microscopy for the laboratory diagnosis of lung tuberculosis in high prevalence countries where it is most needed.

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CONFLICTS OF INTEREST

The authors have no conflict of interest to declare. The society Zeiss has no role in this study which was only supported by Institut Hospitalier Universitaire Méditerranée infection.

REFERENCES

1. **Gagneux S.** 2012. Host–pathogen coevolution in human tuberculosis. Philosophical Transactions of the Royal Society B: Biological Sciences **367:**850-859.
2. **WHO.** 2014. Global Tuberculosis Report 2014.
3. **WHO.** 2014. Global tuberculosis report 2014. World Health Organization.http://www.who.int/tb/publications/global_report/en/ (accessed Dec 12, 2014), Geneva.
4. **Xu DD, Deng DF, Li X, Wei LL, Li YY, Yang XY, Yu W, Wang C, Jiang TT, Li ZJ, Chen ZL, Zhang X, Liu JY, Ping ZP, Qiu YQ, Li JC.** 2014. Discovery and identification of serum potential biomarkers for pulmonary tuberculosis using iTRAQ-coupled two-dimensional LC-MS/MS. Proteomics **14:**322-331.
5. **Mehaffy MC, Kruh-Garcia NA, Dobos KM.** 2011. Prospective on Mycobacterium tuberculosis Proteomics. Journal of Proteome Research **11:**17-25.
6. **Couvin D, Rastogi N.** 2015. Tuberculosis - A global emergency: Tools and methods to monitor, understand, and control the epidemic with specific example of the Beijing lineage. Tuberculosis (Edinb) **95 Suppl 1:**S177-189.
7. **Johnson MM, Odell JA.** 2014. Nontuberculous mycobacterial pulmonary infections. Journal of Thoracic Disease **6:**210-220.
8. **Brown-Elliott BA, Nash KA, Wallace RJ.** 2012. Antimicrobial Susceptibility Testing, Drug Resistance Mechanisms, and Therapy of Infections with Nontuberculous Mycobacteria. Clinical Microbiology Reviews **25:**545-582.
9. **El Khéchine A, Couderc C, Flaudrops C, Raoult D, Drancourt M.** 2011. Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry Identification of Mycobacteria in Routine Clinical Practice. PLoS ONE **6:**e24720.
10. **Escobar-Escamilla N, Ramirez-Gonzalez JE, Gonzalez-Villa M, Torres-Mazadiego P, Mandujano-Martinez A, Barron-Rivera C, Backer CE, Fragoso-Fonseca DE, Olivera-Diaz H, Alcantara-Perez P, Hernandez-Solis A, Cicero-Sabido R, Cortes-Ortiz IA.** 2014. Hsp65 phylogenetic assay for molecular diagnosis of nontuberculous mycobacteria isolated in Mexico. Arch Med Res **45:**90-97.

11. **Falkinham JO, 3rd.** 1996. Epidemiology of infection by nontuberculous mycobacteria. Clin Microbiol Rev **9:**177-215.
12. **Balada-Llasat JM, Kamboj K, Pancholi P.** 2013. Identification of mycobacteria from solid and liquid media by matrix-assisted laser desorption ionization-time of flight mass spectrometry in the clinical laboratory. J Clin Microbiol **51:**2875-2879.
13. **Bownds SE, Kurzynski TA, Norden MA, Dufek JL, Schell RF.** 1996. Rapid susceptibility testing for nontuberculosis mycobacteria using flow cytometry. J Clin Microbiol **34:**1386-1390.
14. **Jang M-A, Koh W-J, Huh HJ, Kim S-Y, Jeon K, Ki C-S, Lee NY.** 2014. Distribution of Nontuberculous Mycobacteria by Multigene Sequence-Based Typing and Clinical Significance of Isolated Strains. Journal of Clinical Microbiology **52:**1207-1212.
15. **Wu X, Zhang J, Liang J, Lu Y, Li H, Li C, Yue J, Zhang L, Liu Z.** 2007. Comparison of Three Methods for Rapid Identification of Mycobacterial Clinical Isolates to the Species Level. Journal of Clinical Microbiology **45:**1898-1903.
16. **Wania R.** 2013. Tuberculosis 2: Pathophysiology and microbiology of pulmonary tuberculosis. SSMJ **6:**10-12.
17. **Lotz A, Ferroni A, Beretti JL, Dauphin B, Carbonnelle E, Guet-Revillet H, Veziris N, Heym B, Jarlier V, Gaillard JL, Pierre-Audigier C, Frapy E, Berche P, Nassif X, Bille E.** 2010. Rapid identification of mycobacterial whole cells in solid and liquid culture media by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol **48:**4481-4486.
18. **Quinlan P, Phelan E, Doyle M.** 2015. Matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry (MS) for the identification of mycobacteria from MBBacT ALERT 3D liquid cultures and Lowenstein-Jensen (LJ) solid cultures. J Clin Pathol **68:**229-235.
19. **Schmidt F, Donahoe S, Hagens K, Mattow J, Schaible UE, Kaufmann SH, Aebersold R, Jungblut PR.** 2004. Complementary analysis of the Mycobacterium tuberculosis proteome by two-dimensional electrophoresis and isotope-coded affinity tag technology. Mol Cell Proteomics **3:**24-42.
20. **Gagneux S, DeRiemer K, Van T, Kato-Maeda M, de Jong BC, Narayanan S, Nicol M, Niemann S, Kremer K, Gutierrez MC, Hilty M,**

Hopewell PC, Small PM. 2006. Variable host–pathogen compatibility in Mycobacterium tuberculosis. Proceedings of the National Academy of Sciences of the United States of America **103:**2869-2873.

21. **de Souza GA, Fortuin S, Aguilar D, Pando RH, McEvoy CRE, van Helden PD, Koehler CJ, Thiede B, Warren RM, Wiker HG.** 2010. Using a Label-free Proteomics Method to Identify Differentially Abundant Proteins in Closely Related Hypo- and Hypervirulent Clinical Mycobacterium tuberculosis Beijing Isolates. Molecular & Cellular Proteomics **9:**2414-2423.
22. **Comas I, Coscolla M, Luo T, Borrell S, Holt KE, Kato-Maeda M, Parkhill J, Malla B, Berg S, Thwaites G, Yeboah-Manu D, Bothamley G, Mei J, Wei L, Bentley S, Harris SR, Niemann S, Diel R, Aseffa A, Gao Q, Young D, Gagneux S.** 2013. Out-of-Africa migration and Neolithic coexpansion of Mycobacterium tuberculosis with modern humans. Nat Genet **45:**1176-1182.
23. **Albrethsen J.** 2013. Proteomic profiling of the Mycobacterium tuberculosis identifies nutrient starvation responsive toxin-antitoxin systems. Molecular and Cellular Proteomics **doi: /10.1074/mcp.M112.018846**.
24. **Mehaffy C, Hess A, Prenni JE, Mathema B, Kreiswirth B, Dobos KM.** 2010. Descriptive proteomic analysis shows protein variability between closely related clinical isolates of Mycobacterium tuberculosis. Proteomics **10:**1966-1984.
25. **Bantscheff M, Schirle M, Sweetman G, Rick J, Kuster B.** 2007. Quantitative mass spectrometry in proteomics: a critical review. Anal Bioanal Chem **389:**1017-1031.
26. **Singhal N, Kumar M, Kanaujia PK, Virdi JS.** 2015. MALDI-TOF mass spectrometry: an emerging technology for microbial identification and diagnosis. Frontiers in Microbiology **6:**791.
27. **Scott JS, Sterling SA, To H, Seals SR, Jones AE.** 2016. Diagnostic performance of matrix-assisted laser desorption ionisation time-of-flight mass spectrometry in blood bacterial infections: a systematic review and meta-analysis. Infect Dis **48:**530-536.
28. **Kruh NA, Troudt J, Izzo A, Prenni J, Dobos KM.** 2010. Portrait of a pathogen: the Mycobacterium tuberculosis proteome in vivo. PLoS One **5:**e13938.

29. **Arthur JM.** 2003. Proteomics. Curr Opin Nephrol Hypertens **12:**423-430.
30. **Brennan PJ.** Structure, function, and biogenesis of the cell wall of Mycobacterium tuberculosis. Tuberculosis **83:**91-97.
31. **Silhavy TJ, Kahne D, Walker S.** 2010. The Bacterial Cell Envelope. Cold Spring Harbor Perspectives in Biology **2:**a000414.
32. **Jarlier V, Nikaido H.** 1994. Mycobacterial cell wall: Structure and role in natural resistance to antibiotics. FEMS Microbiol Lett **123:**11.
33. **Mdluli K, Spigelman M.** 2006. Novel targets for tuberculosis drug discovery. Curr Opin Pharmacol **6:**459-467.
34. **Singh P, Rao RN, Reddy JR, Prasad RB, Kotturu SK, Ghosh S, Mukhopadhyay S.** 2016. PE11, a PE/PPE family protein of Mycobacterium tuberculosis is involved in cell wall remodeling and virulence. Sci Rep **6**.
35. **Neyrolles O, Guilhot C.** Recent advances in deciphering the contribution of Mycobacterium tuberculosis lipids to pathogenesis. Tuberculosis **91:**187-195.
36. **Gunawardena HP, Feltcher ME, Wrobel JA, Gu S, Braunstein M, Chen X.** 2013. Comparison of the membrane proteome of virulent Mycobacterium tuberculosis and the attenuated Mycobacterium bovis BCG vaccine strain by label-free quantitative proteomics. J Proteome Res **12:**5463-5474.
37. **Gu S, Chen J, Dobos KM, Bradbury EM, Belisle JT, Chen X.** 2003. Comprehensive Proteomic Profiling of the Membrane Constituents of a Mycobacterium tuberculosis Strain. Molecular & Cellular Proteomics **2:**1284-1296.
38. **Mehaffy C, Hess A, Prenni JE, Mathema B, Kreiswirth B, Dobos KM.** 2010. Descriptive proteomic analysis shows protein variability between closely related clinical isolates of Mycobacterium tuberculosis. Proteomics **10:**1966-1984.
39. **Ishihama Y, Schmidt T, Rappsilber J, Mann M, Hartl FU, Kerner M, Frishman D.** 2008. Protein abundance profiling of the Escherichia coli cytosol. BMC Genomics **9:**102.
40. **Pleissner KP, Eifert T, Buettner S, Schmidt F, Boehme M, Meyer TF, Kaufmann SH, Jungblut PR.** 2004. Web-accessible proteome databases for microbial research. Proteomics **4:**1305-1313.

41. **Thiede B, Koehler CJ, Strozynski M, Treumann A, Stein R, Zimny-Arndt U, Schmid M, Jungblut PR.** 2013. High Resolution Quantitative Proteomics of HeLa Cells Protein Species Using Stable Isotope Labeling with Amino Acids in Cell Culture(SILAC), Two-Dimensional Gel Electrophoresis(2DE) and Nano-Liquid Chromatograpohy Coupled to an LTQ-OrbitrapMass Spectrometer. Molecular & Cellular Proteomics **12:**529-538.
42. **Wilkins MR, Gasteiger E, Tonella L, Ou K, Tyler M, Sanchez JC, Gooley AA, Walsh BJ, Bairoch A, Appel RD, Williams KL, Hochstrasser DF.** 1998. Protein identification with N and C-terminal sequence tags in proteome projects. J Mol Biol **278:**599-608.
43. **Donnan FG.** 1923. Rays of positive electricity and their application to chemical analyses. By Sir J. J. Thomson, O. M. F.R.S. Second edition. Pp. x + 237. London: Longmans, Green and Co., 1921. Price 16s. Journal of the Society of Chemical Industry **42:**861-861.
44. **Fang J, Dorrestein PC.** 2014. Emerging mass spectrometry techniques for the direct analysis of microbial colonies. Current opinion in microbiology **0:**120-129.
45. **Tanaka K, Waki H, Ido Y, Akita S, Yoshida Y, Yoshida T, Matsuo T.** 1988. Protein and polymer analyses up to m/z 100 000 by laser ionization time-of-flight mass spectrometry. Rapid Communications in Mass Spectrometry **2:**151-153.
46. **Karas MB, D.; Hillenkamp, F.** 1985. Influence of the Wavelength in High-Irradiance Ultraviolet Laser Desorption Mass Spectrometry of Organic Molecules. Anal Chem **78:**53-68.
47. **Karas M, Bachmann D, Hillenkamp F.** 1985. Influence of the wavelength in high-irradiance ultraviolet laser desorption mass spectrometry of organic molecules. Analytical Chemistry **57:**2935-2939.
48. **Cobo F.** 2013. Application of MALDI-TOF Mass Spectrometry in Clinical Virology: A Review. The Open Virology Journal **7:**84-90.
49. **Anhalt JP, Fenselau C.** 1975. Identification of bacteria using mass spectrometry. Analytical Chemistry **47:**219-225.
50. **Bizzini A, Greub G.** 2010. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry, a revolution in clinical microbial identification. Clin Microbiol Infect **16:**1614-1619.

51. **Saleeb PG, Drake SK, Murray PR, Zelazny AM.** 2011. Identification of mycobacteria in solid-culture media by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol **49:**1790-1794.
52. **Fournier P-E, Drancourt M, Colson P, Rolain J-M, Scola BL, Raoult D.** 2013. Modern clinical microbiology: new challenges and solutions. Nat Rev Micro **11:**574-585.
53. **Claydon MA, Davey SN, Edwards-Jones V, Gordon DB.** 1996. The rapid identification of intact microorganisms using mass spectrometry. Nat Biotechnol **14:**1584-1586.
54. **Holland RD, Wilkes JG, Rafii F, Sutherland JB, Persons CC, Voorhees KJ, Lay JO, Jr.** 1996. Rapid identification of intact whole bacteria based on spectral patterns using matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry. Rapid Commun Mass Spectrom **10:**1227-1232.
55. **Panda A, Kurapati S, Samantaray JC, Myneedu VP, Verma A, Srinivasan A, Ahmad H, Behera D, Singh UB.** 2013. Rapid identification of clinical mycobacterial isolates by protein profiling using matrix assisted laser desorption ionization-time of flight mass spectrometry. Indian J Med Microbiol **31:**117-122.
56. **Fournier PE, Drancourt M, Colson P, Rolain JM, La Scola B, Raoult D.** 2013. Modern clinical microbiology: new challenges and solutions. Nat Rev Microbiol **11:**574-585.
57. **Singhal N, Kumar M, Kanaujia PK, Virdi JS.** 2015. MALDI-TOF mass spectrometry: an emerging technology for microbial identification and diagnosis. Front Microbiol **6:**791.
58. **Cain TC, Lubman DM, Weber WJ, Vertes A.** 1994. Differentiation of bacteria using protein profiles from matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Communications in Mass Spectrometry **8:**1026-1030.
59. **Claydon MA, Davey SN, Edwards-Jones V, Gordon DB.** 1996. The rapid identification of intact microorganisms using mass spectrometry. Nat Biotech **14:**1584-1586.
60. **Pignone M, Greth KM, Cooper J, Emerson D, Tang J.** 2006. Identification of mycobacteria by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry. J Clin Microbiol **44:**1963-1970.

61. **Mather CA, Rivera SF, Butler-Wu SM.** 2014. Comparison of the Bruker Biotyper and Vitek MS Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry Systems for Identification of Mycobacteria Using Simplified Protein Extraction Protocols. Journal of Clinical Microbiology **52:**130-138.
62. **Dunne WM, Doing K, Miller E, Miller E, Moreno E, Baghli M, Mailler S, Girard V, van Belkum A, Deol P.** 2014. Rapid Inactivation of Mycobacterium and Nocardia Species before Identification Using Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry. Journal of Clinical Microbiology **52:**3654-3659.
63. **Machen A, Kobayashi M, Connelly MR, Wang YF.** 2013. Comparison of Heat Inactivation and Cell Disruption Protocols for Identification of Mycobacteria from Solid Culture Media by Use of Vitek Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry. Journal of Clinical Microbiology **51:**4226-4229.
64. **Saleeb PG, Drake SK, Murray PR, Zelazny AM.** 2011. Identification of Mycobacteria in Solid-Culture Media by Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry. Journal of Clinical Microbiology **49:**1790-1794.
65. **El Khechine A, Couderc C, Flaudrops C, Raoult D, Drancourt M.** 2011. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry identification of mycobacteria in routine clinical practice. PLoS One **6:**13.
66. **Jiang X, Zhang W, Gao F, Huang Y, Lv C, Wang H.** 2006. Comparison of the proteome of isoniazid-resistant and -susceptible strains of Mycobacterium tuberculosis. Microb Drug Resist **12:**231-238.
67. **Saleeb PG, Drake SK, Murray PR, Zelazny AM.** 2011. Identification of mycobacteria in solid-culture media by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol **49**.
68. **Mather CA, Rivera SF, Butler-Wu SM.** 2014. Comparison of the Bruker Biotyper and Vitek MS matrix-assisted laser desorption ionization-time of flight mass spectrometry systems for identification of mycobacteria using simplified protein extraction protocols. J Clin Microbiol **52**.
69. **Dunne WM, Jr., Doing K, Miller E, Moreno E, Baghli M, Mailler S, Girard V, van Belkum A, Deol P.** 2014. Rapid inactivation of Mycobacterium and nocardia species before identification using

matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol **52:**3654-3659.

70. **Machen A, Kobayashi M, Connelly MR, Wang YF.** 2013. Comparison of heat inactivation and cell disruption protocols for identification of mycobacteria from solid culture media by use of vitek matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol **51:**4226-4229.
71. **Djelouagji Z, Drancourt M.** 2006. Inactivation of Cultured Mycobacterium tuberculosis Organisms Prior to DNA Extraction. J Clin Microbiol **44:**1594-1595.
72. **Somerville W, Thibert L, Schwartzman K, Behr MA.** 2005. Extraction of Mycobacterium tuberculosis DNA: a Question of Containment. J Clin Microbiol **43:**2996-2997.
73. **Seng P, Drancourt M, Gouriet F, Lascola B, Fournier PE.** 2009. Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clin Infect Dis **49**.
74. **Lotz A, Ferroni A, Beretti J-L, Dauphin B, Carbonnelle E, Guet-Revillet H, Veziris N, Heym B, Jarlier V, Gaillard J-L, Pierre-Audigier C, Frapy E, Berche P, Nassif X, Bille E.** 2010. Rapid Identification of Mycobacterial Whole Cells in Solid and Liquid Culture Media by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry. J Clin Microbiol **48:**4481-4486.
75. **Mareković I, Bošnjak Z, Jakopović M, Boras Z, Janković M, Popović-Grle S.** 2015. Evaluation of Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry in Identification of Nontuberculous Mycobacteria. Chemotherapy **61:**167-170.
76. **Kehrmann J, Wessel S, Murali R, Hampel A, Bange F-C, Buer J, Mosel F.** 2016. Principal component analysis of MALDI TOF MS mass spectra separates M. abscessus (sensu stricto) from M. massiliense isolates. BMC Microbiology **16:**1-7.
77. **Balazova T, Makovcova J, Sedo O, Slany M, Faldyna M, Zdrahal Z.** 2014. The influence of culture conditions on the identification of Mycobacterium species by MALDI-TOF MS profiling. FEMS Microbiol Lett **353:**77-84.

78. **Seng P, Rolain J-M, Fournier PE, La Scola B, Drancourt M, Raoult D.** 2010. MALDI-TOF-mass spectrometry applications in clinical microbiology. Future Microbiology **5:**1733-1754.
79. **Yssouf A, Almeras L, Raoult D, Parola P.** 2016. Emerging tools for identification of arthropod vectors. Future Microbiol **11:**549-566.
80. **Yssouf A, Socolovschi C, Flaudrops C, Ndiath MO, Sougoufara S, Dehecq JS, Lacour G, Berenger JM, Sokhna CS, Raoult D, Parola P.** 2013. Matrix-assisted laser desorption ionization--time of flight mass spectrometry: an emerging tool for the rapid identification of mosquito vectors. PLoS ONE **8**.
81. **Emonet S, Shah HN, Cherkaoui A, Schrenzel J.** 2010. Application and use of various mass spectrometry methods in clinical microbiology. Clin Microbiol Infect **16:**1604-1613.
82. **Mediavilla-Gradolph MC, De Toro-Peinado I, Bermúdez-Ruiz MP, García-Martínez MdlÁ, Ortega-Torres M, Montiel Quezel-Guerraz N, Palop-Borrás B.** 2015. Use of MALDI-TOF MS for Identification of Nontuberculous Mycobacterium Species Isolated from Clinical Specimens. BioMed Research International **2015:**854078.
83. **Tudo G, Monte MR, Vergara A, Lopez A, Hurtado JC, Ferrer-Navarro M, Vila J, Gonzalez-Martin J.** 2015. Implementation of MALDI-TOF MS technology for the identification of clinical isolates of Mycobacterium spp. in mycobacterial diagnosis. Eur J Clin Microbiol Infect Dis **34:**1527-1532.
84. **Chen JHK, Yam W-C, Ngan AHY, Fung AMY, Woo W-L, Yan M-K, Choi GKY, Ho P-L, Cheng VCC, Yuen K-Y.** 2013. Advantages of Using Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry as a Rapid Diagnostic Tool for Identification of Yeasts and Mycobacteria in the Clinical Microbiological Laboratory. Journal of Clinical Microbiology **51:**3981-3987.
85. **Amlerova J, Studentova V, Hrabak J.** 2014. [Identification of Mycobacterium spp. isolates using matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS)]. Epidemiol Mikrobiol Imunol **63:**196-199.
86. **Buchan BW, Riebe KM, Timke M, Kostrzewa M, Ledeboer NA.** 2014. Comparison of MALDI-TOF MS With HPLC and Nucleic Acid Sequencing for the Identification of Mycobacterium Species in Cultures Using Solid Medium and Broth. American Journal of Clinical Pathology **141:**25-34.

87. **de Jong BC, Antonio M, Gagneux S.** 2010. Mycobacterium africanum--review of an important cause of human tuberculosis in West Africa. PLoS Negl Trop Dis **4:**e744.
88. **Mokrousov I, Vyazovaya A, Otten T, Zhuravlev V, Pavlova E, Tarashkevich L, Krishevich V, Vishnevsky B, Narvskaya O.** 2012. Mycobacterium tuberculosis Population in Northwestern Russia: An Update from Russian-EU/Latvian Border Region. PLoS ONE **7:**e41318.
89. **Djelouadji Z, Henry M, Bachtarzi A, Foselle N, Raoult D, Drancourt M.** 2009. Pyrosequencing identification of Mycobacterium tuberculosis W-Beijing. BMC Research Notes **2:**239-239.
90. **Dunne WM, Jr., Doing K, Miller E, Miller E, Moreno E, Baghli M, Mailler S, Girard V, van Belkum A, Deol P.** 2014. Rapid inactivation of Mycobacterium and nocardia species before identification using matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol **52:**3654-3659.
91. **Kim BJ, Kim JM, Kim BR, Lee SY, Kim G, Jang YH, Ryoo S, Jeon CO, Jin HM, Jeong J, Lee SH, Lim JH, Kook YH, Kim BJ.** 2015. Mycobacterium anyangense sp. nov., a rapidly growing species isolated from blood of Korean native cattle, Hanwoo (Bos taurus coreanae). Int J Syst Evol Microbiol **65:**2277-2285.
92. **Kim BJ, Hong SH, Kook YH, Kim BJ.** 2014. Mycobacterium paragordonae sp. nov., a slowly growing, scotochromogenic species closely related to Mycobacterium gordonae. Int J Syst Evol Microbiol **64:**39-45.
93. **Kim BJ, Math RK, Jeon CO, Yu HK, Park YG, Kook YH, Kim BJ.** 2013. Mycobacterium yongonense sp. nov., a slow-growing non-chromogenic species closely related to Mycobacterium intracellulare. Int J Syst Evol Microbiol **63:**192-199.
94. **Zingue D, Flaudrops C, Drancourt M.** 2016. Direct Matrix Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry identification of mycobacteria from colonies. European Journal of Clinical Microbiology & Infectious Diseases**:**Ms. No. EJCM-D-16-00565R00561.
95. **WHO.** 2016. Global Tuberculosis Report 2016. WHO, Geneva, Switzerland.

96. **Forero MG, Cristobal G, Desco M.** 2006. Automatic identification of Mycobacterium tuberculosis by Gaussian mixture models. J Microsc **223:**120-132.
97. **Zingue D, Hien H, Meda N, Zida S, Kabore A, Sanou A, Ouedraogo AS, Gomgnimbou M, Diande S, Tarnagda Z, Godreuil S.** 2013. [Advantages and drawbacks of expectoration decontamination methods for tuberculosis and anti-tuberculosis drug resistance diagnosis]. Ann Biol Clin **71:**283-291.
98. **Osibote OA, Dendere R, Krishnan S, Douglas TS.** 2010. Automated focusing in bright-field microscopy for tuberculosis detection. J Microsc **240:**155-163.
99. **Abdelaziz MM, Bakr WM, Hussien SM, Amine AE.** 2016. Diagnosis of pulmonary tuberculosis using Ziehl-Neelsen stain or cold staining techniques? J Egypt Public Health Assoc **91:**39-43.
100. **Gordon C, Van Deun A, Lumb R.** 2009. Evaluating the performance of basic fuchsin for the Ziehl-Neelsen stain. Int J Tuberc Lung Dis **13:**130-135.
101. **Ben-Selma W, Ben-Kahla I, Marzouk M, Ferjeni A, Ghezal S, Ben-Said M, Boukadida J.** 2009. Rapid detection of Mycobacterium tuberculosis in sputum by Patho-TB kit in comparison with direct microscopy and culture. Diagn Microbiol Infect Dis **65:**232-235.
102. **Gupta S, Prasad V, Bairy I, Muralidharan S.** 2009. Comparative evaluation of two cold staining methods with the Ziehl-Neelsen method for the diagnosis of tuberculosis. Southeast Asian J Trop Med Public Health **40:**765-769.
103. **IUATLD.** 2000. Technical guide.Sputum Examination for Tuberculosis by Direct Microscopy in Low Income Countries, vol Fifth edition. International Union Against Tuberculosis and Lung Disease, Paris, France.
104. **Sadaphal P, Rao J, Comstock GW, Beg MF.** 2008. Image processing techniques for identifying Mycobacterium tuberculosis in Ziehl-Neelsen stains. Int J Tuberc Lung Dis **12:**579-582.
105. **Kim TC, Blackman RS, Heatwole KM, Kim T, Rochester DF.** 1984. Acid-fast bacilli in sputum smears of patients with pulmonary tuberculosis. Prevalence and significance of negative smears pretreatment and positive smears post-treatment. Am Rev Respir Dis **129:**264-268.

106. **Levy H, Feldman C, Sacho H, van der Meulen H, Kallenbach J, Koornhof H.** 1989. A reevaluation of sputum microscopy and culture in the diagnosis of pulmonary tuberculosis. Chest **95:**1193-1197.
107. **Rieder HL, Chonde TM, Myking H, Urbanczik R, Laszlo A, Kim SJ, Van Deun A, Trébucq A.** 1998. The public health service national tuberculosis reference laboratory and the national laboratory network. Minimum requirements, role and operation in a low-income country International Union Against Tuberculosis and Lung Disease, Paris, France.
108. **Van Deun A, Salim AH, Cooreman E, Hossain MA, Rema A, Chambugonj N, Hye MA, Kawria A, Declercq E.** 2002. Optimal tuberculosis case detection by direct sputum smear microscopy: how much better is more? Int J Tuberc Lung Dis **6:**222-230.
109. **Nour-Neamatollahi A, Siadat SD, Yari S, Tasbiti AH, Ebrahimzadeh N, Vaziri F, Fateh A, Ghazanfari M, Abdolrahimi F, Pourazar S, Bahrmand A.** 2016. A new diagnostic tool for rapid and accurate detection of Mycobacterium tuberculosis. Saudi Journal of Biological Sciences.
110. **Veropoulos K, Learmonth G, Campbell C, Knight B, Simpson J.** 1999. Automated identification of tubercle bacilli in sputum. A preliminary investigation. Anal Quant Cytol Histol **21:**277-282.
111. **Khutlang R, Krishnan S, Whitelaw A, Douglas TS.** 2010. Automated detection of tuberculosis in Ziehl-Neelsen-stained sputum smears using two one-class classifiers. J Microsc **237:**96-102.
112. **Lewis JJ, Chihota VN, van der Meulen M, Fourie PB, Fielding KL, Grant AD, Dorman SE, Churchyard GJ.** 2012. "Proof-of-concept" evaluation of an automated sputum smear microscopy system for tuberculosis diagnosis. PLoS One **7:**29.
113. **Costa MG, Costa Filho CF, Kimura Junior A, Levy PC, Xavier CM, Fujimoto LB.** 2014. A sputum smear microscopy image database for automatic bacilli detection in conventional microscopy. Conf Proc IEEE Eng Med Biol Soc **4:**6944215.
114. **Ismail NA, Omar SV, Lewis JJ, Dowdy DW, Dreyer AW, van der Meulen H, Nconjana G, Clark DA, Churchyard GJ.** 2015. Performance of a Novel Algorithm Using Automated Digital Microscopy for Diagnosing Tuberculosis. Am J Respir Crit Care Med **191:**1443-1449.
115. **Khutlang R, Krishnan S, Dendere R, Whitelaw A, Veropoulos K, Learmonth G, Douglas TS.** 2010. Classification of Mycobacterium

tuberculosis in images of ZN-stained sputum smears. IEEE Trans Inf Technol Biomed **14:**949-957.

116. **Asmar S, Drancourt M.** 2015. Rapid culture-based diagnosis of pulmonary tuberculosis in developed and developing countries. Frontiers in Microbiology **6:**1184.
117. **Pichat C, Couvin D, Carret G, Frédénucci I, Jacomo V, Carricajo A, Boisset S, Dumitrescu O, Flandrois J-P, Lina G, Rastogi N.** 2016. Combined Genotypic, Phylogenetic, and Epidemiologic Analyses of Mycobacterium tuberculosis Genetic Diversity in the Rhône Alpes Region, France. PLoS One **11:**e0153580.
118. **Guthmann J, Aït Belghiti F, Lévy-Bruhl D.** 2017. Épidémiologie de la tuberculose en France en 2015. Impact de la suspension de l'obligation vaccinale BCG sur la tuberculose de l'enfant, 2007-2015. Bull Epidémiol Hebd **7:**116-126.
119. **Asmar S, Chatellier S, Mirande C, van Belkum A, Canard I, Raoult D, Drancourt M.** 2015. A Novel Solid Medium for Culturing Mycobacterium tuberculosis Isolates from Clinical Specimens. J Clin Microbiol **53:**2566-2569.
120. **Asmar S, Drancourt M.** 2015. Chlorhexidine decontamination of sputum for culturing Mycobacterium tuberculosis. BMC Microbiol **15:**015-0479.
121. **Ghodbane R, Asmar S, Betzner M, Linet M, Pierquin J, Raoult D, Drancourt M.** 2015. Rapid Diagnosis of Tuberculosis by Real-Time High-Resolution Imaging of Mycobacterium tuberculosis Colonies. J Clin Microbiol **53:**2693-2696.
122. **Zingue D, Flaudrops C, Drancourt M.** 2016. Direct matrix-assisted laser desorption ionisation time-of-flight mass spectrometry identification of mycobacteria from colonies. European Journal of Clinical Microbiology & Infectious Diseases**:**1-5.
123. **Veropoulos K, Campbell C, Learmonth G, Knight B, Simpson J.** 1998. The Automated Identification of Tubercle Bacilli using Image Processing and Neural Computing Techniques, p 797-802. *In* Niklasson L, Bodén M, Ziemke T (ed), ICANN 98: Proceedings of the 8th International Conference on Artificial Neural Networks, Skövde, Sweden, 2–4 September 1998. Springer London, London

Figure 5: Automatically store metadata of virtual slides obtained by ZEN imaging software from Carl Zeiss,

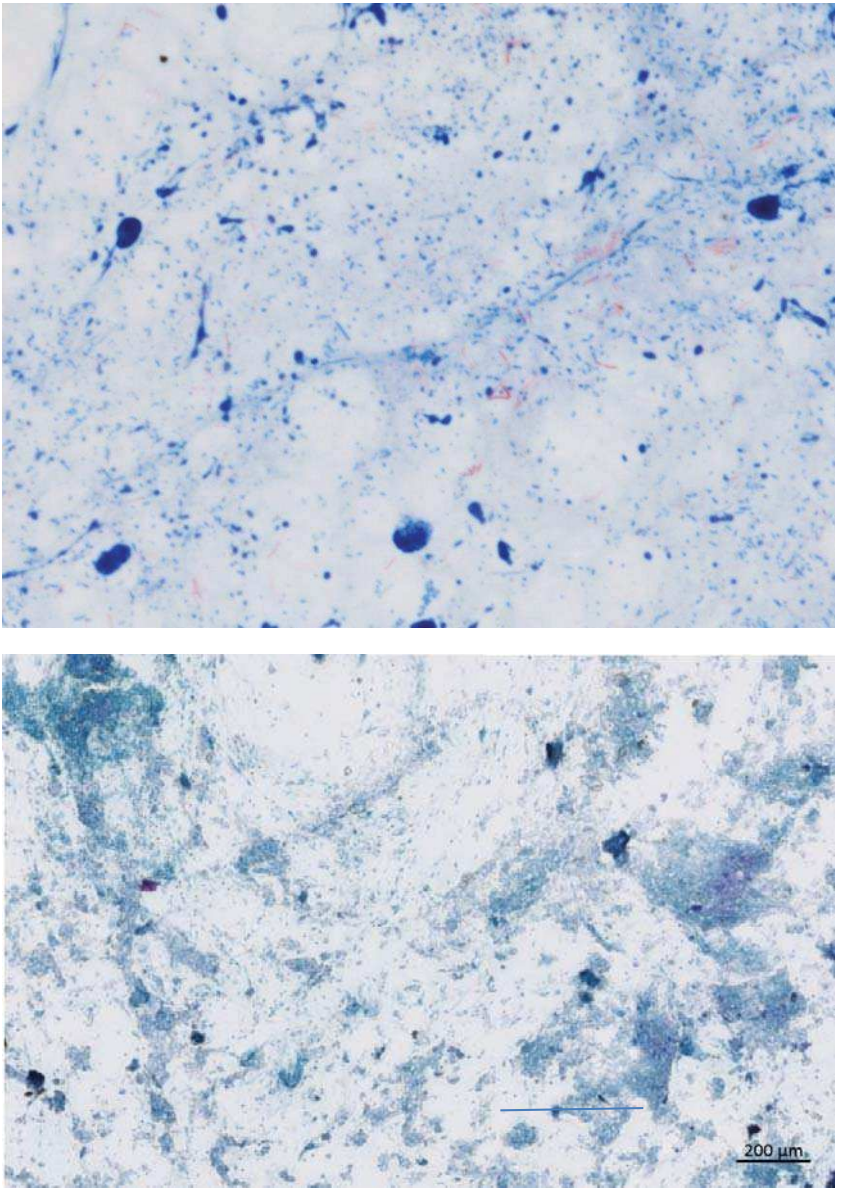


Figure 6: A *M. tuberculosis*-positive smear slide obtained with standard light microscope (up) and Zeiss Axio microscope (below).

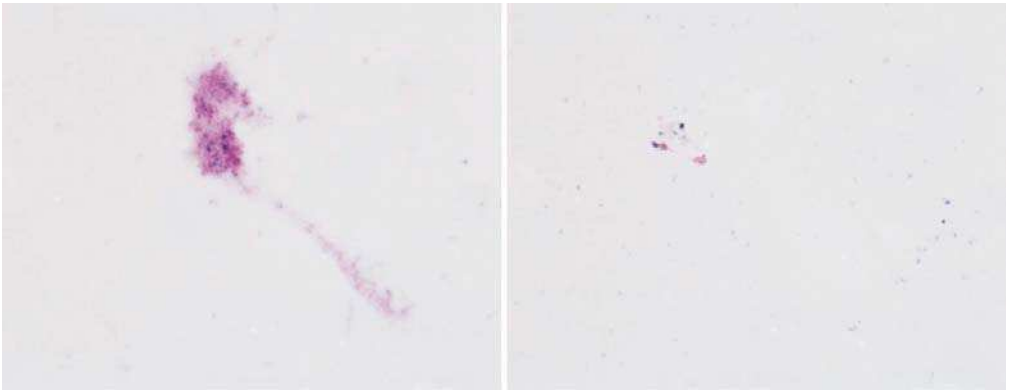
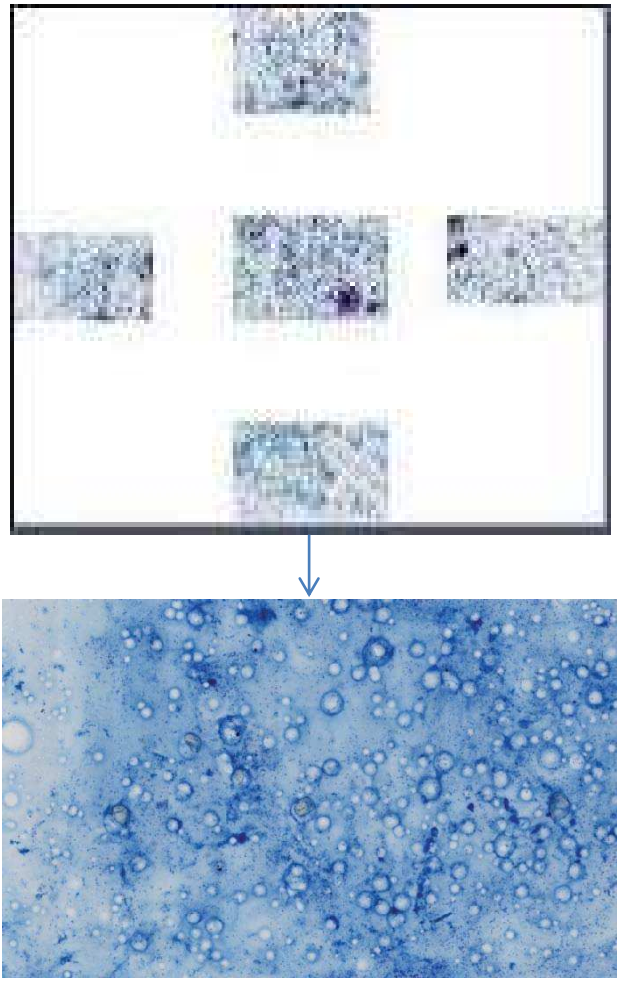


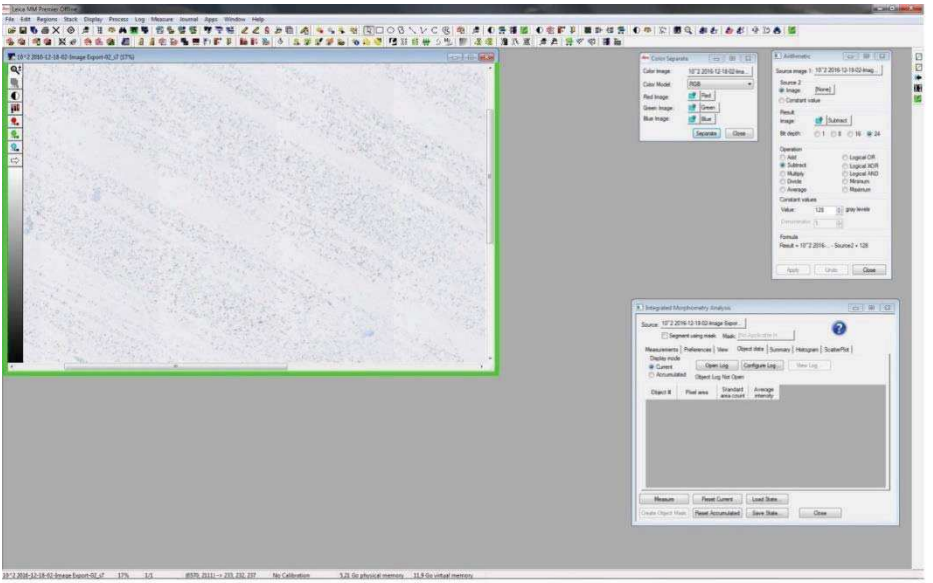
Figure 7: Example of two slides read as false positive by the Zeiss Axio scan microscope and negative by standard microscopy

Supplement 1:data analyzing

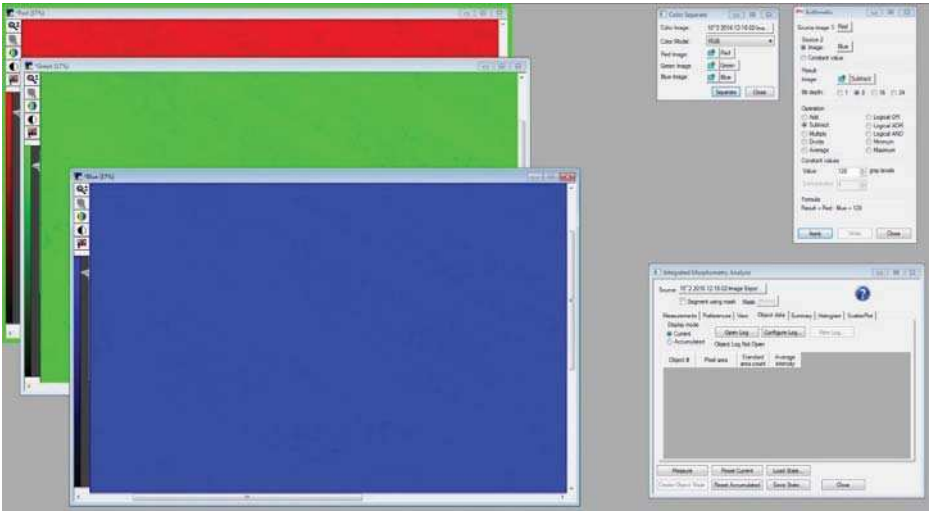


Five fields per slide (The number of fields to be read per slide can be decreased or increased at will)
Analyze with MetaMorph software (Molecular Device)
https://www.moleculardevices.com/systems/metamorph-research-imaging/metamorph-microscopy-automation-and-image-analysis-software
The procedure is described below:

1. **Opening the file:**

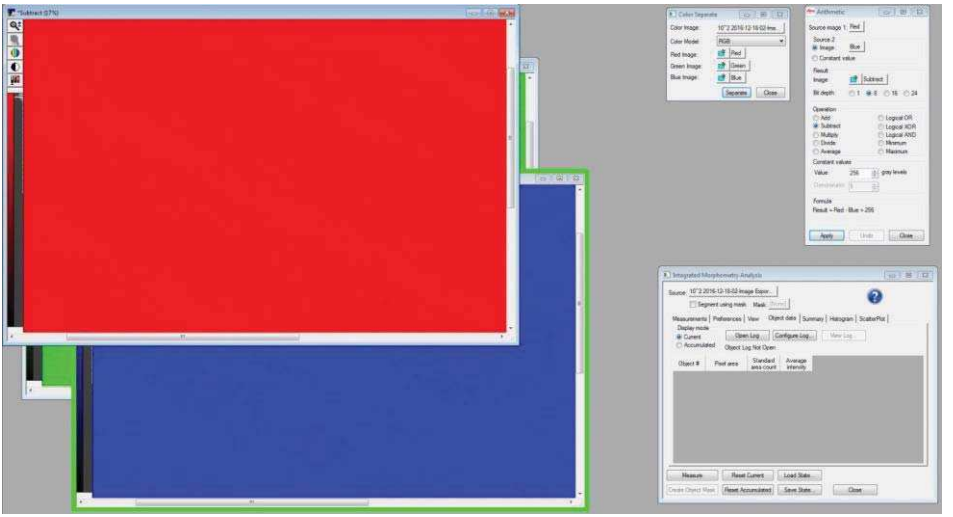


2. **Channel separation in three simple channels: Red, Green, Blue**

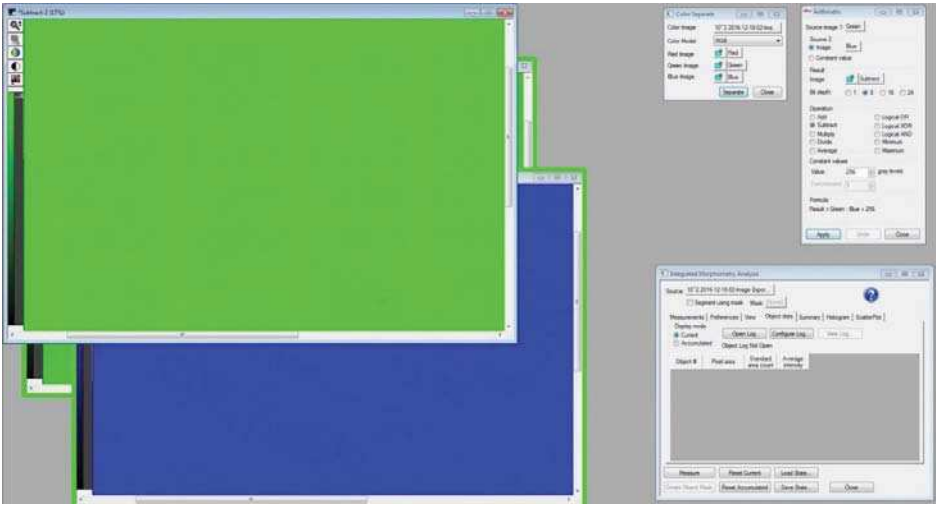


3. Arithmetic Operations:

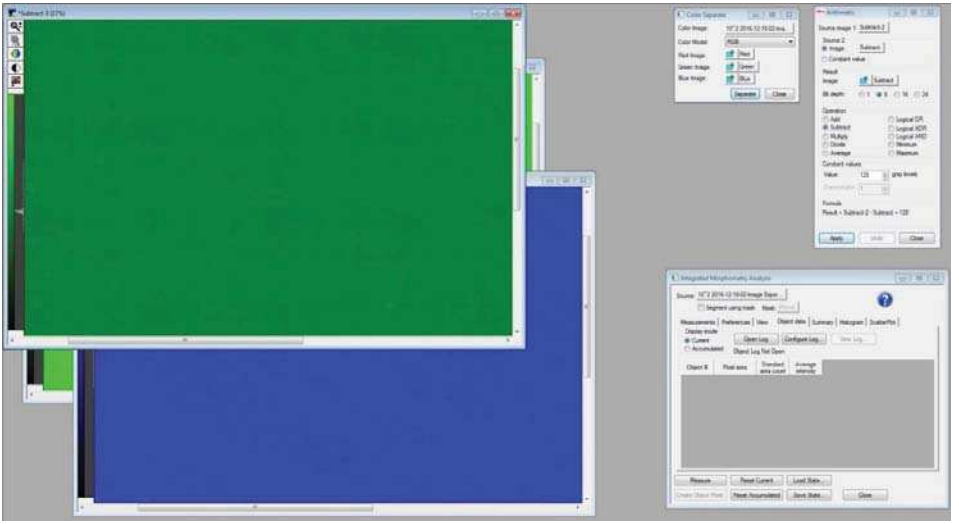
- Red - blue + 256 = substract



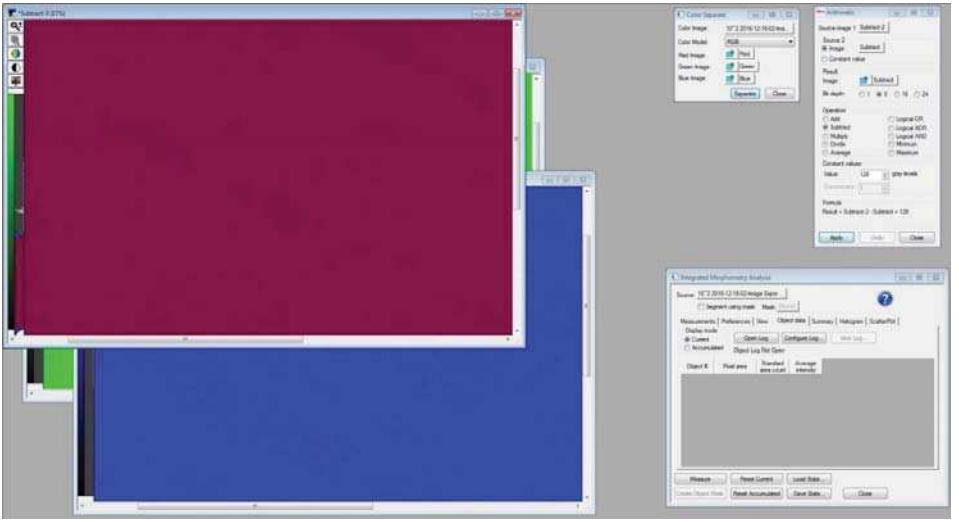
- Green - blue + 256 = substract 2



- Substract2 – substract+128= substract 3



- Thresholding for black objects set to value: 100



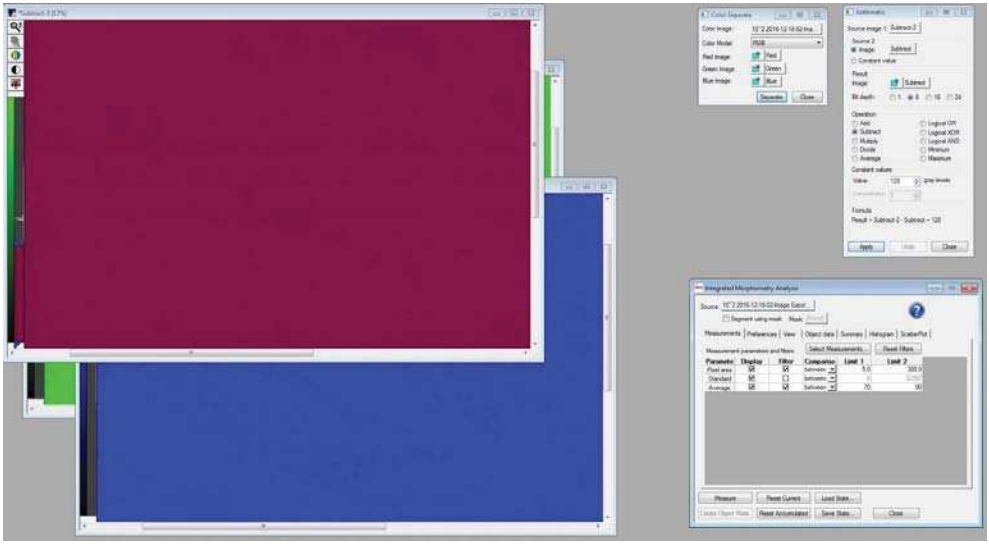
4. Counting :

- Filter Settings Values:

Pixel area: Dispaly on; Filter on, between 5.0 and 300.0

Standard: Diplay on; Filter off; Value set to 50 pixels per bacterium

Average intensity: Display on; Filter on, between 70 and 90



- Counting on : substract 3

Automatically repeated on all fields and on all slides

The software automatically generates an Excel file that will display the name of the slide, the measured values and the

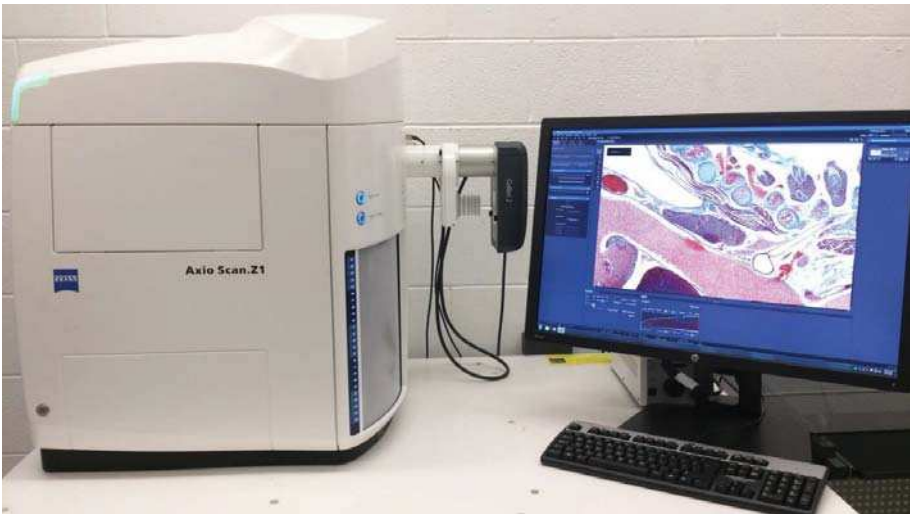
count of detected bacteria. This value is given for information only. A second verification will be necessary

5. **Focus:**

We realized images on negative slides and multiply these acquisitions to refine our values of filters so that a negative sample is systematically negative after analysis via software

The retained values are such that an isolated BK is detected but sometimes epithelial cells are marked when the Ziehl Armand staining.These represent a block of BK we preferred to eliminate them according to their size. It is unlikely that there are only clusters of BK without at least one isolated BK. This choice makes it possible to have false positives and not the reverse.

6. **ZEISS Axio Scan.Z1 Digital Slide Scanner**



This automat can recognize a write or barcode. The lights are LED (light emitting diode). It can save all acquired images in order to re-visualize them and / or re-analyze them. The analysis can be redone on acquisitions

Conclusions and perspectives

This thesis work allowed us to set-up specific methods aimed to improve the diagnosis of Buruli ulcer and the detection of the causative *Mycobacterium ulcerans* in the environment of affected populations in rural regions of western Africa and other countries. Initially, we have developed innovative culture media for the isolation of *Mycobacterium ulcerans* from clinical and environmental samples which decreases the doubling time to less than two days. The innovative culture media combined to a new decontamination protocol allowed the first ever isolation of *M. ulcerans* from *Thryonomys swinderianus* (agouti) feces obtained from western Africa. This observation is in line with recent report from Australia indicating that agouti could be part of the natural chain of transmission of *M. ulcerans* and a potential source of infection for the populations. The high-throughput phenotyping previously performed on *M. ulcerans* strains has allowed us to have a good view on the environmental substrates used by *M. ulcerans* strains for their metabolism and, by extrapolation, for their growth and survival in nature. This

work gave us crucial information on the enrichment pathways of *M. ulcerans* culture media and also guided us on the range of potential reservoirs of *M. ulcerans* in the environment.

In a second step, the identification of mycobacteria by MALDI-TOF-MS was markedly improved. From now on, it is possible to identify the mycobacteria directly from microcolonies taken on a solid culture medium without a step of proteins extraction. This method is currently used routinely for the identification of mycobacteria in our NSB3 laboratories. This innovation is in line with the goal of detecting microcolonies of *M. ulcerans* on our innovative culture medium.These two studies enabled us to considerably reduce the cultivation time of *M. ulcerans* and also the time of identification of mycobacteria.

A third study allowed us to develop a protocol for the automatic reading of the Ziehl Neelsen slides and the counting of the AFB by the ZEISS Slide Scanner Axio Scan.Z1, an automatic system. Once again, this innovation could be applied to the high throughput microscopic detection of *M. ulcerans* in environmental samples after

conventional staining or specific fluorescence *in situ* hybridization.

As a continuation of the development of culture media for *M. ulcerans*, it is in the interest of the entire scientific community to continue this research by testing new components derived from the high-phenotyping of *M. ulcerans*. New crop areas need to be evaluated in larger studies in endemic countries of Buruli ulcer.

To date, important links in the epidemiology of Buruli ulcer are missing, such as the prevalence of skin carriage of *M. ulcerans* in healthy subjects exposed or not to endemic areas. The study of dermal carriers of *M. ulcerans* in healthy subjects should be conducted as part of a multicentre study in endemic areas of Buruli ulcer.

Likewise, the techniques developed during this three-year thesis will be applied to the quest for environmental isolates of *M. ulcerans* in endemic countries, including French Guyane and western Africa countries and Burkina Faso.

Annexe:

Skin carriage of *Mycobacterium ulcerans* project

Projet-Portage cutané de *M. ulcerans*

Comprendre le portage cutané de *Mycobacterium ulcerans* dans les zones à risque de l'ulcère de Buruli au Burkina Faso

Promoteur

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Numéro de projet :	**Projet Portage cutané de *M.ulcerans* PMU-BFI**
Date :	25 avril 2017
Amendement **Version :**	0 1.0
Titre :	Etude du portage cutané de *Mycobacterium ulcerans* dans les zones à risque de l'ulcère de Buruli au Burkina Faso
Pays :	Burkina Faso (BF)
Centres :	**3 sites de recrutement** Région sanitaire des Hauts-Bassins, du Sud-Ouest et des Cascades
Promoteur :	IHU-Méditerranée Infection, Marseille/France
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TABLE DES MATIERES

I. CONTEXTE ET JUSTIFICATION

L'ulcère de Buruli qui fait partie des maladies tropicales les plus négligées, est une infection essentiellement cutanée causée par *Mycobacterium ulcerans*, une bactérie de la même famille que celles responsables de la tuberculose et de la lèpre [1]. Après la tuberculose et la lèpre, l'infection par *M. ulcerans* est la troisième des mycobactérioses les plus courantes chez le sujet immunocompétent [1-6]. L'ulcère de Buruli sévit dans les régions tropicales en foyers endémiques et atteint surtout les populations pauvres en zones rurales [7] . L'ulcère de Buruli a été décrit pour la première fois en 1948 par Mac Callum [8]. Le nom « ulcère de Buruli » vient du district ougandais où l'on a notifié un grand nombre de cas dans les années 1950 [9, 10]. L'ulcère de Buruli sévit en foyers endémiques disséminés, en général situés dans des zones de marais, inondables, à proximité de lacs ou de cours d'eau semble connaître depuis les années 1980 une extension de ses foyers habituels et son incidence. Depuis cette période, elle prend en Afrique de l'Ouest, une expansion telle qu'elle est considérée comme une maladie émergente, malgré sa très faible contagiosité [11]. En 1997, l'Organisation Mondiale de la Santé (OMS) l'a reconnue comme une maladie ré-émergente et en 1998, à Yamoussoukro, un programme de lutte mondiale contre cette affection (Global Buruli Ulcer initiative « GBUI ») a été mis en route par l'OMS [12] . La maladie survient souvent chez les populations vivant à proximité des rivières ou des nappes d'eau stagnante [13]. Les modifications apportées par l'homme à l'environnement comme la construction de systèmes d'irrigation ou de digues, pourraient augmenter la probabilité des contacts des populations avec la mycobactérie et semblent avoir joué un rôle dans la résurgence de cette maladie.

L'infection à *M. ulcerans* entraîne une destruction étendue de la peau et des tissus mous avec la formation d'ulcères, se localisant en général au niveau des jambes et des bras. L'infection peut même atteindre l'os provoquant ainsi une ostéomyélite [14-17]. En l'absence de traitement précoce, la maladie peut laisser des incapacités fonctionnelles durables, comme des restrictions des mouvements articulaires ou des problèmes esthétiques très apparents. La précocité du diagnostic et du traitement est cruciale pour éviter ces incapacités.

Au Burkina Faso la prévalence de l'ulcère de Buruli reste difficile à établir de façon précise malgré des cas déjà notifiés, car ne disposant pas de données épidémiologiques suffisantes sur l'ulcère de Buruli [13, 18] . L'OMS et les autorités sanitaires de chaque pays endémique sont profondément préoccupées par la propagation de l'ulcère de Buruli, notamment chez les enfants, et par ses répercutions sanitaires et socio-économiques sur les communautés rurales défavorisées. Les pays frontaliers du Burkina Faso comme la Côte d'Ivoire, le Ghana, le Togo

et le Bénin sont fortement endémiques de l'ulcère de Buruli qui est souvent classée en 2ème position après la tuberculose relegant la lèpre à la 3ème place parmi les mycobactérioses [19, 20]. Le Burkina Faso entretien avec ces pays de forte endémicité de l'ulcère de Buruli des mouvements de populations intenses. Aussi le Burkina Faso est caractérisé dans sa moitié Sud par un climat humide avec des galeries forestières ; sa moitié Nord est chaude et sèche, avec une savane herbeuse et arbustique [13]. Il convient alors au Burkina Faso d'apprécier l'ampleur de cette infection par des enquêtes épidémiologiques afin de mettre en place une stratégie de prise en charge appropriée [13]. Aussi en 2004 la Fondation Hymne aux enfants qui s'engage en Suisse et au Burkina Faso dans la lutte contre la maladie du Noma a pris en charge des cas d'ulcère de Buruli [21]. Il existe de nombreux moyens diagnostiques qui sont entre autres la recherche de bacilles acido-alcoolo-résistants (BAAR) à l'examen direct, l'examen histologique, la culture, l'inoculation à la souris ainsi que la biologie moléculaire [22-28]. En absence de recherches épidémiologiques et biologiques de grandes envergures sur l'ulcère de Buruli au Burkina, il nous paraît important de faire une étude sur les aspects épidémiologiques ainsi que sur les différentes techniques biologiques susceptibles d'aider au dépistage des cas dans les districts sanitaires du Sud, Sud-Ouest et Ouest, hébergeant probablement les principales zones endémiques de la maladie ainsi qu'au détours des grands barrages aménagés pour l'agriculture et la production de l'électricité. Les zones privilégiées de l'étude seront celles à proximité des marécages ou celles où le réseau hydrographique naturel a été modifié pour les besoins de l'agriculture ainsi que dans les zones rizicoles. Pour mieux cartographier l'aspect épidémiologique de cette pathologie des études clinico-épidémiologiques rétrospectives et prospectives seront d'une importance capitale. Bien que le taux de mortalité soit faible, l'ulcère de Buruli entraîne fréquemment des incapacités et les coûts du traitement et de la réadaptation sont prohibitifs dans les régions les plus touchées [29, 30]. *M. ulcerans* est une mycobactérie environnementale détectée dans le sol, l'eau, les biofilms [31-39], dans les mollusques d'eau [5, 6, 33, 36, 40], les mousses[36] ; les végétaux [35, 36], dans des punaises d'eau [6, 33, 40-45] , chez les petites mammifères et leur fèces [35, 37, 46, 47], les poissons d'eau douce [6, 32, 48], chez la tortue, les crevettes [49, 50] et les grenouilles [51]. L'ADN de *M. ulcerans* a été détecté également chez le chat [52], le cheval [53], le chien [54], l'alpacas [55], les possums [56] et le koala [57-59]. Les petits mammifères vivant à proximité immédiate des êtres humains ainsi que les animaux couramment chassés, les lapins et les rats pourraient être des réservoirs potentiels de *M. ulcerans* [47].

Chez l'homme, *M. ulcerans* est couramment détecté et isolé chez les patients atteints de l'ulcère de Buruli [2-6, 60-62]. La peau dispose d'une protection naturelle contre la

pénétration de bactéries pathogènes, et il est reconnu que *M. ulcerans* infecte l'homme par suite d'une plaie, d'une effraction cutanée. Le mode de transmission le plus plausible chez l'homme est la pénétration dans la peau de *M. ulcerans* après un traumatisme [15, 63, 64] et implique un contact direct avec l'environnement contaminé, les aérosols d'eau et la faune aquatique [5].

A ce jour, aucune donnée existante ne permet de dire qu'il y a un portage cutané à *M. ulcerans* chez les sujets apparemment sains. Une bonne connaissance du portage cutané de *M. ulcerans* permettrait de mieux comprendre l'épidémiologie de cette maladie, de mieux cibler les sujets et milieux à risques en vue d'entreprendre des mesures d'information, d'éducation et de prévention.

Cette étude a pour but d'évaluer le portage cutané de *M. ulcerans* chez les enfants et adultes sains des zones à risque probable de la maladie au Burkina Faso.

II. OBJECTIFS

1. Objectif général

Etudier l'épidémiologie du portage cutané de *M. ulcerans* chez des sujets sains au Burkina Faso.

2. Objectifs spécifiques

1. Déterminer la prévalence du portage cutané chez les enfants et adultes sains en zones rurales de l'Ouest et Sud-Ouest du Burkina Faso
2. Déterminer la répartition des cas de portage cutané en fonction des facteurs intrinsèques (âge, sexe, provenance géographique)
3. Comparer la prévalence du portage cutané entre les populations vivant en zone d'endémie potentielle de l'ulcère de Buruli et les populations vivant hors zones d'endémie.

III. METHODOLOGIE

1. Hypothèse de recherche

Nous émettons l'hypothèse qui stipule qu'il y a un portage cutané de *M. ulcerans* chez les sujets sains vivant en zones à risques de l'ulcère de Buruli.

2. Cadre d'étude

2.1.Champ (lieu) d'étude

Les prélèvements par écouvillonnage de la peau se dérouleront dans les régions sanitaires des Hauts-Bassins, du Sud-Ouest, des Cascades et du Centre Ouest (zone temoin) et ce, dans les villages situés près des cours d'eau (zones marécageuses, retenues d'eau…).

Les deux dernières régions sanitaires sont frontalières au Ghana et au Togo qui sont deux pays où sévit l'ulcère de Buruli.

Le Centre MURAZ est le Centre coordonnateur de l'étude où se déroulera les activités de diagnostic, isolement et identification des espèces mycobactériennes en cause.

Figure 1: Sites de l'étude Portage cutané

2.2.Population d'étude

La population d'étude sera constituée des sujets sains, enfants et adultes sans symptôme connu de l'ulcère de Buruli tous sur une période de un mois.

3. Type d'étude

C'est une étude prospective du portage cutané de *M. ulcerans* dans les trois régions sanitaires

4. Echantillonnage

4.1.Méthodes d'échantillonnage

Nous allons procéder à un échantillonnage exhaustif de tous les volontaires sains consentants durant la période de l'étude.

4.2.Critères d'inclusion

Tout volontaire, enfants et adultes sains ayant donné leur consentement éclairé au cours de la période de l'étude.

4.3.Critères de non inclusion

Sujets ayant une plaie chronique ou sujets ne résidants pas dans la zone de l'étude.

4.4.Nombre de sujets

Un total de 800 sujets sera recruté; équivalent à 200 participants dans chaque direction régionale de la santé.

5. Procédure de collecte et de traitement des échantillons

Les prélèvements seront faits par écouvillonnage cutané, une méthode non invasive. Un questionnaire sera administré pour collecter les données épidémiologiques.

5.1.Collecte des informations épidémiologiques

Les données épidémiologiques : âge, sexe, village de résidence, contact avec le milieu aquatique, types de travaux agricoles,….).

5.2.Prélèvement et traitement des échantillons biologiques

Prélèvements

Les prélèvements seront limités aux seuls écouvillonnages chez les sujets sains.

Transport des échantillons destinés à la biologie moléculaire

Les écouvillons seront mis seront identifiés et placés dans une glacière contenant des ice box bien emballé avec du scotch et expédiés au Centre Muraz où des colis spéciaux seront faits pour leur envoi à l'IHU-Méditerranée Infection où seront réalisés les tests de biologie moléculaires par Mr ZINGUE Dezemon. .

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Réalisation de la PCR en temps réel à l'IHU-Méditerranée infection à Marseille/France

Les écouvillons reçus à Marseille seront repris dans du tampon qui sera utilisé pour l'extraction d'ADN par l'automate EZ1 en utilisant le kit commercial Nucleospin Tissue kit (Macherey-Nagel, Hoerdt, France).

L'amplification de l'ADN sera réalisée grâce au système CFX 96™real time PCR (BIO-Rad, Marnes-la-Coquette, France).

Examen bacilloscopique

Des frottis seront effectués à partir d'une goutte du tampon utilisé pour la récupération du matériel de chaque écouvillon puis une coloration de Ziehl-Neelsen et l'observation microscopique à l'objectif 100 à immersion.

6. Variables de l'étude

- Age
- Sexe
- Zone de résidence
- Contact avec l'eau des rivières

7. Procédures d'assurance qualité

Le questionnaire standardisé fera l'objet d'un test préliminaire. Les agents des services de santé des régons sanitaires sites seront informés sur la méthode d'échantillonnage et sur les outils de collecte des données.

8. Durée de l'étude

Calendrier des activités.

2017										
Activités	jan	Fév	mars	avril	Mai	juin	Juil	août	sept	Oct
Revue de la littérature										
Elaboration du protocole										
Comité d'éthique										
Collecte analyse des échantillons										
Recueil et analyse des données										
Interprétation des résultats										
Elaboration du rapport final-valorisation										

9. Considérations éthiques et démarches administratives

Le protocole sera soumis au comité d'éthique pour la recherche en santé et le comité d'éthique institutionnel du Centre MURAZ pour examen. On s'assurera que le consentement éclairé des enquêtés soit obtenu avant l'administration du questionnaire. La confidentialité des données recueillies sera garantie.

10. Traitement et analyse des données

Les données seront saisies et analysées en utilisant le logiciel Epi info 3.5.1, le traitement et la mise en forme des résultats à l'aide d'Excel 2007.

IV. RESULTATS ATTENDUS

Notre étude nous permettra de déterminer :

- La prévalence du portage cutané de *M. ulcerans* au Burkina Faso;
- Répartition de cette prévalence en fonction de facteurs intrinsèques (âge, sexe, région) ;
- Autres mycobactéries environnementales autre que *M. ulcerans* retrouvées sur la peau
- Transfert de compétence et de méthodes de diagnostic au Burkina Faso

V. EQUIPE PARTICIPANT A L'ETUDE ET PARTENAIRES

- Centre Muraz (Bobo-Dioulasso)
- DRS Hauts-Bassins, Sud-Ouest et Cascades
- IHU-Méditerranée Infection
- MINISTERE DE LA SANTE/BURKINA FASO

VI. BUDGET PREVISIONNEL

1. Nature des dépenses	**Budget prévisionnel**	
	FCFA	**€**
1.1 Ecouvillons stériles, Boîte de 100 (*10)	144320	220
1.2 Gants d'examen latex non poudrés Robé, Boîte de 100 (*10)	65600	100
1.3 Fourniture administrative et Reprographie	100000	152,439024
1.4 Comité d'Ethique	100000	152,439024
1.5 Recrutement de 600 participants	350000	533,536585
1.6 Transfert de prélèvements	200000	304,878049
1.7 Fonctionnement	3000000	4573,17073
1.8 Missions et déplacements	600000	914,634146
1.9 Vacations toute charge comprise	1000000	1524,39024
1.10 Autres dépenses de fonctionnement	940080	1433,04878
Total	6500000	9909

VII. II.13. DIFFUSION DES RESULTATS

- Thèse
- Communications à des conférences scientifiques
- Publications d'articles scientifiques à comité de lecture
- Note de politique au Ministère de la santé

VIII. PERSPECTIVES

- Recherche de *M. ulcerans* dans des prélèvements environnementaux
- Enquête épidémiologique et séro-épidémiologique de l'ulcère de Buruli au Burkina Faso
- Cartographie de l'ulcère de Buruli au niveau national
- Développement des capacités opérationnelles des labortoires

IX. REFERENCE BIBLIOGRAPHIQUE

1. Bratschi MW, Ruf M-T, Andreoli A, Minyem JC, Kerber S, Wantong FG, et al. Mycobacterium ulcerans Persistence at a Village Water Source of Buruli Ulcer Patients. PLoS neglected tropical diseases. 2014;8(3):e2756. doi: 10.1371/journal.pntd.0002756.
2. Johnson PD, Azuolas J, Lavender CJ, Wishart E, Stinear TP, Hayman JA, et al. Mycobacterium ulcerans in mosquitoes captured during outbreak of Buruli ulcer, southeastern Australia. Emerg Infect Dis. 2007;13(11):1653-60. Epub 2008/01/26. doi: 10.3201/eid1311.061369. PubMed PMID: 18217547; PubMed Central PMCID: PMCPmc3375796.
3. Walsh DS, Portaels F, Meyers WM. Buruli ulcer: Advances in understanding Mycobacterium ulcerans infection. Dermatol Clin. 2011;29(1):1-8.
4. Marsollier L, Aubry J, Saint-Andre JP, Robert R, Legras P, Manceau AL, et al. [Ecology and transmission of Mycobacterium ulcerans]. Pathol Biol (Paris). 2003;51(8-9):490-5. Epub 2003/10/22. PubMed PMID: 14568596.
5. Portaels F, Chemlal K, Elsen P, Johnson PD, Hayman JA, Hibble J, et al. Mycobacterium ulcerans in wild animals. Rev Sci Tech. 2001;20(1):252-64. Epub 2001/04/06. PubMed PMID: 11288515.
6. Kotlowski R, Martin A, Ablordey A, Chemlal K, Fonteyne P-A, Portaels F. One-tube cell lysis and DNA extraction procedure for PCR-based detection of Mycobacterium ulcerans in aquatic insects, molluscs and fish. Journal of medical microbiology. 2004;53(9):927-33. doi: doi:10.1099/jmm.0.45593-0.
7. Hayman J. Mycobacterium ulcerans: an infection from Jurassic time? Lancet. 1984;2(8410):1015-6.
8. Mac Callum P, Tolhurst JC, et al. A new mycobacterial infection in man. J Pathol Bacteriol. 1948;60(1):93-122. Epub 1948/01/01. PubMed PMID: 18876541.
9. UBG. The Uganda Buruli Group. BCG vaccination against mycobacterium ulcerans infection (Buruli ulcer). First results of a trial in Uganda. Lancet. 1969;1(7586):111-5.
10. Clancey JK, Dodge OG, Lunn HF, Oduori ML. Mycobacterial skin ulcers in Uganda. Lancet. 1961;2(7209):951-4.
11. Stinear T, Ross BC, Davies JK, Marino L, Robins-Browne RM, Oppedisano F. Identification and characterization of IS2404 and IS2606: two distinct repeated sequences for detection of Mycobacterium ulcerans by PCR. J Clin Microbiol. 1999;37.
12. Asiedu K, Sherpbier R, Raviglione M. Buruli Ulcer Mycobacterium ulcerans infection.Global Buruli Ulcer initiative
Geneva, Switzerland: 2000.
13. Ouoba K, Sano D, Traore A, Ouedraogo R, Sakande B, Sanou A. [Buruli ulcers in Burkina Faso: a propos of 6 cases]. Tunis Med. 1998;76(3):46-50.
14. van der Werf TS, Stienstra Y, Johnson RC, Phillips R, Adjei O, Fleischer B, et al. Mycobacterium ulcerans disease. Bull World Health Organ. 2005;83(10):785-91.
15. Walsh DS, Portaels F, Meyers WM. Buruli ulcer (Mycobacterium ulcerans infection). Trans R Soc Trop Med Hyg. 2008;102(10):969-78.
16. Toll A, Gallardo F, Ferran M, Gilaberte M, Iglesias M, Gimeno JL, et al. Aggressive multifocal Buruli ulcer with associated osteomyelitis in an HIV-positive patient. Clin Exp Dermatol. 2005;30(6):649-51.
17. Hofer M, Hirschel B, Kirschner P, Beghetti M, Kaelin A, Siegrist CA, et al. Brief report: disseminated osteomyelitis from Mycobacterium ulcerans after a snakebite. The New England journal of medicine. 1993;328(14):1007-9. Epub 1993/04/08. doi: 10.1056/nejm199304083281405. PubMed PMID: 8450852.
18. Guimaraes-Peres A, Portaels F, de Rijk P, Fissette K, Pattyn SR, van Vooren J-P, et al. Comparison of Two PCRs for Detection of Mycobacterium ulcerans. Journal of Clinical Microbiology. 1999;37(1):206-8.
19. N'krumah RTAS, Koné B, Tiembre I, Cissé G, Pluschke G, Tanner M, et al. Socio-Environmental Factors Associated with the Risk of Contracting Buruli Ulcer in Tiassalé, South Côte d'Ivoire: A Case-Control Study. PLoS neglected tropical diseases. 2016;10(1):e0004327. doi: 10.1371/journal.pntd.0004327. PubMed PMID: PMC4712845.
20. Amofah G, Bonsu F, Tetteh C, Okrah J, Asamoa K, Asiedu K, et al. Buruli ulcer in Ghana: results of a national case search. Emerg Infect Dis. 2002;8(2):167-70.
21. Enfants FlHa. Rapport d'activité 2004-Burkina Faso. 2004.

22. Josse R, Guedenon A, Darie H, Anagonou S, Portaels F, Meyers WM. [Mycobacterium ulcerans cutaneous infections: Buruli ulcers]. Medecine tropicale : revue du Corps de sante colonial. 1995;55(4):363-73. Epub 1995/01/01. PubMed PMID: 8830223.
23. Portaels F. Laboratory diagnosis of Buruli ulcer: A manual for health-care providers. World Health Organization. Geneva2014. p. 117.
24. WHO. Portaels F, Johnson P, Meyers WM. Buruli ulcer. Diagnosis of Mycobacterium ulcerans disease. A manual for health care providers. http://whqlibdoc.who.int/hq/2001/WHO_CDS_CPE_GBUI_2001.4.pdf. Geneva:: World Health Organization; 2001.
25. Tabah EN, Nsagha DS, Bissek AC, Njamnshi AK, Bratschi MW, Pluschke G, et al. Buruli Ulcer in Cameroon: The Development and Impact of the National Control Programme. PLoS neglected tropical diseases. 2016;10(1).
26. Yotsu RR, Murase C, Sugawara M, Suzuki K, Nakanaga K, Ishii N, et al. Revisiting Buruli ulcer. J Dermatol. 2015;42(11):1033-41.
27. Marion E, Carolan K, Adeye A, Kempf M, Chauty A, Marsollier L. Buruli Ulcer in South Western Nigeria: A Retrospective Cohort Study of Patients Treated in Benin. PLoS neglected tropical diseases. 2015;9(1):e3443. doi: 10.1371/journal.pntd.0003443. PubMed PMID: PMC4287556.
28. Capela C, Sopoh GE, Houezo JG, Fiodessihoué R, Dossou AD, Costa P, et al. Clinical Epidemiology of Buruli Ulcer from Benin (2005-2013): Effect of Time-Delay to Diagnosis on Clinical Forms and Severe Phenotypes. PLoS neglected tropical diseases. 2015;9(9):e0004005. doi: 10.1371/journal.pntd.0004005. PubMed PMID: PMC4565642.
29. van der Werf TS, van der Graaf WT, Tappero JW, Asiedu K. Mycobacterium ulcerans infection. Lancet. 1999;354(9183):1013-8.
30. Asiedu K, Etuaful S. Socioeconomic implications of Buruli ulcer in Ghana: a three-year review. Am J Trop Med Hyg. 1998;59.
31. Vandelannoote K, Durnez L, Amissah D, Gryseels S, Dodoo A, Yeboah S, et al. Application of real-time PCR in Ghana, a Buruli ulcer-endemic country, confirms the presence of Mycobacterium ulcerans in the environment. FEMS Microbiol Lett. 2010;304(2):191-4.
32. Williamson HR, Benbow ME, Nguyen KD, Beachboard DC, Kimbirauskas RK, McIntosh MD, et al. Distribution of Mycobacterium ulcerans in buruli ulcer endemic and non-endemic aquatic sites in Ghana. PLoS neglected tropical diseases. 2008;2(3):e205. Epub 2008/03/28. doi: 10.1371/journal.pntd.0000205. PubMed PMID: 18365034; PubMed Central PMCID: PMCPmc2268743.
33. Portaels F, Elsen P, Guimaraes-Peres A, Fonteyne P-A, Meyers WM. Insects in the transmission of Mycobacterium ulcerans infection. The Lancet. 1999;353(9157):986. doi: 10.1016/s0140-6736(98)05177-0.
34. Marsollier L, Stinear T, Aubry J, Saint André JP, Robert R, Legras P, et al. Aquatic Plants Stimulate the Growth of and Biofilm Formation by Mycobacterium ulcerans in Axenic Culture and Harbor These Bacteria in the Environment. Applied and Environmental Microbiology. 2004;70(2):1097-103. doi: 10.1128/aem.70.2.1097-1103.2004. PubMed PMID: PMC348869.
35. Tian RB, Niamke S, Tissot-Dupont H, Drancourt M. Detection of Mycobacterium ulcerans DNA in the Environment, Ivory Coast. PLoS One. 2016;11(3).
36. Aboagye SY, Ampah KA, Ross A, Asare P, Otchere ID, Fyfe J, et al. Seasonal Pattern of Mycobacterium ulcerans, the Causative Agent of Buruli Ulcer, in the Environment in Ghana. Microbial Ecology. 2017:1-12. doi: 10.1007/s00248-017-0946-6.
37. Narh CA, Mosi L, Quaye C, Dassi C, Konan DO, Tay SC, et al. Source tracking Mycobacterium ulcerans infections in the Ashanti region, Ghana. PLoS neglected tropical diseases. 2015;9(1).
38. Morris A, Gozlan R, Marion E, Marsollier L, Andreou D, Sanhueza D, et al. First detection of Mycobacterium ulcerans DNA in environmental samples from South America. PLoS neglected tropical diseases. 2014;8(1).
39. Hennigan CE, Myers L, Ferris MJ. Environmental Distribution and Seasonal Prevalence of Mycobacterium ulcerans in Southern Louisiana. Applied and environmental microbiology. 2013;79(8):2648-56. doi: 10.1128/aem.03543-12. PubMed PMID: PMC3623173.

40. Marsollier L, Robert R, Aubry J, Saint Andre JP, Kouakou H, Legras P, et al. Aquatic insects as a vector for Mycobacterium ulcerans. Applied and environmental microbiology. 2002;68(9):4623-8.
41. Benbow ME, Williamson H, Kimbirauskas R, McIntosh MD, Kolar R, Quaye C, et al. Aquatic invertebrates as unlikely vectors of Buruli ulcer disease. Emerg Infect Dis. 2008;14(8):1247-54.
42. Konan KL, Doannio JM, Coulibaly NG, Ekaza E, Marion E, Asse H, et al. [Detection of the IS2404 insertion sequence and ketoreductase produced by Mycobacterium ulcerans in the aquatic Heteroptera in the health districts of Dabou and Tiassale in Cote d'Ivoire]. Med Sante Trop. 2015;25(1):44-51.
43. Doannio JM, Konan KL, Dosso FN, Kone AB, Konan YL, Sankare Y, et al. [Micronecta sp (Corixidae) and Diplonychus sp (Belostomatidae), two aquatic Hemiptera hosts and/or potential vectors of Mycobacterium ulcerans (pathogenic agent of Buruli ulcer) in Cote d'Ivoire]. Med Trop. 2011;71(1):53-7.
44. Marsollier L, Severin T, Aubry J, Merritt RW, Saint Andre JP, Legras P, et al. Aquatic snails, passive hosts of Mycobacterium ulcerans. Appl Environ Microbiol. 2004;70(10):6296-8.
45. Marion E, Deshayes C, Chauty A, Cassisa V, Tchibozo S, Cottin J, et al. [Detection of Mycobacterium ulcerans DNA in water bugs collected outside the aquatic environment in Benin]. Med Trop. 2011;71(2):169-72.
46. Dassi C, Mosi L, Akpatou B, Narh C, Quaye C, Konan D, et al. Detection of Mycobacterium ulcerans in Mastomys natalensis and Potential Transmission in Buruli ulcer Endemic Areas in Côte d'Ivoire. Mycobact Dis. 2015;5(3).
47. Narh CA, Mosi L, Quaye C, Dassi C, Konan DO, Tay SCK, et al. Source Tracking Mycobacterium ulcerans Infections in the Ashanti Region, Ghana PLoS neglected tropical diseases. 2015;9(1):e0003437. doi: 10.1371/journal.pntd.0003437.
48. Darie H, Le Guyadec T, Touze JE. [Epidemiological and clinical aspects of Buruli ulcer in Ivory Coast. 124 recent cases]. Bull Soc Pathol Exot. 1993;86(4):272-6.
49. Sakaguchi K, Iima H, Hirayama K, Okamoto M, Matsuda K, Miyasho T, et al. Mycobacterium ulcerans infection in an Indian flap-shelled turtle (Lissemys punctata punctata). J Vet Med Sci. 2011;73(9):1217-20.
50. Luo Y, Degang Y, Ohtsuka M, Ishido Y, Ishii N, Suzuki K. Detection of Mycobacterium ulcerans subsp. shinshuense DNA from a water channel in familial Buruli ulcer cases in Japan. Future Microbiol. 2015;10(4):461-9.
51. Willson SJ, Kaufman MG, Merritt RW, Williamson HR, Malakauskas DM, Benbow ME. Fish and amphibians as potential reservoirs of Mycobacterium ulcerans, the causative agent of Buruli ulcer disease. Infect Ecol Epidemiol. 2013;3(10):22.
52. Elsner L, Wayne J, O'Brien CR, McCowan C, Malik R, Hayman JA, et al. Localised Mycobacterium ulcerans infection in a cat in Australia. J Feline Med Surg. 2008;10(4):407-12.
53. van Zyl A, Daniel J, Wayne J, McCowan C, Malik R, Jelfs P, et al. Mycobacterium ulcerans infections in two horses in south-eastern Australia. Aust Vet J. 2010;88(3):101-6.
54. O'Brien CR, McMillan E, Harris O, O'Brien DP, Lavender CJ, Globan M, et al. Localised Mycobacterium ulcerans infection in four dogs. Aust Vet J. 2011;89(12):506-10.
55. O'Brien C, Kuseff G, McMillan E, McCowan C, Lavender C, Globan M, et al. Mycobacterium ulcerans infection in two alpacas. Aust Vet J. 2013;91(7):296-300.
56. O'Brien CR, Handasyde KA, Hibble J, Lavender CJ, Legione AR, McCowan C, et al. Clinical, microbiological and pathological findings of Mycobacterium ulcerans infection in three Australian Possum species. PLoS neglected tropical diseases. 2014;8(1).
57. Mitchell PJ, Jerrett IV, Slee KJ. Skin ulcers caused by mycobacterium ulcerans in koalas near bairnsdale, australia. Pathology. 1984;16(3):256-60. doi: 10.3109/00313028409068533.
58. Mitchell PJ, McOrist S, Bilney R. Epidemiology of Mycobacterium ulcerans infection in koalas (Phascolarctos cinereus) on Raymond Island, southeastern Australia. J Wildl Dis. 1987;23(3):386-90.

59. McOrist S, Jerrett IV, Anderson M, Hayman J. Cutaneous and respiratory tract infection with Mycobacterium ulcerans in two koalas (Phascolarctos cinereus). J Wildl Dis. 1985;21(2):171-3.
60. WHO. Buruli ulcer (Mycobacterium ulcerans infection). Available at: http://www.who.int/mediacentre/factsheets/fs199/en/2016.
61. Ukwaja KN, Meka AO, Chukwuka A, Asiedu KB, Huber KL, Eddyani M, et al. Buruli ulcer in Nigeria: results of a pilot case study in three rural districts. Infectious Diseases of Poverty. 2016;5:39. doi: 10.1186/s40249-016-0119-8. PubMed PMID: PMC4841952.
62. WHO. World : Distribution of buruli ulcer, 2014. WHO Annual meeting on Buruli ulcer2015.
63. Osei F, B, Duker A, A. Analysis of Buruli Ulcer Prevalence in Amansie West District: A Geostatistical Approach. Austin Biom and Biostat. 2015;2(1):1011.
64. Johnson PD, Stinear TP, Hayman JA. Mycobacterium ulcerans--a mini-review: J Med Microbiol. 1999 Jun;48(6):511-3.

FICHE D'ACCOMPAGNEMENT DES PRELEVEMENTS

Expéditeur
Nom :……..……
Localité/province/Région sanitaire…….....
……

Date : /…./…./…../

Participant inclus
Nom (les trois premières lettres):/…./…../…../
Prénom (les deux premières lettres):/…./…../
Age : …….
Sexe : F □ M □
Ville/Village/Province/Région sanitaire……...
……

Contact régulier
- avec l'eau des rivières/marigot Oui □ Non □
- avec les rizières Oui □ Non □
- les marécages Oui □ Non □
- autres (préciser) …….

Prélèvement
Date du prélèvement :…/…. /….
Ecouvillonnages cutanés réalisés : Oui □ Non □

Contexte épidémiologique
- Provenance géographique actuelle du participant

Préciser (Ville/Village/Province/Région sanitaire) :…….
……

- Provenance d'une zone ayant fait l'objet d'une transmission active :

Oui □ Non □
Préciser :……
……
Si oui, depuis combien de temps vous résider dans la localité actuelle :…..
……
……

NOTICE D'INFORMATION

« Etude du portage cutané de *Mycobacterium ulcerans* dans les zones à risque de l'ulcère de Buruli au Burkina Faso »

Promoteur
IHU - Méditerranée Infection
19 Boulevard Jean Moulin, 13005 Marseille

Investigateurs coordinateurs
ZINGUE Dezemon (Centre Muraz, Bobo Dioulasso, Burkina Faso)
Pr DRANCOURT Michel (**IHU - Méditerranée Infection**, Marseille, France)

de Mr/Mme/Mlle ..... (NOM, Prénom)

Adresse .....

Mr …….. m'a proposé de participer à une recherche organisée par le Centre MURAZ afin d'évaluer la prévalence du portage cutané de *Mycobactérium ulcerans* chez les sujets saints au Burkina Faso.

Nous vous informons que *M. ulcerans* est une mycobactérie appartement à la même famille que les mycobactéries responsables de la tuberculose « sogo-sogo gbê en langue bambara » . la mycobactérie que nous voulons rechercher sur la peau saine est responsable de la maladie qu'on appelle ulcère de Buruli. L'ulcère de Buruli se manifeste par des plaies chroniques sur les membres supérieurs et inférieurs, le tronc, le coup, la tête et peut même se manifester sous forme de plaies chroniques disséminées à différents endroits du corps. Cette maladie dont les plaies peuvent durer sur l'homme si elle n'est pas soignée de façon adéquate peut entrainer des situations invalidantes.
Chaque année, près de 5000 à 6000 personnes sont atteintes de cette maladie et près de 80% des malades se situent en Afrique de l'Ouest. Nos voisins de la Côte d'Ivoire et du Ghana sont les plus atteints dans le monde par cette maladie invalidante qu'est l'ulcère de Buruli.
Au Burkina Faso, ce type de plaies chroniques n'est pas suffisamment étudié. En prélude à des études de plus grandes envergures au Burkina Faso, nous voulons d'abords comprendre certains facteurs de risques de cette maladie. Comme c'est une maladie de la peau et des tissus mous, ce qui veut dire que la mycobactérie peut être sur la peau avant de s'y introduire et de provoquer cette plaie chronique.
Comme c'est une mycobactérie de l'environnement, nous pensons que les malades la contractent après des contacts repétés avec les eaux de rivières, dans les rizières ou contact avec tout échantillon environnemental contaminé.
ob*jectif de l'étude* : Cette étude a pour but d'évaluer le portage cutané de *M. ulcerans* chez les enfants et adultes sains des zones à risque probable de la maladie au Burkina Faso.

Lieu de l'étude :
L'étude se déroulera au dans quatre régions sanitaires du Burkina Faso que sont les régions sanitaires des Hauts-Bassins, du Sud-Ouest, des Cascades et du Centre-Ouest.
Le Centre MURAZ est le centre coordonnateur de l'étude.
Centre MURAZ, *Avenue Mamadou KONATE, Porte 2006 – Lot 218 – 01 B.P. 390 Bobo-Dioulasso 01*

Nombre de sujets participant à l'étude : 800 (200 sujets par région sanitaire)

Durée de l'étude : 06 mois

Déroulement de l'étude :
Les échantillons seront collectés par écouvillonnage sur la peau au niveau des membres supérieurs et inférieurs à raison de deux écouvillons par sujet. Un écouvillon au niveau du bras et un autre au niveau de la jambe.
C'est une méthode non invasive, pas de prélèvements sanguins ni de piqure quelconque.
Les prélèvements seront faits dans vos villages respectifs sous contrôle médical par des infirmiers ou des technologistes biomédicaux de chaque zone. Ensuite ces écouvillons seront envoyés au Centre MURAZ à partir de chaque site de prélèvements. Le Centre MURAZ sera chargé des colis qui seront ensuite envoyés à l'Institut Hospitalo-Universitaire-Méditerranée Infection à Marseille/France. Dans les laboratoires de Marseille, il sera réalisé sur les prélèvements la détection de *M. ulcerans* par une technique de Biologie moléculaire.

Bénéfices de l'étude : Cette étude est à bénéfice individuel direct et permettra de savoir si l'agent causal de l'ulcère de Buruli peut se retrouver sur une peau saine. Ainsi des mesures préventives pourront être entreprises en vue d'empêcher que *M. ulcerans* présent sur la peau ne puisse provoquer la maladie chez l'homme.

Effets indésirables possibles :
Il n'y a pas d'effets indésirables, car le prélèvement est fait par une méthode non invasive qu'est le prélèvement par écouvillonnage.

Impératifs pour participer à cette étude :
Les adultes et les enfants de plus de 5 ans après leur assentiment et le consentement de leur parent ou tuteur seront inclus dans cette étude. Les résultats de détection de *M. ulcerans* sur les écouvillons seront communiqués sauf avis contraire émanant de vous-même par le biais du personnel de santé relevant de votre département.

CEI :
Le protocole de cette étude a été soumis au Comité d'Ethique Institutionnel du Centre MURAZ et a reçu un avis favorable lors de sa séance du _____.

Il m'a été spécifié(e) que cette recherche sera menée conformément à la loi Huriet (loi n°88-1138 modifiée) et conformément à la législation Burkinabé sur le code de la santé publique.

Indemnité compensatrice pour transport :
Chaque sujet inclus recevra une compensation de 500 FCFA en cas de déplacement.

Confidentialité des données :
Certaines informations personnelles sur le sujet inclus (les trois premières lettres de son nom patronymique, les deux premières lettres de son prénom, seront enregistrées sur un registre de laboratoire et sur un fichier automatisé. Les données relatives le concernant sont détruites à l'issue d'un délai de 12 mois suivant le début de la dernière participation à une recherche, sous réserve que la période d'exclusion fixée pour cette recherche soit achevée.
Je peux vérifier auprès du titulaire de l'autorisation de lieux de recherche l'exactitude des données présentes dans le fichier. Je peux également vérifier la destruction de ces données au terme du délai.

Il est prévu que l'analyse de cette recherche biomédicale soit effectuée France et ensuite il y aura un transfert de compétences et de technologies au Burkina Faso pour mieux screener en temps réel les problèmes de santé liés à l'ulcère de Buruli. Les résultats de l'étude pourront être publiés dans des revues médicales, toujours sans que les sujets inclus ne puisse être identifiés.

Données nominatives :
Le fichier informatique utilisé pour réaliser la présente recherche fait l'objet d'une confidentialité au sein de l'équipe de recherche. J'ai été informé(e) de la nature des informations transmises (les trois premières lettres de mon nom patronymiques, les données sur mon lieu de résidence, mes activités clés). J'ai été informé(e) de la finalité du traitement des données (statistiques, analyses des résultats), des personnes physiques ou morales destinataires de ces données (le Promoteur) ainsi que, le cas échéant, les Autorités Sanitaires habilitées. J'ai été informé(e) de mon droit d'accès et de rectification à ces données soit directement, soit indirectement par l'intermédiaire d'un personnel de santé de traitement des données ne pourra permettre mon identification directe ou indirecte.
J'ai été avisé(e) de ce que le Mr Zingué Dezemon a été spécialement désigné par le responsable de la recherche en vue de veiller à la sécurité des informations et de leur traitement ainsi qu'au respect de la finalité de celui-ci.

Assurance :
Vu la spécificité de ce mini-projet, cette étude n'a pas été couverte par une assurance souscrite par le Promoteur.

Participation :
Si, après avoir lu les informations ci-dessus et discuté (e) avec les investigateurs, je décide de ma participation ou de celle de mon enfant à cette recherche biomédicale, je devrai signer le formulaire de consentement et parapher chaque page afin de certifier que j'ai compris les informations reçues.
La participation de mon enfant à cette recherche biomédicale est libre et volontaire et ce, après son assentiment et votre autorisation. Je peux refuser d'y participer ou d'y laisser participer mon enfant ou me (le) retirer de la recherche biomédicale à tout moment, et ce, sans aucun préjudice d'aucune nature et sans nécessairement, si je le souhaite, en motiver la raison.
Il est important que j'informe l'investigateur impliqué dans cette recherche biomédicale de mon éventuel retrait ou refus de participation dès que possible.
Le Promoteur de la recherche biomédicale peut également décider d'arrêter la recherche biomédicale à tout moment et ce, sans mon consentement.

Coordonnées des Investigateurs :
Dezemon ZINGUE
Adresse : Centre MURAZ ; *Avenue Mamadou KONATE, Porte 2006 – Lot 218 – 01 B.P. 390 Bobo-Dioulasso 01*
Téléphone : 20970102
est à ma disposition pour toute information complémentaire que je jugerai utile aussi bien avant que pendant la recherche biomédicale.

Résultats de l'étude :
J'ai été informé(e) que les résultats globaux de l'étude me seront communiqués à votre demande par l'investigateur.

CONSENTEMENT ECLAIRE

« Etude du portage cutané de *Mycobacterium ulcerans* dans les zones à risque de l'ulcère de Buruli au Burkina Faso) ».

Après lecture du formulaire d'information, je déclare :

1. Avoir été informé(e) par Mr _____de la nature du projet de recherche et de ses buts conformément à la déclaration de Helsinki (1964) modifiée à Edimbourg (2000).
2. Avoir reçu toutes les réponses souhaitées à mes questions.
3. Avoir noté que cette étude est réalisée conformément à la législation burkinabé et conformément aux Bonnes Pratiques Cliniques.
4. Avoir noté que cette étude a reçu un avis favorable du Comité d'Ethique Institutionnel du Centre MURAZ de Bobo Dioulasso lors de sa séance du _____.
5. Accepter de faire subir à moi-même ou à mon enfant un prélèvement par écouvillonnage sur la peau.
6. Avoir noté que ma participation ou celle de mon enfant à cette étude est entièrement volontaire et que je suis libre de refuser la participation. Avoir noté que si je décide de participer ou que mon enfant participe, je dois signer ce consentement pour indiquer que j'accepte que mon enfant participe à cette étude. Avoir été informé(e) que je peux me retirer ou retirer à tout moment mon enfant de cette étude sans préjudice d'aucune nature et que, j'informerai l'investigateur de ma décision.
7. Avoir noté que toute nouvelle information survenant en cours d'étude et, susceptible de remettre en cause ma participation ou la participation de mon enfant, me sera communiquée dès que possible.
8. Avoir noté les coordonnées de l'investigateur: Mr Dezemon ZINGUE
   Adresse : Centre MURAZ ; *Avenue Mamadou KONATE, Porte 2006 – Lot 218 – 01 B.P. 390 Bobo-Dioulasso 01*
   Téléphone : 20970102
   .
9. Avoir accepté que les données enregistrées à l'occasion de cette recherche puissent faire l'objet d'un traitement informatisé par le Promoteur. Je pourrais exercer mon droit de rectification auprès de l'investigateur.
10. Les données me concernant ou concernant mon enfant enregistrées dans le fichier automatisé prévu sont détruites à l'issue d'un délai de 12 mois suivant le début de la dernière participation à une recherche, sous réserve que la période d'exclusion fixée pour cette recherche soit achevée. Je peux vérifier auprès du titulaire de l'autorisation de lieux de recherche l'exactitude des données concernant mon enfant présentes dans le fichier. Je peux également vérifier la destruction de ces données au terme du délai prévu à l'article.
11. Accepter que les informations collectées par les personnes accréditées du Promoteur puissent être traitées, transférées et utilisées de façon strictement confidentielle dans un autre pays partenaire de l'étude et sous la responsabilité du Promoteur. Il est prévu que l'analyse de cette recherche biomédicale soit réalisée au Burkina Faso et en France.
12. Avoir été informé(e) que je recevrai une compensation de 500 FCFA comme frais de transport au cas où votre participation demande un déplacement.
13. Avoir été informé(e) que des représentants du Promoteur ou les autorités de tutelle locales ou étrangères peuvent être amenés à mes données de manière confidentielle pour vérifier les données rapportées. En signant ce document, je confirme mon accord pour cette consultation de mes données**.**
14. Avoir été informé qu'à l'issue de l'étude, les résultats me seront communiqués.

J'ACCEPTE MA PARTICIPATION ET/OU « LA PARTICIPATION DE MON ENFANT » A CETTE RECHERCHE DANS LES CONDITIONS PRECISEES CI-DESSUS

Fait en double exemplaires (un remis au volontaire, un pour l'investigateur)

Nom :_____ Prénom :_____

Numéro de sélection du sujet dans l'étude: ⌊___⌋

Date :

Signature du participant adulte ou du parent ou du représentant légal de l'enfant (à défaut l'empreinte de votre index droit):
(Précédée de la mention "lu et approuvé")

Je confirme avoir personnellement expliqué la nature, l'objectif, la durée, ainsi que les effets de l'étude à la personne dont le nom figure ci-dessus.

Nom : _____ Prénom : _____

Date :

Signature de l'investigateur:

Résumé

L'ulcère de Buruli est une maladie infectieuse tropicale présente dans des foyers endémiques et atteignant surtout les populations pauvres en zones rurales. En 1997, l'Organisation Mondiale de la Santé (OMS) a reconnu l'ulcère de Buruli comme une maladie ré-émergente et en 1998, un programme mondial de lutte contre cette infection (Global Buruli Ulcer initiative « GBUI ») a été mis en route lors de la Conférence de Yamoussoukro en Côte d'Ivoire. Cette infection essentiellement cutanée est causée par *Mycobacterium ulcerans*, dérivé d'un ancêtre commun avec *Mycobacterium marinum*, et est la troisième mycobactériose la plus prévalente dans le monde, après la tuberculose et la lèpre. *M. ulcerans* produit la toxine mycolactone qui est responsable du pouvoir pathogène du bacille. Cependant, *M. ulcerans* est un pathogène opportuniste dont le réservoir est environnemental. Notre revue de la littérature a répertorié les sources et hôtes potentiels de cette mycobactérie avec une proéminence du réservoir hydro-tellurique. L'ADN de *M. ulcerans* a été détecté dans le sol, l'eau, les biofilms, les végétaux, les mousses, les fèces ainsi que chez les mollusques d'eau, les punaises d'eau, les poissons d'eau douce, la tortue, les crevettes , les batraciens et les petits mammifères. L'ADN a été détecté également chez le chat, le cheval, le chien, l'alpacas, les possums et le koala. Cependant, seulement cinq souches de *M. ulcerans* ont été isolées à partir de prélèvements de l'environnement en zones d'endémie de l'ulcère de Buruli et l'absence d'une large collection de souches est un frein important à l'investigation des sources et vecteurs de cette mycobactérie. Nous avons montré qu'il existe une corrélation inverse entre réchauffement climatique dans les pays endémiques et incidence de l'ulcère de Buruli, peut-être liée à la sensibilité intrinsèque de *M. ulcerans* aux variations de température et de la lumière, ou bien à des modifications de son écosystème. Dans la perspective d'améliorer les protocoles d'isolement et de culture de *M. ulcerans* à partir de l'environnement, nous avons entrepris une analyse phénotypique à haut débit des substrats carbonés métabolisés par *M. ulcerans* et le profil obtenu nous a orientés après une recherche bibliographique des principales sources environnementales de ces substrats, vers des interactions plus spécifiques de *M. ulcerans* avec les autres bactéries, les algues, les mollusques et les champignons. Les résultats de ce premier travail ont servi de base pour la mise au point de milieux de culture innovants qui, combinés à une méthode originale de décontamination par la chlorhexidine, nous ont permis d'isoler pour la première fois, une microcolonie de *M. ulcerans* à partir de fèces d'agouti collectés en Côte d'Ivoire. Cette microcolonie a été correctement identifiée par amplification de séquences spécifiques et par analyse de son profil peptidique par spectrométrie de masse grâce à un protocole développé au cours de notre thèse. Egalement, nous avons mis au point une méthode de lecture automatisée des échantillons colorés par la coloration de Ziehl-Neelsen à la recherche des mycobactéries, dans une perspective de lecture haut-débit. Notre travail de thèse a produit des protocoles qui ont pour objectif d'être mis en œuvre dans les pays d'endémie Africains dont le Burkina Faso dont nous sommes originaires, pour préciser les sources et modes de transmission de *M. ulcerans* aux populations, par une approche basée sur l'isolement, la culture et la caractérisation des souches.

Mots clés : Ulcère de Buruli, *Mycobacterium ulcerans*, *Mycobacterium marinum*, réchauffement climatique, sources carbonées, culture, agouti, environnement, sources.

Summary

Buruli ulcer is a tropical infectious disease present in endemic foci and mostly affects poor populations in rural areas. In 1997, the World Health Organization (WHO) recognized Buruli ulcer as a re-emerging disease and in 1998 a global program to combat this infection (Global Buruli Ulcer initiative « GBUI ») was launched at the Yamoussoukro Conference in Côte d'Ivoire. This mainly cutaneous infection is caused by *Mycobacterium ulcerans*, which has a common ancestor with *Mycobacterium marinum*, and is the third most prevalent mycobacterial disease in the world after tuberculosis and leprosy. *M. ulcerans* produces mycolactone toxin which is responsible for the pathogenicity of the bacillus. However, *M. ulcerans* is an opportunistic pathogen from the environment. Our literature review has listed the potential sources and hosts of this mycobacterium with a prominence of the hydro-telluric reservoir. The DNA of *M. ulcerans* was detected in soil, water, biofilms, plants, mosses, faeces as well as in water molluscs, water bugs, freshwater fish, turtle, shrimp, amphibians and small mammals. DNA was also detected in cat, horse, dog, alpacas, possums and koala. However, only five strains of *M. ulcerans* have been isolated from environmental sampling in endemic areas of Buruli ulcer and the absence of a large collection of strains is a major obstacle to investigation of the sources and vectors of this mycobacterium.
We have shown that there is an inverse correlation between global warming in endemic countries and incidence of Buruli ulcer, possibly related to the intrinsic sensitivity of *M. ulcerans* to temperature and light changes, or to changes in its ecosystem. In order to improve the isolation and culture protocols of *M. ulcerans* from the environment, we conducted a high-throughput phenotypic analysis of the carbon substrates metabolized by *M. ulcerans* and the profile obtained oriented us afterwards a bibliographic search of the main environmental sources of these substrates, towards more specific interactions of *M. ulcerans* with other bacteria, algae, molluscs and fungi. The results of this first work served as a basis for the development of innovative culture media which, combined with an original method of chlorhexidine decontamination, allowed us to isolate for the first time a microcolony from feces of agouti collected in Côte d'Ivoire. This microcolony was correctly identified by amplification of specific sequences and by analysis of its peptide profile by mass spectrometry through to a protocol developed during our thesis. We also developed a method for automated reading of samples stained by Ziehl-Neelsen staining for mycobacteria in a high-throughput reading perspective. Our thesis work has produced protocols that are intended to be implemented in African endemic countries, including Burkina Faso, in order to clarify the sources and modes of transmission of *M. ulcerans* to populations, an approach based on the isolation, culture and characterization of strains.

Keywords: Buruli ulcer, *Mycobacterium ulcerans*, *Mycobacterium marinum*, global warming, carbon sources, culture, agouti, environment, sources.