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Estimates of Genetic Variability of *Mycobacterium tuberculosis* Complex and Its Association with Drug Resistance in Cameroon

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ABSTRACT

The present study investigates the genetic diversity among *Mycobacterium tuberculosis* complex circulating in the Centre region of Cameroon and analyzes the relationship between genotypes and drug resistance patterns. Spoligotyping was performed by PCR-amplification followed by the reverse hybridization of 298 cultured specimens. Spoligotypes patterns were identified by comparison to reference strains in SPolDB4 database via the MIRU VNTR *plus* web application. About 97.65% of all tuberculosis (TB) cases were attributed to *M. tuberculosis*. A total of 65 different profiles were identified. Of these, 40 were represented as Shared Types (ST) while the others were orphans. LAM10_CAM and Haarlem families were the most prevalent genetic families with 51.01% and 14.09% respectively. ST 61, a member of the LAM10_ CAM family formed the largest cluster with 128 (42.95%) isolates. No association was found between genotypes with regard to drug resistance and HIV sero-status. However, there was a significant association between genotypes and age groups. Patients belonging to 15 - 24 and 35 - 44 age groups were more likely infected by LAM10_CAM strains compared to others. The population structure of *Mycobacterium tuberculosis* complex strains from the Centre region was found to be diverse and the spoligotype 61 of the LAM10_CAM family was highly predominant. Isolates of the LAM10_CAM seem to be not associated with drug resistance.

Keywords: *M. tuberculosis*; Spoligotyping; LAM10_CAM

1. Introduction

Tuberculosis (TB) is a cause of great mortality and suffering, especially in poor and less-developed countries. Its association with the HIV/AIDS pandemic forms a lethal combination. In addition, multidrug resistant (MDR) TB and extensively drug resistant (XDR) TB severely complicate the management and control of the disease worldwide [1]. More recently, the discovery of totally drug resistant (TDR) TB, a deadly form of the disease highlighted a crisis of mismanagement of the disease [2]. Elimination of TB by 2050 is a long-term goal of the World Health Organization (WHO) and their strategy is heavily based on the improvements in the current diagnostics, treatment and vaccination, as well as on the development of new strategies to control and fight the epidemic [3]. Any strategy for combating the epidemic should be based on a thorough appreciation of the problem. Interventions driven by a poor understanding of the pathogen in a specific geographical context will necessarily entail a high risk of failure [4].

Our understanding of the transmission of tuberculosis (TB) has been greatly enhanced since the introduction of DNA fingerprinting techniques for *Mycobacterium tuberculosis* [5]. Spoligotyping is a very practical and reproducible PCR-based method, which assays the presence or the absence of a set of target sequences in the direct repeat (DR) locus [6]. The resulting genotype has a simple binary format, which has recently leaded to the construction of large databases, intended to facilitate recognition of the origin of a particular clinical isolate [7]. Another advantage of spoligotyping is that it can be used simultaneously for the detection and typing of the *M. tuberculosis* complex bacteria in one assay.

As in most resource poor countries, TB epidemiology in Cameroon has so far largely consisted of reporting the

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number of cases detected and their demographic data. Little is known about *Mycobacterium tuberculosis* strains circulating in the Centre region of the country. The objective of the present study was to estimate the genetic variability of *Mycobacterium tuberculosis* complex strains circulating in the Centre region and to analyze the relationship between genotypes and drug resistance.

2. Materials and Methods

2.1. Mycobacterial Isolates

This study included 298 *Mycobacterium tuberculosis* complex isolates. These isolates were selected from a collection of *Mycobacterium tuberculosis* complex strains isolated from smear positive pulmonary tuberculosis patients admitted in Jamot hospital and Mbalmayo District hospital, whose age was ranged from 15 to 85 (mean age, 33.75 years). Among the selected isolates, 28 (10.64%) were phenotypically drug resistant and 3 were multidrug resistant. HIV serology was available for 296 (99.32%), among which 86 (29.05%) were HIV positive.

2.2. DNA Extraction

Mycobacterium tuberculosis complex were scraped from Lowestein-Jensen slopes, collected into Eppendorf tubes containing Tris-EDTA (10 mM, 1 mM, pH 8) and heated for 30 min at 90˚C. After centrifugation at 13,000 xg, the supernatant was collected into a new tube and kept at −20˚C until further use.

2.3. Spoligotyping

All isolates were genotyped with a spoligotyping commercial kit (Isogen Bioscience, BV Maarsen, The Netherlands) according to the protocol previously described by Kamerbeek *et al.* [6]. Briefly, the DR region of the TB genome was amplified using primers DRa, 5'-GGTTTTGGGTCTGACGAC-3' (biotinylated 5' end) and DRb, -CCGA-GAGGGGACGGAAAC-3'.

PCR products were hybridized with a set of 43 spacer oligonucleotides covalently linked to the spoligo-membrane (Isogen Life Sciences, The Netherlands) according to the manufacturer's instructions. The hybridized PCR products were then incubated with a streptavidin-peroxidase conjugate and the membrane exposed to chemiluminescence (Amersham ECL Direct™ nucleic acid labeling and detection system, GE Healthcare Limited, UK). The X-ray film was developed using standard photochemical procedures after 20 minutes exposure. DNA extracts of *M. tuberculosis* H37Rv and *M. bovis* BCG were used as controls.

2.4. Data Analysis

Spoligotype patterns in a binary format were entered in an Excel sheet, and compared with the spoligotype database SpolDB4 using MIRU VNTR plus [8]. The Hunter Gaston Discriminatory Index (HGDI) was used to calculate the discriminatory power of spoligotyping method [9]. The Chi square or Exact Fisher test when necessary were employed to evaluate difference in serology, age group and drug resistance between LAM10_CAM and non LAM10_CAM strains. Values of p of less than 0.05 were considered significant.

3. Results

Of the 298 isolates analyzed, 291 (97.65%) were classified as *Mycobacterium tuberculosis* and 6 (2.03%) as *Mycobacterium africanum* species. The remaining one isolate was identified as *Mycobacterium bovis*.

3.1. Distribution of Different Genetic Families

Among the 298 typed isolates, a total of 65 different profiles clustered into 17 genetic families were identified. Of these, 152 (51.01%) isolates belong to the LAM10_CAM family while 121 (42.7%) were non LAM10_CAM strains. Strains classified into non LAM10_CAM family included strains from the Haarlem family (14.09%), T family (12.75%) and others (**Table 1**).

Table 1. Distribution of different genetic families identified in a collection of 298 *Mycobacterium tuberculosis* complex isolates.

Genetic families	No. isolates	Frequency (%)
LAM10_CAM	152	51.01
H3	42	14.09
T2	22	7.38
U	14	4.70
T1	12	4.03
LAM1	6	2.01
U (likely H)	6	2.01
AFRI_2	3	1.01
H1	3	1.01
LAM9	3	1.01
U (likely H3)	2	0.67
AFRI	1	0.34
AFRI_1	1	0.34
AFRI_3	1	0.34
CAS1_DELHI	1	0.34
T1 (T4-CE1 ancestor0)	1	0.34
T2-T3	1	0.34
T5	1	0.34
T5_MAD2	1	0.34
Non identified	25	8.39
Total	298	100.00

3.2. Predominant Spoligotypes

Of the 65 spoligotypes identified, 40 were represented as Shared Types (ST) according to SpolDB4 while the others were reported for the first time. Among these Shared Types, ST 61 member of the LAM10_CAM and ST 50 member of the Haarlem family respectively represented 42.95% and 11.41% (**Table 2**).

The HGDI value for spoligotyping was low (79.72%), especially for the strains of the LAM10_CAM family (96.71%). No correlation was found between the identified genotypes with regard to drug resistance, and HIV sero-status (**Table 3**). However, a statistical association was found between the LAM10_CAM isolates and age groups. Patients belonging to 15 - 24 and 35 - 44 age groups were more likely infected by LAM10_CAM strains compared to others. In the LAM10_CAM family, the distribution of different Share Types ST403, ST61, ST838, ST850 and ST852 was not associated with HIV sero-status.

Table 2. Distribution of Share Types (ST) identified in a collection of 298 *Mycobacterium tuberculosis* complex isolates.

Genotypes	Share-Types (ST)	Spoligo-patterns	No. isolates	Frequency (%)
AFRI	332		1	0.34
AFRI_1	715		1	0.34
AFRI_2	101		1	0.34
AFRI_3	331		2	0.67
	856		1	0.34
T1	1223		2	0.67
	1324		2	0.67
	144		1	0.34
T1 (T4-CE1 ancestor)	53		7	2.35
	65		1	0.34
T2	1056		2	0.67
	125		1	0.34
	317		2	0.67
	52		8	2.68
	848		1	0.34
	853		8	2.68
T2-T3	73		1	0.34
T5	44		1	0.34
T5_MAD2	1227		1	0.34
LAM1	20		6	2.01
LAM9	42		3	1.01
LAM10_CAM	403		3	1.01
	61		128	42.95
	838		14	4.70
	850		5	1.68
	852		2	0.67
H1	47		3	1.01
H3	316		4	1.34
	49		1	0.34
	50		34	11.41
	75		1	0.34
	840		1	0.34
CAS1_DELHI	99		1	0.34
			1	0.34
U	124		1	0.34
	450		11	11.41
	786		1	0.34
	839		1	0.34
U (likely H)	46		6	2.01
	237		2	0.67

Table 3. Distribution of LAM10_CAM and non LAM10_CAM genotype according to drug resistance, age group and HIV sero-status.

	Total N = 298	LAM10_CAM N = 152	%	Non LAM10_CAM N = 146	%	p-value
DST results						
Resistant	28	17	60.71	11	39.29	0.28
Susceptible	270	135	50.00	135	50.00	
Age groups						
15 - 24	72	46	63.87	26	36.13	0.01*
25 - 34	103	51	49.51	52	50.49	0.70
35 - 44	72	28	38.88	44	61.12	0.02*
45 - 54	28	18	64.28	10	35.72	0.14
55 - 64	23	9	39.13	14	60.87	0.23
HIV sero-status						
Positive	86	50	58.14	36	41.86	0.07
Negative	274	98	35.76	111	64.23	

*Statistically significant.

4. Discussion

It has been reported in some instances that, spoligotyping can distinguish among members of the *M. tuberculosis* complex based on the species-specific presence/absence of spacers [10]. In our study, 3 different species were identified among 298 *M. tuberculosis* complex isolates. With more than 90% cases, *M. tuberculosis* was far the most prevalent species. A similar observation was reported among the *M. tuberculosis* complex isolates collected from the West region of Cameroon [11].

The comparison of spoligotypes found in this study with the International Spoligotyping Database SPolDB4, showed that the most prevalent spoligotype was ST 61 followed by ST 50, which belong respectively to the LAM10_CAM family and Haarlem family. A similar predominance of the LAM10_CAM family was previously described among *Mycobacterium tuberculosis* isolates from Burkina faso [12] and Benin [13]. Although this genotype was described to be prevalent in some countries of the West African coast [11], a study conducted in Sierra Leone revealed only 4% of strains belonging to the LAM10_CAM [14]. The factors that might contribute to the adaptability of *M. tuberculosis* strains or lineages to a particular population or zone are poorly understood. As hypothesized for the *Tunisian* family [15], mass BCG vaccination strictly applied for decades might have profoundly shaped the population structure of *M. tuberculosis* by concurrently favoring the selection and accommodation of particular genotypes, as the LAM10_CAM family in our setting.

W-Beijing family strains were not identified in our isolates. It has been reported that this genotype is very rare in some West African coast countries [12]. A proportion of 10.3% was reported among *M. tuberculosis* from Cotonou (Benin) [13]. As expected, the HGDI value for spoligotyping was low (79.72%). To increase the discriminatory power and for a better understanding of the molecular diversity of the studied population, a more discriminatory technique such as MIRU-VNTR typing is recommended [18].

As it was reported in some instances that, genotypes like W-Beijing family are associated with drug resistance [17], we analyzed the relationship between genotypes circulating in our setting and drug resistance. However, we did not find any statistical association between genotypes and drug resistance ($p = 0.47$) even HIV serostatus ($p = 0.07$) in our study, but only with age groups ($p = 0.01$). The distribution of the LAM10_CAM strains according to age groups followed the trend of HIV infection in our study population. It has been reported that strains well adapted to a specific population like the LAM10_CAM family are more likely to transmit compared to others [18]. Since HIV infection, a known risk factor for tuberculosis was associated with age groups; this could explain the association of LAM10_CAM strains with age groups. Five different Share Types ST403, ST61, ST838, ST850 and ST852 were identified in the LAM10_CAM family. The distribution of these genotypes did not correlate with HIV sero-status. This observation was previously reported in isolates from the West region of the country [11].

The population structure of *Mycobacterium tuberculosis* complex strains from the Centre region was diverse and included 65 different genotypes. The majority of strains belonged to the LAM10_CAM which can be subdivided in 5 spoligotypes. The consequence of this diversity for the TB epidemic are not yet clear and need to be addressed in further studies.

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