First MIRU-VNTR typing of *Mycobacterium ulcerans* in Togo (West Africa)

ABSTRACT

Background: Buruli ulcer is the third most bacterial disease worldwide. Cases most occurs in 30 countries but severe cases occur in West Africa countries such as Benin, Cote d'Ivoire and Togo mainly in rural regions. Early diagnosis may prevent severe disability. Molecular technique seems the best solution and new MIRU VNTR typing method base on variable number tandem repeats (VNTR) and Mycobacterial Interspersed Repetitive Units (MIRU) is most reproducible and propose geographical, inter and intraspecies differentiation and can be used as a diagnosis tool

Objective: The objective of this study was to investigate the molecular diversity by using MIRUVNTR typing in clinical samples of BU patients in Togo

Study design: 64 DNA extracts from clinical samples were collected from BU patients in the two principal endemics districts in Togo (Yoto and Zio) with three less endemic districts (Bas Mono, Lacs and Vo). First we performed IS2404 and KR real time PCR plus IS2606 conventional PCR. In a second step, we have analyzed the strains by PCR typing for five specific and sensitive markers MIRU1, VNTR6, ST1, VNTR19 and VNTR9.

Results and Conclusion: 71.11% were positive for IS2404, 3.13% were positives for PCR-KR and 31.11% for IS 2606. By MIRU-VNTR typing, we have found 48.86% positive for MIRU1 and 25.00%, 20.31%, 18.75% and 14.06% for VNTR6, ST1, VNTR19 and VNTR9 respectively. One of sample was negative for all genotyping markers. Two different genetic profiles were identified by MIRU1, ST1 and VNTR loci by gel-analyzed of the amplified products. The VNTR profile B (3,1,1,2) corresponding of 3 copies MIRU1, 1 copy VNTR6, 1 copy ST-1 and two copies of VNTR19 was detected in 15.63% of samples and the VNTR profile A (1,1,1,2) corresponding of 1 copy MIRU1, 1 copy VNTR6, 1 copy ST-1 and 2 copies of VNTR19 was detected in 3.13% of samples and confirms the West African genotype (3,1,1) in Togo. 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 Togo

Keywords *: Buruli ulcer ; M.ulcerans ; genotyping ; MIRU-VNTR-typing ; diversity ; Togo ; West Africa.*

Introduction

Mycobacterium ulcerans is a slow-growing environmental mycobacterium, which causes significant morbidity and a disfiguring condition known as Buruli ulcer. *M.ulcerans* infections have been found in more than 30 tropical and subtropical countries of Africa, Asia, Latin America and the Western Pacific but are most common and severe in West Africa [1,2] including rural parts of Benin, Cameroon, Côte d'Ivoire, Ghana and Togo. About 48% of reported cases in Africa are in children below 15 years, and both genders are equally affected. Recent reports describe increases in the incidence of BU in Australia [3,4], Benin [5] and Côte d'Ivoire [2]. In Togo, since the early 1990s patients with lesions clinically suspicious for Buruli ulcer disease (BUD) have been treated in Togolese hospitals. The first two laboratory-confirmed and well documented BUD patients from Togo were described in 1996 by Meyers and colleagues [6]. According to data available at the Togolese Ministry of Health, from 1996 through 2004 more than 100 suspected BUD cases were notified and approximately 20% of these were PCR confirmed at the Institute for Tropical Medicine, Antwerp, Belgium. In 2004, a nationwide survey detected 1505 suspected cases of BUD ["Politique Nationale de Lutte contre L'Ulcère de Buruli." Ministère de la Santé, République Togolaise, Lomé 2007] [7] and cases still increasing. The epidemiology of BU is poorly understood, but most foci are associated with slow-flowing or stagnant water; however, the natural reservoir of M. ulcerans remains unknown. M. ulcerans is often difficult to isolate from clinical specimens and usually requires 6 to 8 weeks to produce visible growth in primary culture [8,9]. Definitive identification of M. ulcerans is thus time-consuming; however, it can be recognized by microbiologic methods and classic molecular [10,11]. In the last decade, various DNA-based techniques have been used to classify mycobacteria [12,13]. All such studies have demonstrated a high taxonomic affiliation between *M.ulcerans* and *M.marinum*. Discrimination of genetic variants has become an indispensable tool to unravel the evolution, epidemiology and transmission of pathogenic organisms and to gain insight into host-pathogen interactions [14,15]. In M. ulcerans, such elucidation is impossible due to a remarkable lack of genetic diversity on a local geographic scale [16]. Conventional genetic differentiation tools commonly used for phylogenetic profiling in Mycobacterium tuberculosis, such as restriction fragment length polymorphism, amplified fragment length polymorphism, variable-number tandem repeats (VNTR), and multilocus sequence typing, could distinguish between continental lineages only when applied to *M. ulcerans* [17]. Thus, uncontrolled duplications and insertions of ISEs occur at relatively high frequency in replicating bacteria, leading to genomic insertions, deletions, and rearrangements that have the potential for molecular epidemiological applications. For M. ulcerans, two ISEs were defined, IS2404 and IS2606 [18,19]. Molecular detection for diagnosis based on Insertion Sequence 2404 has good specificity and high sensitivity for M. ulcerans, and then IS2404 is present in over 203 copies in genome [20,17,21]. Previous studies have demonstrated different profiles of M. ulcerans by PCR-restriction profile and 16S rRNA sequencing [8,22]. The use of IS2404 restriction fragment length polymorphism (RFLP) analysis [23] led to the classification of M. ulcerans into six groups, including the isolate from Suriname as M. ulcerans type VI. The availability of complete genome sequences of M. ulcerans [17] allowed the identification of minisatellite markers including MIRUs [16] and other novel tandem repeat loci [18]. Variable number tandem repeats (VNTR) typing is a promising typing tool for M. ulcerans, because analysis based on few loci already has a higher discriminatory power than other standard molecular typing methods. VNTR typing thus revealed for the first time genetic diversity within

isolates from African countries [18]. The high resolution of variable number tandem repeats (VNTR) is very useful in detecting heterogeneity among *M.ulcerans* in clinical and in environmental samples. Previous studies have recommended specific and sensitive VNTR loci to discriminate between M. ulcerans and MPMs [16,21,19,24]. Ecological and Molecular epidemiological studies to identify reservoirs and transmission vectors are important for source tracking infections especially during outbreaks and elucidating transmission routes. Research efforts have therefore focused on genotyping strains of the mycobacteria from clinical and environmental samples. The objective of this study was the molecular characterization of Mycobacterium sp. in clinical samples that circulate in Togo by using MIRU-VNTR typing.

MATERIALS AND METHODS

Ethical Approval

The study was conducted in accordance with the Declaration of Helsinki and received approval from the Bioethics Committee for Health Research (CBRS) of Togo (N ° 21 / 2017 / CBRS of October 8, 2017) and the access to the data was authorized by the board of the National Program against Buruli Ulcer, Leprosis and Pian (Reference: 159/2015/MS/ DGS/ DSSP/ PNLUB-LP of November 20, 2015).

Clinical Sampling

90 DNA extract from Buruli ulcer patients in Togo are selected. All patients were first clinically confirmed for BU by PCR IS 2404 positive and both are Zielh Neelsen positive in Togo. They were treated by recommended double antibiotic therapy. In Institute Pasteur of Cote d'Ivoire, before VNTR-MIRU typing we performed IS 2606, IS 2404 and KR PCR. 64 DNA extract were selected for VNTR-MIRU typing (**Figure 1**)

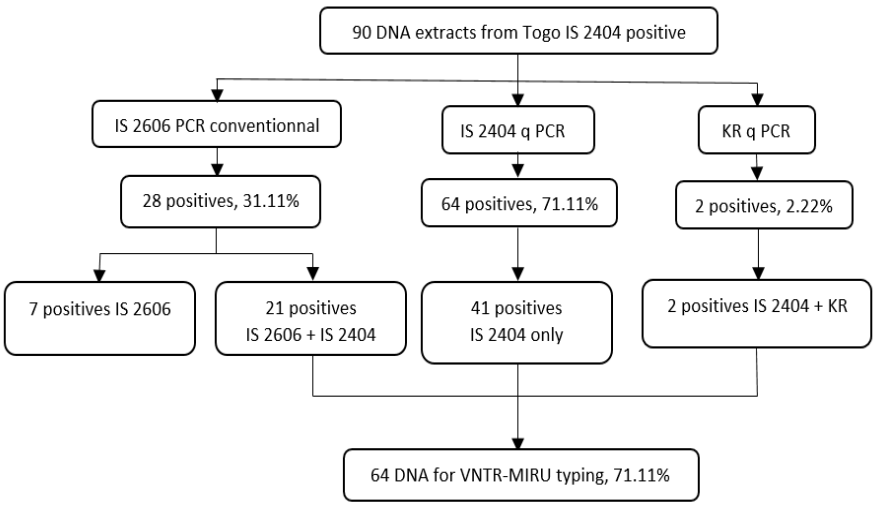

Figure 1: DNA extracts selected for VNTR-MRU typing

64 clinical samples were selected from Maritime Sanitary Region of Togo, mainly 34 from Zio and 27 from Yoto the most endemics districts and 3 from Bas-Mono, Lacs and Vo. 34 from females and 30 from males. All of the isolates were selected to reflect both spatial and temporal diversity in Togo. Length polymorphism was estimated from the size of the PCR product following separation on agarose gel and based on included positive controls as described by previous studies [19,24]. We used *M. ulcerans* strains from Côte d'Ivoire (CI 25/2017) as positive controls for the VNTR typing.

Table 1: Clinical DNA extracts isolated from 2015 to 2017 in Zio, Yoto and Bas-Mono BU endemics districts selected for VNTR-MIRU typing

Strain	Sex	Age	Village	Dis-trict	Year of extraction	Strain	Sex	Age	Village	Dis-trict	Year of extraction
TG0115	F	12	FRANGADUA	ZIO	2015	**TG3315**	F	60	TCHEKPO ANAGALI	YOTO	2015
TG0215	F	50	GATI	ZIO	2015	**TG3416**	M	12	GATI SUN	ZIO	2016
TG0315	F	9	TABLIGBO ADETICOPE	YOTO	2015	**TG3516**	F	14	TCHEKPO DEVE	YOTO	2016
TG0415	M	10	FONGBE APEDOME	ZIO	2015	**TG3616**	F	20	TCHEKPO DEVE	YOTO	2016
TG0515	F	48	FONGBE APEDOME	ZIO	2015	**TG3716**	F	8	TCHEKPO AVEDJI	YOTO	2016
TG0615	M	5	GATI SUN	ZIO	2015	**TG3816**	F	8	GATI AGODOU	ZIO	2016
TG0715	F	5	FONGBE BOETI	ZIO	2015	**TG3916**	M	5	FONGBE ZOGBEDJI	ZIO	2016
TG0815	F	14	GAPE AGOCOPE	ZIO	2015	**TG4016**	M	30	TCHEKPO DEVE AGBEDJIKO	YOTO	2016
TG0915	F	8	GATI SUN	ZIO	2015	**TG4116**	M	2	TCHEKPO DEDEKPOE	YOTO	2016
TG1015	F	7	TCHEKPO DEVE	YOTO	2015	**TG4216**	F	45	TCHEKPO DEVE	YOTO	2016
TG1115	M	8	FONGBE APEDOME	ZIO	2015	**TG4316**	M	48	FONGBE ZOGBEDJI	ZIO	2016
TG1215	M	12	GATI SUN	ZIO	2015	**TG4416**	F	9	FONGBE APEDOME	ZIO	2016
TG1315	M	14	KODZO	ZIO	2015	**TG4516**	M	6	FONGBE APEDOME	ZIO	2016
TG1415	M	15	FONGBE ZOGBEDJI	YOTO	2015	**TG4616**	M	19	GATI SUN	ZIO	2016
TG1515	F	5	TCHEKPO ANAGALI	YOTO	2015	**TG4716**	M	6	TCHEKPO ANAGALI	YOTO	2016
TG1615	M	8	TCHEKPO DEDEKPO	YOTO	2015	**TG4816**	F	32	TCHEKPO DEVE	YOTO	2016
TG1715	M	2	TCHEKPO ANAGALI	YOTO	2015	**TG4916**	M	3	TCHEKPO DEDEKPOE AVEGODOE	YOTO	2016
TG1815	F	15	KODZO	ZIO	2015	**TG5016**	M	12	GATI SUN	ZIO	2016
TG1915	F	11	GATI AGODOU	ZIO	2015	**TG5116**	F	10	GATI SUN	ZIO	2016
TG2015	M	6	TCHEKPO DEDEKPOE	YOTO	2015	**TG5216**	F	19	FONGBE APEDOME	ZIO	2016
TG2115	M	15	AGBATA	LACS	2015	**TG5316**	F	6	GATI SUN	ZIO	2016
TG2215	M	11	GATI AGODOU	ZIO	2015	**TG5416**	F	10	TCHEKPO DEDEKPOE	YOTO	2016
TG2315	F	9	ADANGBE LAKOTO	ZIO	2015	**TG5516**	F	58	TCHEKPO AVEDJI	YOTO	2016
TG2415	M	6	GATI AGODOU	ZIO	2015	**TG5616**	F	70	FONGBE APEDOME	ZIO	2016
TG2515	M	7	FONBGE ZOGBEDJI	ZIO	2015	**TG5716**	F	60	TCHEKPO-DEVE	YOTO	2016
TG2615	F	34	TCHEKPO DEVE	YOTO	2015	**TG5816**	M	62	BATONOU	BAS MONO	2016
TG2715	M	8	YOBO TOUME	ZIO	2015	**TG5917**	M	4	GATI AGODOU	ZIO	2017
TG2815	M	9	TCHEKPO DEDEKPOE	YOTO	2015	**TG6017**	F	13	AKOUMAPE	VO	2017
TG2915	F	50	TCHEKPO ANAGALI	YOTO	2015	**TG6117**	M	13	GATI AGODOU	ZIO	2017
TG3015	F	9	TCHEKPO DEDEKPOE	YOTO	2015	**TG6217**	F	7	FONGBE ZOGBEDJI	ZIO	2017
TG3115	F	10	TCHEKPO ANAGALI	YOTO	2015	**TG6317**	F	14	GATI AKPAFO	ZIO	2017
TG3215	M	11	TCHEKPO DEDEKPOE	YOTO	2015	**TG6417**	M	10	TCHEKPO DEVE	YOTO	2017

DNA Extraction

The swab or FNA samples were first added to 15 µl of Lysozyme and incubated 37°C 1hour in the thermomixor. DNA extraction were performed using extraction reagents Puregene Kit (Qiagen, UK) using the previous protocols [25]. Next added 10 µl of

proteinase K incubated 55°C during 4 hours in the thermomixor or overnight. Next added 230 µl of Protein Precipitation Solution (PPS) in icy bag. The DNA was precipitated by adding 1/10 volume of Sodiumacetat 3M and 2 volumes of 70% Ethanol. The DNA was precipitated by adding 700 µl of isopropanol, 2 µl of glycogen and washed with 700 µl of ethanol 70%. The DNA was eluted in 500 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.

PCR for *Mycobacterium ulcerans*

Target IS2404 located in the genome was specific for *M. ulcerans.* IS 2606 located in the genome was specific for *M. ulcerans* and *M lentiflavum*. KR were in the virulence plasmid (Table 3). Real time PCR and PCR conventional were performed using PCR reagents Kit (Promega, Germany) using the previous protocols [26]. 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). For the conventional PCR, the thermocycler GeneAmp©PCR System 9700 was used.

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, VNTR locus 9 and the VNTR locus 19 in same parameters [27]. For *M. ulcerans* specific-VNTR target ST1, the PCR parameters were described by Hilty et al. [19]. 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 FlexiTaq-Polymerase buffer and 1 Unit Go-Flexi TaqPolymerase (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/Rnasefree water) for the test quality and the detection of contamination. Genomic DNA from ITM 9540 strain and strain from Cote d'Ivoire CI 25/2017 was tested as positive control for the MIRU-VNTR-typing tests.

Visualization of Amplified Products

Each sample was tested in simplex Typing-PCR for the five 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 the 100-bp Ladder (Eurobio, France).

Table 2: Primers used in this study

Target	Oligos	Primer sequence (5'-3')	References
IS2404	IS2404 F	ATTGGTGCCGATCGAGTTG	[21,28,29]
	IS2404 R	TCGCTTTGGCGCGTAAA	
	IS2404probe	6 FAM-CACCACGCAGCATTCTTGCCGT-TAMRA	
IS2606	IS2606 F	CCGTCACAGACCAGGAAGAAG	
	IS2606 R	TGCTGACGGAGTTGAAAAACC	
Keto reductase	KR F	TCACGGCCTGCGATATCA	
	KR R	TTGTGTGGGCACTGAATTGAC	
	KR-probe	6 FAM-ACCCCGAAGCACTG-TAMRA	
MIRU1	MIRU1 F	GCTGGTTCATGCGTGGAAG	
	MIRU1 R	GCCCTCGGGAATGTGGTT	
VNTR6	Locus 6 F	GACCGTCATGTCGTTCGATCCTAGT	
	Locus 6 R	GACATCGAAGAGGTGTGCCGTCT	

ST1	ST1 F	CTGAGGGGATTTCACGACCAG
	ST1 R	CGCCACCCGCGGACACAGTCG
VNTR-19	Locus 19F	CCGACGGATGAATCTGTAGGT
	Locus 19R	TGGCGACGATCGAGTCTC
VNTR-9	Locus 9F	GCCGAAGCCTTGTTGGACC
	Locus 9R	GGTTTCCCGCAGCATCTCG

RESULTS

64 clinical samples were selected from Maritime Sanitary Region of Togo, mainly 53.12% from Zio and 42.19% from Yoto the most endemics districts and 4.69% from Vo, Lacs and Bas-Mono (**Figure 2**). 53.13% from females and 46.87% from males. Patients were 2 to 70 years old.

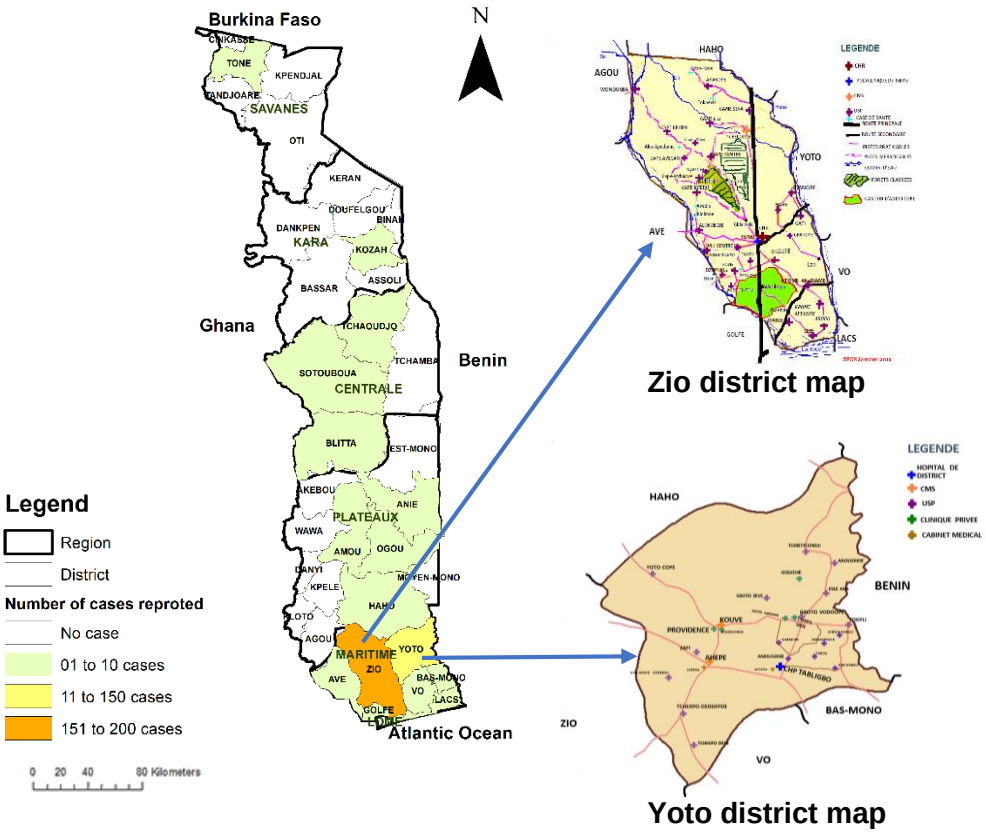

Figure 2: Maps of Togo and the two endemics districts of Yoto and Zio showing the localities from which strains originated

By using real time PCR with target IS2404, 71.11% were found positive for *M.ulcerans*. The detected threshold cycle (Ct) of real time PCR-IS2404 was low (Ct: 3.816 – 26.472) and indicated the high bacterial level of *M.ulcerans* in 14 samples (21.88% of cases) while 50 samples (78.12% of cases) have high detected threshold cycle (Ct: 27.264 – 38.382) indicated low bacterial load. For PCR-KR only two samples (3.13%) were positives with low CT (7.940 and 8.465). They were positives too for IS2404 and the bacterial load were low when the potential mycolacton polyketide producing is high. For PCR-IS2606, conventional PCR were performed and the size of the amplicons revealed positives cases. 28 samples were positives (31.11%) with 21 (32.81%) simultaneously positives to IS2404 (**Table 3**).

Table 3: PCR diagnostics for *M. ulcerans*

N°	Strains	Diagnostics tests	N°	Strains	Diagnostics tests

		PCR IS2404(Ct)	PCR IS 2606	PCR KR (Ct)			PCR IS2404(Ct)	PCR IS2606	PCR KR (Ct)
1	**TG0115**	24,991	POSITIVE	Undetermined	33	**TG3316**	34,411	NEGATIVE	Undetermined
2	**TG0215**	24,312	POSITIVE	Undetermined	34	**TG3416**	31,425	POSITIVE	Undetermined
3	**TG0315**	30,535	NEGATIVE	Undetermined	35	**TG3516**	23,939	POSITIVE	Undetermined
4	**TG0415**	38,065	POSITIVE	Undetermined	36	**TG3616**	31,884	NEGATIVE	Undetermined
5	**TG0515**	23,554	POSITIVE	Undetermined	37	**TG3716**	33,856	NEGATIVE	Undetermined
6	**TG0615**	29,584	POSITIVE	Undetermined	38	**TG3816**	34,600	NEGATIVE	Undetermined
7	**TG0715**	32,255	POSITIVE	Undetermined	39	**TG3916**	30,424	NEGATIVE	Undetermined
8	**TG0815**	22,275	POSITIVE	Undetermined	40	**TG4016**	34,736	NEGATIVE	Undetermined
9	**TG0915**	33,343	NEGATIVE	Undetermined	41	**TG4116**	24,686	NEGATIVE	Undetermined
10	**TG1015**	23,513	POSITIVE	Undetermined	42	**TG4216**	34,197	POSITIVE	Undetermined
11	**TG1115**	32,520	NEGATIVE	Undetermined	43	**TG4316**	33,134	NEGATIVE	Undetermined
12	**TG1215**	32,523	POSITIVE	Undetermined	44	**TG4416**	36,054	POSITIVE	Undetermined
13	**TG1315**	30,062	NEGATIVE	7,940	45	**TG4516**	28,144	POSITIVE	Undetermined
14	**TG1415**	30,095	POSITIVE	Undetermined	46	**TG4616**	27,475	POSITIVE	Undetermined
15	**TG1515**	31,714	NEGATIVE	Undetermined	47	**TG4716**	26,473	POSITIVE	Undetermined
16	**TG1615**	35,324	NEGATIVE	Undetermined	48	**TG4816**	3,817	NEGATIVE	Undetermined
17	**TG1715**	24,089	POSITIVE	Undetermined	49	**TG4916**	34,932	NEGATIVE	Undetermined
18	**TG1815**	32,182	NEGATIVE	Undetermined	50	**TG5016**	33,164	NEGATIVE	Undetermined
19	**TG1915**	22,923	POSITIVE	Undetermined	51	**TG5116**	36,467	POSITIVE	Undetermined
20	**TG2015**	37,196	NEGATIVE	Undetermined	52	**TG5216**	29,872	NEGATIVE	Undetermined
21	**TG2115**	37,602	NEGATIVE	Undetermined	53	**TG5316**	38,383	POSITIVE	Undetermined
22	**TG2215**	24,433	POSITIVE	Undetermined	54	**TG5416**	37,906	NEGATIVE	Undetermined
23	**TG2315**	35,040	POSITIVE	Undetermined	55	**TG5516**	31,719	NEGATIVE	Undetermined
24	**TG2415**	25,658	NEGATIVE	Undetermined	56	**TG5616**	32,962	NEGATIVE	Undetermined
25	**TG2515**	32,572	POSITIVE	Undetermined	57	**TG5716**	37,361	NEGATIVE	Undetermined
26	**TG2615**	25,546	NEGATIVE	Undetermined	58	**TG5816**	37,342	POSITIVE	Undetermined
27	**TG2715**	27,361	POSITIVE	Undetermined	59	**TG5917**	38,140	NEGATIVE	Undetermined
28	**TG2815**	31,505	POSITIVE	8,465	60	**TG6017**	36,471	NEGATIVE	Undetermined
29	**TG2915**	27,265	POSITIVE	Undetermined	61	**TG6117**	30,535	NEGATIVE	Undetermined
30	**TG3015**	35,427	NEGATIVE	Undetermined	62	**TG6217**	37,361	NEGATIVE	Undetermined
31	**TG3115**	35,308	NEGATIVE	Undetermined	63	**TG6317**	38,383	NEGATIVE	Undetermined
32	**TG3215**	31,799	POSITIVE	Undetermined	64	**TG6417**	37,361	NEGATIVE	Undetermined
Positivity rate		**PCR IS 2404**		**PCR IS 2606**				**PCR KR**	
		100.00% (64/64)		**32.81% (21/64)**				**3.13% (2/64)**	

Five MIRU-VNTR genotyping markers for molecular characterization were performed on the 64 DNA extract. MIRU 1 was the most amplified for 48.86% of the samples followed by VNTR locus 6 (25.00%), ST1 (20.31%), VNTR locus 19 (18.75%) and VNTR locus 9 (14.06%). 4.69% (3/64) were positives for 5 genotyping markers, 14.06% (9/64) for 4 genotyping markers and 42.19% (27/64) were negative for all markers (**Table 4**). The detection of MIRU1 has shown two amplified products corresponding to one copy and three copies of 53 bp-repeat sequences. For Locus 6, 100% of the amplified product are one copy (400 bp). The same for ST1, Locus 19 and Locus 9 which amplified products were respectively one copy (500 bp), two copies (500 bp) and two copies (500 bp). Those copies are repeated in the *M.ulcerans* genome. The amplified product's size corresponded to the togolese strains polymorphism and molecular characterization by MIRU-VNTR typing. The VNTR profile (3,1,1,2,-) is the most represented and corresponding to African M.ulcerans strain (3,1,1) and VNTR profile B (3,1,1) for 83.33% (10/12) matching. The VNTR profile (1,1,1,2,-) were represented for 16.67% (2/12) and

were the VNTR profile A. The VNTR profile B were sampling from Yoto district and Zio district and the VNTR profile A were just from Zio district. Others profiles were identified. Three copies for MIRU1 were amplified for different profiles as 20.31% for the profile (3,-,-,-,-), 3.13% for (3,-,-,-,2) and (3,1,-,-,-) and in other hand 1.56% for (3,1,1,-,-). VNTR locus 9 only were matching for 7.81% (5/64) by profile (-,-,-,-,2). The represented profile 28.13% (18/64) were (-,-,-,-,-) with any genotyping marker matching. Both diverse profiles were sampling in all districts of Togo. The control strain from Cote d'Ivoire has shown VNTR-profile C (3,1,2,2) (**Fig 4, Table 4**)

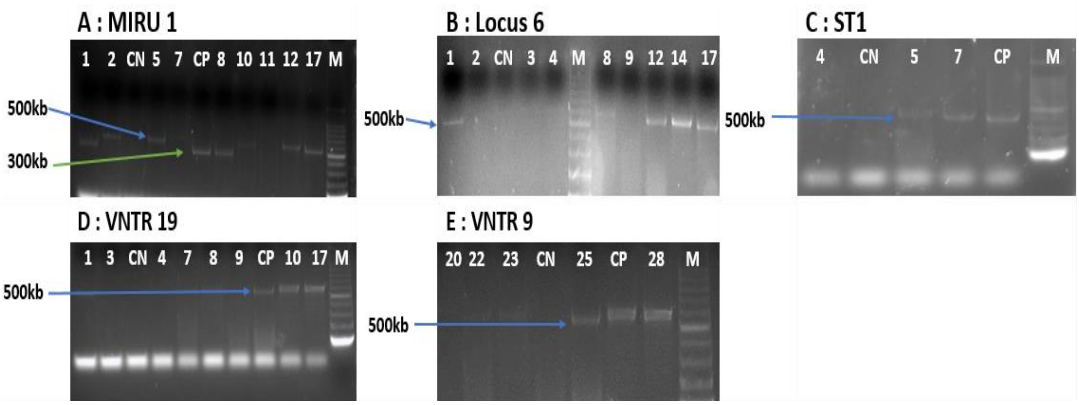

Figure 4: Agarose gel analysis of MIRU-VNTR typing of clinical samples, CN: Negative control DNA/Rnase free water, CP: Positive control DNA CI25/2017, M: 100 bp-DNA Ladder (Promega, Germany)

Table 4: MIRU-VNTR-typing of clinical strains in this study

N°	Strains	Copies detected						VNTR Profile designation	District
		MIRU 1 (509 bp)	LOCUS 6 (500 bp)	ST1 (400 bp)	VNTR 19 (500 bp)	VNTR 9 (550 bp)	VNTR Profile		
1	TG0115	1	1	1	2	-	1,1,1,2,-	A	ZIO
2	TG0215	3	1	1	2	-	3,1,1,2,-	B	ZIO
3	TG0315	-	-	-	-	-		ND	YOTO
4	TG0415	-	-	-	-	-		ND	ZIO
5	TG0515	3	1	1	2	-	3,1,1,2,-	B	ZIO
6	TG0615	-	-	-	-	-		ND	ZIO
7	TG0715	3	1	1		-	3,1,1,-,-	B	ZIO
8	TG0815	1	1	1	2	-	1,1,1,2,-	A	ZIO
9	TG0915	-	-	-	-	-		ND	ZIO
10	TG1015	1	-	1	2	-	1,-,1,2,-	ND	YOTO
11	TG1115	-	-	-	-	-		ND	ZIO
12	TG1215	3	1	-	-	-		ND	ZIO
13	TG1315	-	-	-	-	-		ND	ZIO
14	TG1415	-	1	-	-	-		ND	YOTO
15	TG1515	-	-	-	-	-		ND	YOTO
16	TG1615	-	-	-	-	-		ND	YOTO
17	TG1715	3	1	1	2	-	3,1,1,2,-	B	YOTO
18	TG1815	-	-	-	-	-		ND	ZIO
19	TG1915	3	1	1	2	-	3,1,1,2,-	B	ZIO
20	TG2015	-	-	-	-	-		ND	YOTO
21	TG2115	-	1	-	-	-		ND	LACS
22	TG2215	3	1	1	2	2	3,1,1,2,2	B	ZIO
23	TG2315	-	-	-	-	2		ND	ZIO

24	**TG2415**	3	1	-	-			ND	ZIO
25	**TG2515**	-	-	-	-	2		ND	ZIO
26	**TG2615**	3	-	-	-	-		ND	YOTO
27	**TG2715**	3	-	-	-	-		ND	ZIO
28	**TG2815**	-	-	-	-	2		ND	YOTO
29	**TG2915**	3	-	-	-	2		ND	YOTO
30	**TG3015**	3	-	-	-	-		ND	YOTO
31	**TG3115**	3	-	-	-	-		ND	YOTO
32	**TG3215**	3	-	-	-	2		ND	YOTO
33	**TG3316**	3	-	-	-	-		ND	YOTO
34	**TG3416**	-	-	-	-	-		ND	ZIO
35	**TG3516**	3	1	1	2	-	3,1,1,2,-	B	YOTO
36	**TG3616**	-	-	-	-	-		ND	YOTO
37	**TG3716**	-	-	-	-	-		ND	YOTO
38	**TG3816**	-	-	-	-	-		ND	ZIO
39	**TG3916**	3	-	-	-	-		ND	ZIO
40	**TG4016**		-	-	-	-		ND	YOTO
41	**TG4116**	3	1	1	2	2	3,1,1,2,2	B	YOTO
42	**TG4216**	-	-	-	-			ND	YOTO
43	**TG4316**	-	-	-	-	2		ND	ZIO
44	**TG4416**	-	-	-	-	-		ND	ZIO
45	**TG4516**	3	1	1	2	-	3,1,1,2,-	B	ZIO
46	**TG4616**	-	-	-	-	-		ND	ZIO
47	**TG4716**	3	1	1	2	-	3,1,1,2,-	B	YOTO
48	**TG4816**	-	-	-	-	-		ND	YOTO
49	**TG4916**	-	-	-	-	2		ND	YOTO
50	**TG5016**	-	-	-	-	-		ND	ZIO
51	**TG5116**	-	-	-	-	-		ND	ZIO
52	**TG5216**	3	-	-	-	-		ND	ZIO
53	**TG5316**	-	-	-	-	-		ND	ZIO
54	**TG5416**	-	-	-	-	-		ND	YOTO
55	**TG5516**	3	-	-	-	-		ND	YOTO
56	**TG5616**	3	-	-	-	-		ND	ZIO
57	**TG5716**	3	-	-	-	-		ND	YOTO
58	**TG5816**	-	-	-	-	-		ND	BAS MONO
59	**TG5917**	-	-	-	-	-		ND	ZIO
60	**TG6017**	-	-	-	-	-		ND	VO
61	**TG6117**	-	-	-	-	-		ND	ZIO
62	**TG6217**	3	-	-	-	-		ND	ZIO
63	**TG6317**	3	-	-	-	-		ND	ZIO
64	**TG6417**	3	-	-	-	-		ND	YOTO
Positivity rate		**48.87% (30/64)**	**25.00% (16/64)**	**20.31% (13/64)**	**18.75% (12/64)**	**14.06% (9/64)**			

DISCUSSION

The identification of mycobacterial species constitutes a critical step in patient management because the results obtained influence the choice of appropriate treatment. Classical procedures to establish the species of mycobacteria based on conventional biochemical tests can take several weeks and may generate inaccurate diagnoses. For *M.ulcerans*, there are only a few phenotypic characteristics, making additional molecular

tests essential for conclusive identification. PCR-based methods offer several advantages including speed, sensitivity and specificity [22]. All of our DNA extracts were sampling from endemics districts of Togo by conventional PCR and were from Buruli patients of Centre National de reference d'ulcere de Buruli de Tsevie in Togo. In our study we realized qPCRs for IS 2404 and KR, conventional PCR for IS2606. These three qPCRs served to measure the relative amounts of *M.ulcerans* DNA in the samples and determine the IS2404 : IS2606 copy number ratio which differentiates M. ulcerans from the other MPM. According to Fyfe et al, 2007, *M.ulcerans* susp ulcerans responsible for outbreak in Africa were ΔCt (IS2606-IS2404) ranged from 2.77 (for 104 genomes) to 4.1 (for 0.1 genome) and ΔCt between the KR and IS*2404* assays increased from 1.3 (for 104 genomes) to 4.3 (for 0.1 genomes) but was generally less than that for the ΔCt (IS*2606*-IS*2404*). Although we performed IS2606 conventional PCR, only 32.81% (21/64) samples were positives simultaneously for IS2404 and IS2606 and 67.19% were positives only for IS2404. These results conduct to investigate more about M.ulcerans species whether there are more mycolactone producing Mycobacterium. Relatively the sample's IS2404 Ct, (3.816 – 26.472) and (27.264 – 38.382) for respectively more than 200.000 to 200 copies and 0.2 to 200 copies of genome the genome quantity were few. The few quantity of KR-PCR positive can be associated to the plasmid gene loss. Further investigation may be led to discriminate M.ulcerans to mycolactone producing mycobacteria by Christelle D et al, 2017 [30] based on 16S rRNA and IS2404 sequences confirmed that 94% of the clinical samples contained MPMs. Although q PCR were more 10 to 10000 sensitive than classic PCR, a false-negative PCR may be caused by the time between collection and testing of the specimen or technical limitations [31]. Current typing methods for *M.ulcerans* are capable of resolving only geographical types and are consequently limited in their discriminatory power. The availability of *M.ulcerans* genome sequences presents an enormous resource for the identification of potential markers that are useful for indexing polymorphisms within the species. Since Stragier et al, 2005, MIRUs, besides intraspecies and interspecies differentiation among the *M.ulcerans-M.marinum* complex, MIRU-VNTR typing is a highly reproducible method that can also be applied directly to clinical specimens. All African *M.ulcerans* isolates had three copies of MIRU motifs at locus 1 and differed from all other isolates. Although based on KAKOU et al, 2015 [28] on four specifics MIRU-VNTR typing markers and Lavender et al, 2008 whose included VNTR Locus 9 as specific marker indicated origin of environmental strain in human contaminations, we performed five markers as MIRU1, VNTR locus 6, ST1, VNTR locus 19 and VNTR locus 9 to elucidate the diversity among strains circulate in Togo. Both strains conserved all five loci confirm previous studies that the togolese strains were African strains (3,1,1) when considered in order MIRU1, Locus 6 and ST1. And there were two MIRU-VNTR profiles circulated as A and B when considered in order MIRU1, Locus 6, ST1 and VNTR 9. Our results as Stragier et al, 2006 [29] showed that the profile B as (3,1,1,2) were the predominant. The repeat variations of 1 and 3 for MIRU 1 suggest the presence of genetic diversity in MPM strains from Togo. This length polymorphism in MIRU 1 also explained the limited, but shared homology with isolates from Ghana, as well as the overall genetic differentiation between the two countries. In Ghana, only one allele was reported for MIRU 1 and ST1 in contrast to two for both Locus 6 and Locus 19. In this study, two alleles were seen only in MIRU 1. This suggests that MIRU 1 may be more informative for typing isolates in Togo. However, a larger sample size is needed to estimate the genetic diversity in these MPM populations. An ST1 monomorphic repeat of two is inconsistent with findings in Ghana where there was a polymorphism for the locus. Interestingly, the allelic profile (3,1,1,2) is similar to *M.ulcerans* B genotype, circulating in West Africa. Similar allelic profiles at some loci, e.g., (3,1,1,-) or (3,1,-,-) or (3,-,-,-) suggest orthology to VNTR profile B (3,1,1,2) and (-, 1,-, -) suggest orthology to B (3,1,1,2) or A

(1,1,1,2) carried by some Ghanaian isolates [19]. This may suggest similar strain lineages between Togo and Ghana. A part of MIRU 1, which amplified product reveal polymorphism with two different copies 1 and 3, all the loci reveal an uniformity among togolese strains. Phylogenetic clustering on the basis of genetic diversity in the MIRU 1 locus showed two main *M.ulcerans* lineages in Togo. More again, our study reveal only one allele of Locus 6, ST1, Locus 19 with respectively one copy and two copies.

CONCLUSION

We successfully demonstrated that the shared length polymorphisms at the VNTR loci in Togolese strains and other studies in both Côte d'Ivoire and Ghana suggest common ancestral lineages. Complementation of sequence and length polymorphism data should potentially increase the discriminatory power of the VNTR-typing method. It is envisaged that this approach would be more useful for genotyping *M.ulcerans* and other highly monomorphic species. The allelic copy number in MIRU 1 suggests diversity in *M.ulcerans* strains from Togo. Using standardized genotyping protocols from Cote d'Ivoire and Ghana allowed the comparison of datasets. The latter could help explain the role of different ecologies and demography in BU. This study also suggests that distribution of *M.ulcerans* type in two BU endemic districts not be significantly different.

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