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Genetic pattern of the ST 61 cluster of the Cameroon family among *Mycobacterium tuberculosis* complex strains collected during the CANTAM I project in Cameroon

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Abstract

Tuberculosis (TB) remains one of the most outstanding diseases in global health concern. It is caused by *Mycobacterium tuberculosis* Complex (MTBC) strains. ST 61, a cluster of the LAM10_CAM family has been shown to be the most prevalent cluster implicated in TB transmission in Cameroon. The present study is aimed at revealing the most discriminative loci applied to this cluster and investigating the genetic pattern in the ST 61 cluster clinical samples. This was a 12 month cross- sectional study that included 45 randomly selected clinical specimens previously collected during the CANTAM 1 project. Following DNA extraction, amplification of 12-locus Mycobacterial Interspersed Repetitive Unit-Variable Tandem Repeat (MIRU-VNTR) analysis (MIRU 40, ETR A, QUB 26, Mtub 39, QUB 4156, MIRU26, MIRU 31, MIRU 16, MIRU 02, MIRU 04, MIRU 23, and MIRU 27) PCR assay and gel electrophoresis were performed. The amplicon sizes, the allele assignations, the allelic pattern and the technic discriminative potential were determined using *MIRU-VNTR plus* web application and a dendogram was generated. 45% genetic pattern, and 66,6% clustering rate were reported. Overall, ETR A, MIRU 4, MIRU 16, MIRU 23, MIRU 27 revealed as the most discriminating loci applied to the cluster ST 61. The identification of the most discriminative locus ETRA, MIRU 4, MIRU 16, MIRU 23, and MIRU 27 applied to a precise cluster is a safe economic and time gain option to enhance the difference between strains at the cluster level.

Keywords: Genetic pattern; Spoligotype ST 61; MIRU-VNTR; Tuberculosis; Cameroon

1 Introduction

Regardless of the availability of new generation diagnostic methods for the detection of tuberculosis within populations, tuberculosis (TB) continues to cause great difficulties and increase death rate, especially in poor and developing countries. Almost 1.2 million of cases were reported in 2018, and 230 000 deaths were attributed to the disease worldwide in 2019 [1]. Tuberculosis remains a serious common disease in Cameroon, with an estimated 1.4 million death cases annually similarly to other poor resource countries, and therapeutic decisions are often made by algorithms according to WHO guidelines [2]. Inadequate or insufficient proper descriptions of the causative agent in numerous cases, poor patient compliance and supervision of therapy have led to the development and distribution of resistant strains [3]. Perfect knowledge in tuberculosis (TB) causative agent is a matter of great concern for TB control programs since these strains could spread to the community, stressing the need for early detection with least time, expenses and consequently initiation of adjusted therapy. Conventional diagnostic detection methods of MTB strains rely heavily upon microbial cultures [2]. Where unfortunately results can only be obtained after a given period of time from weeks to months of incubation and as is the case in Cameroon and many other developing countries, lack of resources to establish the stringent laboratories conditions needed for these growth based- method diagnostics. It is known that

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molecular methods are based on assigning the presence or absence of certain mutations in specific positions or genetic locations [4]. Molecular typing of *M. tuberculosis* complex (MTBC) strains can greatly help our understanding of the population dynamic of strains of this disease [5]. In Cameroon, the use of spoligotyping in sparsed studies showed a striking regression of *M. Africanum* as etiological agent of pulmonary TB [6,7,3,5]. while this sub- specie remains endemic in other West and Central African countries among which Nigeria and Chad neighboring Cameroon [8,9]. Therefore, CANTAM (Central African Network for Tuberculosis, HIV/AIDS, and Malaria), an EDCTP (European and Developing Countries Clinical Trials Partnership) funded network [10]. Carried out a previous population based study with the goal to establish a cohort and prepare new sites for conducting future clinical trials for new TB treatments and vaccines in Central African countries [2]. The application of spoligotyping study showed the predominance of a group of strains named "Cameroon Family" strains, representing 35% of tuberculosis cases and designated LAM _10 CAM (ST 61) in SpolDB4. Genetic pattern is defined as the transfer of inherited genes from one population to another. Up to now the data assessing the discriminatory potential and genetic diversity between different originated strains have been done using MIRU/VNTR typing, which have shown to be both reproducible and stable [11]. Since the frequency and type of gene mutation varies greatly among different geographic regions in the world [10]. It becomes important to know precisely the genetic pattern of the most prevalent family of ST 61 strains responsible of the disease. The 12 locus MIRU/VNTR typing method have been used as the first line approach in acquiring genotype information of *M. tuberculosis* [12] and to provide adequate discrimination applied to different clades. The limitation of MIRU/VNTR typing is the dependence of its discriminatory power on a number of loci used. The main problem faced with the disease is the emergency of intrinsic resistance that certain mycobacteria present to any form of treatment with antituberculosis drugs. The affirmation of which there is a total homogeneity within the strains of the ST 61 cluster belonging to the Cameroon family collected during the CANTAM I project should not be a limit. In this study we aimed to establish the genetic pattern of ST 61 cluster of the Cameroon family of MTBC strains collected during the CANTAM I project in Cameroon and hence raise the most predominant loci applied to a precise cluster using a 12 locus MIRU/VNTR typing.

2 Material and methods

2.1 Ethical considerations

This study compiled the standards of the Regional Ethics Committee of University of Douala – Cameroon and obtained institutional permission N° 2455 CEI-Udo /10/2020/ M. All the study procedures were designed according to international standards. The isolates enrolled in this study were obtained from the previous CANTAM I project study after being diagnosed with pulmonary TB.

2.2 Study Design

We conducted a cross sectional study at the LTRP, Laboratory for Tuberculosis Research and Pharmacology of the University of Yaoundé I, Cameroon during 12 months (between October 2019 and January 2020). 45 isolates obtained from a previous study on consecutive TB patients with positive sputum smear collected underwent the following standard procedures: decontamination step with cetylpyridinuim chloride/ NaCl, sputum culture step in Lowenstein Jensen media without pyruvate and another Lowenstein Jensen media supplemented with 0.4 % pyruvate, cultures incubated at 37 °C and all positive cultures were collected, spoligotyped, conserved [2], and later randomly selected for enrollment in our present study.

2.3 DNA extraction

After selection of the clinical samples on the bases of spoligotyping, the extraction of DNA from clinical specimens was limited at the destruction of the cell membrane, followed the inactivation by heating the cell in eppendoff tubes containing Tris EDTA (10Mm, 1Mm, PH 8) at 95 °C for 20min to rupture cell membrane, destruction of the bacteria, centrifugation of the product obtained at 3000trs/ min for 10mins; the supernatant were collected in a new tube and stored at -20 °C until further molecular analysis.

2.4 Molecular analysis

2.4.1 Spoligotyping

Spoligotyping was performed during the CANTAM I project,) [2] as described by Kamenbeerk et al., (1997) [13] using a commercial spoligotyping kit (Isogen Bioscience, BV Maarsen, The Newtherlands) and spoligotypes were assigned.

2.4.2 MIRU- VNTR genotyping

PCRs were carried out using PCR reagents systems (Applied Biosystem UK). Sequences of primers used for amplification of 12 MIRU loci were selected, the conditions for amplification, their standardized designations and the correspondence with alias designations are described in Table 1 as introduced by Supply and colleagues (2006). Five microliters from diluted DNA solutions were added to a final volume of 50µl containing 0.2µl DNA polymerase (1U); 0.2mM each of dATP, dGTP, dCTP, dTTP; 5µl of PCR buffer; 0.4µM of primers; and 1 to 3.5µM of $MgCl_2$. The primer and $MgCl_2$ concentrations used were as described by Mazar et al., (2001) [14]. The isolates were genotyped by PCR amplification of 12 MIRU-VNTR loci. PCR fragments were analyzed by agarose gel electrophoresis with 2.5% agarose. The size of the amplicons was estimated by comparison with -50bp to -100bp ladder. The MIRU copy number per locus was calculated by using the conventions described by Supply et al., (2006) [12]. The reproducibility and accuracy of sizing and the size offsets, which correct differences in relative migration between the size standard and the amplicons depending on the locus and the polymer used were checked and standardized by analyzing selected PCR fragments amplified from *M. tuberculosis* H37Rv as the positive control and deionized water as the negative control and other reference isolates.

Table 1 Locus designations and PCR primers sequences used in this study for the 12-locus set

Locus	Alias(es)	PCR primer pairs (5' to 3' with labelling indicated)	Repeat unit length (bp)
580	MIRU 04	GCGCGAGAGCCCGAACTGC (FAM) GCGCAGCAGAAACGCCAGC	77
2996	MIRU 26	TAGGTCTACCGTCGAAATCTGTGAC CATAGGCGACCAGGCGAATAG (VIC)	51
802	MIRU 40	GGGTTGCTGGATGACAACGTGT (NED) GGGTGATCTCGGCGAAATCAGATA	54
1644	MIRU 16	TCGGTGATCGGGTCCAGTCCAAGTA CCCGTCGTGCAGCCCTGGTAC (VIC)	53
3192	MIRU 31	ACTGATTGGCTTCATACGGCTTTA GTGCCGACGTGGTCTTGAT (NED)	53
2165	ETR A	AAATCGGTCCCATCACCTTCTTAT (NED) CGAAGCCTGGGGTGCCCGCGATTT	75
3690	Mtub 39	CGGTGGAGGCGATGAACGTCTTC (VIC) TAGAGCGGCACGGGGGAAAGCTTAG	58
4156	QUB 4156	TGACCACGGATTGCTCTAGT GCCGGCGTCCATGTT (NED)	59
4052	QUB 26	AACGCTCAGCTGTCGGAT (NED) CGGCCGTGCCGGCCAGGTCCTTCCCGAT	111
154	MIRU 2	TGGACTTGCAGCAATGGACCAACT TACTCGGACGCCGGCTCAAAAT (FAM)	53
2531	MIRU 23	CTGTCGATGGCCGCAACAAAACG (VIC) AGCTCAACGGGTTCGCCCTTTTGTC	53
3007	MIRU 27	TCGAAAGCCTCTGCGTGCCAGTAA GCGATGTGAGCGTGCCACTCAA (NED)	53

Each reaction implemented two primers which are short nucleotides sequences which defines the sequence to be amplified by limiting it in a specific way by complementarity of base pairs; deoxynucleotides triphosphates (dNTPs)(nucleotide bases); an enzyme that catalysis the polymerization of deoxynucleotides in the DNA strand; a PCR buffer that creates an optimum environment for enzyme activity ; enzyme cofactor magnesium chloride; and PCR water that helps complete the reaction volume

2.5 Data Analysis

The MIRU–VNTR allelic diversity at a given locus was calculated as $h = 1 - \Sigma x_i^2 [(n/n - 1)]$, where X_i is the frequency of the *i*th allele at the locus and *n* the number of isolates. Isolates in this study can be classified into two groups, characterized by sub clustered and nonsub-clustered MTB isolates. A possible sub cluster is defined as two or more patients isolates with identical genetic patterns defines by MIRU- VNTR typing applied to a defined cluster; patient's isolates with unmatched genetic profiles were considered nonsub-clustered. Previous literatures have suggested that clusters may be assumed to have arisen from recent transmission; and the clustering rate was used to determine the amount of recent transmission in a population [15,16]. Patient's isolates with the same genetic pattern may represent an epidemiologically linked cluster. Therefore, the minimum estimate of the proportion of *M. tuberculosis* cases related to recent transmission can be obtained as $(n_c - c)/n$, where *n* is the total number of cases in the sample, *c* is the number of genotypes represented by atleast two cases, n_c is the total number of cases in the cluster of two or more patients. The discriminatory power of the MIRU-VNTR typing method was calculated using Hunter and Gaston Discriminatory Index (HGDI) [16].

3 Results

All the Fourty-five isolates of clade ST 61 were randomly chosen and analyzed with MIRU/VNTR typing, which detected 26 different patterns (Haplotypes), comprising 11 sub clusters forms by 30 isolates and 15 unique patterns formed by 15 isolates (Orphans) (Figure 1 and Table 3). The largest sub cluster comprised 6 isolates, with a MIRU–VNTR profile 047924329355 followed by a 4 isolates sub cluster with MIRU–VNTR profile O459434242117. Thus, among the 45 isolates, the MIRU- based clustering rate corresponded to 66.6% (Table 4). Regarding the allelic diversity of MIRU-VNTR loci, the discriminatory power between each locus was calculated using HGDI (summarized Table 2). The allelic diversity of the loci was classified as very discriminant [Hunter and Gaston Discriminatory Index (HGDI) > 0.6], moderately discriminant [Hunter and Gaston Discriminatory Index 0.3 < (HGDI) <0.6], poorly discriminant [Hunter and Gaston Discriminatory Index (HGDI)<0,3]. Among the 12 loci we noted that loci MIRU 4, 16, 23, 27 and ETRA were highly discriminative ((HGDI) > 0.6). Whereas loci MIRU 40 and QUB 4156 were moderately discriminative (0.3 < (HGDI) <0.6) and loci MIRU 2, 26, 31, Mtub 39 and Qub 26 were poorly discriminative ((HGDI)<0,3). Overall the HGDI value in the cluster was also calculated as 0,96%. Two loci were found monomorphic: MIRU 2 and QUB 26. The others were polymorphic.

Table 2 Allelic polymorphism of 12 mycobacterial interspersed repetitive units variable tandem repeat loci (MIRU/VNTR) from 45 MTB isolates from patients

MIRU/VNTR Loci	Allelic diversity (h)	Conclusion
MIRU 04	0.64	Very discriminant
MIRU 26	0.26	Poorly discriminant
MIRU 40	0.46	Moderately discriminant
MIRU 16	0.61	Very discriminant
MIRU 31	0.27	Poorly discriminant
ETRA	0.86	Very discriminant
Mtub 39	0.02	Poorly discriminant
QUB 4156	0.5	Moderately discriminant
QUB 26	-0.07	Poorly discriminant
MIRU 2	-0.02	Poorly discriminant
MIRU 23	1.3	Very discriminant
MIRU 27	0.66	Very discriminant
HGDI = 0.96		

The allelic diversity calculated for each locus is a function of the relative frequency of the alleles revealed during the determination of the number of repetitions. MIRU/VNTR typing was performed on the 45 isolates of the ST 61 cluster of *M. tuberculosis* complex.

From the genotype profile obtained in the form of a 12 – digit number using the appropriate software, a dendogram was generated in order to highlight the phylogenetically close isolates and to show the sub clusters within the samples of the strains studied. UPGMA (Unweighted Pair Group Method with Arithmetic Mean) was used for the construction of the dendogram.

Table 3 Discriminatory Index of MIRU-VNTR using loci MIRU 04, MIRU26, MIRU 40, MIRU 16, MIRU 31, ETR A, Mtub 39, QUB 4156, QUB 26, MIRU 02, MIRU 23, and MIRU 27

Sub clusters	MIRU-VNTR Profile	Number of isolates per sub-cluster
Cs1	012944329337	1
Cs2	027944329347	2
Cs3	027944329356	1
Cs4	047924329355	6
Cs5	047944329354	2
Cs6	042944329254	1
Cs7	043944429254	3
Cs8	183934429264	1
Cs9	183934429264	1
C10	172934425064	2
Cs11	182934425065	2
Cs12	181934425265	2
Cs13	183934425065	3
Cs14	181934425265	1
Cs15	143934025264	1
Cs16	1103934025264	1
Cs17	1104934325264	1
Cs18	114934225264	1
Cs19	015934325264	1
Cs20	005934425264	1
Cs21	0449444242114	2
Cs22	0449444242117	1
Cs23	0459434242117	4
Cs24	0459430252117	1
Cs25	0159430252117	1
Cs26	0059430252117	2
HGDI = 0, 96		

Where Cs = Sub Clusters. The different MIRU-VNTR profiles obtained from the number of repetitions of the 12 loci after data processing.

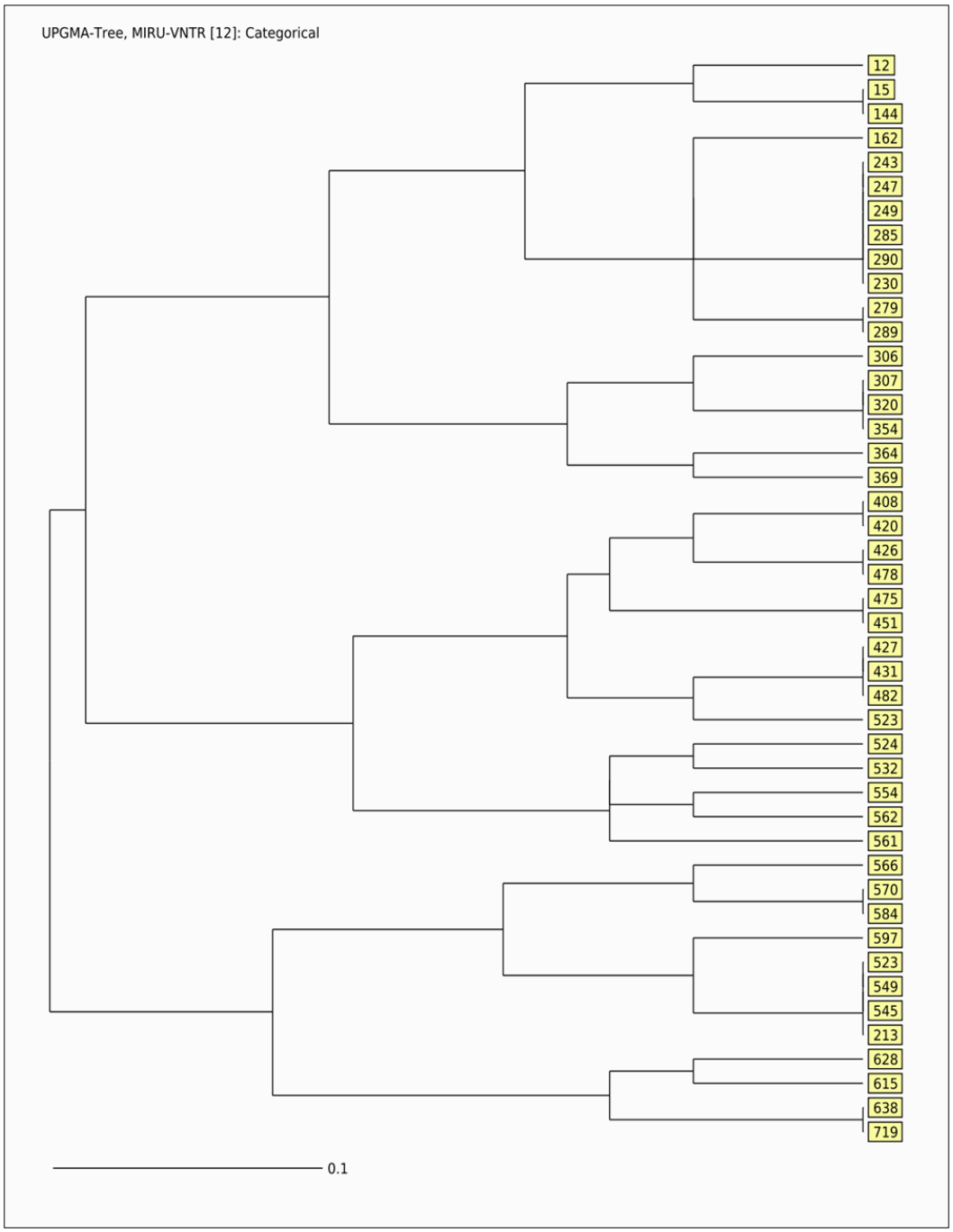

Figure 1 Dendogram of the 45 ST 61 strains, describing the genetic relationships between the 45 isolates. Thus 26 sub- clusters were observed and named (strains with identical genotypes) with the respective proportions of strains

Table 4 Clusterization rate of the 45 isolates obtained by the 12 locus MIRU/VNTR Typing (Cr)

Number of isolates tested (n)	45
Number of identical genotypes for atleast two cases (C)	30
Clusterization rate (Cr)	66 .6%

The clusterization rate observed permits us to estimate the rate of ST 61 strains of the Cameroon family of MTBC showing genetical links between them, or having the same genetic profile. This raises the existence of a genetic bond at 66**. 6%** in the ST61 cluster of the Cameroon family of MBTC.

4 Discussion

Tuberculosis remains a major public health issue in Cameroon with an incidence of 46 000 new cases in 2019 (PNLT, 2022). This is the first study that explores the most discriminative loci applied to a clade using a 12 locus MIRU/VNTR typing method in Cameroon. The drastic change in etiological agent of the disease encountered by precise epidemiological method such as spoligotyping attracted our attention toward the deepened appreciation of the causal pathogen as to evaluate the existence of a homogeneous genetic pattern in the most predominant clade ST 61 which our study has raised collected during the CANTAM project, can be a safe economic and time gain option to enhance the difference between strains and notice the most discriminant loci at the cluster level.

Analysis of the genetic diversity of the population of the strains belonging to this cluster using independent markers (MIRU-VNTR), showed a high genetic pattern of sensitivity 45%. The same result was obtained in the Adamaoua region of Cameroon [17]. This result suggested that this cluster is not the true clone but that some of the strains were not involved in the direct transmission of tuberculosis in population study but it seems to be implicated in the reactivation of cases or in imported cases. However, 26 different patterns (Haplotypes) were observed, comprising 11 sub clusters also called “grapes” which could reflect the implication of strains of these different patterns to different pockets of ongoing TB transmission in the population study similar to results noticed in Adamaoua region [17]. Each grape constitutes between two to six strains, suggesting that the strains of this cluster might be actively implicated in TB transmission. But considering the few number of strains obtained per grape, this assertion needs to be verified with other data and the use of other DNA markers different from the loci used in our present study. The same result was noticed in Adamaoua region Cameroon, and in Nigeria [6]. The fact that 15 unique patterns formed by 15 isolates (Orphans) were not clustered argue in favor of reactivation of the old case than the ongoing transmission of these strains in Cameroon [17]. Moreover, the genetic pattern observed of 45% in our study, is different compared to the value reported in the previous study in Douala [18]. in Adamaoua region [17]. and in the West region of Cameroon [19]. Which might be linked to the system of loci used in each of these studies 5ETR in Douala, 15 Modified Standard set in Adamoua region and 24 complete set in West region of Cameroon. Furthermore, this study reports the locus ETR A as the most discriminative locus applied to the cluster ST 61 which seems different from the most discriminative locus applied at the lineage level as MIRU 40 notified in the Adamaoua [17]. and West region [3]. of Cameroon hence raising the importance of the establishment of such study in the field of genetic pattern at the cluster level.

5 Conclusion

The identification of the most discriminative locus ETRA, MIRU 4, MIRU 16, MIRU 23, and MIRU 27 applied to a precise cluster is a safe economic and time gain option to enhance the difference between strains at the cluster level. Our study provides the first genetic diversity of 45% applied to the cluster ST 61 of the Cameroon family among strains of *M. tuberculosis* complex.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare that they have no competing interests.Statement of ethical approval

The present research was performed under the institutional Ethics Committee of University of Douala – Cameroon N° 2455 CEI-Udo /10/2020/ M.

Statement of informed consent

Informed consent was obtained from all individual participants included in the study.

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