First Insight into a Nationwide Genotypic Diversity of *Mycobacterium tuberculosis* among Previously Treated Pulmonary Tuberculosis Cases in Benin, West Africa

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Background. Molecular studies on tuberculosis (TB) are rare in low-resource countries like Benin, where data on molecular study on previously treated TB cases is unavailable. Materials and Methods. From January to December 2014, all smear- and culture-positive previously treated pulmonary TB patients from all TB clinics were systematically recruited. Drug susceptibility testing and spoligotyping were performed on all isolates. Results. Of the 100 patients recruited, 71 (71.0%) were relapse cases and 24 (24.0%) were failure cases, while 5 (5.0%) were default cases. Resistance rate to any first-line drug was 40.0%, while 12.0% of strains were multidrug-resistant (MDR) and no strain was extensively drug-resistant (XDR). A total of 40 distinct spoligotypes were found to correspond to a genotypic diversity of 40.0%. ST61 was the most predominant spoligotype with prevalence of 33.0%. In all, 31 single spoligotypes and nine clusters were observed with 2 to 33 strains per cluster giving a clustering rate of 69.0%. Euro-American (Lineage 4) was the most prevalent lineage (74.0%) and Lineage 2 was associated with resistance to streptomycin. Conclusion. This first insight into genetic diversity of previously treated pulmonary TB patients in Benin showed a relatively high genetic diversity of *Mycobacterium tuberculosis*. 

1. Introduction

Tuberculosis (TB) remains a global public health problem. According to World Health Organization (WHO), an estimated number of 10.4 million new cases occurred in the world in 2015 [1]. The African Region recorded the highest incidence rate, almost twice that of the world [1]. In Benin in West Africa, 4,092 cases were detected in 2015 [2].

Despite use of standardized treatment regimens and a well-established National TB Program (NTP) in the country, the treatment success rate as well as the number of previously treated cases (failure, relapse, and default) has remained stable over years [2]. In contrast to new cases, previously treated cases are much more likely to harbour multidrug-resistant (MDR) strains, defined as resistance to rifampicin (R) and isoniazid (H), and their characteristics may differ from those of new cases [3, 4].

Molecular tools are useful for better understanding of TB transmission dynamics in a given area. Nevertheless, molecular studies on TB are scarce in high-incidence, low-income countries [5]. In Benin, the only molecular epidemiologic study available to our knowledge recruited only TB new cases in one city [6, 7]. The scarcity of these studies in TB endemic countries is partly due to lack of resources and relative complexity of some molecular techniques. Among them, spoligotyping has the advantage of being relatively simple, inexpensive, and generally sufficient as a first approach of molecular epidemiology of TB [8].

In this study, we aimed to evaluate a nationwide genotypic diversity of *Mycobacterium tuberculosis* complex strains in...
previously treated pulmonary TB patients in Benin, using spoligotyping technique.

2. Materials and Methods

2.1. Setting. Benin is a country with a size of 114,763 square kilometres and an estimated population of 11 million. It has 70 TB facilities spread all over the country and a well-established National TB Program. Every year, about 4,000 TB cases including new and previously treated cases are detected in the country [2].

2.2. Specimens. A total of 100 isolates obtained from 100 sputum samples collected from smear-positive previously treated pulmonary TB patients all over the country were sent to the National Reference Laboratory (NRL) in Cotonou for processing. Previously treated TB patients were from relapse \((n = 71)\), failure \((n = 24)\), and default \((n = 5)\) cases. Two sputum samples were collected (spot and early morning) from each patient, stored at 4°C, and sent in a cool box to the NRL within a week. Upon arrival at the NRL, the two samples were processed for culture but only one strain per patient was used for drug susceptibility testing (DST) and DNA fingerprinting. Samples were systematically collected between January and December 2014, and, for each of them, demographic data was retrieved, while after obtaining consent from each patient, HIV screening was performed on blood using rapid immunochromatography-based tests: Alere Determine HIV-1/2® (Alere Medical, Japan) was used for HIV screening, while samples that were reactive were confirmed by ImmunoComb HIV 1&2 BiSpot® (Origenes, France).

2.3. Culture and DST. Samples were decontaminated using the Petroff method and cultured on Löwenstein-Jensen (LJ) media [9]. The \(M.\) \(tuberculosis\) isolates (one per patient) were tested for susceptibility against rifampicin \((R)\), isoniazid \((H)\), streptomycin \((S)\), and ethambutol \((E)\) using the proportion method on LJ medium at the following concentrations: 40 \(\mu g/mL\), 0.2 \(\mu g/mL\), 4 \(\mu g/mL\), and 2 \(\mu g/mL\), respectively [9]. Internal quality control was routinely performed, while annual external quality assurance was carried out by the WHO Supranational Reference Laboratory at the Institute of Tropical Medicine, Antwerp, Belgium. In case of resistance to \(R\), DST for second-line drugs was performed using the proportion method on LJ medium at the following concentrations: kanamycin \((30 \mu g/mL)\), capreomycin \((40 \mu g/mL)\), amikacin \((40 \mu g/mL)\), and ofloxacin \((2 \mu g/mL)\) [10]. All strains were stored upon routine processing at −80°C and subcultured on LJ for spoligotyping.

2.4. Spoligotyping. DNA was extracted by making a suspension of bacteria with a loop of colonies into 300 \(\mu L\) of molecular grade water followed by heating at 100°C for 20 minutes. Spoligotyping was performed as previously described [11]. \(Mycobacterium\) \(tuberculosis\) H37Rv was used as a positive control, while molecular grade water served as negative control. Spoligotype patterns obtained were then translated into binary code with 1 and 0 for presence and absence of “spacer” and then entered on an Excel file. From these codes, lineages and families of strains were determined using TB lineage database http://tbinsight.cs.rpi.edu/run_tb_lineage.html [12] and the SPOTCLUST database http://tbinsight.cs.rpi.edu/run_spotclust.html [13], respectively. Spoligotype data were compared to the SITVIT WEB database (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/) [14] to determine the Spoligotype International Type (SIT) if already described.

2.5. Data Analysis. Data were analyzed using EpiData 3.1. Chi-square test and Fisher’s exact test were used to compare proportions. \(p\) value < 0.05 was considered significant.

3. Results

In total, 100 viable strains (one single strain per patient) were used for spoligotyping. They were 71, 24, and 5 isolates from relapse, failure, and default patients, respectively. In total, 74 (74.0%) isolates were from male patients, while 26 (26.0%) were from females. HIV positivity rate was 15.2%, all of whom were infected with HIV1.

Resistance pattern to first-line drugs by type of previously treated cases is presented in Table 1. Resistance rate to any first-line drug was 40.0%, while 12.0% of strains were multidrug-resistant (MDR). In addition, two other strains were resistant to \(R\) but not to \(H\); one was monoresistant to \(R\) and another one was resistant to both \(R\) and \(S\). Thus, resistance rate to \(R\) was 14.0%. MDR rates were 20.8% and 9.9% for failure and relapse cases, respectively, while none was found among defaulters. Second-line DST results were available for nine MDR strains, of which six (66.7%) were susceptible, two (22.2%) showed resistance to ofloxacin, and one (11.1%) showed resistance to kanamycin, while none was resistant to both fluoroquinolones and injectable drugs. Thus, no strain was extensively drug-resistant (XDR) (Table 2).

A total of 40 distinct spoligotypes were found to be corresponding to a genotypic diversity of 40.0%. Of these, 21 (52.5%) corresponded to spoligotypes already identified in the SITVIT database and had shared-type (ST) denominations (SIT), while 19 (47.5%) were newly found spoligotypes. ST61, ST53, and ST1 were the most predominant spoligotypes with prevalence rates of 33.0%, 13.0%, and 8.0%, respectively. In this study, 31 single spoligotypes and nine clusters were observed with 2 to 33 strains per cluster, giving a rate of 69.0% (Table 3).

Most prevalent families were LAM 10, T1, and \(M.\) \(africanum\) West-African 1 with prevalence rates of 46.0%, 17.0%, and 12.0%, respectively. For lineages, the more prevalent lineages were Euro-American (Lineage 4), \(M.\) \(africanum\) West-African 1 (Lineage 5), and East-Asian (Lineage 2), with prevalence rates of 74.0%, 12.0%, and 8.0%, respectively. Interestingly, one strain was identified as \(M.\) \(bovis\), representing 1.0% of the total strains tested (Table 4).

By comparing characteristics of patients within lineages, we found no association between sex, HIV status, types of treatment, and lineages; however, drug resistance particularly resistance to \(S\) was associated with lineages distribution. Strains belonging to Lineage 2 were more likely to be resistant to \(S\) than the other strains \((p = 0.001)\) (Table 5).
Table 1: Resistance pattern of strains to first-line drugs.

| Type of resistance | Failure (n = 24) | Relapse (n = 71) | Default (n = 5) | Total (n = 100) |
|--------------------|-----------------|-----------------|----------------|-----------------|
|                    | n (%)           | n (%)           | n (%)          | n (%)           |
| Susceptible to all drugs | 12 (50.0) | 45 (63.4) | 3 (60.0) | 60 (60.0) |
| Monoresistance | | | | |
| H | 1 (4.2) | 1 (1.4) | 0 (0.0) | 2 (2.0) |
| S | 3 (12.5) | 10 (14.1) | 1 (20.0) | 14 (14.0) |
| R | 0 (0.0) | 0 (0.0) | 1 (20.0) | 1 (1.0) |
| E | 1 (4.2) | 1 (1.4) | 0 (0.0) | 2 (2.0) |
| Total | 5 (20.8) | 12 (16.9) | 2 (40.0) | 19 (19.0) |
| Multidrug resistance | | | | |
| HR | 0 (0.0) | 2 (2.8) | 0 (0.0) | 2 (2.0) |
| HRE | 1 (4.2) | 1 (1.4) | 0 (0.0) | 2 (2.0) |
| HRS | 1 (4.2) | 1 (1.4) | 0 (0.0) | 2 (2.0) |
| HRES | 3 (12.5) | 3 (4.2) | 0 (0.0) | 6 (6.0) |
| Total | 5 (20.8) | 7 (9.9) | 0 (0.0) | 12 (12.0) |
| Other patterns | | | | |
| HS | 1 (4.2) | 1 (1.4) | 0 (0.0) | 2 (2.0) |
| HSE | 0 (0.0) | 2 (2.8) | 0 (0.0) | 2 (2.0) |
| RS | 0 (0.0) | 1 (1.4) | 0 (0.0) | 1 (1.0) |
| ES | 1 (4.2) | 3 (4.2) | 0 (0.0) | 4 (4.0) |
| Total | 2 (8.3) | 7 (9.9) | 0 (0.0) | 9 (9.0) |

H: isoniazid; E: ethambutol; S: streptomycin; R: rifampicin.

Table 2: Resistance patterns to second-line drugs on MDR strains.

| Type of resistance | MDR strains (n = 9) |
|--------------------|---------------------|
|                    | n (%)               |
| Susceptible to all second-line drugs | 6 (66.7) |
| Monoresistance | | |
| Ofloxacin | 2 (22.2) |
| Kanamycin | 1 (11.1) |
| Capreomycin | 0 |
| Amikacin | 0 |
| Total | 3 (33.3) |
| XDR | 0 |

XDR: extensively drug-resistant.

4. Discussion

There are still several gaps in understanding TB dynamics in Africa. For example, the reason why *M. africanum* is mainly restricted to the Western and Central parts of the continent remains unclear [5, 7]. Studies using molecular tools may be useful in this respect. Unfortunately, the few molecular studies available either were limited to a city or a region or only focused on new TB cases and if previously treated cases were included, the number was usually low [15, 16].

In this study, we carried out a nationwide molecular study on previously treated pulmonary TB cases detected in Benin over a period of one year. In total, 40 different spoligotypes were found, corresponding to a genotypic diversity of 40.0%. This percentage was higher than the 19.1% found by Ouassa et al. in previously treated cases in Côte d’Ivoire but was quite similar to 35.1% obtained on the genetic diversity in a mixed population of new and previously treated cases in Rwanda [15, 16]. A genotypic diversity of 49.0% was reported in 2005 among new cases in Cotonou, the biggest city in Benin, suggesting that genetic diversities were similar among new and previously treated cases [6]. However, the previous study among new cases was carried out 10 years ago and distribution of spoligotypes in new cases might have changed over time. In addition, the national figure might be different from what was obtained in Cotonou.

This study showed that the most frequent spoligotype was ST61 (33%) belonging to the Latino-American and Mediterranean (LAM) family. This finding was similar to what was previously reported in the same country in 2005, indicating that ST61 was the most prevalent spoligotype in new cases [6]. This same genotype was previously described to be prevalent in countries within the West-African coast [17].

At a lineage level, Lineage 4 was the most prevalent lineage (74.0%). High prevalence of Lineage 4 was also found in both new and previously treated cases at a similar rate in Ethiopia (72.4%) and in Guinea (78.8%) [18, 19]. In comparison with other lineages, Lineage 4 appears to have certain characteristics that promote its rapid expansion. For *M. bovis*, the prevalence rate (1.0%) is similar to those found elsewhere in a mixed population of new and previously
| Family          | Spoligotype | ST | Strains n (%) |
|-----------------|-------------|----|---------------|
| Family33        | 761777767775771 | U  | 1 (1.0%)      |
|                 | 777777777637771 | U  | 1 (1.0%)      |
| Family34        | 7777777770000000 | 46 | 1 (1.0%)      |
| Beijing         | 000000000003771 | 1  | 8 (8.0%)      |
| CAS             | 7037774000171 | 1199 | 1 (1.0%)    |
| LAM1            | 677777607760771 | 20 | 1 (1.0%)      |
| LAM9            | 377777607760771 | 177 | 1 (1.0%)    |
| LAM10           | 777777437607771 | 61 | 33 (33.0%)   |
|                 | 767740741760771 | U  | 1 (1.0%)      |
|                 | 777770343760771 | U  | 1 (1.0%)      |
|                 | 776777437607771 | U  | 3 (3.0%)      |
|                 | 777770343740771 | U  | 2 (2.0%)      |
|                 | 777777434607771 | 772 | 3 (3.0%)    |
|                 | 777777427607771 | U  | 1 (1.0%)      |
|                 | 777777437607731 | 403 | 1 (1.0%)   |
|                 | 777777437407771 | U  | 1 (1.0%)      |
| T1              | 777777777607771 | 53 | 13 (13.0%)   |
|                 | 77777777760731 | 51 | 1 (1.0%)      |
|                 | 73777777760731 | 848 | 1 (1.0%)    |
|                 | 777777757607771 | 44 | 1 (1.0%)      |
|                 | 73777777760531 | U  | 1 (1.0%)      |
| T2              | 777417707000000 | U  | 1 (1.0%)      |
| T4              | 777740017760771 | 159 | 1 (1.0%)    |
| Haarlem1 (H1)   | 777777777020731 | 316 | 1 (1.0%)   |
| Haarlem2 (H2)   | 000000000020731 | U  | 1 (1.0%)      |
| Haarlem3 (H3)   | 777777777720731 | 49 | 2 (2.0%)      |
|                 | 777777777720771 | 50 | 2 (2.0%)      |
| Family36        | 0000000007760771 | 4  | 1 (1.0%)      |
| M. africanum West-African 1 | 774077607777071 | 331 | 3 (3.0%)   |
|                 | 674077717777071 | U  | 1 (1.0%)      |
|                 | 774077400603031 | U  | 1 (1.0%)      |
|                 | 770002607777071 | U  | 1 (1.0%)      |
| M. africanum West-African 2 | 574077607777071 | 319 | 1 (1.0%)   |
|                 | 774077600000071 | U  | 1 (1.0%)      |
|                 | 374077607777031 | U  | 1 (1.0%)      |
|                 | 574077607777071 | U  | 1 (1.0%)      |
|                 | 7740400777707071 | U  | 1 (1.0%)    |
|                 | 774077777777071 | 438 | 1 (1.0%)   |
| M. bovis        | 700003777776771 | U  | 1 (1.0%)      |
|                 | 000040000200000 | U  | 1 (1.0%)      |

ST: shared-type; U: unknown.

A significant association was found between Lineage 2 (Beijing strains) and resistance to streptomycin ($p = 0.001$). This same association was observed in a study of new cases in 2005 in Cotonou, where an outbreak characterised by Information Geographical System was identified [23]. The treated cases in Ethiopia (1.2%), Nigeria (1.0%), and Mali (0.8%) [20–22]. These low proportions could be explained by the fact that *M. bovis* is usually involved in extrapulmonary TB in humans, whereas most of these studies, including the present one, were on pulmonary TB [20–22].
In conclusion, this first insight into the genetic diversity of TB in previously treated cases in Benin showed a genetic diversity of 40.0%, with most strains belonging to Lineage 4, similar to previous data in new TB cases. Occurrence of retreatment cases is more likely to be related to human and environmental factors rather than the intrinsic molecular characteristics of strains.

**Abbreviations**

DST: Drug susceptibility test  
E: Ethambutol  
H: Isoniazid  
L: Löwenstein-Jensen  
LAM: Latino-American and Mediterranean  
MDR: Multidrug-resistant  
NRL: National Reference Laboratory  
R: Rifampicin  
SIT: Spoligotype International Type  
S: Streptomycin  
ST: Shared-type  
TB: Tuberculosis  
WHO: World Health Organization  
XDR: Extensively drug-resistant.

**Conflicts of Interest**

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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