

Original Research Article

Clonality of *Mycobacterium ulcerans* by using VNTR-MIRU typing in Ivory Coast (Côte d'Ivoire), West Africa.

Running Title: Environmental Mycobacteria infections: Buruli ulcer in West Africa.

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ABSTRACT

Background: Buruli ulcer (BU) is neglected skin disease caused by *Mycobacterium ulcerans*. The lack of early diagnosis and treatment causes severe disability. In Central and in West Africa, BU is endemic and its control is difficult because the most cases occur in rural regions. The molecular particularity of *M. ulcerans* was the acquisition of the virulence plasmid pMUM001. Genetic analyses have demonstrated the high diversity with variable number tandem repeats (VNTR) and Mycobacterial Interspersed Repetitive Units (MIRU) in *M. ulcerans* and in mycolactone producing Mycobacteria (MPMs).

Objective: The objective of this study was to investigate the molecular diversity by using MIRU-VNTR method in clinical samples of BU patients in Côte d'Ivoire.

Study Design: 21 clinical samples were collected from BU patients in different sites and were first analyzed in molecular diagnosis of BU using two targets insertion sequence IS2404 and ketoreductase-B-domain (KR). In a second step, we have analyzed the strains by PCR typing for four specific and sensitive markers MIRU1, VNTR6, ST-1 and VNTR19.

Results and Conclusion: 100 % of clinical samples were positive in molecular tests for IS2404 and 95% for KR and confirm *M. ulcerans* in the samples. By PCR typing, we have found 61.9 % positive for MIRU1 and 52%, 85.7%, and 61.9% for VNTR6, ST-1 and VNTR19 respectively. One of sample was negative for all genotyping markers. Two different genetic profiles were identified by MIRU1 and ST-1 loci by gel-analyzed of the amplified products. The VNTR profile C (3,1,1) corresponding of 3 copies MIRU1, 1 copy VNTR6 and 1 copy ST-1 was detected in 28.5% of samples and confirms the West African genotype in Côte d'Ivoire. Different genetic strains of *M. ulcerans* were co-circulated in the same endemic region in the country. This study has described first the circulating of different genetic strains of *M. ulcerans* in Côte d'Ivoire.

Keys words: *M. ulcerans*, genotyping VNTR-typing, diversity, Côte d'Ivoire, Buruli ulcer, West Africa

1. INTRODUCTION

Buruli ulcer (BU) is a neglected skin disease caused by *Mycobacterium ulcerans*. BU is the third most important mycobacterial disease worldwide and occurs in riverine rural regions of West and Central Africa and in coastal Australia, and in Asia (1-3). In Côte d'Ivoire, BU cases were estimated over 1000 each year and the detection of new cases in non-endemic regions represents a new distribution of BU in the country. Because of the low growth (6-10 weeks) of *M. ulcerans*, the clinical and molecular diagnoses have contributed to the general surveillance of BU in Côte d'Ivoire. The virulence gen of *M. ulcerans* coding for mycolactone, is located on a 174-kb plasmid pMUM001 (4). This *M. ulcerans* toxin called "mycolactone" is responsible for the massive tissue destruction in BU. The treatment of BU has recommended the daily administration of rifampicin and streptomycin for 2 months added or not to

62 surgical treatment if there is large skin ulceration. The transmission of BU occurs in the
63 environment, in river, in wetland, or in water bodies. The prevalence of cases of BU in some
64 regions and the detection in aquatic insects, and in animals of *M. ulcerans* DNA (5-6) has
65 demonstrated the environmental facts of *M. ulcerans*. However, the first isolation in pure
66 culture of *M. ulcerans* from an aquatic environment was from an aquatic insect (7) and
67 provides further support for the aquatic transmission of BU. Others Mycolactone Producing
68 Mycobacteria (MPMs) have been found in aquatic environments (8-9). The current mode of
69 transmission and the environmental reservoirs of *M. ulcerans* were not elucidated.

70 Molecular detection for diagnosis based on Insertion Sequence 2404, and has good specificity
71 and high sensitivity for *M. ulcerans*, and then IS2404 is present in over 203 copies in genome
72 (5, 10-11). Previous studies have demonstrated different profiles of *M. ulcerans* by PCR-
73 restriction profile and 16S rRNA sequencing (12-13). Genetic analyses have demonstrated
74 the high diversity with variable number tandem repeats (VNTR) and with Mycobacterial
75 Interspersed Repetitive Units (MIRU) in *M. ulcerans* genome (14-15). By using IS2404
76 restriction fragment length polymorphism (RFLP), *M. ulcerans* is subdivided in six groups
77 related to six geographical regions, including Africa, Australia, Asia, South America, Mexico
78 and Southeast Asia (13). Tandem Repeat (TR) DNA are important to detect polymorphism by
79 different copy numbers of conserved backbones in genome in some species such
80 mycobacteria, and enterobacteria (16-17). A new conserved variable locus called ST1 was
81 identified in *M. ulcerans* in Ghana and in *Mycobacterium marinum*, a fish pathogen and
82 presents three different genotypes in West Africa (18).

83 The accessibility to molecular tools for typing *M. ulcerans* and other environmental
84 mycobacteria allows the discrimination between clinical strains and environmental strains
85 (14-15; 18). In West and in Central Africa, several genotypes or serovars of *M. ulcerans* have
86 been found in clinical and in environmental samples (19). The distribution of mycolactone
87 producing Mycobacteria and non-producing mycolactone strains in endemic and non-
88 endemic sites has indicated no correlation between *M. ulcerans* presence and the disease
89 incidence (19). The high resolution of variable number tandem repeats (VNTR) is very useful
90 in detecting heterogeneity among *M. ulcerans* in clinical and in environmental samples. (14-
91 15; 18). Lavender *et al* 2008 have improved MIRU-VNTR typing for environmental samples
92 and clinical samples (20). Previous studies have recommended specific and sensitive VNTR

loci to discriminate between *M. ulcerans* and MPMs (14-15; 18, 20). The objective of this study was the molecular characterization of *Mycobacterium* sp. in clinical samples from Buruli ulcer patients by using MIRU-VNTR typing.

2. MATERIAL AND METHODS

2.1 Clinical Sampling

21 clinical swabs from chronic ulcers were collected in different endemic regions of Côte d'Ivoire (Ivory Coast) from 2011 to 2012. 10 samples were collected from males and 11 samples from females (Table 1). All patients were first clinically confirmed for BU control and were treated by recommended double antibiotic therapy. The patients were recruited by the National Program of Buruli ulcer surveillance and the protocol was approved by the national ethic committee. Epidemiological data of all samples were not available (unpublished communication of the surveillance program).

Table 1. Clinical strains in this study

Strain	Sex	Location	Year of isolation
2080	M	Djebonoua	2011
2070	F	Djekanou	2011
2084	M	Grand Lahou	2011
2078	M	Sakassou	2011
2081	M	Sinfra	2011
2095	F	nd	2011
2077	M	nd	2011
2076	F	nd	2012
2065	M	Sakassou	2012
2071	F	Djekanou	2012
2082	M	nd	2012
2067	M	nd	2012
2089	F	nd	2012
2108	F	nd	2012

2114	M	nd	2012
2066	F	nd	2012
2079	F	Daloa	2012
2085	M	nd	2012
2090	F	nd	2012
1044	nd	San-Pedro	2012
2086	F	nd	2012

nd: unidentified

2.2 DNA Extraction

The swab samples were first incubated overnight after the addition of 2 ml sterile water at room temperature. The solution was vigorously mixed for 2 min and 200 µl were taken out for the genomic DNA extraction. The solution was centrifuged for 15 min, 15000 rpm at 4°C and the pellet was added to 100 µl NaOH 50mM and incubated at 95°C for 15 min. The neutralization of the DNA was performed by the adding 15 µl Tris-HCl 0.1 M. The DNA was precipitated by adding 1/10 volume of Sodiumacetat3M and 2 volumes of 70% Ethanol. The DNA was washed by 70% Ethanol and was eluted in 50 µl of DNA/Rnase-free water. The controlstrain was *M.ulcerans* (ITM 9540) provided from Institute of Tropical Medicine (ITM),WHO Collaborating Center and Laboratory of Reference for Mycobacteria, Belgium.

2.3 Diagnostics PCR for *Mycobacterium ulcerans*

The targets IS2404 and KR were specifics for *M.ulcerans* and were located in the genome and in the virulence plasmid respectively (Table 2). Real time PCR were performed using PCR reagents Kit (Promega, Germany) using the previous protocols (11; 21-22). Briefly, the 25 µl-reaction contains 5 µl of DNA, 0.3 µM of each primer, 0.25 µM of labelled probe, and PCR-Mix. PCR consisted of 35 cycles of melting at 95°C for 5 sec; annealing and extension at 60°C for 1min. Negative controls were performed with 5 µl of nuclease free-water. Positive controls DNA were tested in duplicate. The real-time PCR machine (Applied Biosystems, USA) was used and the fluorescence of FAM was measured to determine the amplification threshold cycle (Ct).

128 **Table 2. Primers used in this study**

Target	Oligos	Primer Sequence (5'-3')	References
IS2404	IS2404 F	ATTGGTGCCGATCGAGTTG	(21)
	IS2404 R	TCGCTTTGGCGCGTAAA	
	IS2404-		
	probe	6 FAM-CACCACGCAGCATTCTTGCCGT-TAMRA	
Ketoreductase	KR F	TCACGGCCTGCGATATCA	(11)
	KR R	TTGTGTGGGCACTGAATTGAC	
	KR-probe	6 FAM-ACCCCGAAGCACTG-TAMRA	
MIRU1	MIRU1 F	GCTGGTTCATGCGTGGAAG	(14)
	MIRU1 R	GCCCTCGGGAATGTGGTT	
VNTR-6	Locus 6F	GACCGTCATGTCGTTGATCCTAGT	(15)
	Locus 6R	GACATCGAAGAGGTGTGCCGTCT	
ST-1	ST1 F	CTGAGGGGATTTCACGACCAG	(18)
	ST1 R	CGCCACCCGCGGACACAGTCG	
VNTR-19	Locus 19F	CCGACGGATGAATCTGTAGGT	(15)
	Locus 19R	TGGCGACGATCGAGTCTC	

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130 **2.4 MIRU-VNTR Typing**

131 The MIRU-VNTR-typing using PCR was running in a Thermocycler (GenAmp 9700, Applied
132 Biosystems, USA) to amplify the MIRU1, the VNTR locus 6 and the VNTR locus 19 in same
133 parameters (19). For *M. ulcerans* specific-VNTR target ST-1, the PCR parameters were
134 described by Hilty et al 2006 (18). Briefly, 5 µl of extracted DNA were added to 45 µl PCR-Mix
135 containing 0.2 mM dNTPs (Sigma, USA), 0.4 µM each primer, 1.5 mM MgCl₂ (Promega,
136 Germany), 1X Flexi Taq-Polymerase buffer and 1 Unit Go-Flexi Taq-Polymerase (Promega,
137 Germany). All used primers were represented in Table 2. The samples were tested by the
138 1/10 dilution for the detection of inhibitors. All PCR runs have included negative control
139 (sterile Dnase/Rnase-free water) for the test quality and the detection of contamination.
140 Genomic DNA from ITM 9540 strain was tested as positive control for the MIRU-VNTR-typing
141 tests.

2.5 Visualization of amplified products

Each sample was tested in simplex Typing-PCR for the four loci and 15µl amplicons were analyzed in 1.5 % Agarose gel by electrophoresis. The polymorphism was detected in different size of the amplicons. The size of the amplicons was estimated by comparison with the 100-bp Ladder (Eurobio, France).

3. RESULTS

3.1 Diagnosis of BU

21 clinical samples were collected from BU patients in endemic regions of Côte d'Ivoire. 47% were male and 52% were female. Over 50% indicated the location of the patients in Côte d'Ivoire and 47 % of the samples cannot geographically located (Table 1). The detected threshold cycle (Ct) of real time PCR-KR was low (Ct: 21-26) and indicated the high bacterial level of *M. ulcerans* in 11 samples (52% of cases). For low bacterial load, by Ct 27-36, 9 samples (42% of cases) were positive (Table 3). By using real time PCR with target IS2404, all samples were found positive for *M. ulcerans*. High bacterial loads were found in 7 samples (33% of cases) while 14 samples (66% of cases) have low bacterial load by Ct 27-35 (Table 3). The sample 1044 was found negative for the target KR and positive for IS2404 in real time PCR assays.

Table 3. PCR Diagnostics for *M. ulcerans*

Diagnostic tests			
No	Strain	PCR IS2404 (Ct)	PCR-KR (Ct)
1	2080	25.4	21.8
2	2070	24.1	21.8
3	2084	25.3	22.3
4	2078	25.2	22.82
5	2081	24.5	23.6
6	2095	26.3	24.1

7	2077	27.2	24.7
8	2076	26.2	25.3
9	2065	27.6	25.6
10	2071	27.0	26.1
11	2082	28.3	26.2
12	2067	29.3	27
13	2089	31.2	28.9
14	2108	31.8	29
15	2114	30.2	29.3
16	2066	33.5	31.4
17	2079	34.7	31.6
18	2085	34.3	32.4
19	2090	35.6	32.6
20	1044	35.0	-
21	2086	33.4	31.54
%			
Positivity			
Rate			
		100% (21/21)	95% (20/21)

positive control DNA (ITM 9540) by Ct: 22.1; negative control (H₂O): Ct has not been detected.

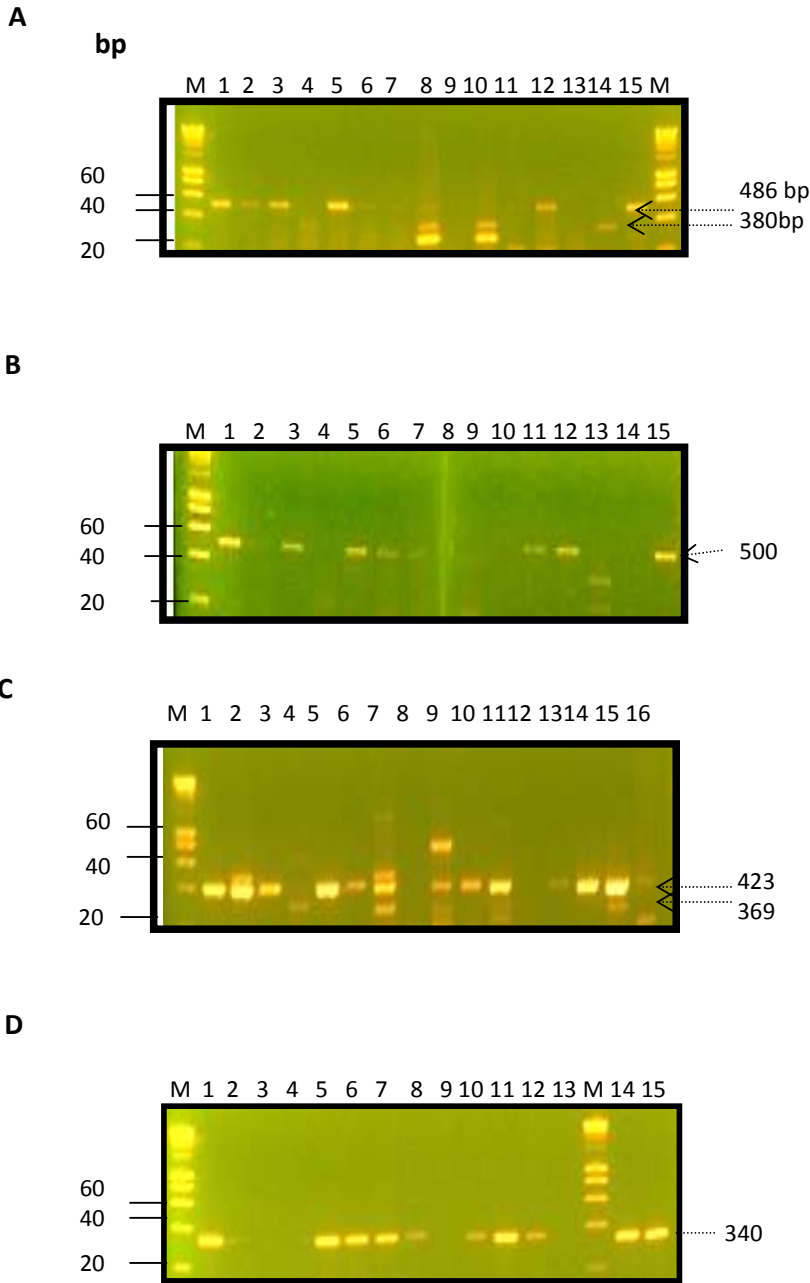
3.2 Genotyping by MIRU-VNTR

From 21 samples collected from BU patients, *M. ulcerans* DNA were analyzed by 4 MIRU-VNTR genotyping markers for molecular characterization. ST-1 was the most amplified locus for 85.7% of the samples, while 61.9% and 57% for VNTR locus 19 and MIRU1 and respectively VNTR locus 6 (Table 4). 4.7 % (1/21) of samples were negative for 4 genotyping loci and 9.5% (2/21) of samples were negative for three loci (Table 4).

The detection of MIRU1 in clinical samples has shown 2 amplified products corresponding to one copy (380 bp) and to 3 copies (486 bp) of the 53 bp-repeat sequences (MIRU1) in the genome of *M. ulcerans*(Fig.1A, Table 4). For VNTR locus 6.57 % (11/21) of samples were amplified only a product-PCR corresponding of 500 bp for one copy of the repeat sequence in the genome (Fig. 1B, Table 4). PCR with target ST-1 has shown 2 amplified products of 369 bp and 423 bp for one copy and 2 copies respectively (Fig.1C, Table 4). PCR targets VNTR locus 19 has amplified one product (340 bp) corresponding to 2 copies in genome (Fig. 1D, Table 4).

Molecular profiles of the strains were elucidated after the amplicon size of genotyping PCR by MIRU-VNTR. The polymorphism of the strains was represented as the copie number of

genotyping markers as VNTR-profile (Table 4). 28.5 % (6/21) of samples have the most VNTR-profile C that represents 3, 1, 2, 2 copies of MIRU1, VNTR6, ST-1 and VNTR19 respectively (Table 4). The strain 2070 with the profile (3,1,1,2) and the strain 2071 with the profile (1,-, 2,2) were collected in the same site Djekanou and have distinct genetic diversity. The strains 2071, 2078, 2085, and 2108 have particular results for the genotyping marker MIRU1, these strains have amplified a 380 bp-PCR-product corresponding to one copy MIRU1 in genome (Fig. 1, Table 4). These results suggest that others VNTR profiles can be implicated for BU infection in Côte d'Ivoire. The control strain ITM 9540 has shown VNTR-profile C (3, 1, 2, 2) first confirmed in West Africa and Central Africa (Fig.1).



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209 **Figure 1. Agarose gel analysis of MIRU-VNTR Typing of clinical samples.** A: MIRU1; B: VNTR
 210 locus 6; C: ST-1; D: VNTR locus 19; M: 100 bp-DNA Ladder (Promega, Germany); 1: positive
 211 control DNA ITM 9540; 2-16: clinical samples.

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218 **Table 4. MIRU-VNTR-typing of clinical strains in this study**

Copies detected							
No	Code Sample	MIRU1	VNTR 6	ST-1	VNTR 19	Profile VNTR	Location
1	2080	3	1	2	2	C	Djebonoua
2	2070	3	1	2	2	C	Djekanou
3	2084	3	1	2	-	C	Grand-Lahou
4	2078	1	-	1	-	-	Sakassou
5	2081	3	1	2	2	C	Sinfra
6	2095	3	1	2	2	C	nd
7	2077	-	1	2	2	-	nd
8	2076	3	-	-	2	-	nd
9	2065	-	-	2	-	-	Sakassou
10	2071	1	-	2	2	-	Djekanou
11	2082	-	1	2	2	-	nd
12	2067	3	1	-	2	-	nd
13	2089	-	-	2	-	-	nd
14	2108	1	-	2	2	-	nd
15	2114	3	1	2	2	C	nd
16	2066	3	-	2	-	-	nd
17	2079	-	-	-	-	-	Daloo
18	2085	1	-	1	-	-	nd

19	2090	-	-	2	-	-	nd
20	1044	-	1	2	2	-	San-Pedro
21	2086	-	1	2	2	-	nd
Total	21	13/21	11/21	18/21	13/21	6/21	
(%)	(100)	(61.9)	(52.3)	(85.7)	(61.9)	(28.5)	

nd: unidentified; the positive control DNA (ITM 9540) has follows results: MIRU positive (3 copies), VNTR 6 positive (1 copy); ST1 positive (2 copies) and VNTR 19 positive (2 copies). Negative control (H₂O) has a negative result for all PCRs.

4. DISCUSSION

Buruli ulcer is an endemic neglected disease in Central and in West Africa. The agent of BU called *M. ulcerans* is an environmental slow grown pathogen and presents different serovars and genotypes. The molecular techniques have presented several advantages to elucidate genomic tools. Previous studies have demonstrated the circulation in West Africa of three VNTR profile A, B and C of *M. ulcerans* strains and VNTR profile D, E, F for MPMs by using MIRU-VNTR typing (19).

For molecular characterization of *M. ulcerans* strains from patients, we have first confirmed 100% of samples by PCR IS2404. The diagnosis using target IS2404 is very consistent and high number copies in genome (10) and is widely used in environmental and clinical samples (11,23). The ketoreductase B-domain is present in 15 copies within the mycolactone synthase gene and the real-time assay has shown good sensitivity in previous assay (21-22). Real-time PCR is more 10-10.000 sensitive than classic PCR, however the PCR is recommended by World Health Organization as the confirmatory test for clinical case diagnosis of *M. ulcerans* infection. This recommendation is appropriate and supports the national surveillance in endemic regions including a multicenter external quality assessment for molecular tests (24). The failure of PCR-KR for BU detection can be explained by the loss of plasmid by genomic extraction or PCR inhibitors. However, all clinical samples were collected from ulcers that involved by mycolactone gene located by plasmid.

To elucidate, the diversity of circulating strains of BU in Côte d'Ivoire, we have using the tandem repeats (TR) as genotyping markers. Among the 59 TR found in MPMs, 19 are consistent in genotyping studies (14-15; 18-19). The findings of Lavender *et al* 2008 support the high specificity and sensitivity of 4 genotyping markers for *M. ulcerans* and others MPMs. Our results have demonstrated by MIRU1 two different profiles that contain three

copies or one copy of the repetitive sequence, that can suggest the VNTR profile A or D. For ST1 locus, we have identified 1 or 2 repeats in genome of clinical strains and these were identical with the previous findings (18). The authors have shown by sequencing analyzing of two alleles (BD for 2 repeats and C for 1 repeat) of ST-1 in clinical samples in West Africa. ST-1 has most amplify in PCR typing by 85.7% of the samples. Unlike others VNTRs, the repeat sequence ST-1 has shown no microdeletion but only single-nucleotide polymorphism in sequence variants (18).

While by the genotyping markers VNTR locus6 and VNTR locus 19, our results have indicated no genomic variation in clinical strains. Hilty *et al* 2006 have shown that the diversity of Mycobacteria strains using MIRU-VNTR typing can be explained by the deleting of nucleotide in repetitive sequence and the consequence of negative results in PCR methods. Previous studies have explored the clonal distribution of African genotype and the association of low rate of genomic drift of *M. ulcerans* (18). The use of minisatellites or tandem repeat (TR) sequences has been described in *Mycobacterium tuberculosis*, in mycolactone producing Mycobacteria (*Mycobacterium marinum*), in *Yersinia pestis* and in *Bacillus anthracis* for the bacterial polymorphism in genome (16; 25-28). Our results have shown that the VNTR profile C is prevalent in Côte d'Ivoire and the circulation of other VNTR-profiles in *M. ulcerans* strains in endemic sites in West Africa. The negative results of strains by genotyping markers are caused by the high variability of TR due to insertion or deletion of nucleotides in the genome and the genome rearrangement for the adaptation in aquatic environment (10). Recently, Narhet *et al* 2015 has demonstrated the discrimination power of VNTR typing to obtain *M. ulcerans* genotypes with similar allelic profiles in BU endemic sites (29). These findings support the evidence of microhabitat preference or pathoadaptation of mycobacteria in BU endemic communities (29).

Our results suggest the circulation of different profiles or genotypes of *M. ulcerans* for BU in Côte d'Ivoire and it might explain the different clinical manifestations of the diseases. In perspectives studies, the whole genome sequencing of *M. ulcerans* will be explored for the clonality of the clinical and environmental strains in Côte d'Ivoire.

5. CONCLUSION

The diagnosis of BU in endemic regions is based mostly on clinical and epidemiological methods. The new implementation of molecular tests has improved the surveillance in endemic regions and should be accessible. Our results confirm first the genomic diversity of the strains of *M. ulcerans* and the circulation of different profiles in Côte d'Ivoire by demonstrating the diversity of *M. ulcerans* for BU patients using MIRU-VNTR typing.

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