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MYCOBACTERIUM TUBERCULOSIS GENETIC DIVERSITY AND DRUG RESISTANCE CONFERRING MUTATIONS IN THE DEMOCRATIC REPUBLIC OF THE CONGO
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ABSTRACT

***Background*: The Democratic Republic of the Congo (DRC) belongs to the 22 tuberculosis (TB) high-burden countries and to the 27 high-burden multidrug-resistant (MDR)-TB countries. To date, there are no data on the genetic diversity of *Mycobacterium tuberculosis* in the DRC.**
***Objective*: To describe the genetic diversity and the distribution of drug resistance conferring mutations of clinical *M. tuberculosis* isolates from the DRC.**
***Design*: We analysed consecutive *M. tuberculosis* single patient isolates cultured in 2010 at the laboratory of the National TB Control Programme in Kinshasa.**
***Setting*: National TB Control Programme in Kinshasa, DRC.**
***Results*: Isolates from 50 patients with pulmonary TB were analysed, including 45 patients (90%) who failed treatment. All isolates belonged to the Euro-American lineage (main phylogenetic Lineage 4). Six different spoligotype families were observed within this lineage, including LAM (20 patients, 40%), T (15 patients; 30%), U (4 patients; 8%), S (3 patients; 6%), Haarlem (2 patients; 4%), and X (1 patient; 2%). No *M. africanum* strains were observed. The most frequently detected drug resistance-conferring mutations were *rpoB* S531L and *katG* S315T1. Various other mutations, including previously unreported mutations, were detected.**
***Conclusions*: The Euro-American lineage dominates in the DRC, with substantial variation in spoligotype families. This study fills an important gap on the molecular map of *M. tuberculosis* in sub-Saharan Africa.**

INTRODUCTION

The Democratic Republic of the Congo (DRC) has an estimated population of 63 million and a gross national income per capita of 280 US$. The DRC is one of 22 tuberculosis (TB) high-burden countries with an estimated incidence of 392 new TB cases per 100,000 per year (1). The country also belongs to the 27 countries with a high burden of multidrug-resistant (MDR) TB, the prevalence of MDR among new cases is 2.3% (2).

Mycobacterium tuberculosis complex (MTBC) has a global phylogeographic population structure consisting of six main phylogenetic lineages (3): Lineage 1 (known as Indo-Oceanic lineage), Lineage 2 (known as East-Asian lineage), Lineage 3 (known as Delhi/CAS), Lineage 4 (known as Euro-American lineage), and Lineages 5/6 (known as West African lineages 1 and 2 also known as *M. africanum*). These bacterial lineages are associated with specific geographic regions and human populations (3-5). Lineage 4 represents the *M. tuberculosis* family with the largest geographical spread and includes spoligotype strain families such as "Haarlem (H)" or "Latin-American-Mediterranean (LAM)". There is increasing evidence that strain diversity in

M. tuberculosis plays a role in the presentation of disease in humans (3,6) and in animal models (7). For example, a study from West Africa found a lineage-dependent rate of progression from infection to active disease comparing *M. africanum* versus *M. tuberculosis* (8). A study from Vietnam showed that a higher proportion of adults with pulmonary TB caused by Lineage 4 (Euro-American lineage) had a consolidation on the chest X-ray radiographs, and that TB meningitis caused by Lineage 2 (East-Asian lineage) was associated with disease progression and leukocyte count in cerebrospinal fluid (9). Another study from Vietnam found that Lineage 4 strains were less likely to cause TB meningitis compared to other lineages (10). Furthermore, *M. tuberculosis* strains belonging to Lineage 2 (which includes the Beijing genotype) are associated with drug resistance and infection with the human immunodeficiency virus (HIV) (7, 11, 12), and have been rapidly emerging in some areas, for example in Cape Town, South Africa (11, 13), or in the Canary Islands in Spain (14). Finally, recent studies have reported associations between *M. tuberculosis* lineages and specific human genetic polymorphisms in genes associated with the immune system (10, 15). Taken together, *M. tuberculosis* is more genetically diverse than previously thought (16) and this genetic diversity may translate into phenotypic effects.

To date, there are no data on the genetic diversity of *M. tuberculosis* or on the distribution of drug resistance conferring mutations in the DRC. Therefore, we characterised clinical *M. tuberculosis* isolates to gain insights into the genetic population structure of *M. tuberculosis* and the frequency of drug resistance conferring mutations in the DRC.

MATERIALS AND METHODS

Study setting: We analysed consecutive *M. tuberculosis* single patient isolates cultured in 2010 at the laboratory of the National TB Control Programme in Kinshasa. Cultures were mainly done in patients failing treatment and when (multi) drug resistance was suspected. The National TB Control Programme in DRC runs TB testing and treatment centres throughout Kinshasa and other parts of the country. Its TB laboratory in Kinshasa acts as the reference laboratory for the country following international guidelines (17). Clinical data were obtained from the National TB Control Programme.

Strain culturing and DNA extraction: Clinical specimens were processed and strains cultured on Löwenstein-Jensen medium according to international guidelines (17) at the TB reference laboratory in Kinshasa. Standard phenotypic drug susceptibility testing (DST) for isoniazid and rifampicin were performed by proportion method according to international guidelines (17).

Molecular investigations: DNA was extracted from subcultures according to standard laboratory procedures. Mutations in *katG*, *inhA*, and *rpoB*, which are known to confer resistance to isoniazid and rifampicin, respectively, were determined using the GenoType MTBDR*plus* line probe assay (Hain Lifescience, Germany; *katG*, *inhA*, *rpoB* genes); and complemented by PCR amplification and direct sequencing of PCR products as described previously (18,19). Determination of the main *M. tuberculosis* lineages was performed by single nucleotide polymorphisms (SNPs) using multiplex real-time PCR with fluorescence-labelled probes (Taqman, Applied Biosystems, USA) as described before (20). The SNP used to define Lineage 4 was originally described by Sreevatsan *et al*. (21) and shown to be specific to this lineage (20). *M. tuberculosis* isolates were further genotyped by spoligotyping as previously described (22). Spoligo-International-Type (SIT) numbers were assigned using data published in the fourth international spoligotyping database (SpolDB4) (22). Spoligotype families were used to define sub-lineages within the main *M. tuberculosis* lineages (23).

Ethical approval: The study was approved by the Ethics Committee of the Kinshasa School of Public Health in Kinshasa, DRC. No informed consent was required by the Ethics Committee. All data and strains were collected in the context of routine care and no additional data collection or contact with patients occurred for this study. The data were completely de-identified before analysis using unique study numbers.

RESULTS

Patient characteristics: A total of 50 pulmonary TB patients were included. Median age was 28 years (interquartile range 26-34 years), 33 (66%) were male, four (8%) were known to be HIV-infected (Table 1). Treatment failure was the most frequent TB category (45 patients; 90%), and the patients were primarily from the capital Kinshasa (33 patients; 66%) and from Lubumbashi (9 patients; 18%). Figure 1 shows the geographical origin of the 50 isolates.

Figure 1

Origin of the clinical Mycobacterium tuberculosis isolates in the Democratic Republic of the Congo (shaded in grey)

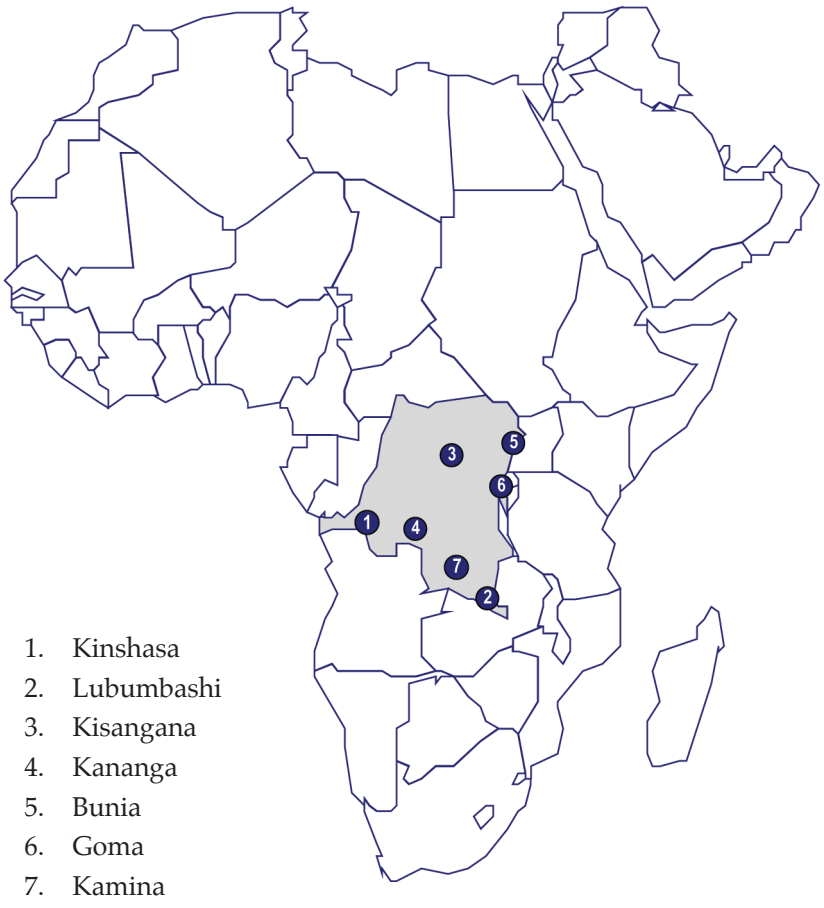

Table 1

Clinical characteristics of the 50 tuberculosis (TB) patients from the Democratic Republic of Congo included in the study

Characteristic	Value
Age, median (interquartile range), years	28(26-34)
Sex, n (%)	
Male	33(66)
Female	17(34)
HIV status, n (%)	
HIV-positive	4(8)
Not determined	46(92)
TB categories, n (%)	
Treatment failure	45(90)
New case	3(6)
Previous TB	1(2)
Recurrent case	1(2)
Origin of patients, n (%)	
Kinshasa	33(66)
Lubumbashi	9(18)
Kisangani	3(6)
Kananga	2(4)
Other regions (Bunia, Goma, Kamina)	3(6)

Genetic diversity of M. tuberculosis: The molecular analyses showed that all isolates belonged to Lineage 4 (Euro-American lineage). Considerable genetic diversity was, however, observed within this lineage based on spoligotyping. The most frequent spoligotype family was the LAM (Latin-American-Mediterranean) family (20 isolates; 40%) and the T-family (the "ill-defined" family; 15 isolates; 30%), followed by the S, U, H and X families (Table 2). One strain belonged to the Cameroon family ("LAM10_CAM"), and two strains to the Zimbabwe family ("LAM11_ZWE") (Table 3). Table 3 details the spoligotyping results according to the classification of SpolDB4, using the SITVIT molecular database, which is available at http://www.pasteur-guadeloupe.fr:8081/SITVITDemo/index.jsp. Spoligotypes are patterns formed by the presence or absence of 43 spacers in the DR locus of Mycobacterium tuberculosis and these patterns (Table 3).

Table 2

Molecular data of the 50 clinical Mycobacterium tuberculosis isolates

Characteristic	n(%)	
	All n=50	MDR n=28
Spoligotyping families, n (%)		
LAM (Latin-American-Mediterranean)	20(40.0)	14(50.0)
T (ill-defined "lineage")	15(30.0)	5(17.8)
U	4(8.0)	3(10.7)
S	3(6.0)	3(10.7)
H	2(4.0)	1(3.6)
X	1(2.0)	1(3.6)
Undesignated	5(10.0)	1(3.6
Main *M. tuberculosis* lineages, n (%)		
Lineage 4 (Euro-American lineage)	50(100)	28(100)
Other lineages	0	0
Drug resistance mutations, n (%)		
rpoB (codon)		
S531L	21(42.0)	17(60.7)
D516V	6(12.0)	4(14.3)
S522Q	3(6.0)	2(7.1)
N518S[1]/L533P	1(2.0)	1(3.6)
H526H[12]	1(2.0)	1(3.6)
H526N	1(2.0)	0
H526D	1(2.0)	0
S531W	1(2.0)	1(3.6)
P535S	1(2.0)	0
Deletion Q-F-M (513-515)[1]	1(2.0)	0
katG (codon)		
S315T1 (AGC>ACC)	31(62.0)	24(85.7)
L333R[1]	2(4.0)	2(7.1)
S315N	2(4.0)	0
M377R[1]	1(2.0)	1(3.6)
inhA Promote		
C15T	4(8.0)	2(71)
T8C	1(2.0)	1(3.6)

MDR, multidrug-resistant as determined by phenotypic drug susceptibility testing
[1]first described in this study
[2]synonymous mutation

Table 3

Spoligotyping results from the 50 clinical Mycobacterium tuberculosis isolates according to the definition in SpolDB4 database using SITVIT2 (http://www.pasteur-guadeloupe.fr:8081/SITVITDemo/index.jsp)

Spoligotype description	SIT	Clade	Spoligo family	n(%)
	42	LAM9	LAM	6(12.0)
	20	LAM1	LAM	5(10.0)
	52	T2	T	5(10.0)
	144	T1	T	5(10.0)
	34	S	S	3(6.0)
	17	LAM2	LAM	2(4.0)
	106	U(LAM3)	U	2(4.0)
	737	LAM9	LAM	2(4.0)
	1548	U	U	2(4.0)
	Orphan	Undesignated	Undesignated	2(4.0)
	Orphan	Undesignated	Undesignated	2(4.0)
	50	H3	H	1(2.0)
	53	T1	T	1(2.0)
	59	LAM11_ZWE	LAM	1(2.0)
	61	LAM10_CAM	LAM	1(2.0)
	73	T2-T3	T	1(2.0)
	95	LAM6	LAM	1(2.0)
	137	X2	X	1(2.0)
	175	T2	T	1(2.0)
	241	T1	T	1(2.0)
	1155	H1	H	1(2.0)
	1284	T1	T	1(2.0)
	1545	LAM9	LAM	1(2.0)
	1549	LAM11_ZWE	LAM	1(2.0)
	Orphan	Undesignated	Undesignated	1(2.0)

SIT, Spoligotype international type

Drug resistance: Phenotypic DST results showed that 28 isolates were MDR strains. Analysis of drug resistance mutations revealed *rpoB* S531L and *katG* S315T1 (AGC>ACC) as the most frequent mutations (Table 2). But other mutations were also detected, including *rpoB* mutations N518S, the *rpoB* deletion 513-515, *katG* mutation L333R and *katG* mutation M377R. We also detected new mutations that had never been reported before according to the TB Drug Resistance Mutation database such as rpoB mutations N518S, the *rpoB* deletion 513-515, *katG* mutation L333R and *katG* mutation M377R. Comparing phenotypically confirmed MDR with non-MDR isolates (with a DST result), the LAM spoligotyping family was more frequent (50.0% versus 16.7%), and T-family less frequent (17.8% versus 50.0%) in MDR isolates (Fisher's exact test p=0.03).

DISCUSSION

This is the first report on the genetic diversity of *M. tuberculosis* in the DRC, one of the largest and most populous countries in sub-Saharan Africa, and a country that is heavily affected by the global epidemic of TB. We found that all *M. tuberculosis* strains belonged to the Euro-American lineage (Lineage 4) but that there was considerable genetic diversity of spoligotyping families such as LAM, T, X, H and S. We also found that *rpoB* S531L and *katG* S315T1 were the most frequent drug resistance-conferring mutations, which is consistent with reports from most other geographic regions.

The Euro-American lineage represents the *M. tuberculosis* family with the largest geographical spread and includes spoligotype families such as "Haarlem" or "LAM" (3). The high proportion of the LAM (Latin-American-Mediterranean) family in our sample is consistent with findings from other countries in Southern Africa, including South Africa (F11 genotype) (24) and Zimbabwe ("Zimbabwe-family") (25), and is possibly linked to the contact with Europeans during colonial times (16). Interestingly, the Cameroon family which

is the major group of *M. tuberculosis* strains in the neighbouring countries of Chad (26) and Cameroon (27,28) does not seem to occur very frequently in the DRC. *M. africanum* lineages have not been detected in the DRC, Angola (29) or Chad (26). However, *M. africanum* was found to be declining in Cameroon in the last three decades, and the same may be true for DRC.

Despite major improvements in treatment and control, TB remains a major public health problem worldwide, particularly in the context of MDR and extensively drug-resistant (XDR) TB (2,30). Drug susceptibility can be tested by culture methods based on bacterial growth, or alternatively by molecular methods detecting drug resistance-associated gene mutations (31). Antibiotic resistance mutations are usually associated with a fitness cost and recent studies suggest that the genetic background in *M. tuberculosis* strains can influence the relative fitness (32-34). Interestingly, *M. tuberculosis* strains with low- or no-cost resistance mutations (e.g. *rpoB* S531L) are also the most frequent among clinical isolates (33). In our study, we found that *rpoB* S531L and *katG* S315T1 were the most frequent drug resistance-conferring mutations in the DRC. We also detected new mutations that have never been reported before, such as *rpoB* mutations N518S, the *rpoB* deletion 513-515, *katG* mutation L333R and *katG* mutation M377R. *rpoB* L533P associated with low-level rifampicin resistance (35) was present in one strain together with *rpoB* mutation N518S. Such low-level drug resistance mutations are difficult to detect using liquid culture based drug resistance testing (35,36). The frequency of these low-level drug resistance mutations in the DRC and the significance of the mutations discovered in this study will need to be evaluated in larger studies.

Our study has several limitations. We could not exclude with certainty that other lineages and less common spoligotyping families or resistance mutations existed due to the relatively small sample size. Secondly, we stress that the strains analysed among selected TB patients failing treatment mainly in Kinshasa did not reflect the *M. tuberculosis* strains causing the disease in the country at large, but will be representative of those sent to the reference laboratory and cultured.

In conclusion, the Euro-American lineage dominates in the DRC, with substantial variation in spoligotype families. To our knowledge, this is the first study on the genetic diversity of *M. tuberculosis* in the DRC, mainly in Kinshasa, one of the largest cities in sub-Saharan Africa which is of great interest due to the high TB and MDR TB burden (1,2). In-depth knowledge of the population biology and epidemiology of *M. tuberculosis* is essential for the development of new TB diagnostics, drugs, and vaccines (3,37,38). Future studies on the molecular epidemiology of TB in DRC, with a special focus on MDR and HIV-infected populations, will provide a more complete picture of the distribution of *M. tuberculosis* genetic diversity in this part of the world.

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