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Phenotypic and Genotypic Characterization of Mycobacteria Isolates from Buruli Ulcer Suspected Patients Reveals the Involvement of Several Mycobacteria in Chronic Skin Lesions

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Abstract Buruli ulcer is a cutaneous mycobacterial disease that occurs in tropical countries in sub-Saharan Africa, South-East Asia, Australia and America. The responsible pathogen is *Mycobacterium ulcerans*. Côte d'Ivoire is the most affected country, with more than 30 endemic health districts reporting a large number of chronic skin lesions. The clinical forms and the severity of ulcers vary from one patient to another. Samples from suspected patients were analyzed by PCR at Pasteur Institute of Côte d'Ivoire, as recommended by WHO. IS2404 sequence was detected in 61% of cases, incriminating *M. ulcerans* in chronic cutaneous lesions. For the other cases the etiology was not identified, thus raising several questions. Are all reported "Buruli ulcer" cases really caused by *M. ulcerans*? Would other mycobacteria be involved in the occurrence of chronic skin lesions considered as "Buruli ulcer"? BU suspected patients were enrolled in endemic areas of Côte d'Ivoire. Samples were collected from cutaneous lesions and transported to the lab at +4°C in 2 ml of Middlebrook 7H9 medium supplemented by Cetylpiridium chloride. The centrifugation pellet was taken with saline buffer to perform microscopic examination and mycobacteria isolation on Lowenstein-Jensen medium. Biochemical characteristics were described by the nicotinic acid detection according to Konno protocol, by the Nitrates reduction test, by the catalase activity detection at 22° and 68°C and the Wayne's Tween 80 hydrolysis test. Genotypic characteristics were determined by PCR with 1 ml of bacterial suspension targeting the insertion sequences (IS6110, IS2404, and IS2606), the plasmid virulence genes and Miru-VNTR loci (Miru-1, VNTR 6, VNTR 19, ST-1). A total of 47 mycobacterial strains were isolated with 3 different types of colonies whose microscopic examination showed Acid-Alcohol-Resistant Bacilli. 65.9% of isolates expressed biochemical characters in favor of *M. ulcerans* strains and 6.4% in favor of *M. marinum* strains. For 29.8% of isolates, the characteristics were related to atypical mycobacterial species. The genotyping targeting the IS6110, IS2606 and IS2404 insertion sequences allowed simultaneous amplification in 53.2% of isolates. IS2404 was amplified in 93.6% of isolates; IS6110 was amplified in 74.5% of isolates and IS2606 was amplified in 70.2% of isolates. Five genotypes were identified corresponding to various species of mycobacteria: genotypes 1 and 2 accounted for 63.8% with all the 3 insertion sequences and biochemical characteristics in favor of *M. ulcerans* strains; genotype 4 accounted for 6.4% of isolates with insertion sequences and biochemical characteristics in favor of *M. marinum* strains; The strains of genotypes 3 and 5 expressed molecular and biochemical characters relating to various *non-M. ulcerans* mycobacteria. Virulence genes were found in 72.3% of isolates corresponding to 90% of *M. ulcerans* strains and 60.7% of *non-M. ulcerans* mycobacteria. This study confirmed the involvement of several genotypes of *M. ulcerans* and other mycobacteria in chronic cutaneous lesions suspected as cases of Buruli ulcer in Côte d'Ivoire.

Keywords: *Buruli ulcer, M. ulcerans, insertion sequences, other mycobacteria, virulence genes*

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1. Introduction

Buruli ulcer (BU) is a cutaneous mycobacterial disease that occurs in thirty tropical countries, especially in sub-Saharan Africa [1,2,3,4,5], South-East Asia [6,7,8,9], Australia [10,11,12] and Latin America [13,14,15]. The responsible pathogen of the disease is *Mycobacterium ulcerans*, a slow-growing mycobacterium. Côte d'Ivoire is one of the most affected countries, with more than 30 endemic health districts whose have been reporting for many years a large number of chronic skin lesions attributed rightly or wrongly to *M. ulcerans* [4]. The evolution of *M. ulcerans* infection occurs in two successive stages: a pre-ulcerative phase marked by the appearance of a nodule, a plaque or an œdema. The pre-ulcerative forms and the severity of ulcers vary from one patient to another. Some patients have small lesions that are easy to cure while others have large ulcers for a long time. Biological confirmation is routinely performed by Polymerase Chain Reaction (PCR) methods targeting the IS2404 insertion sequence and ketoreductase (KR) in clinical samples, as recommended by WHO [16]. Several samples of BU suspected patients were analyzed by the National Buruli Ulcer Reference Center of Pasteur Institute of Côte d'Ivoire. For several years, the molecular diagnosis results incriminated at most *M. ulcerans* in 61% of those samples [4,17]. For the other cases (39%), the etiology was not identified, thus raising several questions. Are all reported BU cases really caused by *M. ulcerans*? Would other mycobacteria be involved in the occurrence of chronic skin lesions liked observed in BU? Would there be a genetic diversity of *M. ulcerans* strains in relation to the clinical polymorphism observed in endemic areas? These questions remained for a long time unanswered, prompted the study of several isolated strains of BU suspected patients in several endemic areas of Côte d'Ivoire. The aim of this work was to study the bacteriological, biochemical and molecular characteristics of isolated mycobacterial strains from cutaneous lesions considered as cases of *M. ulcerans* infection.

2. Patients and Methods

2.1. Patient's Enrollment and Mycobacterial Isolation

BU suspected cases (patients with nodule, plaque, œdema or ulcer) were enrolled in nine endemic areas of Côte d'Ivoire. Epidemiological and clinical informations were collected using UB 01, as recommended by WHO. Samples consisted of exudates or fine needle aspiration fluid [18] were discharged into a tube containing 2 ml of Middlebrook 7H9 medium supplemented by Cetylpiridium chloride (0.5%) and transported to the lab at +4°C. 10 ml of sterile water were added to the samples previously placed in a 15 ml Falcon tube. The mixture was centrifuged at 3000 rpm for 20 minutes

The pellet was resuspended in 1 ml of saline buffer (NaCl, 9‰) to inoculate four tubes of Löwenstein-Jensen medium. The tubes were incubated at 32°C and a daily observation was instituted till the appearance of colonies [19].

2.2. Phenotypic and Molecular Characterization of Strains

Several tests have been performed to describe the phenotypic and genotypic characteristics of isolates. The cultural characteristics were described based on the timing of colony appearance, the pigment secretion, and the morphology of colonies. Microscopic examination to detect Acid-fast bacilli was performed by using dried smears made with 5 µl of bacterial suspension and staining by the Ziehl-Neelsen coloration [20,21]. Biochemical characteristics were described by the Nicotinic Acid detection according to Konno protocol (Niacin-test), by the Nitrates reduction test, by the catalase activity detection at 22° and 68°C, and the Wayne's Tween 80 hydrolysis test [1,21]. The genotypic characteristics were determined by molecular tests using 1 ml of bacterial suspension. The strains were first characterized using insertion sequences (IS6110, IS2404, IS2606) secondly the plasmid virulence factor coding gene (Ketoreductase, KR) and then a VNTR typing. The insertion sequence IS6110 is common to the genus *Mycobacterium* wilton et cousin 1992 [22] while the insertion sequences IS2404 and IS2606 [23] are carried by *M. ulcerans's* genome respectively in about 250 and 100 copies. The third tests targeted minisatellite markers: MIRU-VNTR (Mycobateria Interpersed Repeated Units- Variable Number of Tandem Repeated). The using method is as described by Stragier and several authors [24,25,26,27]. A set of four markers have been used: MIRU1, ST1, VNTR6 and VNTR19. Those markers permitted to distinguish *M.ulcerans* from other mycolactone producing *Mycobacteria* (MPM) and *M.ulcerans* like- strains (*M. marinum, M. Pseudoshotsii*).

PCR were performed in a 9700 PCR system Thermocycler (Applied Biosystems, USA) [28,29,30,31,32]. The IS6110, IS2404, IS2606 and ketoreductase (KR) were amplified according to the following program: a pre-denaturation phase at 94°C for 2 minutes, followed by 35cycles consisted of a denaturation phase at 94°C for 1 minute, an annealing at 66°C for 1 minute and extension phase at 72°C for 1 minute. A final extension at 72°C for 5 minutes stopped the reaction. PCR products were revealed on a 1.5% gel electrophoresis containing Sybr Green. A 100 bp molecular weight marker was used to evaluate PCR products size. For Genotyping, the thermocycler (GeneAmp 9700) was programmed as following: an initial denaturation for 5 min at 95°C, followed by 40 cycles of 94°C for 1 min, 58°C for 1 min (MIRU-1, locus 6, VNTR 19) or 65°C (for ST-1) and 72°C for 1 min. DNA inhibition was tested by serial dilution of the DNA extract. Positive DNA samples of M. ulcerans and M. marinum from the Institute of Tropical Medicine were tested for each amplification.

The GoTaq G2 Flexi DNA polymerase kit (Promega Corporation, USA) was used for the PCR mixes containing 0.2µM of each primer, 1.5µM, MgCl, 0.1µM dNTPs, 1 unit Taq polymerase, 1X of buffer and 3µl of DNA template for a final volume of 50µl.The revelation was made on a GelDoc Bioanalyzer (BioRad) after electrophoresis.

The size of the amplicons was used to determine the copy number of the targeted sequence and the profile result was given in code form characterizing one type of polymorphism [25,26].

Table 1. Methods, targets and primer's sequences used for molecular characterization

Targets	Primers	Direct and indirect Sequences	Amplicons size (pb)	References
Mu5/6	IS2404	GAT CAA GCG TTC ACG AGT GA GGC AGT TAC TTC ACT GCA CA	600	Ross et *al*., 1997
Mu7/8	IS2606	CCG TCA CAG ACC AGG AAG AAG TGC TGA CGG AGT TGA AAA ACC	332	Stinear et al, 1999
Mycgen-F/R	IS6110	AGA GTT TGA TCC TGG CTC AG TGC ACA CAG GCC ACA AGG GA	1030	Wilton et Cousin (1992)
KR-F/R	PKS	TCA CGG CCT GCG ATA TCA TTG TGT GGG CAC TGA ATT GAC		Fyfe et *al*., 2007
MIRU1 F/R	MIRU1	GGC TTG ACT CAT GTC ACG TAA G GCT GGT TCA TGC GTG GAA G GCC CTC GGG AAT GTG GTT		
VNTR6 F/R	LOCUS 6	GAC CGT CAT GTC GTT CGA TCC TAG T GAC ATC GAA GAG GTG TGC CGT CT		Stragier et al., 2004
VNTR19 F/R	LOCUS 19	CCG ACG GAT GAA TCT GTA GGT TGG CGA CGA TCG AGT CTC		
ST1 F/R	ST1	CTG AGG GGA TTT CAC GAC CAG CGC CAC CCG CGG ACA CAG TCG		

Table 2. Data from different reference strains used in the study

Reference strains	Origin	IS2404	IS2606	KR	Miru-1	ST-1	Locus 6	Locus 19
ITM 9540	Australia (Queensland)	Positive	Positive (300bp)	Positive	Positive (400bp)	Negative	Positive (400bp)	Positive (350bp)
ITM 94-0821	Côte d'Ivoire	Positive	Positive	Positive	Positive (500bp)	Positive (400bp)	Positive (400bp)	Positive (380bp)
ITM 98-912	China	Positive	Positive	Positive	Positive (400bp)	Positive (400bp)	Positive (500bp)	Positive (500bp)
ITM 842	Suriname	Positive	Positive	Positive	Negative	Positive (400bp)	Positive (480bp)	Positive (400bp)
ITM 97-483	Ghana	Positive	Positive	Positive	Positive (500bp)	Positive (400bp)	Positive (500bp)	Positive (350bp)
ITM 99-0006	Australia (Queensland)	Positive	Positive	Positive	Positive (500bp)	Positive (400bp)	Positive (500bp)	Positive (400bp)

The data of these methods applied to the reference strains are recorded in Table 2.

3. Results

3.1. Microbiological and Biochemical Characteristics of Isolates

47 isolates were obtained on Löwenstein-Jensen medium from 9 endemic areas. Microscopic examination showed Acid-Alcohol-Resistant Bacilli. Three types of colonies were described among the isolates. The first type accounted for 65% of the isolates and consisted of firm, rounded, bulging, yellow-gold colonies. The colonies appeared after 3 to 6 weeks of incubation at 32°C (Figure 1A). The second type accounted for 25% of the isolates and consisted of rough, irregular-shaped, cauliflower-like, whitish, brittle-like colonies. The colonies appeared between 1 and 3 weeks of incubation at 32°C (Figure 1B). The third type of culture represented 10% of the isolates and associated rough, whitish, cauliflower-like colonies and rough yellow-gold colonies with curved surfaces. The colonies appeared after 3 weeks of incubation at 32°C (Figure 1C).

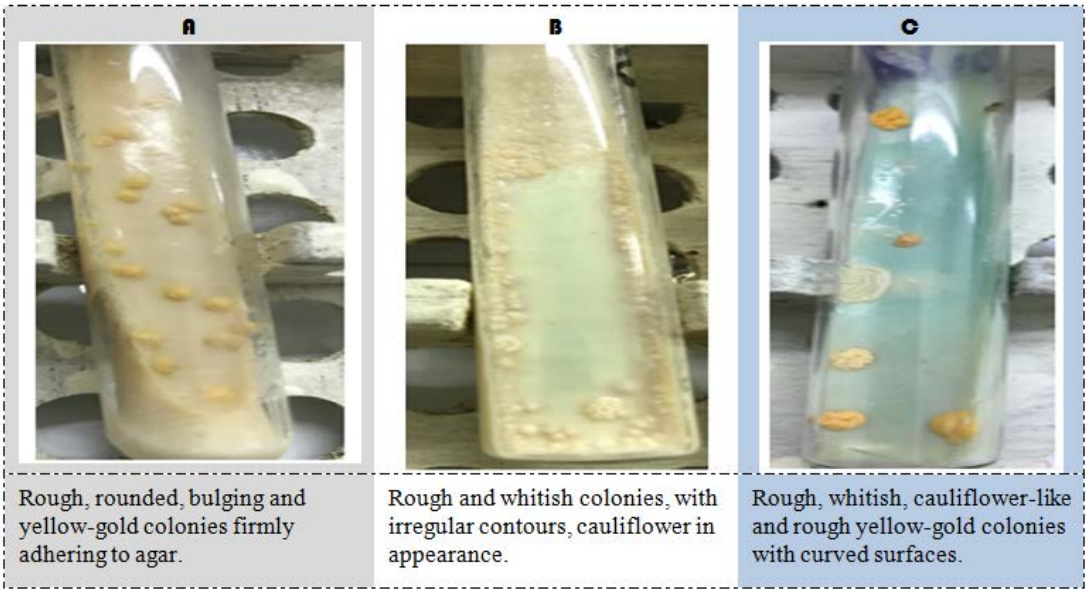

Figure 1. Phenotypic characteristics of bacterial colonies described on Löwenstein-Jensen medium

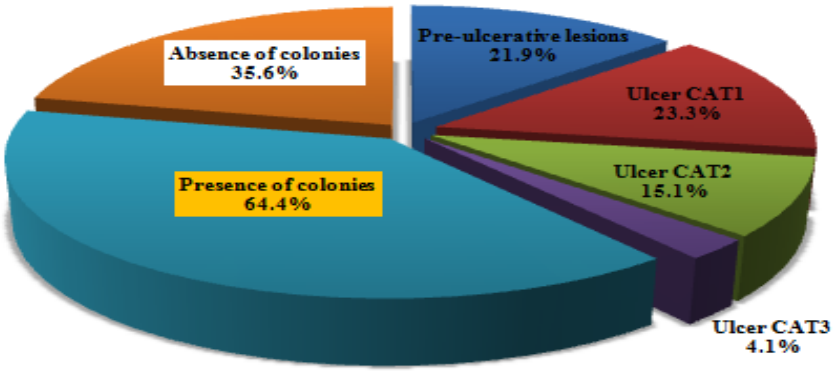

Figure 2. Performance of Mycobacteria isolation on Löwenstein-Jensen medium

Table 3. Microbiological, biochemical and molecular characteristics of mycobacterial isolates

Endemic areas	Isolates (N=47)	C22	C68	NP	NR	HT80	PSA+	IS6110	IS2606	IS2404	Mycobacteria Species
Tonkpi	4	+	-	-	-	-	+	+	-	+	*M. smegmatis*
	5	+	+	-	-	-	+	+	+	+	*M. ulcerans*
Lôh Djiboua	2	+	-	+	-	-	+	-	-	+	*M. chelonae*
	1	+	+	-	-	-	+	+	+	+	*M. ulcerans*
Mé	1	+	-	+	-	-	+	-	-	+	*M. chelonae*
	2	+	+	-	-	-	+	+	+	+	*M. ulcerans*
	1	+	+	-	-	-	+	-	+	+	*M. ulcerans*
	1	+	+	+	-	-	+	+	+	-	*M. marinum*
Gbêkê	2	+	-	-	-	-	+	-	+	+	*M. ulcerans*
	3	+	+	-	-	-	+	+	+	+	*M. ulcerans*
Marahoué	1	+	+	-	+	+	+	+	-	+	*M. smegmatis*
	2	+	+	-	-	-	+	+	-	+	*M. smegmatis*
	1	+	-	-	-	-	+	+	+	+	*M. ulcerans*
	1	+	+	-	-	-	+	-	+	+	*M. ulcerans*
Bélier	2	+	+	+	-	-	+	+	+	-	*M. marinum*
	5	+	-	-	-	-	+	+	+	+	*M. ulcerans*
Agnéby-Tiassa	2	+	+	+	-	-	+	-	-	+	*M. chelonae*
	2	+	-	-	-	-	+	+	+	+	*M. ulcerans*
	1	+	+	-	-	-	+	-	+	+	*M. ulcerans*
Sud-Comoé	3	+	-	-	-	-	+	+	+	+	*M. ulcerans*
Gôh	2	+	+	+	-	-	+	-	-	+	*M. chelonae*
	3	+	-	-	-	-	+	+	+	+	*M. ulcerans*

C22 : Catalase at 22°C ; C68 : Catalase at 68°C ; NP : Nicotinic acid Production ; NR : Nitrate reductase; HT80: Hydrolysis of Tween 80; PSA+: Growth on medium with Para amino Salicylic Acid.
30 strains of *M. ulcerans* (63.8%) ; 3 strains of *M. marinum* (6.4%); 7 strains of *M. chelonae* (14.9%); 7 strains of *M. smegmatis* (14.9%).

The rate of mycobacteria isolation on Löwenstein-Jensen medium was 64.4%. The majority of isolates (45.2%) were obtained with samples collected on pre-ulcerative lesions and ulcer of category 1. Mycobacterial isolation was lower in ulcers of categories 2 and 3, with rates of 15.1% and 4.1%, respectively. In 35.6% of samples from BU suspected patients, no mycobacteria were isolated after 12 weeks incubation at 32°C (Figure 2).

Three groups of isolates were identified based on biochemical characters detected. Most of isolates (31/47) expressed biochemical characters in favor of a *M. ulcerans* strain. In 6.4% of the isolates (3/47), the biochemical characteristics were in favor of a *M. marinum* strain. For 27.6% of the isolates (13/47), the biochemical characteristics were related to atypical mycobacterial species (Table 3).

3.2. Molecular Characteristics of the Isolates

Molecular characterization of mycobacteria with the inserted elements IS6110, IS2606 and IS2404 allowed simultaneous amplification in 53.2% of the isolates (Table 3). Specifically, IS2404 was amplified in 93.6% of the isolates (44/47); IS6110 was amplified in 74.5% of isolates (35/47) and IS2606 was amplified in 70.2% of isolates (33/47). Depending on the number of amplified insertion sequences and biochemical characters expressed, five different genotypes were identified corresponding to various species of mycobacteria (Table 3). The strains of genotype 1 accounted for 53% of the isolates that possessed the three insertion sequences and biochemical characteristics in favor of *M. ulcerans* strains. Genotype 2 is composed of 10.6% of the isolates (5/47) with the IS2606 and IS2404 insertion sequences and expressed biochemical characteristics of *M. ulcerans* strains. Both genotypes 1 and 2 accounted for 63.8% of isolates (30/47) from suspicious lesions of Buruli ulcer. Genotype 4 composed of strains with sequences IS6110 and IS2606 accounted for 6.4% of the isolates. Their microbiological and biochemical characteristics were identical to those of *M. marinum* strains. Genotype 3 accounted for 14.9% of isolates (7/47)

and had sequences IS6110 and IS2404. Genotype 5 also accounted for 14.9% of the isolates (7/47) and had only IS2404 sequence. However, both genotypes 3 and 5 expressed biochemical characters relating to various strains of mycobacteria. The association of microbiological, biochemical and genotypic characters allowed the identification of *M. chelonae* and *M. smegmatis* species isolated (Figure 3).

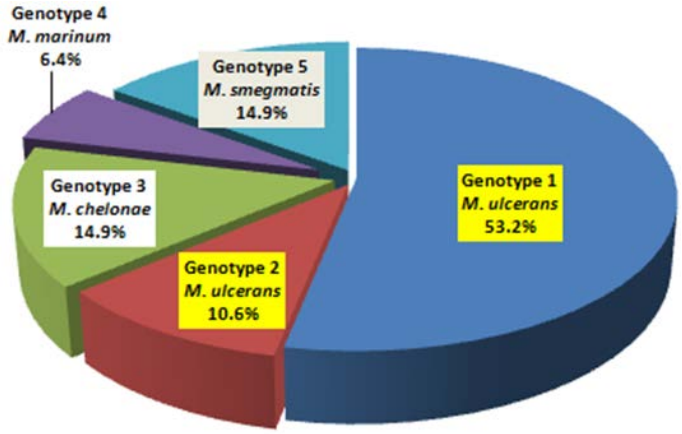

Figure 3. Genotypes identified by targeting IS6110, IS2404 and IS2606 insertion sequences

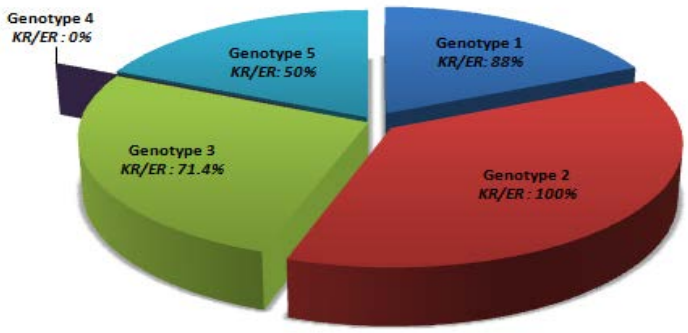

Figure 4. Mycobacterial strains possessing virulence genes among genotypes

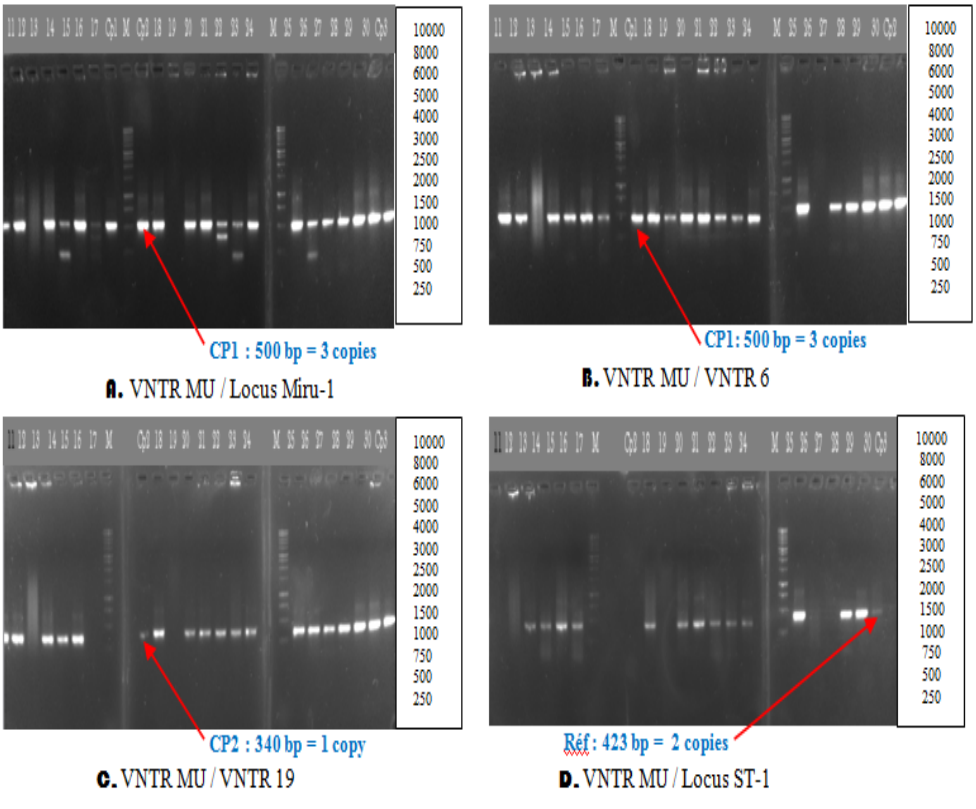

Figure 5. VNTR profiles defined from Miru-1, VNTR 6, VNTR 19 and ST-1 loci typing.

Table 4. Geographical distribution of mycobacterial isolates and VNTR profiles

Régions	Isolats	Séquences d'insertion		Marqueurs MIRU-VNTR				Profil déterminé
		IS2606	IS2404	Miru-1	Locus 6	Locus 19	ST-1	
Tonkpi (n=9)	CI-29 ; CI-37	-	+	-	-	+	+	[0012]
	CI-11 ; CI-08 CI-35 ; CI-28	+	+	+	+	+	+	[3312]
	CI-18	+	+	+	+	-	+	[3302]
	CI-36	-	+	-	+	-	+	[0302]
	CI-13	-	+	-	-	-	-	[0000]
Lôh Djiboua (n=3)	CI-09	-	+	-	+	-	+	[0302]
	CI-10	+	+	+	+	+	+	[3312]
	CI-27	-	+	-	+	-	-	[0300]
Mé (n=5)	CI-06	-	+	-	-	-	-	[0000]
	CI-01 ; CI-02	+	+	+	+	+	+	[3312]
	CI-03	+	+	+	+	-	+	[3302]
	CI-04	+	-	-	+	-	+	[0302]
Gbêkê (n=6)	CI-12 ; CI-22	+	+	+	+	+	+	[3312]
	CI-23 ; CI-24 ; CI-31	+	+	+	+	-	+	[3302]
	CI-52	-	+	-	+	-	+	[0302]
Marahoué (n=4)	CI-39	-	+	-	-	-	+	[0002]
	CI-41	+	+	+	+	-	+	[3302]
	CI-42	-	+	-	-	-	-	[0000]
	CI-40	+	+	+	+	+	+	[3312]
Bélier (n=7)	CI-25 ; CI-47	+	-	-	+	-	-	[0300]
	CI-44 ; CI-45 CI-46 ; CI-51 ; CI-53	+	+	+	+	+	+	[3312]
Agnéby-Tiassa (n=5)	CI-13/21	-	+	-	-	-	+	[0002]
	CI-13/29	-	+	-	-	-	-	[0000]
	CI-36/16	+	+	+	+	+	+	[3312]
	CI-37/16	+	+	-	+	-	+	[0302]
	CI-64	+	+	+	+	-	+	[3302]
Sud-Comoé (n=3)	CI-60 ; CI-55 ; CI-56	+	+	+	+	+	+	[3312]
Gôh (n=5)	CI-72	-	+	-	-	-	-	[0000]
	CI-15/02 ; CI-101	+	+	+	+	+	+	[3312]
	CI-34/16	+	+	-	+	-	+	[0302]
	CI-153	-	+	-	-	-	+	[0002]

M. ulcerans VNTR profile [3312] ; *M. ulcerans* VNTR profile [3302] ;
M. ulcerans VNTR profile [0302]; VNTR profiles of others atypical mycobacteria

72.3% of the isolates were carried the KR gene coding for the plasmid of virulence. 27 out of 30 *M. ulcerans* strains (90%) were amplified the KR gene. 71.4% of isolates from genotype 3 and 50% isolates from genotype 5 possessed the virulence genes. But, the virulence factor genes were not found in the strains from genotype 4 (Figure 4).

The rate of MIRU-VNTR loci amplification varied from one locus to another. ST-1marker was the most amplified locus by 80.8% of the isolates (38/47) with an amplification size of 340 base pairs (bp) corresponding to two replicates (Figure 5D). VNTR 6 locus was amplified by 78.7% of the isolates (37/47) and by two reference controls at a length of 500 bp, corresponding to three replicates (Figure 5B). In the third position, Miru-1 locus was amplified by 61.7% of isolates (29/47) and by all 3 controls, with 3 tandem repeats at a length of 500 bp (Figure 5A). VNTR 19 was the least amplified locus by 51% of the isolates (24/47) according to the reference controls at a length of 340 bp, corresponding to one tandem repeat (Figure 5C).

Seven VNTR profiles were identified among the mycobacteria isolates (Table 4). Three different profiles were determined in *M. ulcerans* strains based on the number of amplified loci. In profile 1, all targeted VNTR loci were amplified with 3 copies of Miru-1 and VNTR 6, 1 copy of VNTR 19 and 2 copies of ST-1. This profile named [3312] was identified in 44.7% of isolates (21/47) and in 70% of *M. ulcerans* strains (21/30). In profile 2, the VNTR 19 locus was not amplified. That profile named [3302] consisted of 23.3% of *M. ulcerans* strains (7/30). In profile 3, the Miru-1 and VNTR 19 loci were not amplified, unlike the reference control [0302]. It was identified in 6.7% of *M. ulcerans* strains (2/30) and in 17.6% of the *non-M. ulcerans* mycobacteria isolates (3/17). Four profiles were only identified in *non-M. ulcerans* mycobacteria isolates. The profiles [0300] and [0002] were found in 6.4% of the isolates (3/47), where only the VNTR 6 and ST-1 loci were respectively amplified. The profile [0012] was identified in 4.3% (2/47) of *non-M. ulcerans* mycobacteria isolates, where both VNTR 19 and ST-1 loci were simultaneously amplified. In 10.6% of the mycobacteria isolates (5/47), none targeted locus was amplified [0000].

4. Discussion

The rate of bacterial isolation was different according to the stage of the lesions. It was better when lesions were early (45.2%) and the skin lesions were not subject to any antibiotic treatment. So the bacilli were more viable to grow easily on Löwenstein-Jensen medium. Conversely, the isolation rate was low when lesions were old, which had mostly undergone antibiotic treatments that destroyed or weakened the bacilli. In 2009, Ahoua and al. obtained an isolation rate of 22.1%. However, she didn't refer to either the stage of the lesions or the antibiotic treatments received by the patients [17].

8.5% of the strains were isolated from lesions after the end of antibiotic treatment according to WHO protocol. That treatment combining Rifampicin and Streptomycin was directly and daily administered by health workers. Mycobacteria isolation after 8 weeks treatment appeared abnormal and caused suspicions of strains that may have particular characteristics. Different phenotypes were identified among the isolates based on microbiological characters described. For the dominant phenotype (65.9%), bacteriological and biochemical characteristics were identical to those of a *M. ulcerans* strain. 6.4% of the isolates expressed bacteriological and biochemical characteristics in favor of a *M. marinum* strain. For 27.6% of the isolates, these characteristics were associated with atypical mycobacteria, such as *M. chelonae*, *M. smegmatis* (Table 3).

The results of molecular typing targeting the insertion sequences IS6110, IS2606 and IS2404 showed differences between the isolates. Indeed, those targets were amplified in different proportions. IS2404 was amplified in the majority of the isolates (93.6%), followed by IS6110 (74.5%) and IS2606 (70.2%), respectively. Five genotypes were identified according to the number of amplified sequences. A typical *M. ulcerans* strain possesses both IS2606 and IS2404 insertion sequences, as described by Stinear in 1999 [23]. In this study, only the strains of genotype 1 (53.2%) and 2 (10.6%) had microbiological, biochemical and molecular characteristics identical to those of a *M. ulcerans* strain. Thus in our context, *M. ulcerans* was responsible for 63.8% of skin lesions treated as "Buruli ulcer". The strains of the genotype 4 had IS6110 and IS2606 sequences and expressed microbiological and biochemical characteristics identical to those of a *M. marinum* strain (6.4%). The strains of genotypes 3 and 5 (29.8%) expressed microbiological, biochemical and molecular characteristics relating to various atypical mycobacteria, such as *M. smegmatis* and *M. chelonae* (Table 3). Because of the possession of IS2404 sequence, those pathogens were reported and treated as *M. ulcerans* strains. But they were not.

Geographical distribution of the isolates showed a different circulation of genotypes in the endemic areas of Côte d'Ivoire. *M. ulcerans* strains of genotype 1 were found everywhere patients were enrolled. In some localities of the Sud-Comoé region (Bonoua and Adiahou), the genotype 1 was the only one detected. Both localities are in agricultural areas drained by the Comoé River and numerous wetlands. *M. ulcerans* strains of genotype 2 were found in central (Gbêkê, Marahoué) and southern (Mé, Agnéby-Tiassa) regions of Côte d'Ivoire. The geographical distribution of genotypes 1 and 2 provided information on the circulation of *M. ulcerans* in endemic areas of Cote d'Ivoire. Other mycobacteria of genotypes 3 and 5 with IS2404 sequence were found in almost areas (7/9). Their presence could explain the high number of cases reported as "Buruli ulcer" in many localities, which may be mistaken for BU hyper-endemic areas. Some of those mycobacteria were isolated from patients after 8 weeks of well-conducted specific antibiotic therapy. This may explain the slow-healing lesions where Mycolactone and viable bacilli persisted after several weeks of antibiotic therapy [19,33,34].

The molecular typing targeting Miru-VNTR loci confirmed the variety of strains by showing the circulation of three VNTR profiles among *M. ulcerans* strains. Our results were similar to those found by Quinet and al. in 2017 [35]. The same loci were amplified in clinical samples and isolates from regions of Côte d'Ivoire in the same order of magnitude: ST-1 locus (57.4%), MIRU-1 locus (42.3%), VNTR 6 locus (30.8%) and VNTR 19 locus (23.1%). By contrast, Kakou [36] had amplified in 2015 those four loci but in proportions different from ours. In fact, she had found ST-1 in 85.7%, MIRU-1 and VNTR 19 in 61.9% and VNTR 6 in 52% of the samples tested, respectively. The MIRU-VNTR are variable repeat sequences with a new deletion or insertion in repetition loci. These different profiles would be evidence of a mutation in MIRU-VNTR loci in human strains. RFLP typing with better resolution showed differences between environmental mycobacterial strains and human *M. ulcerans* strains. Variations of MIRU-VNTR profiles could be a marker of adaptation of mycobacteria genotypes during the transition from the environment to humans. On the one hand, these studies confirmed a diversity of VNTR profiles between human *M. ulcerans* strains, and on the other hand between strains of atypical mycobacteria isolated from human skin lesions.

90% (27/30) of *M. ulcerans* strains expressed virulence genes. The lack of amplification of the virulence genes could result from the loss of the plasmid by strains under the isolation or conservation conditions in the lab. In contrast, virulence genes were amplified in 63.6% of non-*M. ulcerans* strains possessing IS2404 sequence. This sequence is also carried by the plasmid pMUM001 as described by Stinear [23,37,38]. Its presence as well as that of the virulence genes could translate an acquisition of the plasmid by other mycobacteria living in the same ecosystem as *M. ulcerans*. Our results corroborated those reported by Vakou et al. [39] which isolated environmental fast-growing mycobacteria with both IS2404 and ketoreductase sequences. Transfer of the plasmid from *M. ulcerans* to environmental mycobacteria could confer virulence or enhance pathogenicity leading to necrotic skin lesions observed in patients. Conversely, virulence genes were not detected in the *M. marinum* strains identified in patients.

Molecular diagnosis of *M. ulcerans* infection based on IS2404 sequence and Ketoreductase gene detection may present limitations. The first limitation is that this method doesn't allow a differentiation between *M. ulcerans* strains and fast-growing or slow-growing mycobacteria, possessing IS2404 sequence or having acquired the plasmid pMUM001. Many cutaneous lesions probably caused by other mycobacteria could be attributed to *M. ulcerans*. The

second limitation of this method is that it does not target IS2606 sequence found in *M. marinum* also involved in chronic skin ulcerations. The third limitation is that it could target environmental mycobacteria that contaminate skin ulcers. The best molecular diagnostic approach would be to target both insertion sequences IS2404-IS2606 and ketoreductase gene. On the one hand, this algorithm would discriminate between *M. ulcerans* and other environmental mycobacteria that also possess IS2404 sequence. On the other hand, it would determine the involvement of *M. marinum* in chronic skin lesions.

5. Conclusion

Mycobacterium ulcerans is an environmental mycobacterium that causes an endemic skin disease called "Buruli ulcer". Every year several health districts in Cote d'Ivoire report a large number of chronic ulcers, rightly or wrongly, as cases of Buruli ulcer.

Phenotypic and genomic characterization of strains isolated from Buruli ulcer suspected cases showed the presence of different mycobacteria species: *M. ulcerans* (63.8%), *M. marinum* (6.4%) and other atypical mycobacteria, such as *M. chelonae* and *M. smegmatis* (29.8%). Most of *M. ulcerans* strains (90%) and atypical mycobacterial strains (63.6%) expressed genes encoding mycolactone bio synthesis, with the exception of *M. marinum* strains. Geographical distribution of strains showed that *M. ulcerans* co-circulated with other mycobacteria strains in the same ecosystems. Some of those *non-M. ulcerans* mycobacteria possessed insertion sequences (IS2404, IS2606) and virulence genes.

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