Epidemiological, Bacteriological And Pathogenicity Profile Of Mycobacterium Ulcerans Infection At The Buruli Ulcer Detection And Treatment Centre In Pobè, South-East BENIN

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***Abstract*—Buruli ulcer (BU), a Mycobacterium ulcerans infection, is the third most common mycobacterial disease in the world and has been emerging rapidly since 1980, mainly in sub-Saharan African countries. Until now, epidemiological knowledge on BU was based on series of clinical cases not confirmed by laboratory. We constituted a cohort of 655 laboratory-confirmed cases between October 2014 and February 2017; to describe the epidemiology, bacteriological profile and pathogenicity of Mycobacterium Ulcerans infection.**

Patients with BU are children (median age at diagnosis 12 years), presenting with a single (96%), large (more than 15 cm, 36%), ulcerative (66%) lesion of the lower limb (60%). We report an atypical clinical presentation of BU, in which patients present exclusively with M. *ulcerans* osteomyelitis. The sex ratio varies with age: boys predominate among children (57% male patients in children under 15 years old), and women among adults (33% male patients). Clinical presentation depended on age and sex. 9% of male patients had osteomyelitis compared to 4% of female patients. One year after the end of treatment, 22% of patients had fixed functional sequelae. A clinical presentation involving an edematous, bony, large lesion or several lesions was significantly associated with the development of functional sequelae (OR 7.64, IC95% [1]).

Understanding the pathophysiology of the bacteriological features and pathogenicity of *M. ulcerans* infection is crucial to generate new therapeutic and vaccine leads.

***Keywords*—Epidemiological profile; bacteriological profile; Buruli ulcer; pathogenicity; Mycobacterium ulcerans**

Introduction

The term "neglected tropical disease", which emerged in 2006 at the initiative of the World Health Organization (WHO), represents a diverse group of communicable diseases prevalent in tropical and subtropical settings in 149 countries and affecting more than one billion people. These infections are a consequence of environmental and socio-economic conditions. Indeed, they mainly affect populations living in poverty, without adequate sanitation and in close contact with parasitic vectors and infectious bacteria. Currently, about fifteen diseases are considered as neglected tropical diseases. They are the subject of global control plans coordinated by WHO, with the aim of preventing, controlling, eliminating or eradicating them. They include widespread diseases such as leprosy, rabies and dengue fever, as well as much less publicized diseases such as Buruli ulcer. Buruli ulcer has been prioritized by the WHO because of the limited knowledge about it and the social and economic burden it places on affected developing regions.

Buruli ulcer (BU) is a necrotizing infection of mucocutaneous, subcutaneous and bony tissues; caused by Mycobacterium ulcerans.

Globally, it is the third most common mycobacterial disease after tuberculosis and leprosy. It is endemic in more than 30 countries in Africa, Latin America, Oceania and Asia [2]. In some African countries, such as Benin, BU has become the second most common disease caused by mycobacteria after tuberculosis [2,3].

The objectives of our study are to describe the epidemiological profile, the bacteriological profile and the pathogenicity of M. ulcerans.

Frame and Material

The CDTUB (Buruli Ulcer Detection and Treatment Centre) in Pobè, financed by the Raoul Follereau France association, was founded in 2003 and employs about fifty people, including three doctors and a surgeon. It is located in a rural area in eastern Benin, near the Nigerian border. The hospital has a capacity of 58 beds. The operating theatre operates twice a week. The CDTUB has an analysis laboratory where the main biological tests can be carried out. X-rays are carried out at the nearby hospital in Pobè. The CDTUB coordinates inpatient and outpatient care in Pobè and in the 15 outposts in the heart of the endemic villages within a radius of about 30 kilometres. The organization of care includes three weekly medical rounds in the endemic villages. The annual number of patients treated is around 200. All patients are reviewed at a distance from the treatment to detect relapses and evaluate functional after-effects.

Patient and Methods

The retrospective; prospective; descriptive and analytical study took place from the period of October 2014 to February 2017 at the CDTUB of Pobè, at the Laboratory of Biology and Molecular Typing in Microbiology and at the Laboratory of Research and Services in Clinical Biochemistry and Microbiology, Hospital Hygiene and Biosafety where complementary examinations were performed. We have worked on 238 cases of BU whose results are confirmed by PCR. They are divided in table 1 according to the different stages of evolution of the infection.

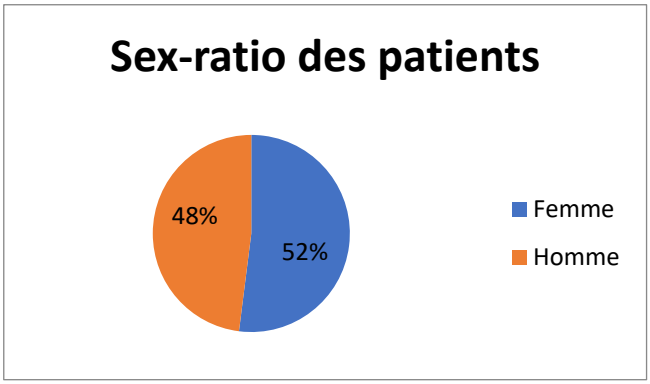
Clinical epidemiological profile of BU

Table 1: Distribution of patients according to the different stages of M. ulcerans infection

Stages of M. ulcerans infection	Number of patients at this stage of the infection	Percentage
Ulceration	200	42%
Plates	116	25%
Edema	72	15%
Nodules	53	11%
Osteomyelitis	35	7%
Total	476	100%

Table 2: Sex ratio of patients, their number and percentage

Sex ratio of patients - number and percentage			
Women	248	52%	< 15 years
Men	228	48%	15 years old
Total	476	100%	



Clinical features

M. ulcerans is essentially a skin-tropic germ. However, in recent years it has been implicated in apparently primary osteomyelitis.

The cutaneous forms evolve in three stages

Pre-ulcer phase

Early, painless, it is often neglected by patients. It can take several forms.

1° The nodular form: the most frequent, it is characterized by a single hard nodule adhering to the skin, painless and sometimes pruritic. The epidermis covering this nodule is often hyper-pigmented.

2° The oedematous form: less frequent but immediately more serious, presents as an oedema of sudden or progressive appearance. The tissues are infiltrated. Sometimes hot and painful, it tends to spread, involving a segment of limb or even the whole limb. It is a necrotizing panniculus of immediate concern [4].

3° Other rarer manifestations of the onset of Buruli ulcer can be observed. The papular and bullous forms and the banal cutaneous placard resting on an oedematous base and within which an ulceration will appear.

The ulcerative phase

Progressively, over a period of several weeks to several months, the nodule spreads, the epidermis softens and in a few days necrosis appears, giving rise to an ulceration which progresses centrifugally. The dermis and the deep fascia are invaded, and the subcutaneous fatty tissue becomes infected. The lesion oozes a necrotic fluid.

The ulceration that has formed has a characteristic appearance: its edges are irregular and largely detached from the underlying musculoaponeurotic

plane, which means that the actual ulceration has a much larger surface area than the apparent skin ulceration.

The bottom of the ulcer is more or less clean depending on the degree of superinfection. It is not very painful and is not accompanied by general signs. It is often at this stage that the patient comes to consult. Biopsy of peripheral skin tissue or swabbing under the edges of the ulcer reveals acid-fast bacilli. Several lesions may coalesce and extend to an entire limb.

Healing phase

In the absence of treatment, after a phase of variable extension, fleshy buds appear at the bottom of the ulceration. The lesions stop spreading and healing begins. Healing is slow and may lead to recovery in a few months, with sequelae that are all the more serious because the lesions were more extensive. They put at risk the functional and aesthetic prognosis. In other cases, the lesions evolve in a chronic way with frequent relapses.

Bone forms: arthritis, osteitis, osteomyelitis

They are not rare. While it is easy to explain osteoarticular infections that develop near an ulceration, it is more difficult to explain the genuine primary osteomyelitis that has been described [5]. Indeed, diffusion by the hematogenous or lymphatic route into the depths of the bone is incompatible with the growth temperature of *M. ulcerans, and it* is possible that the strain has adapted to higher temperatures. The same phenomenon has been noted with a number of deep infections caused by *M. marinum.* However, these bone infections can lead to amputation [6].

Clinical features		Explanatory variable and percentage related to the clinical characteristic		Univariate analysis
		Age	continuous variable	- <10-4
Gender Male		> 15 ≤ 15	33% (n= 157) 57% (n= 271)	1 - - 2.59 [2.04-3.30] <10-4
Location of the upper part of the body		> 15 ≤ 15	32% (n= 152) 68% (n= 324)	1 - - 2.00 [1.58-2.55] <10-4
	Female gender		57%(n= 271)	1 - -
	Male gender		43% (n= 205)	0.91 [0.73-1.14] 0.4241
Ulcer		> 15 ≤ 15	32% (n= 152) 68% (n=324)	1 - - 0.68 [0.53-0.88] <0.0024
	Female gender		53%(n= 252)	1 - -
	Male gender		47% (n= 224)	1.24 [0.98-1.57] 0.0731
Plate		> 15 ≤ 15	45% (n= 214) 55% (n= 262)	1 - - 1.90 [1.51-2.40] <10-4
	Female gender		57%(n=271)	1 - -
	Male gender		43% (n=205)	0.85 [0.68-1.06] 0.1491
Edema		> 15 ≤ 15	37% (n= 176) 63% (n= 300)	1 - - 1.33 [1.01-1.75] 0.0390
	Female gender		55%(n=252)	1 - -
	Male gender		45% (n=214)	1.00 [0.77-1.30] 0.9863
Nodule		> 15 ≤ 15	41% (n= 195) 25% (n= 119)	1 - - 0.75 [0.40-1.41] 0.3639
	Female gender		58%(n= 276)	1 - -
	Male gender		42% (n= 200)	0.53 [0.27-1.01] 0.0528
Osteomyelitis		> 15 ≤ 15	47% (n= 224) 53% (n=252)	1 - - 1.46 [0.91-2.41] 0.1181
	Female gender		61%(n= 290)	1 - -
	Male gender		39% (n= 186)	2.21 [1.39-3.59] 6.8x10-4
Range ≥ 15 cm		> 15	42% (n=200)	1 - -

		≤ 15	58% (n=276)	0.78 [0.61-0.99] 0.0422
	Female gender		54%(n= 257)	1 - -
	Male gender		46% (n= 219)	1.18 [0.93-1.49] 0.1746
Multifocal disease		> 15	4% (n= 19)	1 - -
		≤ 15	7% (n= 33)	1.23 [0.69-2.24] 0.4878
	Female gender		6%(n= 28)	1 - -
	Male gender		4% (n= 19)	1.05 [0.60-1.82] 0.8654
Severe form		> 15	52% (n= 248)	1 - -
		≤ 15	48% (n= 228)	0.90 [0.71-1.13] 0.3537
	Female gender		5%(n= 24)	1 - -
	Male gender		7% (n= 33)	1.22 [0.97-1.53] 0.0842
Occurrence of functional sequelae		> 15	20% (n= 95)	1 - -
		≤ 15	23% (n= 109)	1.20 [0.88-1.63] 0.2479
	Female gender		20% (n= 95)	1 - -
	Male gender		23% (n= 109)	1.30 [0.97-1.75] 0.0783
Deaths occurring PLWHIV and sepsis and toxemia Hepatitis C and Hepatitis B		> 15	2,51% (n= 12)	
		≤ 15	0%	
	Female gender		1,47% (n= 7)	
	Male gender		1,05% (n= 5)	

Table 3: Impact of age and gender on the clinical presentation of BU

Morphological and cultural characteristics.

Microbiological diagnosis of *M. ulcerans* infections

Microscopic examination of a pathology product is the first step in the bacteriological diagnosis of *M. ulcerans* infections and sometimes the only one in developing countries. It is performed directly on the smear of a purulent or hemorrhagic patch of the pathology product.

To detect mycobacteria, their acid-fastness is used, i.e. their ability to form stable complexes with basic dyes, fuchsin or phenic fluorochromes, which persist despite the double action of alcohol and diluted strong acids [7]. The main methods using fuchsin are the Ziehl-Neelsen method and its variants including the Kinyoun method.

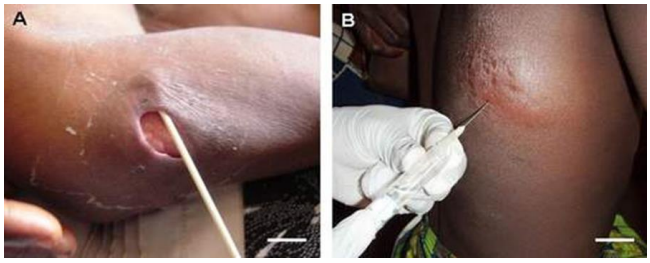


Figure 1: Fine needle aspiration of a nodule or plaque

(A) Swabbing of an ulcerated form. The swab should be taken deep under the detached edges. It is advisable to take several swabs from different locations.

(B) Fine needle aspiration. This minimally invasive and painless sampling method is recommended for closed lesions. *Photos and comments: courtesy of Dr A. Chauty*

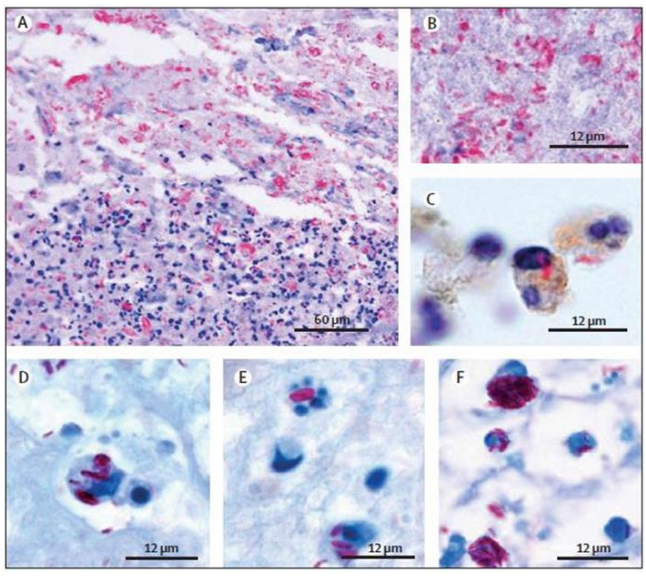


Figure 2. Pathological aspects of *M. ulcerans* infection

Biopsy of an African patient: Anatomopathology

(A) BAARs are mainly found in the upper necrotic region, while the lower region shows an inflammatory infiltrate.

(B) A detail of the necrotic region shows extracellular BAARs.

(C) Intramacrophagic bacillus from the inflammatory region in panel A.

(D-E) Intracellular bacilli, causing apoptosis

(E, core fragmentation)

(F) Massive clustering of intracellular bacilli

Bacteriological characteristics

M. ulcerans is a very slow-growing, non-pigmented species that is classified in Runyon group III.

- After Ziehl's staining, the bacilli vary in size from 3 to 10 µm in the samples, and are often grouped in clusters comparable to the globi observed in leprosy.

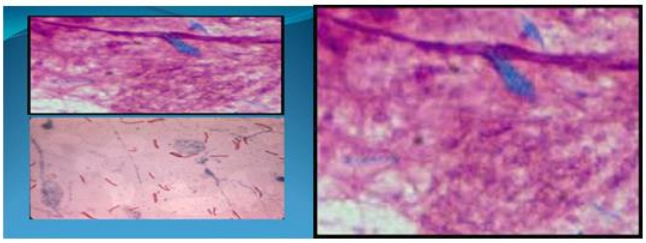


Figure 3: Observation with a 100 mm immersion microscope stained by the Ziehl-Neelsen method

- The cultural characteristics are important for the identification of the germ. The culture is difficult, slow (from 6 weeks to several months). *M. ulcerans* does not grow at 37° but between 29 and 32°. On egg medium *(Löwenstein Jensen),* the colonies are rough, slightly yellow pigmented. This scotochromogenic pigmentation is not constant. The same culture may give rise to pigmented and unpigmented colonies. *M. ulcerans* grows on 7H12B medium but does not grow faster than on egg medium.

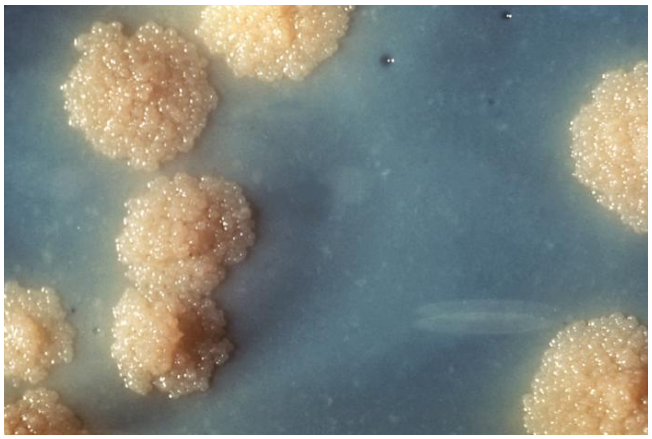


Figure 4: Cauliflower-like appearance of M. ulcerans colonies on löwesten Jensen egg medium

- Biochemical identification characteristics are of great importance but vary according to the geographical origin of the strains. All strains grow in the presence of TCH. When they possess catalase, it is thermostable. African and Australian strains grow in the presence of 250 µg hydroxylamine. African strains can produce an acid phosphatase. Urease and nicotinic acid accumulation are possible but not common [8].

Media should be incubated for at least 8 weeks and preferably for 12 weeks. Samples that were microscopically positive and do not show cultures should be stored even longer.

- Inoculation of ^{104} germs into the footpad of the mouse or into the tail is followed by a significant inflammatory reaction which appears 4 to 5 weeks after inoculation of American strains and later (8 to 9 weeks) in the case of African strains. Ulcerations develop and the animal dies around the 12th week due to superinfection. This pathogenicity is due to toxigenic strains. Non-toxigenic mutants are non-pathogenic [9]. The demonstration of experimental pathogenicity in mice is a good test for identification.

a) Reading cultures

Daily monitoring of cultures during the first week of incubation allows contaminated tubes to be quickly discarded and re-decontamination or re-sampling requested. It also allows the detection of fast-growing mycobacteria.

For slow-growing mycobacteria, i.e. group Terrae or group III to which *M. ulcerans* belongs, the reading is taken once a week. The characteristic cauliflower-like appearance of *M. ulcerans* colonies is rarely recognized before 21 to 28 days of incubation.

The culture is usually declared negative if after 12 weeks of incubation there has been no colony development. It is noted that this result may be questioned if a growth occurs before the end of the incubation period.

Mycobacteria of the terrae complex are susceptible to only a few antibiotics in vitro. In Smith's study [10], all strains tested were sensitive to azithromycin MIC < 32 µg/ml to clarithromycin, only 57% to amikacin and 50% to streptomycin.

Virtually all are resistant to rifampicin, izoniazid, clofazimine and fluoroquinolones.

In practice, treatment should always combine surgery with antibiotic therapy, which could include: macrolide, azithromycin or clarithromycin combined with ethambutol or rifampicin.

Table 4: Biochemical characteristics of species belonging to the Terrae Complex.

	M. non chromogenicum	***M. terrae***	***M. trivial***
Culture 25°C	+	+	+
Culture 37°C	+	+	+
Culture 45°C	-	-	-
Catalase > 45 mm	+	+	+
Thermostable Catalase	+	+	+
Hydrolysis Tween 80	+	+	+
Acid phosphatase	+	+	±
Betagalactosidase	+	+	+
Aryl sulgatase 10d	+	±	±
Nitrate reductase	-	-	+
Nacl 5% culture	-	-	+
Tellurite reduction 10d	-	+	-

- **Culture in liquid media**

Figure 5: Liquid seeded media

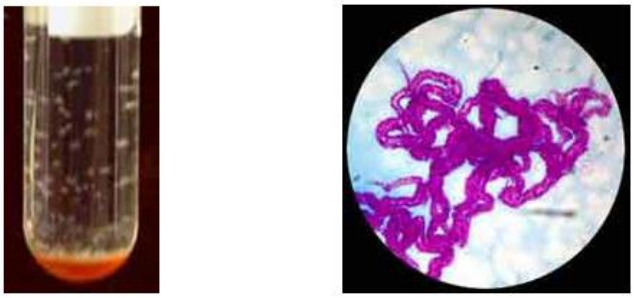


Figure 6: Colonies of *M. ulcerans* in liquid media.

Culture and histology

The search for M. ulcerans must be done by taking samples in situ at the lesion and must be repeated because the number of germs is often small. A well performed biopsy, under local anaesthesia and involving the deep dermis, is the best sample. The detection of extracellular acid-fast bacilli on direct examination of a sample from the ulcer is positive in one third to two thirds of cases, depending on the series. The histology is characteristic of the extent of inflammation and necrosis of the hypodermis by septal and lobular panniculitis and leucocytoclassic vasculitis. The bacilli are well stained by the Zielh-Neelsen method and are objectified in the collagen of the fascia or within the fatty lobules at the edge of the ulceration. Obtaining colonies on Löwenstein-Jensen or Coletsos medium is difficult and time consuming but does not exceed 10 weeks to 30. The colonies obtained are eugonic and colourless.

	M. marinum M	*M. ulcerans* Agy99
Genome size and arrangement	• Circular chromosome; 6,637 kb • Circular mercury resistance plasmid (pMM23; 23 kb)	• Circular chromosome; 5,632 kb • Circular mycolactone-associated plasmid (pMUM001; 174 kb)
Number of genes	5,424	4,160
Number of pseudogenes	65	771
Number of insertion sequences (ISs)	7 ISs; *Myma01* (7 copies), *Myma02* (7 copies), *Myma03* (4 copies), *Myma04* (5 copies) and *Myma05*, *Myma06* and *Myma07* (2 copies of each)	IS*2404* (213 copies) and IS*2606* (91 copies)
Number of PE and PPE genes	175 PE genes and 106 PPE genes	69 PE genes and 46 PPE genes
Number of ESX secretion systems	5	3
Number of ESX effectors	18 *espA* paralogues and 31 *esx* paralogues	2 *espA* paralogues and 14 *esx* paralogues
Number of phospholipase C genes	Seven phospholipase C paralogues (*plcB*, *plcB1*, *plcB2*, *plcB3*, *plcB4*, *plcB5* and *plcB6*)	One gene that encodes phospholipase C (*plcB*); others have become pseudogenized or lost by DNA deletion
Number of lipoproteins	88 genes that encode lipoproteins	77 genes that encode lipoproteins; *lipY* has been lost by deletion
Phenolic glycolipids	Mycoside M	Phenolphthiodiolone lipid backbone cannot be glycosylated as the glycosyl transferase (MUL_1998) has been pseudogenized
Mycolactone	Not produced	Mycolactone A and B

Figure 7: *Comparison of the M. Ulcerans genome to that of M. marinum*

(A) Phylogenetic tree of mycobacteria (16S rRNA).

(B) Point-by-point comparison of *M. ulcerans* and *M. marinum*.

The bacteria are derived from the same ancestor and have a genetic humology of 98.3%.

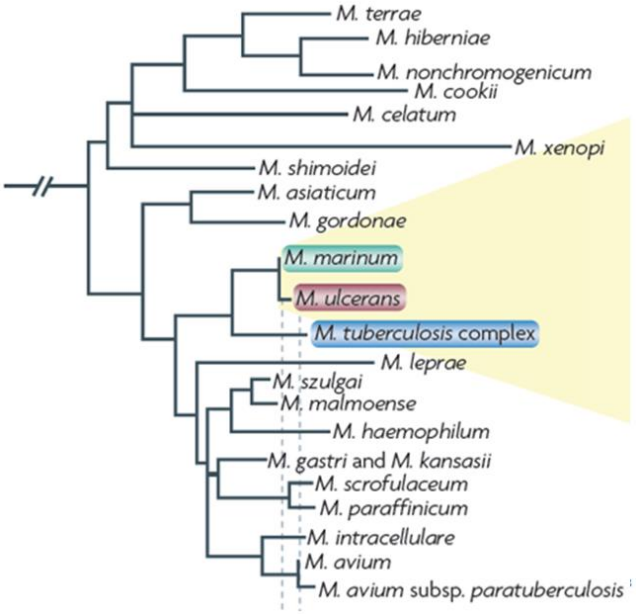


Figure 8: Family tree of *M. Ulcerans*

Phenotypic method

The individualization of species of the Terrae complex will be carried out by traditional techniques; the following will be taken into account: cultural characteristics, time of appearance of colonies, rough or smooth appearance, biochemical characteristics: niacin test, reduction of nitrates, sensitivity to

thiophene-2-carboxylic acid hydrazide 2 mg/L (T.C.H.), to cycloserine 30 mg/L or to pyrazinamide 200 mg/L (P.Z.A.). These tests require rich cultures and can therefore only be performed after several days or weeks. These tests are controlled by positive and negative controls performed with reference strains. The interpretation is made according to the results shown in table 5.

Table 5: Characteristics used to differentiate species of the *tuberculosis* complex from atypical Mycobacteria including *M. ulcerans*

Aspect Colonies	***M. tuberculosis***	***M. bovis***	***M. africanum***	**BCG**	***M. atypical***
	Rough	Smooth	Rough	Rough	Rough/Smooth
Pigmentation	Not pigmented	Not pigmented	Not pigmented	Not pigmented	+/- pigmented
Time limits for culture	10 à 20 days	30 à 60 Days	30 à 60 days	10 à 20 Days	4 à 30 days or more
Niacin test	Positive	Negative	Negative *	Negative	Negative
Nitrate reductase	Positive	Negative	Negative * or positive	Negative	Positive/ Negative
TCH	R	S	S*	S	R/S
PZA	S	R	S	R	
Cycloserine	S	S	S	R	

R: resistant / S: sensitive.

Technical details of the microscopic examination

- **Performing the smear test**

From the pathological product, the sampled product is examined, with a looped loop, a part is taken. It will be spread on a slide, carefully degreased and previously identified with the name or number of the corresponding patient. The smear will be spread at the end of the slide by a back and forth movement of the loop on the slide so that an area of 20 minutes long and 10 minutes wide is covered. A smear of the correct thickness should enable the characters of a newspaper to be distinguished when viewed through a transparency. After Ziehl's staining, such a smear will appear slightly blue in colour. The smear is air-dried; the use of a hot plate accelerates the drying process.

- **Fixation**

Before staining, the product deposited on the slides is fixed. Several methods are used: heat and methyl alcohol. Their purpose is the same: to denature the proteins of the product to allow a better adhesion to the support. The safest methods consist in covering the smear held on the hot plate with methyl alcohol until complete evaporation or by cold immersion for 10 minutes. Heat fixation involves passing the slide rapidly three times through the flame of a Bunsen burner so that the face of the smear is directly exposed to the flame. The method of covering the slide with alcohol and igniting it is a compromise between the two previous methods.

- **Colouring of Ziehl Neelsen**

Hot is the reference method. It can also be performed cold, in which case the slides are immersed in a fuchsin bath for at least three hours. It is convenient but less sensitive than the original hot method.

The decolourisation can be done in different ways with a strong diluted acid and alcohol. Amand's blue or Fraendel Gabbet's reagent, which combines acid, alcohol and methylene blue in the same reagent, allows decolourisation and counter-colourisation to be carried out in the same operation, but the results obtained are of lesser quality.

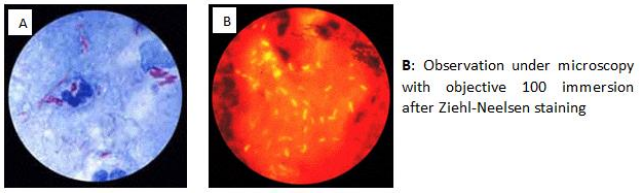


Figure 9: Observation by microscopy with a 100 mm immersion objective stained by the Ziehl-Neelsen method

- **Auramine staining by fluorescence microscopy**

The BAARs are observed on a bright yellow background against the dark red background of the preparation; they are easily seen at 400 magnification (40X objective, 10X ocular). The microscopic field scanned is then 5 times larger than in immersion. Experienced techniques use the 250 magnification, the field observed is then about 16 times larger than that examined in immersion. Variations exist which use rhodamine, acridine orange. In our work, we used **Ziehl Neelsen** staining and florescence.

Expression of results

The result of the microscopic examination is expressed quantitatively, which makes it possible to control the progressive disappearance of the bacilli under treatment.

Table 6: Expression of results of microscopic examination

Number of observed BAARs		
Absence	After fuchsin staining (X 1000)	After staining with fluorochrome (X 250)
Absence	0	0
Doubtful	1-2/200 fields	1-9/frottis
1+	1-9/100 fields	1-9/10 fields
2+	1-9/10 fields	1-9/field
3+	1-9/field	10-99/field
4+	10/field	100/field

Cultivation

- **Solid media**

Solid culture media are agar or egg media.

Figure 10: *M. ulcerans* culture gallery

- **Atypical mycobacteria**

- **Atypical mycobacteria**

Bacteriologically, atypical mycobacteria differ from *M. tuberculosis* in colony appearance, pigmentation, growth rate and biochemical properties.

Right: Various cultural aspects on Loewenstein-Jensen medium and after varying incubation times.

Figure 11: Gallery of *M. ulcerans* culture on solid media cast in tubes for 30 weeks

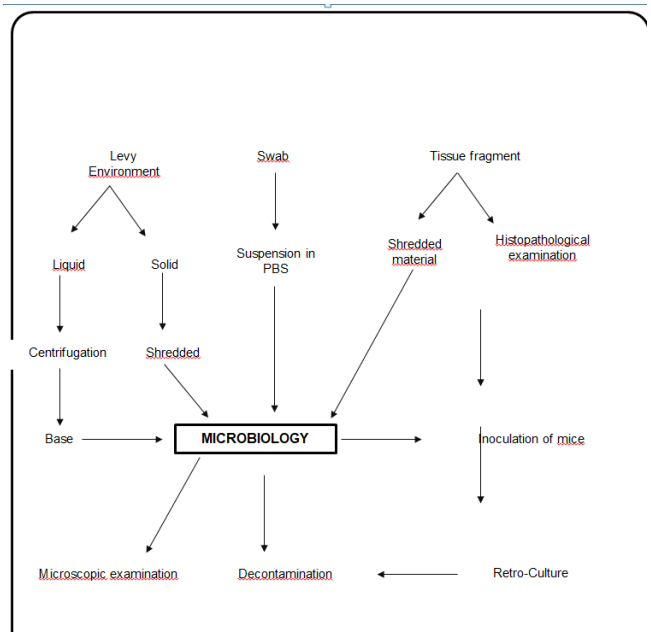


Figure 12: Implementation of microbiological diagnostic procedures on samples that may harbour M. ulcerans.

Diagnosis of *M. ulcerans* infections by PCR

Genetically, the three species in the complex have a 16S RNA profile that clearly differentiates them from other species, and within the complex from each other. Thus, using the PRA method, it is possible to differentiate the species.

BstE II	***Hae III***	
No digestion	180/140	*Mr. Trivial*
325/120	190/140	*M. terrae*
245/120/30	155	*M. non chromogenicum II*
245/120/80	160	*M. non chromogenicum I*

Molecular aspects: Molecular identification has become the preferred method. It is based on the characterization of nucleic acid sequences amplified then hybridized with probes (GeneProbe® technique, Innolipa® technique).

GenProbe® Technique

Innolipa® Technique

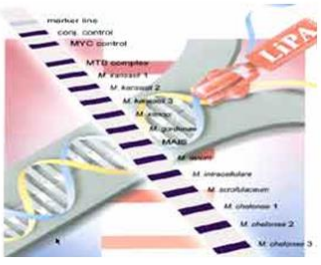


Figure 13: Molecular identification of the insertion sequence: IS2404

Pathogenicity of M. Ulcerans

Pathophysiology of M. ulcerans infection

M. ulcerans is related to M. marinum, but differs significantly from it in several aspects [10]. Clinically, M. marinum causes rare localized skin infections such as swimming pool granuloma [11]. Microbiologically, M. ulcerans has a doubling time of 50 hours compared to 4 to 10 hours for M. marinum, and does not produce photochromogenic pigments [12]. The composition of the mycobacterial wall appears to be significantly altered in M. ulcerans [13]. M. Marinum has an intracellular lifestyle, while M. ulcerans has a mixed intracellular and extracellular lifestyle. M. ulcerans has the unique characteristic among mycobacteria to produce a hydrophobic toxin, mycolactone.

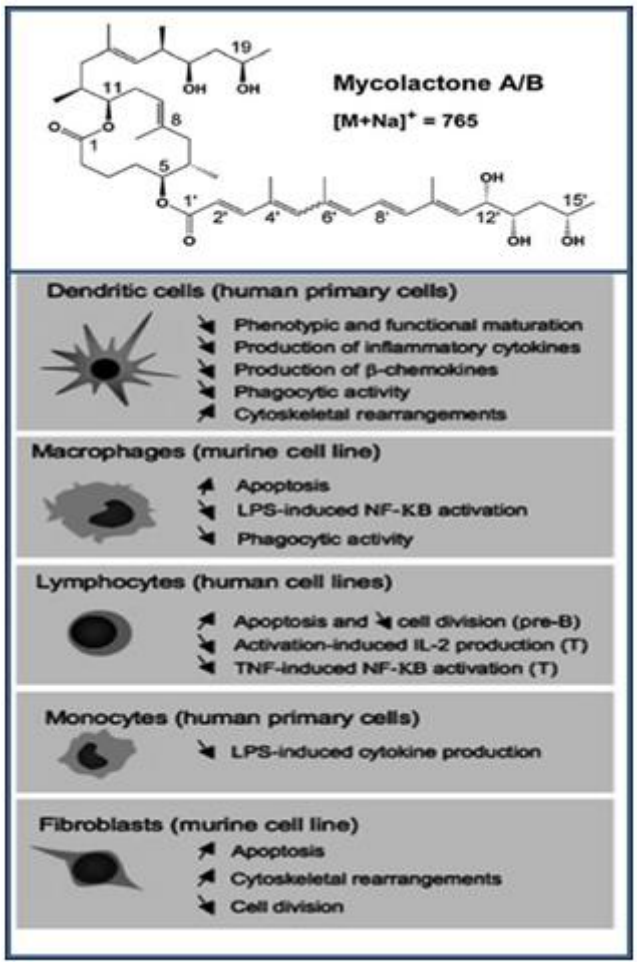


Figure 14: Mycolactone, a diffusible cytotoxic and immunomodulatory toxin of *M. ulcerans*

(A) Chemical structure of mycolactone A/B, with its lactone ring and two side chains

(B) Summary of cytotoxic and immunomodulatory activities of mycolactone *in vitro*

These gene losses reveal adaptation to a particular ecological niche, probably made possible by the acquisition of a giant 174 kilobase (kb) plasmid, pMUM, encoding several enzymes required for mycolactone production [14]. Different strains of M. ulcerans produce different mycolactones (A to F), the most active being mycolactone A/B, produced by African strains. They all share a cyclic lactone ring, classifying the mycolactone in the macrolide family, and two polyketide side chains [15].

Mycolactone: a major virulence factor of M. ulcerans

The typical histology of M. ulcerans infection shows large areas of fat necrosis in the subcutaneous tissue, in which impressive quantities of predominantly extracellular bacilli proliferate, clustered by the production of an extracellular matrix, contrasting with a sparse peripheral inflammatory infiltrate [16]. Vasculopathy with endarteritis and thrombosis is also observed. Mycolactone is the main cause of these lesions. In guinea pigs, subcutaneous injection of purified mycolactone reproduces the lesions caused by subcutaneous inoculation of M. ulcerans, both clinically and microscopically, whereas a mutated strain of M. ulcerans that does not produce the toxin causes no clinical lesions [17].

Mycolactone secreted by M. ulcerans diffuses into the lesions and can be identified in the blood of infected patients [18]. At the cellular level, it diffuses passively across membranes into the cytoplasm and shows in vitro (keratinocytes, fibroblasts, adipocytes, lymphocytes, monocytes, macrophages, dendritic cells, neutrophils) and in vivo (mice, guinea pigs, humans) highly cytotoxic and pro-apoptotic properties [19]. In epithelial cells, this cytotoxicity is mediated by the binding of mycolactone to the N-WASP protein, which triggers a major reorganization of the cytoskeleton, leading to a loss of cell adhesion and the initiation of a cell death program [20].

In addition to its cytotoxicity, mycolactone has at lower concentrations, in vitro, a highly polymorphic immunomodulatory role [22]), affecting monocytes, macrophages, dendritic cells and T cells. Mycolactone can inhibit the production of the proinflammatory cytokine TNFα by monocytes and macrophages and modulate the production of multiple cytokines by monocytes and dendritic cells [23]. The efficiency of phagocytosis by macrophages and dendritic cells is impaired by mycolactone, as well as the transfer of dendritic cells and naive T cells to the lymph nodes, and thus the activation of the adaptive immune response [24]. The in vivo biological relevance of these effects remains unclear because the actual concentration of mycolactone in the lesions is unknown. Infection with M. ulcerans triggers an immune response that allows control of the infection even in the absence of treatment. Notably, while mycolactone is thought to have an immunosuppressive effect [25], there are no opportunistic infections in the natural history of BU. In mice, it has been shown that the immunosuppressive effect is actually local (at the lesion) and regional (at the lymph nodes) but not systemic [26].

Mycolactone is also involved in the physiology of one of the most striking features of BU. The lesions caused by M. ulcerans are painless despite their

sometimes spectacular extension. This characteristic is the cause of significant delays in consultation and management. Mycolactone caused a hyperpolarization of the neuron membrane inducing analgesia. This hyperpolarization follows activation of the angiotensin 2 receptor, AT2R, by mycolactone. Activation of the arachidonic acid pathway by AT2R results in hyperpolarizing potassium efflux via TRAAK-type potassium channels [27]. Fifteen years after the identification of mycolactone, two molecular targets have been identified, the WASP/N-WASP proteins involved in programmed cell death in epithelial tissues and AT2R responsible for the analgesia observed in BU.

- Cytology of M. ulcerans

M. ulcerans: an intracellular and extracellular germ

Mycobacteria are generally considered a prototype of facultative intracellular pathogens [28]. The extracellular predominance of M. ulcerans on histological sections has raised fundamental questions about the biology of the bacillus, the type of immune response it induces, and therefore the most likely effective vaccination model to use against it. However, it seems clear that the bacterium has an intracellular stage of development, as suggested by histological, immunological and clinical findings.

There is evidence of intracellular invasion by the bacterium in mice and humans throughout the course of infection [29]. In vitro, the mycobacterium multiplies in a murine macrophage model, and in vivo we observe macrophages with cytoplasm saturated with mycobacteria, also suggesting intracellular multiplication [30]. Histologically in humans, healing lesions show a significant inflammatory infiltrate, the formation of gigantocellular granulomas without caseous necrosis, and the intracellular presence of the bacteria [31]. The adaptive immune response against M. ulcerans is primarily cellular and has a TH1 profile that is more commonly associated with intracellular organisms [32]. It has been shown in a mouse model of the interferon-gamma gene that this cytokine controls infection with a strain of M. ulcerans provided that the strain does not produce mycolactone. Mycolactone secretion would inhibit phagolysosome acidification and could represent a new mechanism of immune evasion by an intracellular pathogen [33]. Finally, clinically, a delayed hypersensitivity reaction, a marker of cellular adaptive immunity targeting an intracellular germ, in this case provoked by the intradermal injection of a M. ulcerans sonicate, burulin, is observed in 70 to 100% of patients, depending on the series [34].

The identification of intracellular bacilli and a cell-like immune response leading to granuloma formation and delayed hypersensitivity are arguments for considering M. ulcerans as a facultative intracellular pathogen. Its physiology is nevertheless unique following the acquisition of the pMUM plasmid and the production of mycolactone, a molecule with multiple fascinating properties (cytotoxicity, immunomodulation, analgesia, possibly antisepsis). These properties sometimes appear contradictory for an understanding of the biology of M. ulcerans (e.g. inhibition of phagocytosis and cytotoxicity compatible with an extracellular lifestyle vs. inhibition of phagolysosome acidification compatible with an intracellular lifestyle).

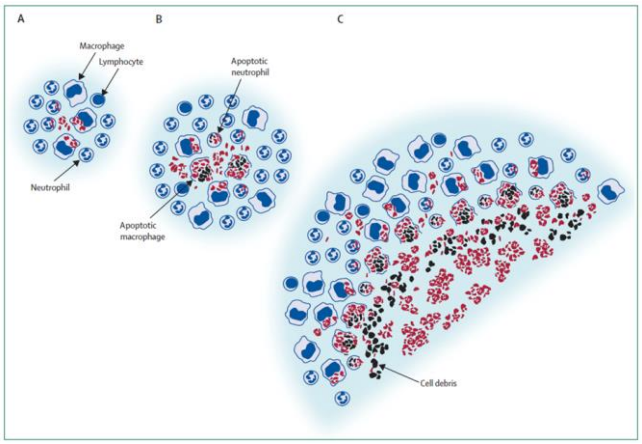


Figure 15: Pathophysiological model of M. ulcerans infection

(A) Early stage of infection: phagocytosis of *M. ulcerans* by macrophages and neutrophils

(B) Intermediate stage: continued phagocytosis; apoptosis of early phagocytic cells;

production of mycolactone by extracellular bacilli

(C) Advanced stage, typical of anatomopathological diagnostic lesions: in the centre of the necrotic lesion, large number of extracellular bacilli; at the margin of the necrosis, inflammatory infiltrate including intracellular bacilli

Discussion

Despite its rapid progression since the 1980s, BU remains a neglected infectious disease. The CDTUB of Pobè is currently at the forefront of clinical and basic research on this disease [7,9,10,13,25,30,34]. Our epidemiological study benefits from several fundamental characteristics whose combination is unprecedented in the context of BU: (i) it is a study with a meticulous prospective data collection leading to a low proportion of missing data. We described the clinical presentation of BU, its variation according to age and sex of the patients and its impact on the occurrence of functional sequelae. Several epidemiological features of BU consistently reported by previous studies were confirmed, supporting the generalizability of our results: BU is a predominantly pediatric disease (median age at diagnosis 12 years); lesions are overwhelmingly unifocal (>90%) [19,27,29-31]; they predominate on the lower limbs (≅60%) [35]; ulceration is the most common clinical form (≅70%) [35]; a significant proportion of patients present with osteomyelitis (>5%) [35]; functional sequelae are common (>20%) [35].

In our study, the risk of developing *M. ulcerans* osteomyelitis was significantly higher in male patients (OR=2.21, 95%CI [36]) and tended to be higher in younger patients. Interestingly, the effect of age on the risk of osteomyelitis was significant when the sample size was increased by considering all patients, regardless of their diagnostic confirmation by laboratory examination.

We report for the first time cases of exclusive osteomyelitis in patients without active or past skin lesions of BU, which represent 17% of *M. ulcerans* osteomyelitis. We applied stringent criteria to define exclusive osteomyelitis, which implies that this estimate represents a lower bound. This is also the conclusion of the specific study carried out by Virginie Pommelet, who estimates that 25% of osteomyelitis is exclusive [37]. These patients came to the clinic with a clinical osteomyelitis of non-specific appearance and the infection by *M. ulcerans* was only identified through complementary examinations. Our observation would remain of great clinical, epidemiological and pathophysiological relevance.

CONCLUSION

The clinical relevance is obvious: *M. ulcerans* is an etiological candidate in the face of osteomyelitis in a UB-endemic area, which implies sampling for the bacterium and possible therapeutic adaptation. As far as pathophysiology is concerned, these exclusive cases are proof that osteomyelitis in BU does not necessarily result from uncontrolled multiplication of the bacteria in its tissue of choice, the skin. Some individuals are susceptible to bone invasion by moderate amounts of bacteria without contiguous tissue destruction. It is important to note that we do not question the mode of transmission of *M. ulcerans*: we do not assert that patients have not been bitten or bitten by an insect vector, or that a skin abrasion has not been infected by contact with contaminated water. We report that some patients develop bone lesions of BU in the absence of a massive skin reservoir for the mycobacterium. Given the local clonality of the bacterium [38,39], this observation raises questions about the source of interindividual variability in the immune response to this pathogen. This is an important point that could be explored by different studies, in particular genetic ones. Finally, this observation is in favour of a mechanism of haematogenous dissemination of the bacterium from the site of inoculation.

We systematically evaluated the impact of clinical presentation on the fixation of functional sequelae. Four elements of the clinical presentation of BU were associated with a significantly longer recovery time and were at risk of functional sequelae. We therefore propose an operational definition of a severe case of BU as a clinical course involving an edematous form, a bony form, an extensive lesion (≥15 cm) or multiple lesions (multifocality). The strength of the association between the severity of the clinical presentation of BU as defined here and the development of functional sequelae (OR=7.6) is of unquestionable clinical significance. Patients with one of these four types of injury should benefit from specific clinical care, with close follow-up, intensive functional rehabilitation and optimal surgical reconstruction management. Our observation also raises the question of why some patients develop a severe form of BU. As the time to diagnosis is difficult to estimate, we can conjecture that a longer diagnostic delay would explain the severity of the clinical presentation in some patients. Some severe forms, such as the edematous form, are nevertheless potentially rapid in progression. The identification of patients who have experienced a rapid progression of BU to a severe form would be interesting to explore inter-individual variations in susceptibility to BU.

Several elements in our study suggest an underestimation of the incidence of BU in Africa. The CDTUB is located a few kilometers from the Nigerian border and receives Nigerian BU patients every year. While the annual number of BU cases reported by Nigeria to WHO is usually less than ten (e.g., 7 in 2010), about 20 cases are treated each year at the CDTUB. The comparison of the areas of Benin and Nigeria suggests that the underestimation of the number of BU cases in Nigeria is major. In addition, the lack of diagnosis of BU in some atypical presentations, such as exclusive osteomyelitis, also contributes to the underestimation of BU incidence.

Our study also highlighted some areas where data collection needs to be more precise to differentiate between different situations, particularly with regard to recovery from BU. It is not clear how to differentiate between a recurrence (a short-term infectious relapse due to a living population of *M. ulcerans population* not completely eradicated), a paradoxical reaction (a non-infectious relapse due to excessive immunological reactivity, possibly related to the lifting of local immunosuppression by mycolactone), a reactivation (a long-term infectious relapse due to the emergence of *M. ulcerans* from latency) and a reinfection (an independent episode of BU). We have observed about ten patients in whom new BU lesions appeared several years after the healing of the initial episode. By analogy with tuberculosis, this could be a reinfection or reactivation of latent bacteria. Characterizing these patients would be particularly important for understanding the immune response to *M. ulcerans* with a view to developing a vaccine and new therapies. [40].

The most sensitive method, 98% and 100% specific in the absence of contamination of the material by previous samples, but often difficult to access in endemic areas, is PCR amplifying the IS2404 insertion sequence [41]. It should be noted, however, that this method does not allow follow-up of treatment, since it remains positive by amplification of microbial debris, long after clinical recovery. Finally, pathological examination of a biopsy can reveal characteristic aspects of M. ulcerans infection with a high sensitivity of about 90%. These include massive

necrosis of the subcutaneous fatty tissue (with ghost cells - necrotic adipocytes), a large number of mainly extracellular BAARs, a weak inflammatory infiltrate compared to the bacillary load, restricted to the borders of the lesion, and thrombotic inflammatory vascular involvement [42]. Furthermore, the diagnosis of paradoxical reaction is based on (i) the secondary unfavourable evolution of a UB lesion treated with antibiotics, or the appearance of a new lesion at a distance from the initial site and (ii) a pathological examination identifying an intense inflammatory infiltrate [43].

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