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(Research Article)

Evaluation of polymorphism of *Mycobacterium tuberculosis* complex and its association with age group and HIV sero-status in Ngaoundéré-Cameroon

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Abstract

Pulmonary tuberculosis is a millennial scourge that is still current, and considered one of the most communicable diseases in the world. This study aimed to assess the genetic diversity of isolates of the *Mycobacterium tuberculosis* complex (MTBC) from pulmonary tuberculosis patients in Ngaoundéré. We estimates it from newly diagnosed sputum smear-positive patients by spoligotyping method. In total we obtained 21 different profiles including 9 orphan profiles and 12 clusters grouping from 2 to 54 strains. The comparison of the profiles of the strains of Ngaoundéré studied here with those of the strains of the genetically known families and listed in the SpolDB4 database reveals 14 profiles. Of these, 73 (45.6%) isolates belong to the LAM10_CAM family while 67 (41.9%) were non LAM10_CAM strains. Strains classified into non LAM10_CAM family included strains from the T family 38 (23.75%), Uganda family 15 (9.37%), Haarlem family 10 (6.25%), and others. Among the Shared Types, ST 61 member of the LAM10_CAM represented 54 (36.98**%)** and ST 53 member of the T family represented 24 (16.49%). No correlation was found between the genotypes identified and both the serological status and sex. The results show that *M. tuberculosis* was the only species incriminated with a strong predominance of ST 61 clones from the LAM 10_CAM family and ST53 from the T family.

Keywords: Polymorphism; *M. tuberculosis*; Spoligotyping; Ngaoundéré; Cameroon

1 Introduction

Pulmonary tuberculosis is a millennial scourge that is still current, and considered as one of the most communicable diseases in the world [1]. This infectious and contagious disease is a global public health problem classified as the second leading cause of death due to infectious diseases after COVID-19 and before HIV/AIDS. In 2020, 9.9 million people worldwide contracted tuberculosis with 5.5 million men, 3.3 million women and 1.1 million children [2]. Lethality rate (the global proportion of people with TB dying from the disease) varied from under 5% in a few countries to more than 20% in most countries of the WHO African Region [3]. The causative agent of tuberculosis, *Mycobacterium tuberculosis* complex (MTBC), is made up of a group of phylogenetically very similar mycobacteria with a greater than 90% DNA-DNA homology and an average nucleotide identity of ≥ 99% thus making it difficult to differentiate them from one

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another [4]. Nowadays, progress on standardized molecular typing methods using so-called genetic fingerprinting or sequencing techniques offers a large number of epidemiological advantages and contributes to differentiation between strains of the same species [5]. 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 [5].

These typing tools are widely used in the investigation of TB epidemics for the detection of recent transmission, the identification of the dissemination of a mycobacterial clone, as well as for the distinction of cases of endogenous reactivation versus exogenous reinfection of TB. Thus the evaluation of the genetic diversity of the strains of the MTBC could allow the improvement of the therapeutic management of patients and targeted antibiotic therapy in order to fight against the emergence of forms of resistance, a major obstacle to the eradication of the disease by WHO in 2050 [6]. In this context, spoligotyping has proven to be a very practical and reproducible PCR-based method. This method assays the presence or the absence of a set of target sequences in the direct repeat (DR) locus [7]. 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 [8]. 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. Cardinal studies in the field of genotyping of bacteria of the *Mycobacterium tuberculosis* complex with the MIRU-VNTR 24 loci and spoligotyping method revealed that the majority of isolates in circulation in Cameroon were of the LAM10_CAM family, T and Haarlem3 [9]. However, very few studies have been carried out in recent years in Ngaoundere, a town populated by a people where pastoral activity (cattle breeding) occupies a prominent place in an overall context of regression in the frequency of *Mycobacterium bovis* and *Mycobacterium africanum* strains in favor of *Mycobacterium tuberculosis* strains. Hence, this study aimed to assess the genetic diversity of isolates of the MTBC from pulmonary tuberculosis patients in Ngaoundere and to better understand their transmission dynamics using the spoligotyping method.

2 Material and methods

2.1 Study Design and Population

2.1.1 Type and period of study

This is a cross-sectional descriptive study of clinically suspected tuberculosis patients with positive sputum microscopy conducted in Ngaoundéré from March 15, 2019 to March 15, 2021.

2.1.2 Study population

Our study population was made up of people who had come for a sputum examination (microscopy and culture) in the two Tuberculosis Diagnosis and Treatment Centers (CDT) in the city of Ngaoundéré and who had given informed consent for their participation in the study. Sputum samples that were positive by microscopy were kept at +4°C and transported twice weekly to the Laboratory for Tuberculosis Research and Pharmacology (LTRP) of the Biotechnology Center of the University of Yaoundé 1 (BTC UYI).

2.2 Biological Tests

2.2.1 HIV test

One blood sample of approximately 5 ml from a venous puncture was collected to perform HIV.

2.2.2 Mycobacterial Isolates, DNA Extraction and Spoligotyping

Sputum Culture and Identification

After centrifugation, three to four drops of the suspended decontaminated sputum sediment, was used to inoculate two Lowenstein Jensen (LJ) media slants, one without pyruvate and the other supplemented with 0.4% pyruvate. The cultures were incubated at 37 °C, and growth was monitored weekly by counting colonies. A sample was considered negative when no colony was obtained after 8 weeks of incubation. For positive cultures, *M. tuberculosis* species were identified based on growth rate and colony morphology observation.

DNA Extraction

A loop full of mycobacterial colonies scraped from Lowenstein-Jensen slopes using a sampling loop was introduced 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 ×g, the supernatant was collected in a new tube and stored at -20°C until further use.

Spoligotyping

All isolates were genotyped with a commercial spoligotyping kit (Isogen Bioscience, BV Maarsen, The Netherlands) according to the protocol previously described by Kamerbeek *et al.* [**7**]. Briefly, the DR region of the TB isolates was amplified using the primers DRa, 5'-GGTTTTGGGTCTGACGAC-3' (biotinylated5'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 chemi- luminescence (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 positive controls.

2.3 Data Analysis

Spoligotype patterns in binary format were entered in an Excel sheet, and compared with the spoligotype data-base SpolDB4 using MIRU VNTR *plus* [**10**]. When necessary, Fisher's Chi-square or Exact Test was used to evaluate the difference in serology and age between LAM10_CAM and non LAM10_CAM strains. P-values less than 0.05 were considered significant.

2.4 Research Ethics

This study was conducted in accordance with the ethical guidelines for human research in Cameroon. Ethical clearance was also obtained from the Institutional Ethics Committee of the University of Douala N° 2080 CEI-UD/01/2020/T. Patients who agreed to participate in the study gave their informed consent after being informed of the strict confidentiality of the use of their results, the risks involved and the purpose of the study.

3 Results

3.1 Socio-demographic characteristics of the study subjects

Out of a total of 160 tuberculosis patients included in this study, 98 **(**61.25**%**) were males, while 62 (38.75 %) were females, with a male to female ratio of 1.58. The average age of the patients was (35.52 ±1.25 years), with minimum age of 9 years. The TB-HIV coinfection rate was 36 (22.50%).

3.2 Distribution of Different Genetic Families

A usable spoligotype profile was obtained for the 160 strains. In total we obtained 21 different profiles including 9 orphan profiles and 12 clusters grouping from 2 to 54 strains.

The comparison of the profiles of the strains of Ngaoundéré studied here with those of the strains of the genetically known families and listed in the SpolDB4 database reveals 14 profiles.

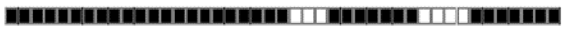
Of these, 73 (45.6%) isolates belong to the LAM10_CAM family while 67 (41.9%) were non LAM10_CAM strains. Strains classified into non LAM10_CAM family included strains from the T family 38 (23.75%), Uganda family 15 (9.37%), Haarlem family 10 (6.25%), and others (**Table 1**).

Table 1 Distribution of different genetic families identified in a collection of 160 *Mycobacterium tuberculosis* complex isolates

Especies	Genetic families	No isolates	Frequency (%)
Mycobacterium tuberculosis	LAM 10_CAM	73	45.60%
	T1	32	20.00%
	T2	6	3.75%
	U	4	2.50%
	H3	10	6.25%
	H1	6	3.75%
	U Likely H3	1	0.62%
	U Likely H	10	6.25%
Mycobacterium bovis	/	0	0%
Mycobacterium africanum	/	0	0%
Orphan		18	11.25%

3.3 Predominant Spoligotypes

Table 2 Families and lineage among the 160 *M. tuberculosis* complex strains isolated from Ngaoundéré.

Genotype	Share-type (ST)	Spoligo-patterns	No isolates	Frequency (%)
LAM _10 CAM	61		54	36.98%
	838		12	8.21%
	403		1	0.68%
	852		4	2.74%
T1	74		4	2.74%
	1688		4	2.74%
	53		24	16.49%
T2	317		1	0.68%
	52		6	4.10%
	118		1	0.68%
U	172		1	0.68%
	523		1	0.68%
	602		1	0.68%
U LIKELY H3	237		1	0.68%
U LIKELY H	46		10	6.85%
H3	316		10	6.85%
H1	919		6	4.10%

Of the 160 spoligotypes identified, 142 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 represented 54 (36.98**%)** and ST 53 member of the T family represented 24 (16.49%) (**Table 2**).

3.4 Distribution of LAM 10_CAM and no LAM 10_CAM genotype according to age group and HIV sero-status

No correlation was found between the genotypes identified and both the serological status and sex. However, a statistically significant association was found between the Lam 10_ CAM isolates and the 21-29 age group. (**Table 3**).

Table 3 Distribution of LAM 10_CAM and no LAM 10_CAM genotype according to age group and HIV sero-status

		TOTAL	LAM 10 CAM	%	NO LAM 10 CAM	%	P VALUE
SEX	FEMALE	64	34	53,1	24	37,5	
	MALE	96	39	40,6	45	46,9	0,297
	11 - 20	36	20	55,6%	12	33,3%	
	21-29	32	12	37,5	18	56,3	0,047
AGE	30-39	44	16	36,4	22	50	
	40-49	20	14	70	4	20	
	50-59	12	4	33,33	4	33,33	
	60-69	10	3	30	7	70	
	ABOVE 70	6	4	66,7	2	33,33	
	POSITIVE	44	23	52,3	13	29,5	0,056
HIV	NEGATIVE	116	50	43,1	56	48,3	

4 Discussion

The results obtained in this work made it possible to identify 142 isolates all belonging to the species *Mycobacterium tuberculosis* (88.75%) and 18 orphan isolates (11.25%) were highlighted; they also revealed the absence of strains belonging to the *Mycobacterium bovis* species, which is nevertheless strongly suspected due to the involvement of local populations in cattle breeding. The results of this study show that tuberculosis infection in the town of Ngaoundere in Cameroon is mainly caused by the species *M. tuberculosis*, yet this region is the seat of large cattle breeding sites intended for consumption. These results are in line with the observations made in the Littoral by Thumamo [1**]. which found 82% strains of *M. tuberculosis* and 0% *M. bovis*, in Central, West, South Cameroon [**1**]. who detected 81% strains of M. tuberculosis and 0% *M. bovis*. The lack of *M. bovis* isolates suggests several hypotheses; this absence could be explained by the fact that the genotypes of all *M. bovis* isolates circulating in Cameroon have not yet been entered into the SpolDB4 database for this reason it is likely that the orphan isolates found in this study belong to this species, this can also be explained by the fact that tuberculosis infections due to *M. bovis* are most often located outside the lungs **[11**], or by the fact that the systematic introduction of the BCG vaccine in newborns in Cameroon may have selected certain species (notably *M. tuberculosis*) over others **[12**]. As for *M. africanum,* its decline as an etiological agent in favor of *M. tuberculosis* was observed in 2011 in other Cameroonian regions **[13]**. This phenomenon of regression had already been mentioned by **[14**], who revealed that the involvement of *M. africanum* fell from 56% in the 1970s to 9% in 2003. Although the factors that may justify this change of etiological agent are not fully known, the treatment protocol currently applied could undoubtedly play an essential role.

In total we obtained 21 different profiles including 9 orphan profiles and 12 clusters grouping from 2 to 54 strains.

The comparison of the profiles of the strains of Ngaoundéré studied here with those of the strains of the genetically known families and listed in the SpolDB4 database reveals 14 profiles.

Of these, 73 (45.6%) isolates belong to the LAM10_CAM family while 67 (41.9%) were non LAM10_CAM strains. Strains classified into non LAM10_CAM family included strains from the T family 38 (23.75%), Uganda family 15 (9.37%), Haarlem family 10 (6.25%), and others.

Regarding the polymorphism within the families of spoligotypes, the results of this study show a diversity of moderate importance. Each identified family is represented by a single spoligotype.

These results clearly show that the population structure of *M. tuberculosis* in the city of Ngaoundere is dominated by the LAM10_CAM family. Equivalent results have been reported in Cameroon in the Adamaoua [13], Center, West, South and East [11]. This observation is also made in Nigeria [15] and Chad [16] countries bordering Cameroon. This family is very widespread in Northern Europe, as well as in Central Africa where it is believed that it was introduced during European colonization. This family is represented by the ST 61 which is the most common spoligotype as described in other regions of Cameroon by [13], in Burkina Faso by [12], in West Africa (Nigeria, Benin, Senegal and Cote d'Ivoire). This spoligotype is characterized by the absence of spacers 23-25 and 33-36, this predominance in the regions of Central Africa and West Africa can be explained by frequent cross-border movements [17]. However, the reasons that would justify this selection and dissemination remain unknown. The T1 family of the T superclade is represented in this area by the spoligotype 53. Still called the Ghana family, this spoligotype circulates in most countries of Central Africa. In Nigeria a molecular study of tuberculosis revealed that the 2nd largest cluster was also that of ST53 of the T family (22.4%) [15].This spoligotype showed an increase in Cameroon and Chad, and a very high rate in Niger and Ghana. The least represented family is the U family (Likely H3) with one isolate. It has also been found in the South and East [11]. This rare family is only described in few countries such as Nigeria and the United States, the presence of this family is probably due to the migratory trade between Nigeria and Cameroon or to indirect zoonosis since this family has been identified in strains implicated in bovine TB in the Adamaoua region [13].

5 Conclusion

Our study provides a genetic structure of *Mycobacterium tuberculosis* complex strains in the city of Ngaoundéré-Cameroon. The results show that *M. tuberculosis* was the only species incriminated with a strong predominance of ST 61 clones from the LAM 10_CAM family and ST53 from the T family No correlation was found between the genotypes identified and both the serological status and sex.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare that they have no competing interests.

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