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

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Authors' contributions

This work was carried out in collaboration between all authors. Author KNES designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors KNES, CND and VS managed the analyses of the study. Authors AN and VS managed the technical analysis. Authors AS and DM were carried out in administration proof. All authors read and approved the final manuscript.

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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).

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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 keto reductase-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.

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

Molecular detection for diagnosis based on Insertion Sequence 2404 and has good specificity and high sensitivity for *M. ulcerans*, and then IS2404 is present in over 203 copies in genome [5,10,11]. 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 lengh polymorphism (RFLP), *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 copy numbers 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 *M. ulcerans* and other environmental mycobacteria allows the discrimination between 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) is very useful in detecting 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* sp. in clinical samples from Buruli ulcer patients by using MIRU-VNTR typing.

2. MATERIALS 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 50 mM 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 Sodiumacetat 3M 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 control strain 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 1 min. 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).

2.4 MIRU-VNTR Typing

The MIRU-VNTR-typing using PCR was running in a Thermocycler (GenAmp 9700, Applied Biosystems, USA) to amplify the MIRU1, the VNTR locus 6 and the VNTR locus 19 in same parameters [19]. For *M. ulcerans* specific-VNTR target ST-1, the PCR parameters were described by Hilty et al. [18]. Briefly, 5 µl of extracted DNA were added to 45 µl PCR-Mix containing 0.2 mMd NTPs (Sigma, USA), 0.4 µM each primer, 1.5 mM $MgCl_2$ (Promega, Germany), 1X Flexi Taq-Polymerase buffer and 1 Unit Go-Flexi Taq-Polymerase (Promega, Germany). All used primers were represented in Table 2. The

samples were tested by the 1/10 dilution for the detection of inhibitors. All PCR runs have included negative control (sterile Dnase/Rnase-free water) for the test quality and the detection of contamination. Genomic DNA from ITM 9540 strain was tested as positive control for the MIRU-VNTR-typing tests.

2.5 Visualization of Amplified Products

Each sample was tested in simplex Typing-PCR for the four loci and 15 µl 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 the100-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 Cote d'Ivoire and 47% of the samples cannot geographical 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 7samples (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.

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 copienumber of genotyping markers as VNTR-profile (Table 4).

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	
Keto reductase	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	GACCGTCATGTCGTTCGATCCTAGT	[15]
	Locus 6R	GACATCGAAGAGGTGTGCCGTCT	
ST-1	ST1 F	CTGAGGGGATTTCACGACCAG	[18]
	ST1 R	CGCCACCCGCGGACACAGTCG	
VNTR-19	Locus 19F	CCGACGGATGAATCTGTAGGT	[15]
	Locus 19R	TGGCGACGATCGAGTCTC	

Table 3. PCR diagnostics for *M. ulcerans*

No	Strain	Diagnostic tests	
		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 (H_20): Ct has not been detected

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).

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 keto reductase B-domain is present in 15 copies within the mycolactone synthase gen 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 clinicalcase 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 gen 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

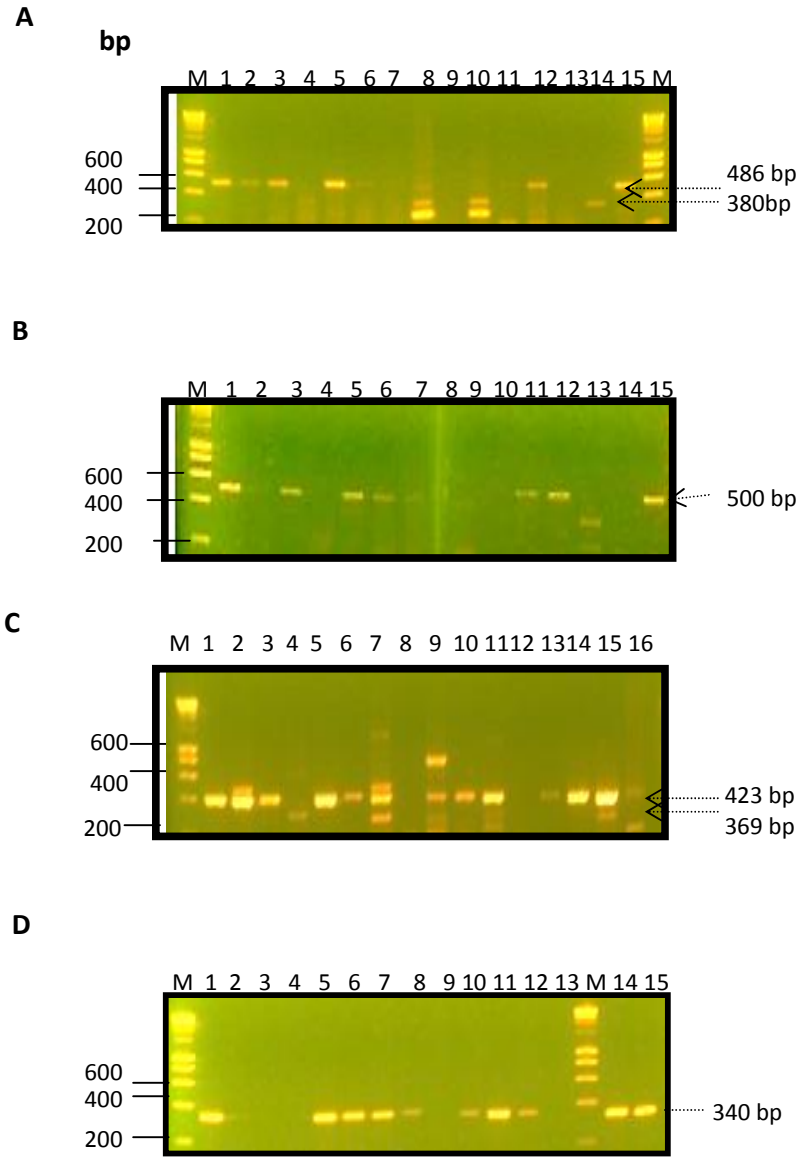

Fig. 1. Agarose gel analysis of MIRU-VNTR typing of clinical samples

A: MIRU1; B: VNTR locus 6; C: ST-1; D: VNTR locus 19; M: 100 bp-DNA Ladder (Promega, Germany); 1: positive control DNA ITM 9540; 2-16: clinical samples

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].

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	-	-	-	-	-	Daloa
18	2085	1	-	1	-	-	nd
19	2090	-	-	2	-	-	nd
20	1044	-	1	2	2	-	San-Pedro
21	2086	-	1	2	2	-	nd
Total (%)	**21(100)**	**13/21(61.9)**	**11/21(52.3)**	**18/21(85.7)**	**13/21(61.9)**	**6/21(28.5)**	

nd: unidentified; the positive control DNA (ITM 9540) has follows results: MIRU1 positive (3 copies), VNTR 6 positive (1 copy); ST-1 positive (2 copies) and VNTR 19 positive (2 copies).Negative control (H_2O) has a negative result for all PCRs

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 causedby 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, Narh 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'Ivoireby demonstrating the diversity of *M. ulcerans* for BU patients using MIRU-VNTR typing.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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