Genotyping of *Mycobacterium tuberculosis* isolates from a low-endemic setting in northwestern state of Paraná in Southern Brazil

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The purpose of this study was to provide information about the genetic diversity and prevalent genotype of Mycobacterium tuberculosis *in a low-endemic setting in northwestern state of Paraná in Southern Brazil. We employed spoligotyping and mycobacterial interspersed repetitive units-variable number tandem repeat (MIRU-VNTR) techniques to genotype* M. tuberculosis *isolates from patients with pulmonary tuberculosis (TB). The 93 isolates analyzed by spoligotyping were divided into 36 different patterns, 30 of which were described in the SITVIT database. Latin American and Mediterranean, Haarlem and T families were responsible for 26.9%, 17.2% and 11.8% of TB cases, respectively. From the 84 isolates analyzed by MIRU-VNTR, 58 shared a unique pattern and the remaining 26 belonged to nine clusters. The MIRU loci 40, 23, 10 and 16 were the most discriminatory. A combination of MIRU-VNTR and spoligotyping resulted in 85.7% discriminatory power (Hunter-Gaston index = 0.995). Thus, combining spoligotyping and MIRU-VNTR typing proved to be most useful for epidemiological study in this low-endemic setting in Southern Brazil. The current study demonstrated that there is significant diversity in circulating strains in the city of Maringá and the surrounding regions, with no single genotype of* M. tuberculosis *predominating.*

Key words: *Mycobacterium tuberculosis* - tuberculosis - molecular epidemiology - spoligotyping - MIRU

Tuberculosis (TB) is the leading cause of death by an infectious agent and thus an important public health problem worldwide, with an estimated 9.27 million new cases in 2007. In Brazil, the estimated incidence rate is 48 cases per 100,000 inhabitants (WHO 2009). Brazil has five distinct geographic regions, among which the distribution of TB cases varies greatly. In the state of Paraná (PR) in Southern Brazil, the average rate of TB is 27.52 cases per 100,000 inhabitants (Malaghini et al. 2009). Maringá and the small cities surrounding it form a low-endemic area of TB in PR, with an incidence rate of 22.62 cases per 100,000 in 2007 (personal communication).

Genotyping methods have been applied extensively in the epidemiological study of TB worldwide (Candia et al. 2007, Sharma et al. 2008, Valcheva et al. 2008). These studies have been based on the assumption that patients with genotypically clustered strains are epidemiologically linked and represent recent transmissions. In contrast, patients infected with different types of strains are not considered indicative of recent transmission. Genetic markers need to be both sufficiently polymorphic to distinguish unrelated strains and stable enough to identify isolates of the same strains. Furthermore, the typing methods employed must be reproducible, discriminatory and easy to perform.

Some polymerase chain reaction (PCR)-based techniques are now being used to differentiate *Mycobacterium tuberculosis* isolates. Currently, genotyping approaches targeting the analysis of the variable number of tandem repeats (VNTR), based on mycobacterial interspersed repetitive units (MIRU), are the most promising. This technique is based on the variability found at 12 specific loci interspersed throughout the mycobacterial genome (Supply et al. 2000). Recently, MIRU-VNTR genotyping strategies using 15 or 24 loci (Supply et al. 2006, Oelemann et al. 2007a, Alonso-Rodríguez et al. 2008) were evaluated and applied to molecular epidemiological typing in mycobacteria.

Spoligotyping is the second most widely used method for *M. tuberculosis* complex genotype after IS*6110*-based fingerprinting (Prodinger 2007). It is based on the presence or absence of a set of target sequences in the direct repeat (DR) locus in the *M. tuberculosis* complex genome (Kamerbeek et al. 1997) that in combination with MIRU has been used to replace typing via restriction fragment length polymorphisms (RFLP) based on the insertion sequence IS*6110*. The IS*6110* method has been considered the "gold standard" for genotyping *M. tuberculosis* since 1993, but it is an expensive, laborious and lengthy methodology that requires weeks of *M. tuberculosis* culturing and specific software to analyze the RFLP band-patterns, all of which make it difficult to interpret and exchange data. In addition, the IS*6110*

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method has limited use in genotyping *M. tuberculosis* isolates that contain few copies of IS*6110*.

The aim of our study was to use spoligotyping and MIRU-VNTR typing to provide preliminary information about the genetic diversity and prevalent genotype of *M. tuberculosis* isolates in a low-endemic setting in northwestern PR.

PATIENTS, MATERIALS AND METHODS

Study isolates - From November 2005-June 2008, a total of 93 *M. tuberculosis* isolates were obtained from sputum of patients suspected of TB who were seen at the Clinical Bacteriology Laboratory, Department of Clinical Analysis of the State University of Maringá. This lab is a reference TB laboratory that handles patients from Maringá and other cities in northwestern PR. All isolates were cultured in Difco™ Lowenstein Medium Base (Becton, Dickinson and Company, Sparks, MD, USA) and identified as *M. tuberculosis* using the conventional biochemical tests (Kent & Kubica 1985) and molecular biology (van Embden et al. 1993). The following retrospective demographic and epidemiological data were collected for all patients through a review of a national TB notification database (National Diseases Notification System): city and zip code of residence at the time of diagnosis, age, sex, ethnicity, HIV status, sample susceptibility profile, alcoholism and the occurrence of other diseases.

DNA extraction - DNA from *M. tuberculosis* was extracted from a subculture on Difco™ Lowenstein Medium Base, as described by González-y-Merchand et al. (1996), with minor modifications. Briefly, a loopful of bacteria was suspended in 6 M guanidine hydrochloride (Sigma Chemical Co, St. Louis, MO, USA) and bacilli were lyzed by freezing at -20°C for 30 min followed by heating at 100ºC for 10 min. This procedure was repeated twice. DNA was further extracted with two volumes of phenol-chloroform-isoamyl alcohol (25:24:1, v/v) followed by two steps of extractions with chloroform-isoamyl alcohol (24:1, v/v). DNA was purified by ethanol precipitation, dissolved in 50 µL of Tris-ethylenediamine tetraacetic acid (TE buffer), pH 8.0, and stored at -20ºC until use. DNA concentration was determined by ultraviolet spectrophotometry.

Spoligotyping - Spoligotyping was performed in all 93 *M. tuberculosis* isolates, using the standard method, to detect the presence or absence of 43 spacers (Molhuizem et al. 1998). Briefly, the DR region was amplified from 1 µL of mycobacterial DNA (10 ng) in 24 µL of a reaction mixture containing 0.4 µM of each primer and PCR Master Mix (Promega Corporation, Madison, WI, USA) according to manufacturer's instructions. The primer sequences were Dra, 5'-GGTTTTGGGTCTGACGAC-3' (biotinylated 5' end) and DRb, 5'-CCGAGAGGGGACGGAAAC-3' (Integrated DNA Technologies, Inc Coralville, USA). The DNA amplification was carried out in a TC-512 thermal cycler (Techne, UK). 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. Bound fragments were detected by chemiluminescence after incubation with a streptavidin-peroxidase conjugate (Boehringer Ingelheim, Germany) and assessed by an enhanced chemiluminescence system (GE Healthcare UK Limited, Buckinghamshire, UK). Spoligotypes were reported using an octal code in which the 43-digit binary representing the 43 spacers ("1" is hybridization and "0" is no hybridization) was divided into 14 sets of three digits (spacers 1-42) plus one additional digit (spacer 43). Each three-digit set was converted to octal code (000 = 0, 001 = 1, 010 = 2, 011 = 3, 100 = 4, 101 = 5, 110 = 6 and 111 = 7), with the final digit remaining either 1 or 0, yielding a 15-digit octal designation (Dale et al. 2001).

MIRU-VNTR typing - MIRU-VNTR typing was performed in only 84 of the 93 *M. tuberculosis* isolates at the Laboratory of Mycobacteria Dr. Hugo David in the School of Pharmaceutical Science, São Paulo State University, Araraquara, São Paulo (SP), Brazil. MIRU-VNTR was not performed in nine of the isolates because there was insufficient DNA for analysis.

The isolates were genotyped by PCR amplification of the original 12 MIRU-VNTR loci (2, 4, 10, 16, 20, 23, 24, 26, 27, 31, 39, 40) as described by Supply et al. (2000) and Mazars et al. (2001) in a PTC-100 thermal cycler (MJ Research, Ramsey, Minnesota, USA). Each locus was amplified individually from 2 µL of mycobacterial DNA (20 ng) in 23 µL of a reaction mixture containing 0.4 µM of loci-respective primers and PCR Master Mix (Promega Corporation, Madison, Wisconsin, USA) according to the manufacturer's instructions. The PCR conditions for each set of primers have been described elsewhere (Pandolfi 2006). PCR products were subjected to electrophoresis in a 2% weight/volume agarose gel (Invitrogen Life Technologies, SP, Brazil). Both 50 and 100-bp DNA Ladders (Invitrogen Life Technologies, SP, Brazil) were used as molecular markers. The gels were stained with ethidium bromide and visualized under ultraviolet light, then photodocumented with an Alpha-imager 2200 (Alpha Innotech Corporation, San Leandro, CA, USA). PCR fragment size was determined by visual comparison with the molecular markers and the MIRU allele scoring was determined according to Mazars et al. (2001) and Supply et al. (2001). The results from each of the 12 loci were combined to create the 12-digit allelic profiles.

Interpretation of genotyping results - The observed spoligotypes were compared to the international database, SITVIT, which is an updated version of the published SpolDB4.0 database (Brudey et al. 2006) and it is available at pasteur-guadeloupe.fr:8081/SITVITDemo/. For previously unreported spoligopatterns in the SpolDB4.0, the "Spotclust" database was used (Vitol et al. 2006). This model takes into account knowledge of the evolution of the DR region and assigns spoligopatterns to families and subfamilies, using a computer algorithm based on studies of SpolDB3 (cgi2.cs.rpi.edu/~bennek/SPOTCLUST.html). BioNumerics software (version 4.45; Applied Maths, Sint-Martens-Latem, Belgium) was used for analysis of spoligotyping and MIRU-VNTR patterns. Dendrograms were constructed for spoligotyping, MIRU-VNTR and the combination of both methodolo-

gies. The genetic distance was built using the unweighted pair group method with arithmetic mean (UPGMA) algorithm (Sneath & Sokal 1973). The evaluation of the discriminative power of each typing method alone as well as the two in combinations was undertaken using the Hunter-Gaston index (HGI) (Hunter & Gaston 1988). HGI is based on the probability that two unrelated strains sampled from the population will be placed into different typing groups. Allelic diversity of each locus' MIRU-VNTR was classified as "highly discriminant" (HGI > 0.6), "moderately discriminant" (0.3 ≤ HGI ≤ 0.6) and "poorly discriminant" (HGI < 0.3) (Sola et al. 2003).

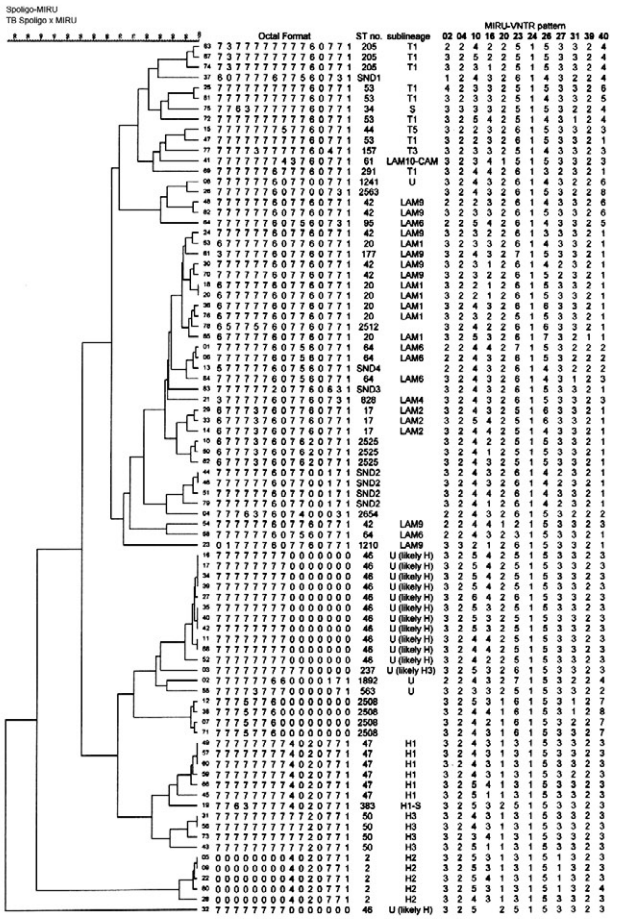
RESULTS

The study population belonged to eight cities (Maringá and seven other small, neighboring cities) and consisted of 78 males (83.9%) and 15 females (16.1%). The mean patient age was 40 years. The results of HIV-testing were available for 43 patients (46.2%) and, among these, four (9.3%) tested positive for HIV. Data regarding the use of alcohol and the presence of other diseases were not obtained.

Spoligotyping - Of the 93 *M. tuberculosis* isolates, 36 different spoligotyping patterns were observed. Based on these spoligotypes, distinct families of TB were identified: Haarlem (H), Latin American and Mediterranean (LAM), undesignated (U), T family (modern TB strains), East-African Indian (EAI) and S lineage (Figure).

The spoligopatterns of 83 isolates (89.2%) were classified according to the SITVIT database and, of these, 65 isolates were clustered into 12 Shared International Types (ST), with each cluster containing between three and 13 isolates. The remaining 18 isolates showed unique STs. The main STs found in this study were ST46 (n = 13, 15.7%), ST20 (n = 6, 7.2%), ST42 (n = 6, 7.2%) and ST47 (n = 6, 7.2%).

Of the 83 *M. tuberculosis* isolates identified in the SITVIT database, 72 were classified into families and sublineages according to SpolDB4. The number of these 72 isolates that ranked in the LAM, U, H and T families were, respectively, 25 (34.7%), 17 (23.6%), 16 (22.2%)



Combined numerical analysis of 84 *Mycobacterium tuberculosis* clinical isolates from Maringá and other cities in the northwest of the state of Paraná, Brazil, using spoligotyping and mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTR) typing. The dendrogram was obtained using the BioNumerics. CAM: Cameroun; H: Haarlem; LAM: Latin American and Mediterranean; S: S lineage; SND: spoligotype not described; ST: Shared International Types; T: modern tuberculosis strains; U: undesignated.

TABLE I

Spoligopatterns and family assignment of 10 isolates of *Mycobacterium tuberculosis* not described in SITVIT database

	Spoligotype	Spotclust ID	Spotclust probability	Isolates with identical pattern n
SND1	607777677560731	T1	0.99	1
SND2	777777607700171	LAM9	0.99	4
SND3	777777207760631	LAM9	0.99	1
SND4	577777607560771	LAM9	0.99	2
SND5	774337606560771	LAM9	0.99	1
SND6	777757777760471	T1	0.99	1

ID: identification; LAM: Latin American and Mediterranean; SND: spoligotype not described; T: modern tuberculosis strains.

and 11 (15.3%). The minor families observed in our study were EAI (n = 1, 1.4%), S lineage (n = 1, 1.4%) and H1-S (n = 1, 1.4%). Eleven *M. tuberculosis* isolates were not classified into families according to SpolDB4, but did have attributed ST numbers according to the SITVIT database (ST2508, ST2512, ST2525, ST2563 and ST2654).

Ten isolates (10.8%), comprising six spoligotyping patterns not found in the SITVIT database, were assigned to families and subfamilies by "SpotClust". The family assignment of these remaining isolates revealed that 80% belonged to the LAM family and 20% belonged to the T family (Table I). These six new spoligotypes have been submitted to the SITVIT database (pasteur-guadeloupe.fr:8081/SITVITDemo/).

MIRU-VNTR typing - MIRU-VNTR typing was conducted for 84 clinical isolates and a total of 67 distinct MIRU patterns were obtained. Of the 84 isolates, 58 (69%) were orphans and the remaining 26 (31%) were grouped into nine clusters, each comprising 2-6 isolates (Figure).

Allele polymorphism analysis of 12 MIRU loci revealed that MIRU locus 40 was the most discriminatory locus with eight alleles, followed by MIRU loci 23, 10 and 16. In MIRU locus 40, the presence of three alleles was most frequent, followed by a single copy of an allele. The MIRU 20, 26 and 31 loci were moderately discriminant. Other loci were less polymorphic, with only two alleles for MIRU loci 4, 20 and 39 and one allele for MIRU locus 24, for which a single copy was present in all 84 *M. tuberculosis* isolates analyzed (Table II).

Combining spoligotyping and MIRU-VNTR typing - Among the 84 *M. tuberculosis* isolates analyzed by spoligotyping and MIRU-VNTR typing, 72 (85.7%) distinct genotypes were obtained, resulting in eight clusters with 100% similarity. By considering similarity indices of at least 69%, we observed three distinct clonal groups that represented 92.9% of the isolates analyzed (Figure). Table III summarizes the discriminative power of each typing method alone and of the two methods combined, according to the HGI.

TABLE II

Allelic polymorphism of 12 mycobacterial interspersed repetitive units (MIRU) loci from the 84 *Mycobacterium tuberculosis* isolates from patients with tuberculosis in Maringá and region in the northwest of the state of Paraná, Brazil

	Allele number										
MIRU number	0	1	2	3	4	5	6	7	8	HGI	Conclusion
2	-	1	10	72	1	-	-	-	-	0.254	Poorly discriminant
4	-	-	82	2	-	-	-	-	-	0.047	Poorly discriminant
10	-	-	6	14	40	23	1	-	-	0.673	Highly discriminant
16	1	9	9	45	20	-	-	-	-	0.641	Highly discriminant
20	-	21	63	-	-	-	-	-	-	0.380	Moderately discriminant
23	-	-	1	16	-	29	35	3	-	0.678	Highly discriminant
24	-	84	-	-	-	-	-	-	-	0.000	Poorly discriminant
26	-	-	-	2	16	61	4	1	-	0.439	Moderately discriminant
27	-	4	6	74	-	-	-	-	-	0.219	Poorly discriminant
31	-	4	12	68	-	-	-	-	-	0.326	Moderately discriminant
39	-	1	83	-	-	-	-	-		0.024	Poorly discriminant
40	-	26	5	34	8	2	4	3	2	0.732	Highly discriminant

the allelic diversity of the loci was classified as highly discriminant [Hunter-Gaston index (HGI) > 0.6], moderately discriminant ($0.3 \leq HGI \leq 0.6$) and poorly discriminant (HGI < 0.3), according to Sola et al. (2003).

TABLE III

Discriminatory power of spoligotyping and mycobacterial interspersed repetitive units (MIRU) typing, alone and in association

Methodologies	Distinct patterns n	Clusters n	Clustered isolates n	HGI
Spoligotyping	33	13	64	0.954
MIRU	67	9	26	0.991
Spoligotyping + MIRU	72	8	20	0.995

HGI: Hunter-Gaston index.

DISCUSSION

Molecular methods have been used for epidemiological studies of TB in developed countries, but this kind of study is scarce in developing countries. Particularly, there are few studies of this nature in Brazil (Borsuk et al. 2005, Cafrune et al. 2006, Oelemann et al. 2007b, Malaspina et al. 2008, Malaghini et al. 2009). To explore the molecular epidemiology of TB in northwestern PR, we chose to work with two effective and fast methodologies: spoligotyping and MIRU-VNTR typing (Malaghini et al. 2009).

The distribution of 93 isolates analyzed by spoligotyping in our study revealed 36 distinct patterns, six of which have not yet been described in SITVIT (10.8% of isolates). These new STs have been submitted to the database by our group. The LAM, H and T families were the largest families observed in our study and they are the three genotypic families most frequently found in Africa, Central America, Europe and South America (Brudey et al. 2006).

Previous studies analyzing *M. tuberculosis* isolates from Araraquara and the state of Rio Grande do Sul (RS) in Southern Brazil reported the prevalence of ST53 (T1 sublineage) (Borsuk et al. 2005, Malaspina et al. 2008). In contrast, in our study, ST53 was only representative of 5.4% (5/93) of the isolates, while ST46 (U, likely H lineage) was the most prevalent and accounted for approximately 14% (13/93) of the isolates.

One isolate detected in our study belonged to the EAI lineage (ST48, EAI1_SOM), which is typically more prevalent in southeast Asia. This lineage has also been detected in another study in Northern Brazil. ST1892 (U lineage) and ST2512, both characterized in our study, are known to have exclusive geographic distribution in Brazil. ST61 (LAM10_CAM sublineage), ST563 (U lineage) and ST2654 were also identified in our study but had not yet been described in our country. The other STs, which had already been described in the SITVIT database (pasteur-guadeloupe.fr:8081/SITVITDemo/), have been previously identified in Brazil, but this is the first report of their presence in our state, probably because of the limited study in our region previously.

When MIRU-VNTR was applied to the study of *M. tuberculosis* isolates, the most allelic diversity was observed for MIRU loci 40, 23, 10 and 16 (HGI = 0.732, 0.678, 0.673 and 0.641, respectively), while moderate polymorphisms were found in MIRU loci 26, 20 and 31 (HGI = 0.439, 0.380 and 0.326, respectively). Conversely, Kovalev et al. (2005) observed the most allelic diversity for MIRU loci 26, 31 and 10 in *M. tuberculosis* isolated from the Ural region of the Russian Federation. Sharma et al. (2008), studying isolates from Kanpur, India, and working with only six MIRU loci (MIRU 4, 10, 16, 26, 39 and 40), observed that the most discriminatory loci were, in order of diversity, 26, 10, 16 and 40. Despite the high discriminatory power of MIRU locus 40 as observed in this study (8 alleles), it is possible that a higher discriminatory power can be obtained with another study population.

MIRU locus 24 was present in a single copy in all isolates analyzed in our study, which is in agreement with Kovalev et al. (2005). However, that study found MIRU locus 23 to have the second-lowest discriminatory power, which differs from our finding that MIRU locus 39 was the second-least discriminating.

MIRU-VNTR significantly reduced the number of epidemiological links among the isolates studied, while spoligotyping overestimated these links. Thus, MIRU-VNTR demonstrated a superior capacity to differentiate *M. tuberculosis* clinical isolates in our study. We believe that using the newly developed 15 or 24-locus MIRU-VNTR (Supply et al. 2006, Oelemann et al. 2007a, Alonso-Rodríguez et al. 2008) could further increase the discriminatory power of this method.

Despite the high discriminatory power of MIRU-VNTR typing observed in our study, a few isolates clustered by this method and, in some cases, the clustered isolates were discriminated by spoligotyping. This result demonstrates the need to combine the two methodologies to provide a higher discriminatory power for epidemiological study. According to Cowan et al. (2005), when combining these two methodologies, the clustering rate is similar to that of IS*6110*-RFLP fingerprinting.

The results obtained with spoligotyping in our study suggested a possible epidemiological link between two patients who were prisoners in the same prison at the same time and whose isolates displayed the same molecular pattern (ST46). However, when these isolates were typed by MIRU-VNTR, they showed different patterns. In other *M. tuberculosis* isolates that were clustered by combining spoligotyping and MIRU-VNTR typing, no correlation with retrospective epidemiological data was apparent.

Significant diversity in circulating strains was observed in our study of *M. tuberculosis* isolates from what is considered to be a low-endemic TB setting in Brazil, using a combination of spoligotyping and MIRU-VNTR typing. These methodologies allowed 85.7% differentiation of the *M. tuberculosis* isolates analyzed, which is in agreement with a recent study conducted in the city of Curitiba (the capital of PR) that found 93.5% differentiation using Mixed-linker PCR DNA fingerprinting (Malaghini et al. 2009). Alternatively, an analysis of *M. tuberculosis* isolates from three regions of RS found 66% clonal differentiation using IS*6110*-RFLP and spoligotyping (Cafrune et al. 2006), while isolates from two cities in RS that were analyzed by IS*6110*-RFLP and spoligotyping revealed high clonal diversity of *M. tuberculosis* (Borsuk et al. 2005).

In summary, the present study offers the first insight about the genetic diversity of *M. tuberculosis* isolates from patients with TB in cities from the northwest region of PR. This kind of study is important for tracing relationships among the strains and may also help to interrupt the chain of transmission between different communities and aid in recognition of the principal clades responsible for spreading the disease. Of course, additional studies with more isolates over longer time periods will be critical if we are to fully understand the epidemiology of TB in this region.

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