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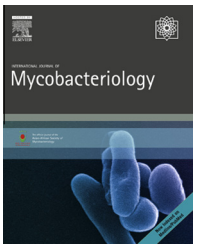

Short Communication

Genetic diversity of *Mycobacterium tuberculosis* complex strains isolated from patients with pulmonary tuberculosis in Anambra State, Nigeria

Gertrude N. Uzoewulu [a], *Lovett Lawson* [b], *Ibeh S. Nnanna* [c], *Nalin Rastogi* [d,*], *Madhu Goyal* [e,*]

[a] *Nnamdi Azikiwe University Teaching Hospital, Nnewi, Anambra State, Nigeria*
[b] *Zankli Medical Centre, Abuja, Nigeria*
[c] *University of Benin, Edo State, Nigeria*
[d] *World Health Organization TB Supranational Reference Laboratory, Institut Pasteur de Guadeloupe, Les Abymes, Guadeloupe, France*
[e] *University of Hertfordshire, Hatfield, England, UK*

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ABSTRACT

In this study, we analyzed *Mycobacterium tuberculosis* complex (MTC) genetic diversity in Anambra State, Nigeria based on spoligotyping followed by 5-loci exact tandem repeats (ETRs). Spoligotyping of 180 MTC strains isolated in 2009–2011 from pulmonary tuberculosis (TB) patients led to a total of 31 distinct patterns. A comparison with the SITVIT2 international database showed that all the 31 patterns could be classified as Shared-types (SITs) in this database; briefly, 26/31 SITs (n = 174 isolates) matched a preexisting shared-type in the database, whereas 5/31 SITs (*n* = 6 isolates) were newly created due to 2 or more strains belonging to an identical new pattern within this study (SIT3396) or after a match with an orphan in the database (SIT3397, SIT3398, SIT3399 and SIT3400). A total of 18/31 SITs containing 167 or 92.8% isolates were clustered within this study (2–89 isolates per cluster) while 13/31 SITs contained unique strains. Using VNTR typing, a total of 36 distinct patterns were identified; 27 patterns (*n* = 157 isolates) matched a pattern already reported in the SITVIT2 database. Combination of both the methods generated 47 combined patterns for the 180 strains: 17 belonged to clustered isolates (*n* = 127 isolates or 70.5%) while 30 corresponded to as many unique strains (note 23 strains could not be typed using 5-loci ETRs). No correlation was found between the spoligotyping pattern and the HIV status of the patient or drug sensitivity of the strain. This study showed that the LAM10-CAM prototype SIT61 accounted for highest number of isolates (*n* = 89) in Anambra State, showing its relative contribution to the TB burden in the study.

* Corresponding authors at: Institut Pasteur de Guadeloupe, BP 484, F97183 Les Abymes, Guadeloupe, France (N. Rastogi). School of Life and Medical Sciences, University of Hertfordshire, Hatfield, England, UK (M. Goyal).
E-mail addresses: nrastogi@pasteur-guadeloupe.fr (N. Rastogi), m.goyal@herts.ac.uk (M. Goyal).
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Introduction

Nigeria, with a population of over 150 million, is among the high-tuberculosis (TB)-burden countries and ranks 13th in the world [1]. Multiple-drug-resistant TB (MDR-TB) is another problem, and in a recent study, it has been found that as much as 8% of all cultured specimens were MDR-TB positive in three states in Nigeria [2]. The information available on the incidence, drug susceptibility, and genotyping of the *Mycobacterium tuberculosis* complex (MTC) in Nigeria is limited [3–6]. Additional data are needed to explore the population structure of strains of MTC to identify specific endemic strains in the study area; monitor transmission dynamics to link outbreak cases in communities, hospitals, or institutions; and for better treatment.

Many molecular-typing techniques have been used to differentiate strains of MTC involved in TB infection, among which the spoligotyping method based on the polymorphism of the direct repeat locus is a widely used first-line typing method [2,6]. However, when used alone, the lower discriminatory power of spoligotyping requires that it is ideally used in association with 12, 15, or 24-loci mycobacterial interspersed repetitive unit–variable number of tandem repeats (MIRU–VNTRs) for *M. tuberculosis* molecular epidemiology, or at minima in association with a more convenient five-loci exact tandem repeats (ETRs, [7]) that have been successfully used to improve the potential of spoligotyping for studying the genetic diversity of TB [7–9]. The present study constitutes a first attempt to describe the genetic population structure of MTC circulating in Anambra State, Nigeria using spoligotyping and five-loci ETRs.

Materials and methods

Setting, clinical isolates, and molecular characterization

The study was conducted among patients between the ages of 10 years and 82 years with pulmonary TB attending Nnamdi Azikiwe University Teaching Hospital and different peripheral DOTS clinics in Anambra State during the period 2009–2011. Data regarding the patients' gender, human-immunodeficiency-virus (HIV) status, and age were collected. MTC strains were isolated and identified from 550 sputum samples of suspected TB patients after smear microscopy by the Ziehl–Neelsen method at Nnamdi Azikiwe University Teaching Hospital, Nnewi, and cultured on Löwenstein–Jensen medium at Zankli TB laboratory, Abuja. DNA was extracted using the classical cetyl-trimethyl-ammonium-bromide method as described previously [8,10], and sent to the University of Hertfordshire, Hatfield, England for molecular typing. Spoligotyping was performed using a commercially available kit (Ocimum Biosolutions, Hyderabad, India), following the manufacturer's instructions, and previously described methodology [11], shown to be useful to study the transmission of *M. tuberculosis* [12]. Five-loci ETR (A, B, C, D, and E) typing was performed, as described by Frothingham and Meeker-O'Connell [7]. The exact number of tandem repeats at each locus was analyzed for each strain using polymerase chain reaction.

Database comparison

The identified spoligotypes and five-loci ETR patterns were analyzed using the BioNumerics software (BioSystematica), and compared with the SITVIT2 proprietary database of the Institut Pasteur de Guadeloupe, which is an updated in-house version of the recently released SITVITWEB database [13], available online at http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/. In this database, spoligotype international type (SIT) and VNTR international type (VIT) designate spoligotype and five-loci ETR patterns shared by two or more patient isolates, as opposed to "orphan," which designates patterns reported for a single isolate. Major phylogenetic clades were assigned according to the signatures provided in the database defining 62 genetic lineages/sublineages. These include various MTC members, such *as Mycobacterium bovis, Mycobacterium caprae, Mycobacterium microti, Mycobacterium canettii, Mycobacterium pinnipedii*, and *Mycobacterium africanum*, as well as rules defining major lineages/sublineages for *M. tuberculosis* sensu stricto. These include the Beijing clade, the Central-Asian clade and two sublineages, the East-African–Indian clade and nine sublineages, the Haarlem clade and three sublineages, the Latin-American–Mediterranean (LAM) clade and 12 sublineages (note that two sublineages, LAM7-TUR and LAM10–CAM, were reclassified as Turkey and Cameroon lineages), the ancestral "Manu" family and three sublineages, the S clade, the IS6110-low-banding X clade and three sublineages, and an ill-defined T clade with five sublineages.

The description of predominant clusters in this study (four or more isolates) and their worldwide distribution was studied in function of their reported numbers in various macrogeographical regions in the SITVIT2 database (reported for regions with more than 3% of a given shared type). The definition of macrogeographical regions and subregions (http://unstats.un.org/unsd/methods/m49/m49regin.htm) was according to the United Nations scheme (regions: AFRI [Africa], AMER [Americas], ASIA [Asia], EURO [Europe], and OCE [Oceania], subdivided in E [eastern], M [middle], C [central], N [northern], S [southern], SE [southeastern], and W [western]). Note that, in this scheme, CARIB (Caribbean) belongs to Americas, while Oceania is subdivided in four subregions: AUST (Australasia), MEL (Melanesia), MIC (Micronesia), and POLY (Polynesia). Furthermore, Russia was attributed a new subregion by itself (Northern Asia), instead of including it among the rest of Eastern Europe, reflecting its geographical localization, as well as due to the similarity of specific TB genotypes circulating in Russia (a majority of Beijing genotypes) with those prevalent in Central, Eastern, and Southeastern Asia. Finally, the three-letter country codes were according to http://en.wikipedia.org/wiki/ISO_3166-1_alpha-3.

Ethical considerations

An ethical clearance was granted by the hospital ethical committee, and informed consent was obtained from each patient.

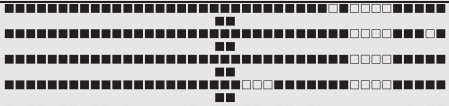
Results and discussion

Out of 550 suspected patients sputa screened for acid-fast bacilli, only 180 sputum samples were culture positive for MTC, giving a culture-positive rate of 33% among the suspected cases, which is significantly higher in the study area as compared with the 6% culture-positive rate among the suspected patients in Nigeria ($p < .05$). The available demographic data for the 180 patients showed that 84% were new TB cases, while 16% were previously treated; 61% males versus 39% females (male-to-female-sex ratio of 1.56), with a mean age of 35 years. Regarding HIV serology, 81% were negative versus19% being HIV positive.

Spoligotyping of 180 MTC strains led to a total of 31 distinct patterns (Table 1). A total of 26 out of 31 SITs containing 174 isolates matched a preexisting shared type in the database, whereas five out of 31 SITs ($n = 6$ isolates) were newly created. A total of 18 out of 31 SITs containing 167 isolates were clustered within this study (2–89 isolates per cluster), while 13 out of 31 SITs contained unique strains (Table S1). In Table 1, SITs followed by an asterisk indicate "newly created" SITs due to two or more strains belonging to an identical new pattern within this study or after a match with an orphan in the database, (SIT3397, SIT3398, and SIT3399 matched a single isolate from, Cameroon, Metropolitan France, and Germany, while SIT3400 matched two strains—one each from Austria and USA.).

The description of predominant clusters containing four or more isolates in this study and their worldwide distribution in the SITVIT2 database is illustrated in Table 2. It corresponded to a total of seven clusters (in decreasing number of cluster size) as follows: SIT61/LAM10–CAM, $n = 89$ (49.44%); SIT331/AFRI_2, $n = 16$ (8.89%); SIT838/LAM10–CAM, $n = 13$ (7.22%); SIT403/LAM10–CAM, $n = 8$ (4.44%); SIT523/Manu_ancestor, $n = 7$ (3.89%); SIT53/T1, $n = 5$ (2.78%); and SIT50/H3, $n = 4$ (2.22%). With the exception of ubiquitous pattern SIT53/T1 and SIT50/H3 (the latter is prevalent in North and South America, and Europe), all other patterns belonging to

Table 1 – Description of 31 shared types and Corresponding spoligotyping-defined lineages/sublineages starting from a total of 180 *Mycobacterium tuberculosis* clinical isolates from patients residing in Anambra State, Nigeria.

SIT*	Spoligotype description	Octal number	Number (%) in study	Percent in study versus database	Lineage	Clustered versus unique patterns
50		777777777720771	4 (2.22)	.13	H3	Clustered
52		777777777760731	3 (1.67)	.38	T2	Clustered
53		777777777760771	5 (2.78)	.09	T1	Clustered
61		777777743760771	89 (49.44)	11.29	LAM10-CAM	Clustered
168		777777777720671	1 (.56)	4.35	H3	Unique
181		770777777777671	1 (.56)	.38	AFRI_1	Unique
191		177777777760771	2 (1.11)	10	T1	Clustered
316		777777770020731	3 (1.67)	6.82	H3	Clustered
319		574077607777071	1 (.56)	7.69	AFRI_2	Unique
320		770003606377071	1 (.56)	10	AFRI_2	Unique
331		774077607777071	16 (8.89)	27.59	AFRI_2	Clustered
373		777777767760771	2 (1.11)	3.17	T1	Clustered
403		777777743760731	8 (4.44)	18.18	LAM10-CAM	Clustered
523		777777777777771	7 (3.89)	14	Manu_ancestor	Clustered
838		777777743760751	13 (7.22)	48.15	LAM10-CAM	Clustered
846		760777777760731	1 (.56)	16.67	T2	Unique
848		737777777760731	2 (1.11)	7.14	T2	Clustered
1057		000003743760771	2 (1.11)	40	LAM10-CAM	Clustered
1069		771777777760771	1 (.56)	4.17	T1	Unique
1580		777777747760771	2 (1.11)	11.11	T	Clustered
1684		776000000000000	1 (.56)	33.33	Unknown	Unique
1783		776377743760771	3 (1.67)	50	LAM10-CAM	Clustered
1907		777777777774771	2 (1.11)	33.33	Unknown	Clustered
2832		577777743760771	1 (.56)	33.33	LAM10-CAM	Unique
2900		777777763760771	2 (1.11)	25	T1	Clustered
2984		770003607777071	1 (.56)	20	AFRI_2	Unique
3396*		777777743760711	2 (1.11)	66.67	LAM10-CAM	Clustered
3397*		706377777760771	1 (.56)	50	S	Unique
3398*		776377773760771	1 (.56)	50	S	Unique
3399*		740000000000001	1 (.56)	50	Unknown	Unique
3400*		777770343760771	1 (.56)	50	LAM10-CAM	Unique

Note. SIT = spoligotype international type.
*SITs followed by an asterisk indicate "newly created" SITs due to two or more strains belonging to an identical new pattern within this study or after a match with an orphan in the database.

Table 2 – Description of predominant clusters containing four or more isolates in this study, and their worldwide distribution in the SITVIT2 database (predominant shared types are shown in decreasing order of their occurrence)*.

SIT (lineage) octal number spoligotype description	Number (%) in study	Percent in study versus database	Distribution in regions with ≥3% of a given SIT	Distribution in countries with ≥3% of a given SIT
61 (LAM10-CAM) 777777743760771 ■■■■■■■■■■■■■■■■■■■■■■□□□■■■■■■■□□□□■■■■■■■	89 (49.44)	11.29	AFRI-W 36.17, AFRI-M 22.34, AMER-N 10.15, EURO-W 8.88, ASIA-W 5.58, EURO-S 5.08	NGA 33.63, CMR 21.45, USA 10.15, SAU 5.58, FXX 5.08, ITA 3.3
331 (AFRI_2) 774077607777071 ■■■■■■■□□□□■■■■■■■■□□□□■■■■■■■■■■■■■□□□■■■■	16 (8.89)	27.59	AFRI-W 74.14, AMER-N 17.24, AFRI-M 5.17	NGA 67.24, USA 17.24, CMR 5.17, CIV 5.17
838 (LAM10-CAM) 777777743760751 ■■■■■■■■■■■■■■■■■■■■■■□□□■■■■■■■□□□□■■■■□■■	13 (7.22)	48.15	AMER-S 29.92, AMER-N 15.39, EURO-S 11.75, AFRI-N 10.13, EURO-W 6.46, AFRI-E 3.4, AFRI-S 3.74	USA 14.01, BRA 12.0, MAR 8.33, COL 7.23, ITA 6.18, ESP 3.95, VEN 3.92, ZAF 3.74
403 (LAM10-CAM) 777777743760731 ■■■■■■■■■■■■■■■■■■■■■■□□□■■■■■■■□□□□■■■□■■■	8 (4.44)	18.18	AFRI-W 40.91, AFRI-M 34.09, EURO-W 6.82, AMER-N 6.82, EURO-S 4.55, ASIA-S 4.55	NGA 40.91, CMR 34.09, USA 6.82, ITA 4.55, IND 4.55
523 (Manu_ancestor) 777777777777771 ■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■	7 (3.89)	14.0	ASIA-E 26.0, AFRI-W 20.0, ASIA-SE 16.0, AMER-N 10.0, EURO-W 8.0, ASIA-W 8.0, AFRI-S 4.0	NGA 18.0, USA 10.0, SAU 8.0, JPN 8.0, CHN 8.0, MYS 6.0, KOR 6.0, ZAF 4.0, VNM 4.0, TWN 4.0, THA 4.0, BEL 4.0
53 (T1) 777777777760771 ■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□■■■■■■■	5 (2.78)	.09	AMER-N 18.35, AMER-S 14.17, EURO-W 11.4, EURO-S 10.2, ASIA-W 7.66, EURO-N 5.99, AFRI-S 5.58, AFRI-E 5.01, AFRI-N 3.95, ASIA-E 3.74	USA 14.83, ITA 5.99, BRA 5.64, ZAF 5.46, TUR 3.9, AUT 3.85, MEX 3.2
50 (H3) 777777777720771 ■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■■■■■■■	4 (2.22)	.13	AMER-N 20.14, AMER-S 18.13, EURO-W 13.91, EURO-S 12.61, EURO-E 5.84, EURO-N 4.7, AFRI-N 4.67, AFRI-S 4.44, CARI 3.79, ASIA-W 3.05	USA 19.26, BRA 7.65, AUT 6.68, ITA 5.97, ESP 5.97, ZAF 4.44, PER 4.05, CZE 4.02, SWE 3.11

Note. SIT = spoligotype international type.
*SIT and lineage designations are shown following SITVIT2 proprietary database of Institut Pasteur de Guadeloupe. Note that countrywide distribution is only shown for SITs with ⩾3% of a given SIT as compared to their total number in the SITVIT2 database.

LAM10–CAM, AFRI_2, and Manu_ancestor sublineages showed relatively high phylogeographical specificities for Africa (and/or Nigeria, in particular). Regarding their classification in lineages (and not shared types), the 180 MTC isolates were assigned into six major phylogeographical clades, the LAM10–CAM being the most predominant ($n = 119$ or 66%), followed by various ill-defined T sublineages grouped together ($n = 20$ or 11%), AFRI-2 sublineage ($n = 19$ or 11%), Haarlem sublineage H3 ($n = 8$ or 4.4%), and Manu_ancestor ($n = 7$ or 4%). Minor families corresponded to the S family ($n = 2$ or 1%), AFRI-1 ($n = 1$ or 0.5%), and three spoligotype patterns containing four or 2% isolates with signatures designated as "unknown."

Thirty-six different patterns from 157 isolates were observed starting from 180 samples analyzed by the five-loci ETRs; note that the five-loci ETR patterns could not be successfully amplified for some strains ($n = 23$ strains) for which no more DNA was available afterward. All the 36 patterns matched existing patterns in the SITVIT database, and were assigned a VIT designation (see Table S1). Ten VIT patterns containing 140 isolates were clustered (2–69 isolates per cluster), while 17 VIT patterns corresponded to unique isolates. Twenty-seven (75%) VIT patterns containing 157 isolates matched the SITVIT2 database. Finally, the combination of spoligotyping and five-loci ETRs generated a total of 47 SIT/VIT patterns—30 patterns corresponded to unique patterns, while 17 SIT/VIT patterns corresponded to a total of 127 clustered isolates (with 2–61 strains per cluster), which correspond to a clustering rate of 70.5%, with SIT61/LAM10–CAM being the main genotype with 89 isolates. (The three other LAM10–CAM isolates showed unique patterns.) Thus, the LAM10–CAM lineage with phylogeographical specificity for Cameroon and the neighboring countries in West Africa accounted for the majority of isolates, 119 (66%), which is the most predominant clade circulating within the Nnewi community ($n = 70$ or 39%), followed by Onitsha ($n = 48$ or 27%) and Awka ($n = 1$ or .5%) communities (but not observed in the Ihiala community), indicating the endemicity of the LAM10–CAM clone in the Anambra State, the southeast geopolitical zone of Nigeria.

These results are similar to the study by Lawson et al. [2], which showed similar prevalence (66%) of CAM clade in their study from Nigeria. The LAM10–CAM strains have been isolated from TB patients in other neighboring countries, such as Chad, Sierra Leone, and Burkina Faso, as well as from Nigeria [3,14–16]. The LAM10 lineage as the predominant type (76%) was also found in Jos, Nigeria [6]. The high prevalence of LAM10–CAM indicates that this family is spreading rapidly and is associated with recent transmission in Anambra State, indicating an evolutionary advantage of this genotype over other genotypes [6]. Whether bacillus Calmette–Guérin vaccination plays a role in the selection and expansion of

the LAM10–CAM family, as shown previously for the Beijing family [17], remains speculative, but should be studied in future studies.

The result of the comparison of spoligotyping versus VNTRs demonstrated that spoligotyping analysis alone was less discriminative than when used in association with five-loci ETRs; for example, isolates with identical spoligotype pattern SIT61 were all split into different VIT patterns: VIT58, VIT180, VIT299, VIT406, VIT407, and VIT409, and four distinct orphan patterns (Table S1). Thumamo et al. [17] also showed higher discrimination using 12-loci MIRU typing as compared to spoligotyping. Hence, future studies using 15- and/or 24-loci MIRUs might even lead to a much higher discrimination and more conclusive results in our study area.

A significant number of *M. africanum* AFRI_2 sublineage ($n = 19$) were found among pulmonary TB patients attending the Nnamdi Azikiwe University Teaching Hospital ($n = 13$) and Onitsha ($n = 6$), underlining that *M. africanum* also plays an important role in the prevailing TB epidemic in Nigeria. Interestingly, in our study, we had only one isolate belonging to the AFRI_1 sublineage. In a recent study, as high as 33.3% isolates from the Cross River State, Nigeria were shown to belong to the AFRI_2 sublineage [17]. These results are in contrast to the study by Lawson et al. [2], where 13% of the isolates belonged to the AFRI_1 sublineage. Cadmus et al [3] also showed that 13% of TB cases in Nigeria were caused by *M. africanum* (essentially AFRI_1) and *M. bovis*. In another study from Guinea-Bissau, almost all the *M. africanum* strains belonged to the AFRI_1 sublineage [18]. However, Thumamo et al [17] underlined that misidentification of *M. africanum* strains due to substantial heterogeneity leading to their erroneous identification as *M. bovis* and/or *M. tuberculosis* is more common than thought, which requires careful reexamination of the trends of prevalence of the *M. africanum* sublineages in different parts of Africa. Last, but not least, in addition to the LAM10–CAM and AFRI_2 lineages, we also found 4.4% of isolates belonging to the Haarlem sublineage H3 and 4% isolates typed as Manu_ancestor; these results are in agreement with other studies in Nigeria showing a small percentage of isolates belonging to these genotypes [2,6].

Although drug-susceptibility testing was not systematically performed, the partial drug-susceptibility-testing data available ($n = 87$ isolates) showed a relatively high proportion of MDR-TB cases ($n = 14$ or 16%). However, no correlation was found between the spoligotyping pattern and the HIV status of the patient, or drug sensitivity of the strain. However, tracing the route of infection and the risk factors for TB transmission based on patient records, we suggest that TB in clustered patients most probably resulted from recently acquired infection [19,20] versus reactivation among patients infected by unique patterns [20,21]. Last, but not least, out of the total of seven strains belonging to the SIT523/Manu_ancestor lineage (all the 43 spacers present by spoligotyping), only one strain could be typed by VNTRs and belonged to VIT153; the six remaining strains could not be amplified (Table S1). It will be important to look for such strains in future investigations to verify if they did not derive from mixed-strain infections as revealed recently by a novel computational approach [22]. Whether it is the case or not would require extended MIRU–VNTR typing to exclude multiple bands.

Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijmyco.2015.06.008.

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