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Population structure and circulating genotypes of drug-sensitive and drug-resistant *Mycobacterium tuberculosis* clinical isolates in São Paulo state, Brazil

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Abstract

São Paulo is the most populous Brazilian state and reports the largest number of tuberculosis cases in the country annually (over 18,500). This study included 193 isolates obtained during the 2nd Nationwide Survey on *Mycobacterium tuberculosis* Drug Resistance that was conducted in São Paulo state and 547 isolates from a laboratory based study of drug resistance that were analyzed by the Mycobacteria Reference Laboratory at the Instituto Adolfo Lutz. Both studies were conducted from 2006 to 2008 and sought to determine the genetic diversity and pattern of drug resistance of *M. tuberculosis* isolates (MTC) circulating in São Paulo. The patterns obtained from the spoligotyping analysis demonstrated that 51/740 (6.9%) of the isolates corresponded to orphan patterns and that 689 (93.1%) of the isolates distributed into 144 shared types, including 119 that matched a preexisting shared type in the SITVIT2 database and 25 that were new isolates. A total of 77/144 patterns corresponded to unique isolates, while the remaining 67 corresponded to clustered patterns ($n = 612$ isolates clustered into groups of 2–84 isolates each). The evolutionarily ancient PGG1 lineages (Beijing, CAS1-DEL, EAI3-IND, and PINI2) were rarely detected in São Paulo and comprised only 13/740, or 1.76%, of the total isolates; all of the remaining 727/740, or 98.24%, of the MTC isolates from São Paulo state were from the recent PGG2/3 evolutionary isolates belonging to the LAM, T, S, X, and Haarlem lineages, i.e., the Euro-American group. This study provides the first overview of circulating genotypes of *M. tuberculosis* in São Paulo state and demonstrates that the clustered shared types containing seven or more *M. tuberculosis* isolates that are spread in São Paulo state included both resistant and susceptible isolates.

Keywords

Mycobacterium tuberculosis; Population-structure; Spoligotyping; Brazil; Database

1. Introduction

Tuberculosis (TB) continues to be a major cause of disease and death and is a significant economic burden in the Americas. According to the World Health Organization's (WHO) report on global TB control from 2009, Brazil had an estimated 92,102 new cases of TB in 2007 and an estimated incidence of 48/100,000 in the population. These cases accounted for 31% of all TB cases in the WHO's Latin American Region (http://www.usaid.gov/our_work/global_health/id/tuberculosis/countries/lac/brazil_profile.html). São Paulo state had over 18,500 cases and a reported incidence of 40.5/100,000 in 2010 (CVE-São Paulo, 2011). Although the incidence of TB in São Paulo state is close to the average for Brazil (Bombarda and Galesi, 2006; WHO, 2011), it nonetheless requires special attention because of the high number of patients; the increased number of multidrug-resistant (MDR) tubercle bacilli cases, which are defined by simultaneous drug resistance to isoniazid (I) and rifampin (R) (i.e., $n = 1056$ cases in 2007); and the relatively high prevalence of HIV (12%) in patients with TB in Brazil (http://www.stoptb.org/assets/images/about/tbl_burden.gif). As of October 2008, extensively drug-resistant TB (XDR-TB, which is defined as MDR-TB, i.e., resistance to I and R and simultaneous resistance to any of the fluoroquinolones and at least one of the three injectable second-line drugs) had also been found in Brazil (Araújo-Filho et al., 2008).

Global and local surveillance of *Mycobacterium tuberculosis* drug resistance is beneficial to TB control programs because information regarding resistance is important for effective control of new TB transmissions. The first World Survey covering the period from 1995 to 1996 showed that MDR-TB represented 1.3% of total TB cases detected in Brazil (WHO, 1997). The 2nd Nationwide Survey on *M. tuberculosis* Drug Resistance, which covered the period from 2006 to 2008, found that MDR-TB accounted an estimated 0.90% of all newly notified TB cases (ranging from 0.60% to 1.4%; WHO, 2011). Similar observations in the last decade have highlighted the global increase in drug-resistant tubercle bacilli, thereby facilitating and promoting the use of molecular markers to study TB epidemiology. The global TB control objectives were recently redefined to add an active research component to routine TB diagnostics and a treatment approach because a better understanding of the disease and the bacterium that causes it may facilitate the design of new approaches to control TB (<http://www.stoptb.org/global/plan>).

Molecular typing of *M. tuberculosis* greatly assists our understanding of the epidemiology and diversity of this pathogen. Restriction fragment length polymorphism analysis using IS6110 (IS6110-RFLP), PCR-based methods, such as mycobacterial interspersed repetitive units-variable-number tandem repeats (MIRU-VNTR) and Spacer Oligonucleotide Typing (spoligotyping) are considered standard methodologies for elucidating the dissemination of *M. tuberculosis*. The spoligotyping which is an easy method has replaced the cumbersome IS6110-RFLP method because it allows simple determination of the circulating clades (genotypic families, lineages) of tubercle bacilli in a specific region versus their worldwide circulation (van Embden et al., 1993; Brudey et al., 2006).

Among these newer approaches, a fine study of the macropopulation structure of *M. tuberculosis* clinical isolates is now possible thanks to new innovations that were developed to study TB molecular epidemiology (reviewed in García de Viedma et al., 2011) and the existence of huge international *M. tuberculosis* genotyping databases, such as the SITVIT2 proprietary database at the Pasteur Institute of Guadeloupe, which was recently used in the first nationwide study to describe the spoligotyping-based genetic diversity of *M. tuberculosis* complex clinical isolates in Brazil (Gomes et al., 2011). Although the emerging drug-resistant strains of TB may further exacerbate the already complex TB situation in Brazil (Bombarda and Galesi, 2006; Dalcolmo et al., 2007; WHO, 1997), this preliminary

study from Gomes et al. (2011) did not explore the distribution of drug resistance patterns in relation to genotypic lineages. Because a correlation between genotype, the resistance data, and the respective prevalence of clones region by region may provide more insight into the global circulation of complex *M. tuberculosis* genotypic clones (Borsuk et al., 2005; Ritacco et al., 2011), we decided to analyze by spoligotyping the population structure of *M. tuberculosis* clinical isolates and the existence of resistance-associated genotypes. In our opinion, this is an important step in understanding TB and in the implementation of the appropriate control measures in São Paulo, Brazil.

2. Material and methods

2.1. Patients and bacterial isolates

This study included a total of 740 *M. tuberculosis* complex isolates; 193 isolates were obtained during the 2nd Nationwide Survey on *M. tuberculosis* Drug Resistance that was conducted in São Paulo state between 2006 and 2008 and 547 isolates were obtained from a laboratory based study of drug resistance analyzed by the Mycobacteria Reference Laboratory at Institute Adolfo Lutz during the same time period.

2.2. Drug susceptibility testing

The BD BACTEC-Mycobacteria Growth Indicator Tube MGIT960 system (MGIT 960)TM was used to establish the susceptibility patterns to I (0.1 µg/ml), R (1.0 µg/ml), ethambutol – E (5.0 µg/ml) and streptomycin – S (1.0 µg/ml). For each isolate, 0.5 ml of bacterial inoculum was added into four MGIT 960 tubes containing the drugs and one growth control MGIT 960 tube without drug. All of the inoculated tubes were placed into the fluorometric BACTEC MGIT 960 system and the instrument reported the susceptibility results automatically. (Giampaglia et al., 2007) As previously reported, the susceptibility to pyrazinamide (P) was determined using a pyrazinamidase test with a medium containing pyrazinamide 0.100 g/L. After de bacterial growth in the medium, 1 ml of 1% freshly prepared ferrous ammonium sulfate was added in the tube. A reading was made after 15 min, a pink color indicate pyrazinamidase positive test (OPAS–OMS, 1986).

2.3. Spoligotyping and database analysis

Spoligotyping was performed using a previously published method that is based on DR locus polymorphisms (Kamerbeek et al., 1997). Briefly, the entire DR locus in the *M. tuberculosis* genome was amplified with the Dra and Drb primers, and the Dra primer was biotinylated at the 5' end. The amplified products were hybridized to a set of 43 oligonucleotides that were covalently bound to a nylon membrane. The hybridized DNA was detected using chemiluminescence, the presence of a spacer was visualized on X-ray film, and spoligotypes were recorded in a binary format as black (positive hybridization) or empty (negative hybridization) squares. For database analysis, spoligotypes were converted to octal codes and entered into the SITVIT2 proprietary database at the Pasteur Institute of Guadeloupe, which is an updated version of the previously released SpolDB4 database (Brudey et al., 2006). During the present study, SITVIT2 database contained genotype information on approximately 87,000 *M. tuberculosis* clinical isolates from 160 countries. In this database, SIT (Spoligotype International Type) designates spoligotypes that are shared by two or more patient isolates as opposed to “orphan” isolates, which are single isolates. The major phylogenetic clades were assigned according to the signatures provided in the SpolDB4 database, which defined 62 genetic lineages/sub-lineages; these include specific signatures for various *M. tuberculosis* complex (MTC) species such as *Mycobacterium bovis*, *Mycobacterium microti*, *Mycobacterium caprae*, *Mycobacterium pinipedii* and *Mycobacterium africanum* and rules that define the major lineages/sublineages for *M. tuberculosis* sensu stricto. The latter include the Beijing clade, the Central Asian (CAS)

clade and two sublineages, the East African–Indian (EAI) clade and nine sublineages, the Haarlem (H) clade and three sublineages, the Latin American–Mediterranean (LAM) clade and 12 sublineages, the “Manu” family and three sublineages, the S clade, the IS6110-low-banding X clade and four sublineages and an ill-defined T clade (defined by default) and five sublineages.

2.4. Distinction between ancestral, ancient, and modern lineages

The lineages were tentatively linked to evolutionary “ancient” and “recent” lineages of tubercle bacilli as defined by the Principal Genetic groups (PGG), which are based on *KatG463–gyrA95* polymorphisms (Sreevatsan et al., 1997) that were inferred from the reported linking of specific spoligotype patterns to PGG1, -2 or -3 (Soini et al., 2000; Rastogi and Sola, 2007). Furthermore, based on the previously reported presence or absence of an *M. tuberculosis* specific deletion 1 (TbD1, a 2-kb sequence), the lineages were classified as “ancestral” (TbD1+) or “modern” TbD1-negative (TbD1-) MTC isolates. This classification superimposes well with the previous PGG classification (Brosch et al., 2002). As reviewed recently, all MTC species other than *M. tuberculosis* sensu stricto and the EAI lineage are TbD1+; hence, only two groups of MTC are considered to be the classical human pathogens. The EAI and the *M. africanum* lineages are considered ancestral (Rastogi and Sola, 2007). Thus, the MTC isolates that were used in this study were tentatively identified as TbD1+/PGG1 (ancestral: *M. africanum* and the EAI lineage of *M. tuberculosis*), TbD1-/PGG1 (ancient: Beijing, CAS), and TbD1-/PGG 2–3 (modern/evolutionary recent: LAM, T, S, X, Haarlem). Last but not least, as described recently (Gagneux et al., 2006; Gagneux and Small, 2007), the latter subgroup (TbD1-/PGG 2–3) was also found to superimpose perfectly with the “Euro-American” phylogenetic group based on large sequence polymorphisms (LSP). These terms are used throughout this paper to denote the lineages observed in the present study.

2.5. Quality control

M. tuberculosis H37Rv (ATCC 27294) and *M. bovis*–BCG (obtained from the culture collection of the Mycobacteria Reference Laboratory at the Institute Adolfo Lutz, São Paulo state, Brazil) were included as quality controls for the phenotypic and genotypic tests.

3. Results

This study included 740 isolates, 483 (65.3%) of which were obtained from the Metropolitan São Paulo area, 125 (16.9%) of which were obtained from 64 interior towns, 58 (17.8%) were obtained from metropolitan Baixada Santista area and 74 (10.0%) of which were obtained from the prisons in São Paulo state. In addition to a high proportion of resistant isolates from the laboratory-based studies (547–73.9%), we also analyzed 193 (26.1%) isolates from the 2nd Nationwide Survey of Drug-Resistant TB in São Paulo.

Among the isolates from the laboratory based studies of resistance, 308 (56.3%) isolates were detected in the metropolitan São Paulo, 173 (31.6%) in the interior towns and 66 (12.1%) were detected in the prisons of São Paulo state. The majority of the isolates obtained during the 2nd Nationwide Survey of Drug Resistance were from the metropolitan São Paulo area [175 (90.7%)], and the remaining isolates were from interior towns [10 (5.2%)] and the prisons in São Paulo state [8 (4.1%)].

When the patterns obtained from the spoligotyping analysis of these isolates were compared with those described in the SITVIT2 database, a total of 51/740 (6.9%) of the isolates corresponded to orphan patterns (Supplemental Table S1). Lineage interpretations for these orphan patterns were performed manually by expert-based interpretations using revised SpoIDB4 rules; this method designated the lineages of these orphan isolates as follows:

LAM $n = 21$, T $n = 14$ (including 1 isolate from the T5-Madrid2 sublineage), Haarlem (H) $n = 4$, S family $n = 1$, EAI (EAI5 sublineage) $n = 1$, and unknown signatures, $n = 10$ (Supplemental Table S1). The remaining 689/740 (93.1%) of the isolates were distributed into the 144 shared types including 119 patterns that matched a preexisting shared type in the SITVIT2 database and 25 that were newly created (Supplemental Table S2); 77/144 of the patterns corresponded to unique isolates, while the remaining 67 patterns corresponded to clustered patterns ($n = 612$ isolates clustered into groups of 2–84 isolates each). Thus, the total level of clustering corresponded to 612/740 strains, or 82.7% of all isolates. The analysis of these isolates by shared types/sublineages showed that only four sublineages contained >5% of the isolates [SIT50/H3 ($n = 84$, 11.35%), SIT 53/T1 ($n = 62$, 8.38%), SIT 42/LAM9 ($n = 48$, 6.49%), and SIT17/LAM2 ($n = 46$, 6.22%)].

When the isolates were analyzed by lineage (including the orphans), the following distribution pattern was observed for the 740 isolates: Beijing ($n = 7$, 0.95%), CAS1-DEL ($n = 1$, 0.13%), EAI3-IND ($n = 4$, 0.54%), Haarlem ($n = 153$, 20.68%), LAM ($n = 331$, 44.73%), PINI2 ($n = 1$, 0.13%), S ($n = 11$, 1.49%), T ($n = 148$, 20%), X ($n = 24$, 3.24%), and unknown ($n = 60$, 8.11%). The evolutionarily ancient PGG1 lineages (Beijing, CAS1-DEL, EAI3-IND, and PINI2) were rarely detected and made up only 13/740, or 1.76%, of the isolates; all of the remaining 727/740, or 98.24%, of the MTC isolates in São Paulo state belonged to the recent evolutionary PGG2/3 isolate lineages LAM, T, S, X, and Haarlem, i.e., the Euro-American group (see Supplemental Table S3 for details).

The most predominant lineages and shared types in our study were the following: H1/SIT47 $n = 24$, H3/SIT50 $n = 84$, LAM1/ SIT20 $n = 20$, LAM2/SIT17 $n = 46$, LAM3/SIT33 $n = 36$, LAM4/SIT60 $n = 23$, LAM6/SIT64 $n = 31$, LAM9/SIT42 $n = 48$, T1/SIT53 $n = 62$, T1/ SIT51 $n = 21$, and X2/SIT317 $n = 13$. Thus, very high spoligotype diversity was documented for the LAM lineage. Out of the 12 sublineages that have been reported worldwide for the LAM family (Brudey et al., 2006), a total of eight sublineages were observed in our recruitment sample. Similarly, all of the T sublineages that have been reported were present in our study sample.

Lastly, the 25 newly created SITs that matched another orphan patterns in the database or the SITs that were composed of two or more strains belonging to a new pattern within our study were as follows (Supplemental Table S2): SITs 3113, 3114, 3116, 3117, and 3119–3123 contain two or more isolates in the present study, as opposed to SITs 3112, 3115, 3144, 3147, and 3150, which contain 1–3 isolates in the present study and matched an orphan pattern from some region in Brazil. The remaining shared types (SITs 3124–3128, 3142, 3143, 3145, 3146, 3148, and 3149) contained 1–2 isolates in the present study and matched an orphan pattern from another country (see Supplemental Table S2 for details).

Table 1 presents the description of clusters containing seven or more isolates, 15 SITs (453 isolates – 61.2%), and their worldwide distribution in the SITVIT2 database. The distribution (3%) of these SITs by countries showed that 14 of them had already been described in Brazil, with the exception of SIT 1-Beijing. The majority (53.0%) of clustered isolates belonged to four SITs: 50-H3, 53-T1, 42-LAM9 and 17-LAM2.

Table 2 shows that the drug susceptibility pattern of clustered spoligotypes correlated with gender and age. The mean age of the patients ranged from 31.0 years (SIT 4-unknown) to 46.0 years (SIT 137-X2). The male/female sex ratio observed ranged from 1.2 (SIT 1905-T1) to 12.0 (SIT 137-X2).

The drug susceptibility pattern of clustered isolates showed 349 (77.1%) drug-resistant isolates that contained a significant percentage of MDR (31.1%) and mono-resistant isolates

(40.4%). In the following five SITs, MDR isolates made up more than 40.0% of the SIT: SIT 1-Beijing, SIT 4-unknown, SIT 60-LAM 4, SIT 177-LAM 9, and SIT 137-X2.

SIT 51-T1 had the lowest percentage of MDR isolates (4.8%) but the highest percentage of mono-resistant isolates (71.4%), primarily with resistance to S. Over 45.0% of the isolates in the following three SITs were mono-resistant: SIT 1905-T1 63.6% (only I), SIT 53-T1 51.6% (I = 16.1%, S = 30.6%), and SIT 47-H1 45.8% (I = 25.0%, S = 8.3%, R = 8.3%).

The 25 newly created SITs included 46 (6.2%) isolates. Twelve (26.1%) of the isolates were susceptible to all drugs tested, 9 (19.6%) of the isolates were MDR, 20 (43.5%) of the isolates were mono-resistant (10 to S, 7 to I, 2 to R, 1 to E) and 5 (10.9%) of the isolates were resistant to other drugs. Among the 13 (52.0%) new SITs (presenting 2–5 isolates), the designated lineages were LAM-12 isolates, T and S – 4 isolates each, H3 – 2 isolates and unknown – 12 isolates. The resistance profile contained the following six MDR isolates: SIT 3113-H3 (1), SIT 3114-LAM 4 (1), SIT 3119-LAM2 (2), SIT 3122-S (1), and SIT 3124-LAM3 (1).

The 51 orphan isolates contained 10 (19.6%) isolates that were susceptible to all drugs tested, 8 (15.7%) isolates that were MDR, 28 (54.9%) isolates that were mono-resistant (19 to I, 6 to S, 2 to R, and 1 to P) and 5 (9.8%) isolates were resistant to other drugs.

The analysis of 547 isolates from laboratory based study of resistance showed that 11 isolates showed no resistance to the drugs tested. The 536 remaining isolates showed 210 (39.2%) MDR isolates with the following profiles: IR – 94 (17.5%), IRS – 34 (6.3%), IRP – 26 (4.8%), IRE – 19 (3.5%), IRPS – 15 (2.8%), IRES – 8 (1.5%), IRPE – 8 (1.5%) and IRPES – 6 (1.1%). The 281 mono-resistant isolates presented I – 134 (47.7%), S – 109 (38.8%), R – 28 (9.9%), E and P – 5 (1.8% each). The other-resistances were composed by 45 (8.4%) isolates with the following profiles: double resistance IS – 25 (4.7%), IP – 6 (2.1%), IE – 5 (1.8%), RS – 2 (0.7%), RP – 1 (0.3%) and triple resistance IES – 4 (1.4%), IPS – 2 (0.7%).

Among resistant isolates, 321 (59.9%) were found in the clustered SITs and 134 (25.0%) of them were MDR isolates and showed the following profiles IR – 57 (42.5%), IRS – 26 (19.4), IRP – 21 (15.7%), IRPS – 11 (8.2%), IRPE – 6 (4.5%), IRPES – 5 (3.7%), IRE and IRES – 4 (3.0%) each.

Isolates from the 2nd Nationwide Survey of Drug-Resistant TB in São Paulo included 156 (80.8%) susceptible isolates and 37 (19.2%) isolates with various resistant profiles. Twenty-seven isolates (73.0%) were mono-resistant [I-14 (51.8%), S-8 (29.6), E-2 (7.4%), P-2 (7.4%), R-1 (3.7%)] eight (21.6%) were double resistant [IR-2, ES-1, and IS-5] and two (5.4%) isolates were triple resistant [IRP-1, and IES-1]. The SITs containing the three MDR isolates were 42, 91 (IR) and orphan (IRP).

4. Discussion

This study provides the first overview of circulating genotypes of drug-sensitive and drug-resistant *M. tuberculosis* in São Paulo state and shows the predominance of the LAM lineage, which accounts for 44.5% of the isolates. This percentage is similar to that described in South America (45.0%) by Ritacco et al. (2011) and to the average obtained by Gomes et al. (2011) from 11 states in Brazil (46.2%). However, it differs from that described by other studies in the same country. Mendes et al. (2011) reported a frequency of 36.1% of LAM among 93 isolates from the metropolitan São Paulo area, and Gomes et al. (2011), who provided the first insight into the population structure of TB isolates in Brazil, reported that the lowest percentage of the LAM lineage (35.0%) had been observed among 180

isolates from São Paulo state. Noguti et al. (2010) showed the lowest percentage (34.7%) of this lineage in Maringá (93 isolates), and Miranda et al. (2011) showed the highest percentage (55.3%) of this lineage in Minas Gerais (114 isolates).

In this study, the H and T lineages accounted for 21.5% and 18.7% of the 740 isolates, respectively, which is different from the rates described by Mendes et al. (2011), who studied isolates from the metropolitan São Paulo region and found that T accounted for 33.0% and H accounted for 15.3% of the isolates. However, our results are similar to those described by Noguti et al. (2010), who studied isolates from Maringá and found that H accounted for 22.5% of the isolates and T accounted for 15.3% of the isolates. Other studies also showed a higher percentage of the T lineage when compared to the H lineage. In particular, Miranda et al. (2011) described the lowest percentage of the H lineage (7.0%) in Minas Gerais.

Although SIT 1905-T1 accounted for a relatively low percentage (1.49%) of clustered isolates in our study, it showed a high phylogenetical specificity for São Paulo since 55.0% of all SIT1905 isolates in the SITVIT2 databases were from the present study; further countrywide distribution showed that 85.0% of such isolates were described from Brazil (Table 1).

As cited by Ritacco et al. (2011) the frequency of the Beijing genotype was found to be below 1.0% in most of the surveyed South American countries. This study showed a low percentage of this lineage in São Paulo state, which is similar to the results observed in previous studies from other states in Brazil. Nevertheless, Mendes et al., (2011) reported that the Beijing genotype comprised 5.2% of the lineages in the metropolitan São Paulo area.

A group of SITs found in Brazil at a very low frequency could represent imported genotypes due to the international migratory flux (Gomes et al., 2011). Some genotypes were observed at a very low frequency in our study, six had already been described in Brazil, including SIT 11 (EA13-IND), SIT 18 (X2), SIT 58 (T5-Madrid2), SIT 254 (T5-RUS1), SIT 863 (PIN12), and SIT 1983 (EA13-IND). However, three of the SITs were described for the first time in this study, including SIT 22 (CAS1-Delhi), SIT 39 (T4-CEU1), and SIT 209 (LAM 12-Madrid1).

The results from laboratory based study showed a higher percentage of some clusters in the metropolitan São Paulo area when compared to the interior towns: SIT 53 (56.8%), SIT 1 and 17 (60%), SIT 20 (64.7%), SITs 137, 177, 1905 (66.7%), and SIT 64 (71.4%) and SIT 60 (85.7%).

The incidence of TB in the metropolitan Baixada Santista area is twice the average of the incidence in the interior towns. Isolates from this area comprised 40.0% of the isolates in SIT 33 and 18.2% of the isolates in SIT 50. However, despite this, the laboratory-based study showed no isolates from SITs 1, 42, 177, and 1905.

We observe that SIT 137 included isolates detected primarily in males (92.3%) with mean age of 40 years, but detailed patient records were not available to conclude the association of clustering with gender.

On the other hand, the analysis of 193 isolates from the 2nd Nationwide Survey showed that 74 (47.4%) of the susceptible isolates were distributed among clusters composed of seven or more isolates. No resistant isolates were found in the clustered SIT 1, 60, 137 and 177. Eighteen (48.6%) resistant isolates were distributed in 10 SITs that were primarily mono-resistant to I (8 isolates) and S (5 isolates). Two isolates that were mono-resistant to I were detected in SIT 50, and two isolates resistant to S were detected in SIT 51. Only one MDR

isolate was included in the clustered SIT 42-IR. The MDR-TB rate of 1.5%, as reported by the WHO (2011) for Brazil, suggests low transmission of MDR strains among newly diagnosed patients. Among the three MDR isolates, two were from the interior towns of Santos (SIT 42) and São José dos Campos (orphan isolate) and one was from prison (SIT 91).

In a nutshell, a comparison of the spoligotyping patterns obtained in our study with those described in the SITVIT2 database as well as with similar data presented in other Brazilian studies highlights: (i) the predominance of LAM lineage, (ii) the high phylogenetical specificity of a minor clone for São Paulo area (SIT1905-T1; 1.49% of all isolates in our study which represented 55% of all SIT1905 isolates in the SITVIT2 database), (iv) overall similar percentages of resistant and susceptible isolates among clustered SITs, (v) a higher percentage of some clusters in the metropolitan São Paulo area when compared to the interior towns, e.g., the incidence of TB in Santos is twice the average of the incidence in the interior towns (with a predominance of SIT 137 in males, 92.3%; with a mean age of 40). Nonetheless, some limitations of our study need to be acknowledged when interpreting the results. The study was performed with *M. tuberculosis* isolates obtained from the 2nd Nationwide Survey of Drug Resistant TB in São Paulo and high proportion of resistant isolates from the laboratory-based studies which can cause a selection bias. In São Paulo the culture for mycobacteria and susceptibility testing is performed according to criteria defined by Program to Control Tuberculosis such as history of MDR-TB contact, previous treatment, homeless, prisoner, occupational healthy and of HIV patients. Despite these limitations, our results are consistent with the literature, and they allowed showing the population structure of *M. tuberculosis* clinical isolates of São Paulo.

An additional comment may be made regarding the choice of spoligotyping used alone for genotyping that may not be sufficient to make definite conclusions about the probable subdivision of the clusters containing seven or more *M. tuberculosis* isolates spread in São Paulo state (Table 2), including both resistant and susceptible isolates, as well as acquisition of drug-resistance during treatment. Indeed, the results obtained with the isolates from laboratory-based studies of resistance showed the diversity of *M. tuberculosis* genotypes causing resistant tuberculosis with high percentage of isolates with resistance to I, S and MDR distributed in the clustered SITs. Among the MDR isolates, 42.5% showed resistance only to IR and 57.5% showed resistance to one or more drugs in addition to IR, which suggests the acquisition of resistance during treatment. In this regard, it is worth mentioning that the isolates from the 2nd Nationwide Survey showed a similar percentage of resistant and susceptible isolates distributed in the clustered SITs. As 90% of these isolates were obtained from new cases (according to principles of patient recruitment in this global project); this result may indicate that both resistant and susceptible isolates are now circulating among newly infected patients. However, only future studies using MIRU-typing would allow to split some of the big spoligotyping defined clusters found in this study, and shed new light not only on genotypes with potential epidemiological links, but also to pinpoint the proportion of primary drug resistance in newly-infected TB patients versus secondary drug-resistance that is emerging due to a lack of observance during treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.meegid.2012.10.015>.

Description of clusters containing seven or more isolates in this study, and their worldwide distribution in the SIVTIT2 database (predominant shared-types are shown in decreasing order of their occurrence).

[illegible]

SIT (Lineage) octal number	spoligotype description ^a	Number (%) in study	% In study versus database	Distribution in regions with 3% of a given SIT ^b	Distribution in countries with 3% of a given SIT ^b
1905 (T1)	77777777460771	11 (1.49)	55	AMER-S 95.0, EURO-S 5.0	BRA 85.0, PER 5.0, ITA 5.0, ARG 5.0
177 (LAM9)	377777607760771	9 (1.22)	11.39	AMER-S 60.76, EURO-S 17.72, AFRI-N 6.33, ASIA-W 3.8, AMER-N 3.8	BRA 49.37, ESP 11.39, ITA 6.33, USA 3.8, SAU 3.8, PRY 3.8, MAR 3.8
1 (Beijing)	0000000000003771	7 (0.95)	0.11	AMER-N 30.23, ASIA-SE 13.68, AFRI-S 12.451, ASIA-E 11.01, ASIA-N 8.22, ASIA-S 5.13, ASIA-W 3.89, EURO-N 3.19	USA 29.79, ZAF 12.45, RUS 8.22, JPN 8.05, VNM 5.86, MYS 3.60

¹ST and lineage designations are shown following SITVT2 proprietary database of Institut Pasteur de la Guadeloupe. Worldwide distribution is reported for regions with more than 3% of a given SITs compared to their total number in the SITVT2 database. The definition of macro-geographical regions and sub-regions (<http://unstats.un.org/unsd/methods/m49/m49regin.htm>) is according to the United Nations (UN) classification scheme. Regions: AFRI (Africa), AMER (Americas), ASIA (Asia), EURO (Europe), and OCE (Oceania), subdivided in: E (Eastern), M (Middle), C (Central), N (Northern), S (Southern), SE (South-Eastern), and W (Western). Furthermore, CARIB (Caribbean) belongs to Americas, while Oceania is subdivided in four sub-regions, AUST (Australasia), MEL (Melanesia), MIC (Micronesia), and POLY (Polynesia). Note that in our classification scheme, Russia has been attributed a new sub-region by itself (Northern Asia) instead of including it among rest of the Eastern Europe. It reflects its geographical localization as well as due to the similarity of specific TB genotypes circulating in Russia (a majority of Beijing genotypes) with those prevalent in Central, Eastern and South-Eastern Asia.

^b The three letter country codes are according to http://en.wikipedia.org/wiki/ISO_3166-1_alpha-3; countrywide distribution is only shown for SITs with 5% of a given SITs as compared to their total number in the SITVIT2 database.

Drug susceptibility patterns of clusters containing seven or more *M. tuberculosis* isolates, correlated with gender and age of the patients from São Paulo state, Brazil.

Table 2

SIT (lineage) – No. of isolates	Drug resistant patterns, No. (%)				Other-resistance		Susceptible		Gender, No. (%)		Age (years)	
	MDR		Monoresistance		I	R	S		Male	Female	Mean	Median
1 (Beijing) – 7	3 (42.8)	1 (14.3)	0	1 (14.3)	0	0	2 (28.6)	5 (71.4)	2 (28.6)	41.0 (25–58)	37.0	
177 (LAM9) – 9	4 (44.4)	1 (11.1)	0	1 (11.1)	0	0	3 (33.3)	7 (77.8)	2 (22.2)	38.0 (26–50)	38.0	
1905 (T1) – 11	3 (27.3)	7 (63.6)	0	0	0	0	1 (9.1)	6 (54.5)	5 (45.5)	38.0 (17–54)	51.0	
4 (U) – 12	7 (58.3)	4 (33.3)	0	0	0	0	1 (8.3)	8 (66.7)	4 (33.3)	31.0 (20–51)	29.0	
137 (X2) – 13	6 (46.1)	4 (30.8)	0	2 (15.4)	0	0	1 (7.7)	12 (92.3)	1 (7.7)	46.0 (20–71)	48.0	
51 (T1) – 21	1 (4.8)	3 (14.3)	0	12 (57.1)	1 (4.8)	0	4 (19.0)	16 (76.2)	5 (23.8)	40.0 (25–66)	41.0	
60 (LAM4) – 23	15 (65.2)	5 (21.7)	0	0	0	0	3 (13.0)	13 (56.5)	10 (43.5)	33.0 (10–62)	31.0	
47 (H1) – 24 ^a	4 (16.7)	6 (25.0)	2 (8.3)	2 (8.3)	3 (12.5)	0	6 (25.0)	18 (75.0)	6 (25.0)	34.3 (20–54)	33.0	
20 (LAMI) – 26	6 (23.1)	4 (15.4)	2 (7.7)	3 (11.5)	3 (11.5)	0	8 (30.8)	18 (69.2)	8 (30.8)	39.8 (21–69)	40.0	
64 (LAM6) – 31	9 (29.0)	11 (35.5)	0	1 (3.2)	1 (3.2)	0	9 (29.0)	20 (64.5)	11 (34.5)	38.3 (16–74)	37.0	
33 (LAM3) – 36	11 (30.5)	4 (11.1)	3 (8.3)	7 (19.4)	2 (5.5)	0	9 (25.0)	20 (55.6)	16 (44.4)	39.7 (17–70)	40.0	
17 (LAM2) – 46	18 (39.1)	14 (30.4)	0	3 (6.5)	1 (2.2)	0	10 (21.7)	36 (78.3)	10 (21.7)	36.5 (15–76)	33.0	
42 (LAM9) – 48	13 (27.1)	7 (14.6)	2 (4.2)	5 (10.4)	6 (12.5)	0	15 (31.2)	32 (66.7)	16 (33.3)	36.5 (12–63)	35.5	
53 (T1) – 62 ^a	10 (16.1)	10 (16.1)	2 (3.2)	19 (30.6)	3 (4.8)	0	17 (27.4)	44 (71.0)	18 (29.0)	35.0 (15–66)	34.0	
50 (H3) – 84 ^b	31 (36.9)	16 (19.0)	2 (2.4)	14 (16.7)	5 (5.9)	0	15 (17.8)	65 (77.4)	19 (29.6)	36.3 (16–64)	34.0	

Abbreviations: I, isoniazid; R, rifampin; SM, streptomycin; MDR, multidrug-resistant.

^aOne isolate with monoresistance to ethambutol.

^bOne isolate with monoresistance to pyrazinamide.