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Genetic diversity and resistance profile of mycobacterial strains isolated in Senegal

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Tuberculosis remains a major public health problem in Senegal. The aim of this study was to determine the resistance and molecular profiles of mycobacterial strains isolated over a four-year period in Senegal. Mycobacterial isolates worked on were obtained at the National Reference Laboratory of the National Antituberculosis Program (NAP) in Senegal. These strains were cultured on Lowenstein-Jensen (LJ) medium or on LJ medium supplemented with pyruvate, between January 2011 and December 2014. Depending on the availability of reagents, the drug susceptibility tests were performed by the proportion method or molecular methods. Strains were characterized by spoligotyping. This study focused on 208 samples with 160 strains and 48 sputa. Antibiotic resistance testing was carried out on 151 samples among which 32 (21.2%) came from new patients and 119 (78.8%) from previously treated patients. Seventy (43.35%) MDR strains were detected. Spoligotyping were performed on 64 strains, essentially MDR strains which mostly belong to three families: T, Beijing and Cameroon. In Senegal, anti-tuberculosis drug resistance rates are very high among retreatment patients with a high percentage of MDR strains in patients with treatment failure. The strains belong to three main families: T superfamily, Beijing and Cameroon. Multidrug resistance compromises the effectiveness of tuberculosis treatment with a high risk of spread of these MDR strains in the community, hence, the importance of strengthening the fight against tuberculosis in general and MDR-TB in particular.

Key words: *Mycobacterium tuberculosis*, spoligotypes, resistance, Senegal.

INTRODUCTION

With an annual incidence exceeding 300 cases per 100,000 inhabitants in some countries, particularly in sub-Saharan Africa, tuberculosis remains a major public health problem (Mbatchou et al., 2008). The World Health Organization (WHO) reported 13,117 cases of tuberculosis in Senegal (including new cases and relapses) for 2016, 0.9% of which were multidrug-resistant tuberculosis (MDR-TB) among new cases and 17% in retreatment patients (WHO, 2017). Resistance to anti-tuberculosis drugs is therefore a major public health problem worldwide.

The strategy adopted in Senegal for the detection of

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cases of MDR-TB is a systematic cultivation for retreatment patients (failure, relapse and repeating treatment), tuberculosis contacts for MDR-TB, people living with HIV/AIDS (PLWHA) and health personnel with tuberculosis (NAP, 2013).

In Senegal, the national resistance screening algorithm in use since 2014 recommend the use of the Xpert MTB/RIF as the first-line diagnostic tool. The Xpert MTB/RIF test uses Polymerase Chain Reaction (PCR) to amplify directly the *rpoB* gene of tuberculosis complex mycobacteria present in the sample. It can also be used to detect the mutations associated with Rifampicine (RIF) resistance (WHO, 2011; FIND, 2015).

As the worldwide emergence of multidrug-resistant strains has become one of the greatest public health concerns, understanding the genetic backgrounds of circulating drug-resistant strains is crucial (Affolabi et al., 2009). Unfortunately, as in many African countries, few molecular typing data are available in Senegal for the strains involved.

In order to bridge this gap, this study was carried out with the objective to determine the molecular profiles of mycobacterial strains isolated over a four-year period in Senegal.

MATERIALS AND METHODS

Patients and strains

Mycobacterial isolates obtained at the National Reference Laboratory of the National Antituberculosis Program (NAP) in was worked on Senegal. These strains were isolated between January 2011 and December 2014, mostly from patients experiencing treatment failure or relapse, from Dakar and other regions of Senegal. The clinical data were collected from analysis reports and included the following items: Surname, first name, age, sex, clinical diagnosis, history of treatment with antituberculous drugs and the healthcare structure of origin.

Culture and tests of susceptibility to antituberculous drugs (drug susceptibility tests- DSTs)

DSTs were performed at the reference laboratory of the NAP in Senegal. Isolates were cultured on Lowenstein-Jensen (LJ) medium or on LJ medium supplemented with pyruvate. The strains were identified on the basis of the time taken for the colonies to appear, the results of the niacin test, and tests for heat-labile catalase (at 22 and 70°C) and for nitrate reductase. Depending on the availability of reagents, the DSTs were performed by the proportion method (Canetti et al., 1963) or by molecular methods.

For antibiogram by the proportion method, the samples were decontaminated with 4% NaOH and centrifuged.

The resulting pellet was then used to inoculate plain LJ medium, LJ medium supplemented with pyruvate and LJ medium supplemented with 5 mg/mL thiophene-2-carboxylic acid hydrazide (TCH). The molecules tested were: Ethambutol (EMB, at a concentration of 2 µg/L), streptomycin (SM, 4 µg/L), isoniazid (INH, 2 µg/L) and rifampicin (RIF, 40 µg/L). A strain was considered to be resistant to a drug if its growth in media containing this drug was ≥1% that of the control.

Molecular DSTs were based on two tests recommended by the WHO (WHO, 2011; Find, 2015) for identifying mycobacteria of the tuberculosis complex and the mutations most frequently conferring resistance to RIF and INH: The line probe assay (LPA) (Find, 2015) and the Xpert MTB/RIF assay (WHO, 2011). The samples were thus tested with the Xpert MTB/RIF assay, the LPA, or both. In cases in which both tests were used, the LPA was used, above all, to check the susceptibility to INH of strains resistant to RIF with the Xpert MTB/RIF assay.

Molecular typing of strains

This part of the work was carried out in the reference laboratory for mycobacteria in Cotonou, Benin. Spoligotyping was used for molecular characterization. The standardized method was used as described by Kamerbeek et al. (1997), based on the detection of polymorphism of the direct repeat (DR) region. This molecular typing method is based on the polymorphism of nucleotide sequences located between the identical 36-base pair sequences of the DR region present only in members of the *M. tuberculosis* complex. The number of DR sequences can vary between strains of the same species. These 36 bp sequences are separated by 36 to 41 bp non-repetitive DNA sequences known as inter-DR sequences or spacers, which display limited variation. Forty-three spacer sequences were synthesized and immobilized on a commercial nylon membrane (Isogen Bioscience B.V., BT Maarsen (The Netherlands). Two primers complementary were used to conserve part of the DR region, directed towards the exterior, to amplify the spacers. Genomic profiles were determined by hybridizing the membrane bearing all 43 spacer sequences with these probes. Spoligotypes were then defined on the basis of the presence or absence of several spacers.

Ethical considerations

This retrospective study included data collected during routine diagnosis and treatment, so it did not require ethical approval.

Data analysis

The data were entered into the computer and analyzed with EPI-INFO version 7 software (Centers for Disease Control and Prevention, Atlanta, GA, United States).

RESULTS

The work focused on 208 patients, aged 9 to 89 years with an average age of 35.8 years and a sex ratio (M/F) of 2.7. These patients come from the regions of Dakar (89.0%), Thies (3.7%), Louga (4.6%), Diourbel (1.8%), Kaolack (0.9%). Of these, 41 (37.3%) had treatment failure and 69 (62.7%) relapsed.

208 samples including 160 strains and 48 sputa were focused on. These strains came from patients who had therapeutic failure (19.7%), from relapsed patients (33.2%) and from new patients who have not had any TB treatment before (15.4%).

Antibiotic resistance testing was carried out on 151 samples among which 32 (21.2%) came from new patients and 119 (78.8%) from previously treated patients.

Table 1. Spoligotypes and resistance profile of the strains circulating in Senegal.

Clade	SIT	Wild-Type	SM-R	EMB-R	MR	Not tested	Total (%)
T	*53*	2			10	1	13 (20.3%)
T	*1580*				1		1 (1.6%)
T	*181*				1		1 (1.6%)
Beijing	*1*				9		9 (14.1%)
Cameroon	*61*	1			2		3 (4.7%)
Cameroon	*37*	1			2		3 (4.7%)
LAM	*42*				3		3 (4.7%)
LAM4	*60*					2	2 (3.1%)
CAS1-Delhi	*26*	1			2		3 (4.7%)
H3	*183*					2	2 (3.1%)
H2	*2*	1				1	2 (3.1%)
AFRI_1	*187*	1					1 (1.6%)
AFRI_1	*326*	1					1 (1.6%)
T5-RUS1	*765*				1		1 (1.6%)
Orphans	/	2	1	1	12	3	19 (29.7%)
Total	/	10	1	1	43	9	/

SM: Streptomycine; EMB: Ethambutol; SIT: shared-type number in the SITVIT database; R: Resistant; H2: Harlem 2; H3: Harlem 3; LAM: Latin American and Mediterranean; T: T superfamily; AFRI: Africanum.

Table 2. Rate of resistance to anti-tuberculosis medication in patients with therapeutic failure or relapse.

Antibiotics	Patients with therapeutic failure			Patients in relapse			Total strains tested
	R	N	%	R	N	%	
RIF	21	28	75	10	43	23.25	**71**
INH	22	28	78.57	11	43	25.58	**71**
EMB	18	23	78.26	15	41	36.58	**64**
SM	20	24	83.33	17	42	40.47	**66**

RIF: Rifampicine; INH: Isoniazide; SM: Streptomycine; EMB: Ethambutol; R: Resistant; N: Number; %: Percentage.

Results of spoligotyping

Spoligotyping were performed on 64 strains, essentially MDR strains which mostly belong to three families: T, Beijing and Cameroon (Table 1).

Drug susceptibility tests

Global results of drug susceptibility tests

Antibiotic resistance testing was carried out on 151 biological samples, 103 of which was tested by proportions method, 32 by molecular methods and 16 by molecular and proportions methods. In total 70 (43.3%) MDR strains were detected, among which 57 (37.4%) detected by proportions method and 13 (8.6%) by Xpert MTB/RIF. Among these MDR strains, 53 (35.1%) were also resistant to SM and 48 (31.8%) to SM and EMB. Five MDR strains were isolated from new patients (15.6% (5/32)).

Drug susceptibility tests by proportions methods

In total, one hundred and nineteen (78.8%) samples were tested by proportions method. High resistance rates were noted with streptomycine (56.1%), ethambutol (51.6%), isoniazide (46.5%) and rifampicin (43.7%). The highest resistance rates were noted in patients with treatment failure and in relapsed patients (Table 2).

Drug susceptibility tests by molecular methods

Forty-eight sputa samples were tested by molecular methods. Thirteen of these samples were tested only with the Xpert MTB/RIF, 16 were tested only with the LPA and 19 were tested with both.

Table 3. Classification of the isolates according to Xpert MTB/RIF and LPA results.

LPA	Xpert MTB/RIF		Total
	RIF R	RIF S	
INH R/RIF R	13	0	13
INH S/RIF R	2	0	2
INH S/ RIF S	0	4	4
Total	15	4	19

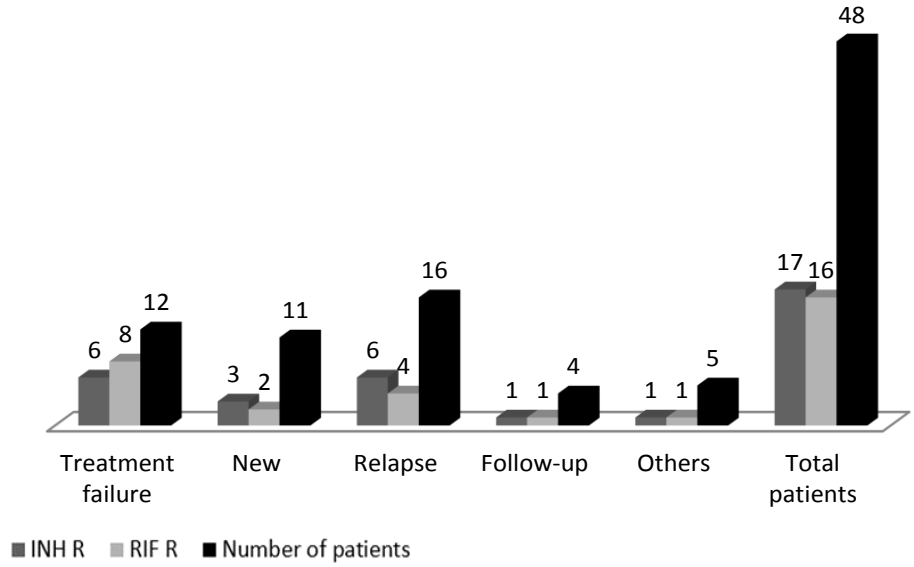

Figure 1. Distribution of strains resistant to RIF and INH by patients category.

Of the samples tested with the Xpert MTB/RIF, one was found to be resistant to RIF. The LPA identified three isolates as INH-R/RIF-R, three as INH-R/RIF-S, nine as INH-S/RIF-S and one sample negative. By combining Xpert MTB/RIF and LPA results, there were identified two isolates (4.2%) susceptible to INH and resistant to RIF (INH-S/RIF-R) (Table 3). In total, 16 MDR isolates were detected (33.3%). The rates of resistance were high among patients with relapses (37.5% resistant to INH and 25% resistant to RIF) or treatment failure (50% resistant to INH and 66.7% resistant to RIF) (Figure 1).

DISCUSSION

Epidemiological and clinical data

This study, on biological specimen obtained at the National Reference Laboratory for Mycobacteria of the National Antituberculosis Program in Senegal. Most of the samples came from young male patients, as reported in other studies (Traore et al., 2014). Most presented treatment failure or relapse, potentially accounting for the high frequency of MDR strain detection in this study (46.3%).

Indeed, this frequency is higher than that reported for the Central African Republic (40%) (Minime et al., 2010). However, it is lower than frequencies reported in Burkina Faso (50.5%) (Sangare et al., 2010).

Molecular typing of strains

This study is, to our knowledge, the third to focus on the molecular profile of the mycobacterial strains circulating in Senegal (Niang et al., 1999).

We used spoligotyping which is known as an excellent method to determine the molecular profile of our strains. However, the association of spoligotyping with a more discriminatory method, such as MIRU-VNTR or IS6110-RFLP, is recommended to improve the study of clusters (Affolabi et al., 2009; Moström et al., 2002).

Data analysis and comparison with international databases, particularly the SITVIT2 and SpolDB3

databases identified three major spoligotypes: ST53, ST1 and ST61, corresponding to the T superfamily, the Beijing family and the Cameroon family respectively.

The T superfamily remains poorly defined. It is ubiquitous (Brudey et al., 2006), but more frequent in Europe than elsewhere (Bezanahary et al., 2008).

The strains of this family are the most widespread worldwide (Arora et al., 2014). Furthermore, their rapid propagation, for multiple reasons (demographic changes, globalization), in certain contexts, suggests that the members of this family are intrinsically virulent (Bezanahary et al., 2008; Affolabi et al., 2009). The presence of strains from this family in Senegal may be linked, as previously suggested (Bezanahary et al., 2008), to immigrants originating from the Far East. The percentage of Beijing strains identified in this study (14.1%) is greater than that found in Benin (10.3%) (Affolabi et al., 2009).

The third spoligotype in this study is ST61. ST61 has been described as prevalent in the coastal countries of West Africa (Affolabi et al., 2009), but accounted for only 4.5% of the MDR isolates. It does not therefore seem to be very frequent in Senegal. This spoligotype also accounts for the majority of strains in Burkina Faso and Cameroon, where it has been referred to as the "Cameroon family" (Godreuil et al., 2007).

Strains belonging to the Latin American and Mediterranean (LAM) family (ST 42) was also found. Like ST53 (T superfamily), ST42 is ubiquitous, but more frequent in Europe than elsewhere (Brudey et al., 2006; Bezanahary et al., 2008). Its presence in Africa reflects the impact of colonization and past migration (Brudey et al., 2006).

Two spoligotypes rarely found in West Africa was detected: ST765 (1.56%) and ST26 (4.69%) corresponding to the T5-RUS1 and Central Asia (CAS1-Delhi) types. Type T5-RUS1 (ST765), previously known as "non-LAM families (T1 or T5-RUS)" and recently reclassified as belonging to the LAM family originated in the European part of Russia (Mokrousov et al., 2014). The Central Asia type (CAS1-Delhi) may be geographically linked to North India or Pakistan, or to other countries or regions, such as Sudan, Libya or East Africa (Bezanahary et al., 2008).

Resistance profile of mycobacterial strains

Detection of resistance by proportions methods

For a long period, the proportions method remained the only method available for the DST. But it is fastidious and requires a high level of biosecurity (Canetti et al., 1963). Overall, the high resistance rates that were obtained by the proportion method are related to the profile of the selected patients who are in treatment failure or relapse, therefore strongly suspected to have infection with resistant strains.

The overall rate of MDR strains that found (43.7%) is comparable to that found in the Central African Republic (40% of MDR strains in patients with relapse or treatment failure) (Minime et al., 2010) It is, however, below that found in Burkina Faso (50.5%) (Sangare et al., 2010).

This percentage of MDR strains was higher in patients with treatment failure with a rate of 75% versus 23.2% in patients with relapse. A previous study on Senegal reported 1% of MDR-TB among new patients and 11% in patients treated (Chevalier et al., 2010). The WHO report on Senegal for 2016 reported 0.9% of MDR-TB among new patients and 17% in retreatment patients (WHO, 2017). Our numbers are logically higher than those of the WHO report (WHO, 2017) linked to the profile of our patients who are in treatment failure or relapse, therefore strongly suspected of anti-tuberculosis drug resistance.

The analysis of the literature shows that the best factor associated with the rate of MDR-TB for a country is the failure rate to retreatment while the incidence of tuberculosis or co-infection with HIV in Africa do not appear to be significantly associated with multidrug resistance (Abdelhadi et al., 2012).

Detection of resistance by molecular methods

This study used two molecular techniques recommended by the WHO (WHO, 2011; FIND, 2015) and suitable for use directly on samples and in laboratories in the field, because they do not require the extensive biosafety precautions needed for culture of the tuberculosis bacillus.

In Senegal, the national resistance screening algorithm in use since 2014 involves the use of the Xpert MTB/RIF as the first-line diagnostic tool. Consequently, the NAP has equipped all the regions with Xpert MTB/RIF machines, to facilitate the detection of MDR strains (NAP, 2013). Three INH-R/RIF-S isolates were identified, consistent with the suggestion that RIF is often the last antituberculous drug to be affected by resistance. Nevertheless, two isolates (4.2%) resistant to RIF but susceptible to INH (RIF-R/INH-S) were found. Other studies have reported the occurrence of such isolates, including that of Kurbatova et al. (2012).

A comparison of the results with published findings (Kurbatova et al., 2012) suggested that testing for resistance to RIF may not necessarily be the best approach to the diagnosis of probable MDR tuberculosis, with implications for the use of tests identifying only DNA mutations associated with RIF resistance (Kurbatova et al., 2012).

Indeed, RIF and INH do not act on *M. tuberculosis* in the same way. INH has powerful bactericidal activity against *M. tuberculosis*. This prodrug is activated by the KatG enzyme of *M. tuberculosis*, a catalase-peroxidase (Brossier, 2011). INH inhibits the synthesis of the

mycobacterial cell wall, leading to cell death. About 80% of the strains resistant to INH carry point mutations or partial or complete deletions of the *katG* gene. Resistance to RIF is conferred by mutations of the *rpoB* gene (Kurbatova et al., 2012; Brossier, 2011).

Spoligotypes and resistance profile

Most of the trains were MDR strains (43 MDR strains). Two major spoligotypes were found: ST53 and ST1, corresponding to the T superfamily and the Beijing family, respectively. In Guadeloupe, if all strains (MDR and non-MDR) are considered together, the T superfamily is also the major family identified (Brudey et al., 2006). By contrast, in the French territories in the Americas (Guadeloupe, Martinique and French Guiana), the X and LAM lineages predominate among resistant and MDR isolates, accounting for 10.5 and 42.3% of MDR isolates, respectively (Millet et al., 2014).

The second major family of MDR strains identified in this study was the Beijing family. The strains of this family, the most widespread family worldwide (Arora et al., 2014), are known to be associated with multidrug resistance to antituberculous drugs (European Concerted Action, 2006). The third most frequent spoligotype among our multiresistant isolates was ST42, which belongs to the Latin American and Mediterranean (LAM) family.

The strains belonging to the LAM family are often associated with resistance. Indeed, in the French territories in the Americas (Guadeloupe, Martinique and French Guiana), the LAM lineage is overrepresented among MDR strains with respect to strains with other resistance profiles and susceptible strains (Millet et al., 2014).

ST61 accounted only for only 4.5% of MDR isolates. It does not therefore seem to be strongly linked to multidrug resistance to antituberculous drugs in Senegal.

A large number of MDR isolates in this study were grouped into clusters (40/64 or 62.5%). In total, nine clusters were identified, the two principal clusters being ST53 (n=13) and ST1 (n=9). The clustering of isolates in a given population is thought to reflect recent transmission. The other cases, corresponding to strains with a distinctive genomic fingerprint, are thought to result from the reactivation of older infections (Alland et al., 1994; Small et al., 1994). However, the association of spoligotyping with a more discriminatory method, such as IS6110-RFLP and MIRU-VNTR, is recommended to improve the study of clusters (Brudey et al., 2006, Affolabi et al., 2009).

Conclusion

This study provided a snapshot of the resistance profile and the spoligotypes of *M. tuberculosis* circulating in Senegal. Findings demonstrate that some strains may be resistant to RIF but susceptible to INH, resulting in their misclassification as MDR strains if testing for MDR strains is based exclusively on resistance to RIF. Two families accounted for most of the strains: T superfamily and Beijing followed by Cameroon and LAM families. Spoligotypes generally considered to be “European” or “Asian” were found among the MDR isolates: T5-RUS1 and CAS1-Delhi. A large study covering the entire country would provide us with a clearer idea of the molecular profiles of the *M. tuberculosis* strains circulating in Senegal.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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