

Original Research Article

Molecular diversity of environmental *Mycobacterium* ssp : Buruli ulcer infection in Ivory Coast, Côte d'Ivoire, West Africa.

Running Title: Mycobacterial Infections in Humans: Buruli Ulcer in West Africa

ABSTRACT

Background: Buruli ulcer (BU) is neglected skin disease caused by mycobacterium called *Mycobacterium ulcerans*. The lack of early diagnosis and treatment causes severe disability. In Central and in West Africa, BU is endemic and the control of the disease 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 analyzing in diagnosis molecular tests of BU using two targets insertion sequence IS2404 and ketoreductase-B-domain (KR). In a second step, we have analyzing the strains by PCR typing for four specific and sensitive markers MIRU1, VNTR6, ST1 and VNTR19.

Results and Conclusion: 100 % of clinical samples are 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, ST1 and VNTR19 respectively. One of sample was negative for all genotyping markers. Two different genetic profiles were identified by MIRU1 and ST1 loci by gel-analyzing of the amplified products. The VNTR profile

29 C (3,1,1) corresponding of 3 copies MIRU1, 1 copy VNTR6 and 1 copy ST1 was detected in
 30 28.5% of samples and confirms the West African genotype in Côte d'Ivoire. Different genetic
 31 strains of *M. ulcerans* were co-circulated in the same endemic region in the country. This
 32 study described first the circulating of different genetic strains for BU in Côte d'Ivoire and
 33 can explain the high prevalence of BU in the country.

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

36 1. INTRODUCTION

37 Buruli ulcer (BU) is a neglected skin disease causing by *Mycobacterium ulcerans*. BU is the
 38 third most important mycobacterial disease worldwide and occurs in riverine rural regions of
 39 West and Central Africa and in coastal Australia, and in Asia (1-3). In Côte d'Ivoire, BU cases
 40 were over 1000 each year and the detection of new cases in non-endemic regions represents
 41 a new distribution of BU in the country. Because the low growth (6-10 weeks) of *M. ulcerans*,
 42 the clinical and molecular diagnosis have contributed to the general surveillance of BU in
 43 Côte d'Ivoire.

44 The virulence gen of *M. ulcerans* is coding for mycolactone and is located on a 174-kb plasmid
 45 pMUM001 (4). The toxin mycolactone is responsible for the massive tissue destruction in BU.
 46 The treatment of BU has recommended the daily administration of rifampicin and
 47 streptomycin for 2 months and surgical intervention by invasive ulcers of the skin.

48 The transmission of BU occurs in the environment in river, in wetland, or in water bodies.
 49 The prevalence of infected cases of BU in some regions and the detection in aquatic insects,
 50 and in animals of *M. ulcerans* DNA (5-6) has demonstrated the environmental facts of
 51 *M. ulcerans*. However, the first isolation in pure culture of *M. ulcerans* from an aquatic
 52 environment was from an aquatic insect (7) and provides further support for the aquatic
 53 transmission of BU. Others Mycolactone Producing Mycobacteria (MPMs) have been found
 54 in aquatic environments (8-9). The actual mode of transmission and the environmental
 55 reservoirs of *M. ulcerans* were not elucidated.

56 Molecular detection for diagnostic is based on Insertion Sequence 2404, specific and high
 57 sensitive for *M. ulcerans*, and then IS2404 was present in over 203 copies in genome (5, 10)

Some molecular assays were not able to discriminate between *M.ulcerans* and closely related mycolactone-producing mycobacteria by using targets IS2404 or IS2606 (11). Previous studies have demonstrated different profiles of *M.ulcerans* by PCR-restriction profile and 16S rRNA sequencing (12-13). Genetic analyses have demonstrated the high diversity with variable number tandem repeats (VNTR) and with Mycobacterial Interspersed Repetitive Units (MIRU) in *M.ulcerans* genome (14-15). By using IS2404 restriction fragment length polymorphism (RFLP) fingerprint, *M.ulcerans* is subdivided in six groups related to six geographical regions, including Africa, Australia, Asia, South America, Mexico and Southeast Asia (13). Tandem Repeat (TR) DNA are important to detect polymorphism by different copies number of conserved backbones in genome in some species such Mycobacteria, and Enterobacteria (16-17). A new conserved variable locus called ST1 was identified in *M.ulcerans* in Ghana and in *Mycobacterium marinum* a fish pathogen and presents three different genotypes in West Africa (18).

The accessibility to molecular tools for typing of *M.ulcerans* and of all environmental Mycobacteria allow the discrimination within clinical strains and environmental strains (14-15; 18). In West and in Central Africa, several genotypes or serovars of *M.ulcerans* have been found in clinical and in environmental samples (19). The distribution of mycolactone producing Mycobacteria and non-producing mycolactone strains in endemic and non-endemic sites has indicated no correlation between *M.ulcerans* presence and the disease incidence (19). The high resolution of variable number tandem repeats (VNTR) has very useful in detecting of heterogeneity among *M. ulcerans* in clinical and in environmental samples. (14-15;18). Lavender *et al* 2008 have improved MIRU-VNTR typing for environmental samples 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 msssp* in clinical samples from Buruli ulcer patients by using MIRU-VNTR typing.

2. MATERIALS AND METHODS

2.1 Clinical Sampling

21 clinical schwab from chronic ulcers were collected in different endemic regions of Côte d'Ivoire (Ivory Coast) in 2011-2012. 10 samples were collected from male and 11 samples

88 from female (Table 1). All patients were first confirmed clinical for BU and were treated by
 89 recommended antibiotic therapy. The patients were recruited by the National Program of
 90 Buruli ulcer surveillance and the protocol was approved by the national ethic committee.
 91 Epidemiological data of all samples were not available (unpublished communication of the
 92 surveillance program).

93 **Table 1. Clinical strains in this study**

Strain	Origin	Sex	Location	Year of isolation
2080	Human	M	Djebonoua	2011
2070	Human	F	Djekanou	2011
2084	Human	M	Grand Lahou	2011
2078	Human	M	Sakassou	2011
2081	Human	M	Sinfra	2011
2095	Human	F	nd	2011
2077	Human	M	nd	2011
2076	Human	F	nd	2012
2065	Human	M	Sakassou	2012
2071	Human	F	Djekanou	2012
2082	Human	M	nd	2012
2067	Human	M	nd	2012
2089	Human	F	nd	2012
2108	Human	F	nd	2012
2114	Human	M	nd	2012
2066	Human	F	nd	2012
2079	Human	F	Daloa	2012
2085	Human	M	nd	2012
2090	Human	F	nd	2012
1044	Human	nd	San-Pedro	2012
2086	Human	F	nd	2012

94 *nd: Not done*

95

96 2.2 DNA Extraction

97 The **Schwab** samples were first incubated overnight after the addition of 2 ml sterile water at
 98 room temperature. The solution was vigorously **mixed 2 min** and 200 µl were taken out for
 99 the **genomic extraction**. The solution was centrifuged for 15 min, 15000 rpm at 4°C and the
 100 pellet was added to 100 µl NaOH 50mM and incubated at 95°C for 15 min. The neutralization
 101 of the DNA was performed by the adding of 15 µl Tris-HCl 0.1 M. The DNA was precipitated
 102 by adding of 1/10 volume of **CH₃CO₂Na** 3M and 2 volumes of 70% Ethanol. The DNA **was**
 103 **washing** by 70% Ethanol and was eluted in 50 µl of DNA/Rnase-free water. The control strain
 104 of *M.ulcerans* (ITM 9540) **strainwas** provided from Institute of Tropical Medicine (**ITM**),WHO
 105 Collaborating Center and Laboratory of Reference for Mycobacteria, Belgium.

106 2.3 Diagnostics PCR for *Mycobacterium ulcerans*

107 The targets IS2404 and KR were specific for *M.ulcerans* and were located in the genome and
 108 in the virulence plasmid respectively (Table 2). Real time PCR were performed using PCR
 109 **regeants** Kit (Promega, Germany) using the previous protocols (11; 21-22) Briefly, the 25 µl-
 110 reaction contains 5 µl of DNA, 0.3 µM of each primer, 0.25 µM of labelled probe, and PCR-
 111 Mix. PCR consisted of 35 cycles of melting at 95°C for 5 sec, **annealing** and extension at 60°C
 112 for 1min. Negative controls were performed with 5 µl of nuclease free-water. Positive
 113 controls DNA were tested in duplicate. The 7300 real-time PCR (Applied Biosystems, USA)
 114 was used and the fluorescence of FAM was measured to determine the amplification
 115 threshold cycle (Ct).

116 **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)
	MIRU 1 R	GCCCTCGGGAATGTGGTT	
VNTR-6	Locus 6F	GACCGTCATGTCGTTTCGATCCTAGT	(15)
	Locus 6R	GACATCGAAGAGGTGTGCCGTCT	
ST1	ST1 F	CTGAGGGGATTTCACGACCAG	(18)
	ST1 R	CGCCACCCGCGGACACAGTCG	
VNTR-19	Locus 19 F	CCGACGGATGAATCTGTAGGT	(15)
	Locus 19R	TGGCGACGATCGAGTCTC	

117

118 2.4 MIRU-VNTR Typing

119 The MIRU-VNTR-typing using PCR was running in a Thermocycler (GenAmp 9700, Applied
 120 Biosystems, USA)to amplify the MIRU1, the VNTR locus 6 and the VNTR locus 19 in same
 121 parameters (19). For *M. ulcerans* specific-VNTR target ST-1, the PCR parameters were
 122 described by Hilty et al 2006 (18). Briefly, 5µl of extracted DNA were added to 45 µl PCR-Mix
 123 containing 0.2 mMdNTPs (Sigma, USA), 0.4 µM each primer, 1.5 mM MgCl₂ (Promega,
 124 Germany), 1X Flexi Taq-Polymerase Puffer and 1 Unit Go-Flexi Taq-Polymerase (Promega,
 125 Germany).All used primers were represented in Table 2. The samples were tested by the
 126 1/10 dilution for the detection of inhibitors. All PCR runs have included negative control
 127 (sterile Dnase/Rnase-free water) for the test quality and the detection of contamination.
 128 Genomic DNA from ITM 9540 strain was tested as positive control for the MIRU-VNTR-
 129 typing tests.

130 2.5 Visualization of amplified products

131 Each sample was tested in simplex Typing-PCR for the four loci and 15µl amplicons were
 132 analyzed in 1.5 % Agarose gel by electrophoresis. The polymorphism was detected in
 133 different size of the amplicons. The size of the amplicons was estimated by comparison with
 134 the100-bp Ladder (Eurobio, France).

135

136 3. RESULTS

3.1 Diagnosis of BU

21 clinical samples were collected from BU patients in endemic regions of Côte d'Ivoire. 47% were female and 52% were male. Over 50% indicated the location of the patients in Côte d'Ivoire and 47 % of the samples cannot be **geographical located** (Table1).

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 (52%) samples. For low bacterial load, by Ct 27-36, 9 (42%) samples 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 (33%) **sampleswhile** 14 (66%) samples have low bacterial load by Ct 27-35 (Table3). 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)

148

149 3.2 Genotyping by MIRU-VNTR

150 From 21 samples collected from BU patients, *M. ulcerans* DNA were analyzing by four MIRU-
 151 VNTR genotyping markers for molecular characterization. ST1 was the most amplified locus
 152 by 85% of the samples, while 61.9% and 57% for VNTR locus 19 and MIRU1 and respectively
 153 VNTR locus 6 (Table 4). 4.7 % (1/21) of samples were negative for 4 genotyping loci and 9.5%
 154 (2/21) of samples were negative for three loci (Table 4).

155 The detection of MIRU1 in clinical samples has shown 2 amplified products corresponding of
 156 one copy (380 bp) and of 3 copies (486 bp) of the 53 bp-repeat sequence (MIRU1) in the
 157 genome of *M. ulcerans*(Fig.1A, Table 4). For VNTR locus 6, 57 % (11/21) of samples were
 158 amplified only a product-PCR corresponding of 500 bp for one copy of the repeat sequence
 159 in the genome (Fig. 1B, Table 4). PCR with target ST1 has shown 2 amplified products of 369
 160 bp and 423 bp for one copy and 2 copies respectively (Fig.1C, Table 4). PCR targets VNTR
 161 locus 19 has amplified one product (340 bp) corresponding to 2 copies in genome (Fig. 1D,
 162 Table 4).

163 Molecular profiles of the strains were elucidated after the amplicon size of genotyping PCR
 164 by MIRU-VNTR. The polymorphism of the strains was represented as the copie number of
 165 genotyping markers as VNTR-profile (Table 4). 28.5 % (6/21) of samples have the most VNTR-
 166 profile C that represents 3, 1, 2, 2 copies of MIRU1, VNTR6, ST1 and VNTR19 respectively
 167 (Table 4).

168 The strain 2070 with the profile (3,1,1,2) and the strain 2071 with the profile (1,-, 2,2) were
 169 collected in the same site Djekanou and have distinct genetic diversity.

170

171 The strains 2071, 2078, 2085, and 2108 have particular results for the genotyping marker
 172 MIRU1, these strains have amplified a 380 bp-PCR-product corresponding to one copy
 173 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)

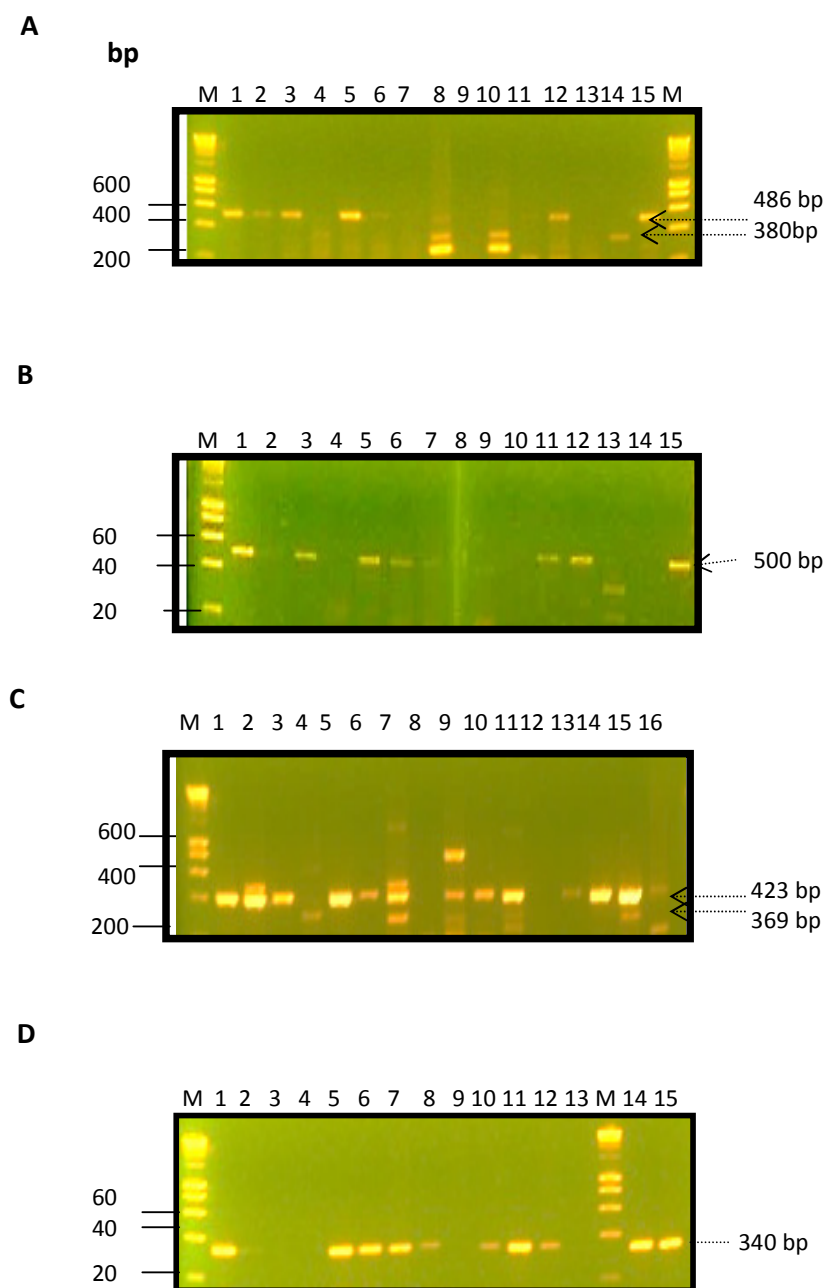


Figure 1. Agarose gel analysis of MIRU-VNTR Typing of clinical samples. A: MIRU1; B: VNTR 6; C: ST1; D: VNTR19; M: 100 bp-DNA Ladder (Promega, Germany); 1: positive control DNA ITM 9540; 2-16: clinical samples.

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

Copies detected						
No	Code Sample	MIRU1	VNTR 6	ST1	VNTR 19	Profile VNTR
1	2080	3	1	2	2	C
2	2070	3	1	2	2	C
3	2084	3	1	2	-	C
4	2078	1	-	1	-	-
5	2081	3	1	2	2	C
6	2095	3	1	2	2	C
7	2077	-	1	2	2	-
8	2076	3	-	-	2	-
9	2065	-	-	2	-	-
10	2071	1	-	2	2	-
11	2082	-	1	2	2	-
12	2067	3	1	-	2	-
13	2089	-	-	2	-	-
14	2108	1	-	2	2	-
15	2114	3	1	2	2	C
16	2066	3	-	2	-	-
17	2079	-	-	-	-	-
18	2085	1	-	1	-	-
19	2090	-	-	2	-	-
20	1044	-	1	2	2	-
21	2086	-	1	2	2	-
Total	21	13/21	11/21	18/21	13/21	6/21
(%)	(100)	(61.9)	(52.3)	(85.7)	(61.9)	(28.5)

202

203 4. DISCUSSION

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

210 For molecular characterization of *M. ulcerans* strains from patients, we have first confirmed
 211 100% of samples by PCR IS2404. The diagnostic using target IS2404 is very consistent and
 212 specific because the high number copies in genome (10) and is widely used in environmental

213 and clinical samples (11,23). The ketoreductase B-domain is present in 15 copies within the
214 mycolactone synthase *gen* and the real-time assay has shown *gut sensitivity* in previous
215 assay (21-22). Real-time PCR is more 10-10.000 sensitive than classic PCR, however the PCR
216 is recommended by World Health Organization as the confirmatory test for clinical diagnosis
217 of *M. ulcerans* infection. This recommendation is appropriate and supports the national
218 surveillance in endemic regions and including a multicenter external quality assessment for
219 molecular tests (24). The failure of PCR-KR for BU detection can be explain by the loss of
220 plasmid by genomic extraction or PCR inhibitors. However, all clinical samples were collected
221 from ulcers that involved by mycolactone *gen* located by plasmid.
222 To elucidate, the diversity of circulating strains of BU in Côte d'Ivoire, we have using the
223 tandem repeats (TR) as genotyping markers. Among the 59 TR found in MPMs, 19 are
224 consistent to be use in genotyping studies (14-15; 18-19). The findings of Lavender *et al* 2008
225 support the high specificity and sensitivity of four genotyping markers for *M. ulcerans* and
226 others MPMs.

227 Our results have demonstrated by MIRU1 two different profile strains that contain three
228 copies or one copy of the repetitive sequence, that can suggest the VNTR profile A or D.

229 For ST1 locus, we have identified 1 or 2 repeats in genome of clinical strains and these were
230 identical with the previous findings (18). The authors have shown by sequencing analyzis of
231 two alleles (BD for 2 repeats and C for 1 repeat) of ST1 in clinical samples in West Africa. ST1
232 has most amplify in PCR typing by 85% of the samples. Unlike others VNTRs, the repeat
233 sequence ST1 has shown no microdeletion but only single-nucleotide polymorphism in
234 sequence variants (18).

235 While by the genotyping markers VNTR locus6 and VNTR locus 19, our results have indicated
236 no genomic variation in clinical strains. Hilty *et al* 2006 have shown that the diversity of
237 Mycobacteria strains using MIRU-VNTR typing can be explain by the deleting of nucleotide in
238 repetitive sequence and the consequence of negative results in PCR methods. Previous
239 studies have explored the clonal distribution of African genotype and the association of low
240 rate of genomic drift of *M. ulcerans* (18). The use of minisatellites or tandem repeat (TR)
241 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 the most 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 can be explain by the high variability of TR by insertion or deletion of nucleotides in the genome and the genome rearrangement for the adaptation in aquatic environment (10).

Our results suggest the circulation of different profiles or genotypes of *M. ulcerans* for BU in Côte d'Ivoire that can explain the endemicity of the disease in the country. In perspectives studies the whole genome sequencing of *M. ulcerans* will be explore 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. In this study, we have demonstrated the diversity of *M. ulcerans* for BU patients using MIRU-VNTR typing. Our results confirm first the genomic diversity of the strains of *M. ulcerans* and the circulation of different profiles in Côte d'Ivoire.

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