

Molecular profiling of drug resistant isolates of *Mycobacterium tuberculosis* in the state of Santa Catarina, southern Brazil

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Drug resistance is a global threat and one of the main contributing factors to tuberculosis (TB) outbreaks. The goal of this study was to analyse the molecular profile of multidrug-resistant TB (MDR-TB) in the state of Santa Catarina in southern Brazil. Fifty-three MDR Mycobacterium tuberculosis clinical isolates were analysed by spoligotyping and a partial region of the rpoB gene, which is associated with rifampicin resistance (RMP-R), was sequenced. Some isolates were also distinguished by their mycobacterial interspersed repetitive units (MIRU). S531L was the most prevalent mutation found within rpoB in RMP-R isolates (58.5%), followed by S531W (20.8%). Only two MDR isolates showed no mutations within rpoB. Isolates of the Latin American Mediterranean (LAM) family were the most prevalent (45.3%) found by spoligotyping, followed by Haarlem (9.4%) and T (7.5%) families. SIT106 was found in 26.4% of isolates and all SIT106 isolates typed by MIRU-12 (5 out of 14) belong to MIT251. There was a high correlation between the S531W mutation and the LAM family mainly because all SIT2263 (LAM9) isolates carry this mutation. Among isolates with the S531W mutation in rpoB MIRU demonstrates a cluster formed by four isolates (SIT2263 and MIT163) and very similar profiles were observed between eight of the nine isolates. Better characterisation of TB isolates may lead to new ways in which to control and treat TB in this region of Brazil.

Key words: tuberculosis - MDR-TB - rpoB - spoligotyping - MIRU

Among the many infectious diseases, tuberculosis (TB) remains the world's leading cause of death. Despite the advances in TB therapy over the past century, there were an estimated nine million new cases and 1.1 million deaths in 2013 (WHO 2014). Mycobacterial drug resistance is a global threat and one of the main problems associated with this illness, as evidenced by multidrug-resistant TB (MDR-TB). For MDR-TB, multidrug resistance is defined as resistance to isoniazid (INH) and rifampicin (RMP), the two key drugs used to treat TB. Thus, MDR-TB requires second-line drugs for treatment, which are more toxic, expensive and less efficient (Dye & Williams 2000, Espinal 2013).

The acquisition of resistance by *Mycobacterium tuberculosis* occurs spontaneously due to the natural mutation rate of genomic DNA and there are no known plasmids that confer resistance (Riska et al. 2000). The molecular basis of RMP resistance is mainly due to several mutations within the 81-bp hotspot region of the rpoB gene (corresponding to codons 507-533), most fre-

quently at codons 531, 526 and 516. This gene encodes the β -subunit of the *M. tuberculosis* RNA polymerase (RNAP), the target of RMP (Telenti et al. 1993). Mutations in this hotspot region are found in almost 96% of RMP-resistant strains, making rpoB the molecular marker of multidrug resistance and, in fact, the majority of the RMP-resistant isolates are MDR-TB (Somoskovi et al. 2001, Sam et al. 2006, Zhang & Yew 2009).

Genotyping tools are used to elucidate the distribution of TB and its transmission dynamics in the population (Demay et al. 2012). Spoligotyping is a genotyping technique based on the polymorphism of the direct repeat (DR) locus present within *M. tuberculosis*. The DR contains 36-bp well-conserved repeat sequences interspersed with 34-41-bp nonrepetitive spacers. The profile of the presence/absence of the 43 spacers is compared to more than 58,000 strains deposited in the SITVIT-WEB database (pasteur-guadeloupe.fr:8081/SITVITD) (Kamerbeek et al. 1997, Demay et al. 2012). Another important genotyping method is based on the copy number of the variable number tandem repeats of mycobacterial interspersed repeat units (MIRU-VNTR) (Supply et al. 2000). In fact, out of the 41 loci that contain MIRUs, the most prevalent VNTRs are the 12, 15 and 24-loci.

Despite the relevance of drug resistance to the treatment and control of TB and MDR-TB, few studies have been published about circulating resistant strains in Brazil. Thus, the aim of this study was to analyse the molecular profile of MDR-TB strains in the state of Santa Catarina (SC), Brazil.

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SUBJECTS, MATERIALS AND METHODS

Bacterial isolates and DNA extraction - From March 2008-March 2012, 53 MDR *M. tuberculosis* isolates were obtained from clinical specimens (only 1 isolate per patient) during routine work at the Central Laboratory of the State of Santa Catarina, the reference laboratory for TB diagnosis in SC. SC has an estimated population of 6,727,148 inhabitants and a demographic density of 65.27 inhabitants per km² (IBGE 2015). These strains represent all strains detected by the reference laboratory in that period. Susceptibility testing to INH, RMP, ethambutol (EMB) and streptomycin (SM) was performed using BACTEC-960™-MGIT™ according to the manufacturer's instructions. The H37Rv strain was used as a RMP-susceptible control.

Liquid cultures from BACTEC-960™-MGIT™ were transferred to Ogawa-Kudoh medium and, after four weeks at 36°C, DNA was extracted using the CTAB method (van Soolingen et al. 1994).

PCR and DNA sequencing - A partial region of *rpoB* (411 bp) was sequenced using primers described by Telenti et al. (1993). A 50 µL polymerase chain reaction (PCR) was assembled with 1U Taq DNA polymerase, 25 pmol of each primer, 200 µM dNTPs, 1.5 mM MgCl₂, 20 mM Tris-HCl (pH 8.4) and 50 mM KCl. The following cycling protocol was applied: 94°C for 1 min, 55°C for 1 min and 72°C for 1 min for 40 cycles.

The *rpoB* PCR fragments were purified by PureLink® Invitrogen PCR purification kit. Sequencing was performed in an Applied Biosystems ABI model 3730 A automated DNA Sequencer (ThermoFisher Scientific, USA). Sequences were aligned and compared to the H37Rv strain sequence (GenBank accession U12205 and our H37Rv sequencing) using BioEdit 7.1.11 software.

Spoligotyping - Spoligotyping was carried out as described by Kamerbeek et al. (1997) using commercially available membranes (Ocimum Biosolutions, India). Hybridising fragments were detected by chemiluminescence using peroxidase-labelled streptavidin and ECL detection reagents.

Binary formats of spoligotypes were compared to those stored in the SITVITWEB database. Within the SITVITWEB database, spoligotype international type (SIT) designates patterns shared by two or more patient isolates, whereas "orphan" designates patterns reported for a single isolate (Demay et al. 2012).

MIRU - MIRU-12 was performed using agarose gel electrophoresis as referenced by Supply et al. (2000). The results were compared with those stored in the SITVITWEB database. MIRU international type (MIT) designates patterns shared by two or more patient isolates in the SITVITWEB database (Demay et al. 2012).

Data analysis - Nominal data were expressed as absolute or relative numbers. A cluster is defined as two or more isolates with identical spoligotypes and MIRU patterns. Comparisons between categorical variables were performed using the Fisher exact test. *p* values < 0.05 were considered statistically significance.

Ethics - Samples were collected with approval by the Research Ethical Committee of the Federal University of Santa Catarina (process 204.297).

RESULTS

Characterisation of samples - The median age of patients at the time of diagnosis was 36 ± 10 years with a male:female ratio of 2.3. Approximately 60% of cases were either new or relapsed; the remaining cases consisted of retreatments after dropout. Among the 53 MDR-TB isolates, 41 were resistant only to INH and RMP, eight to INH, RMP and EMB, three to INH, RMP and SM and only one to INH, RMP, EMB and SM.

Mutations associated with RMP resistance - The evaluation of 53 MDR isolates identified eight different mutations within *rpoB*. S531L was the most prevalent mutation, which was found in 31 isolates. The second most frequent mutation was S531W, which was detected in 11 isolates. Other single mutations were found in codons 526, 516 and 572. No mutations were found in two MDR isolates (Table I).

Spoligotyping patterns - Spoligotyping showed 22 different profiles, including 17 shared types, four unknowns and one orphan type (Table II). Between shared types, eight subfamilies and three families were found. As expected, Latin American Mediterranean (LAM) (47.2%) was the most prevalent family, followed by Haarlem (H) (9.4%) and T (7.5%) families. SIT106 represented 26.4% of the isolates and was considered a different group because it is not related to a specific family. The five main shared types were SIT106 (26.4%), SIT42 (13.2%), SIT2263 (13.2%), SIT50 (5.7%) and SIT73 (5.7%) (Figure).

MIRU-12 and associations between mutations and molecular typing methods - Isolates with enough DNA from two groups were subjected to MIRU-12; these isolates were those with the S531W mutation (9/12) and isolates from SIT106 (5/14). Among 14 SIT106 isolates, it was possible to type five isolates by MIRU-12 analysis. These

TABLE I
Frequencies of mutated *rpoB* gene found in 53 multidrug-resistant isolates of *Mycobacterium tuberculosis*

Amino acid change	Nucleotide change	Strains (n)
S531L	TCG → TTG	31
S531W	TCG → TGG	11
H526Y	CAC → TAC	3
H526D	CAC → GAC	2
D516V	GAC → GTC	1
F505V/D516F	TTC → GTC/GAC → TTC	1
S512G/H526N	AGC → GGC/CAC → AAC	1
I572V	ATC → GTC	1
No mutation	-	2

TABLE II

Spoligotyping profiles found in 53 multidrug-resistant tuberculosis isolates from the state of Santa Catarina, Brazil

[illegible]

H: Haarlem; LAM: Latin American Mediterranean; SIT: spoligotype international type.

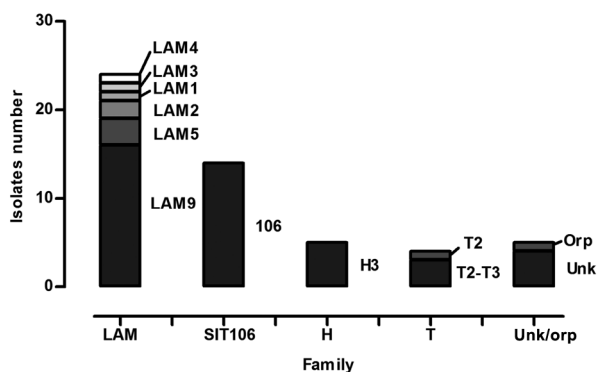
five isolates belonged to the MIT251 (224326133323). Interestingly, SIT106 showed six different mutations in *rpoB*: S531L (10 isolates), S531W (1 isolate), D516V (1 isolate) and H526D (1 isolate), with D516F and F505V mutations being located in the same isolate.

The S531W mutation showed significant association with the LAM family ($p < 0.001$) as shown in Table III. This mutation was present in 40% of LAM isolates and 7.2% of SIT106 isolates and was not found in the other families identified in this study. Among the isolates with the S531W mutation, seven belong to SIT2263, two to SIT42, one to SIT50 and one to SIT106. These isolates, excluding SIT106, belong to the LAM9 subfamily. MIRU-12 analysis defined a cluster formed by four isolates (SIT2263 and MIRU 224126152321; isolates 5, 6, 8 and 9) (Table IV) and very similar profiles between eight of the nine isolates that were possible apply MIRU (isolates 1-9) (Table IV).

DISCUSSION

RMP represents a great advance in TB treatment because the drug enables short-course chemotherapy *via* fast bactericidal effects on TB bacilli. However, treatment and control of RMP-resistant TB, mainly MDR-TB, remain a challenge in the XXI century.

Analysis of mutations in the *rpoB* hotspot region revealed 10 different mutations in 96.2% of resistant strains included in our study. Single nucleotide polymorphisms in codon 531 were responsible for 79.2% of all *rpoB* mutations. S531L was the most prevalent mutation found in



Spoligotyping profiles of 53 multidrug-resistant tuberculosis isolates grouped by families. H: Haarlem; LAM: Latin American Mediterranean; SIT: spoligotype international type; Unk/orp: unknown and orphan.

TABLE III
Association between *rpoB* S531W mutation
and spoligotyping families

Spoligotyping family	<i>rpoB</i> S531W		p
	Mutated (n)	Nonmutate (n)	
SIT106	1	13	0.11
Non-SIT106	10	29	
H	0	5	0.30
Non-H	11	37	
LAM	10	14	< 0.001
Non-LAM	1	28	
T	0	4	0.43
Non-T	11	38	
Unk/orp	0	6	0.23
Non-unk/orp	11	36	

H: Haarlem; LAM: Latin American Mediterranean; SIT: spoligotype international type; unk/orp: unknown/ohphan.

58.5% of RMP-R isolates, which is in agreement with several studies from Brazil (Hofling et al. 2005, Perizzolo et al. 2012) and other countries (Hilleman et al. 2005, Prammananan et al. 2008, Bolotin et al. 2009, Imperiale et al. 2012, Tang et al. 2013) where prevalence ranged from 40-70%. S531W was the second most common mutation in the *rpoB*, as verified in our study and was detected in 20.8% of our RMP-R isolates. Araya et al. (2011) found S531W mutations in 28% of RMP-R strains from Chile. Clemente et al. (2008) described the same mutation in 14.3% of RMP-R isolates from Brazil. In contrast, several studies from other Brazilian regions and other countries observed this mutation in less than 10% of RMP-R isolates (Bodmer et al. 1995, Spindola de Miranda et al. 2001, Cavusoglu et al. 2002, Hofling et al. 2005, Lee et al. 2005, Alves et al. 2011, Tang et al. 2013). The high prevalence of S531W mutations is likely due to a clonal expansion of the LAM9 subfamily (discussed below).

All strains with *rpoB* mutations showed changes in one of 12 amino acids that surround the RMP binding pocket, which contains the RNAP active site. These mutations cause a modification in the active site of RMP because they lead to the replacement of an amino acid with a compact side chain with an amino acid containing a large side chain (Campbell et al. 2001). Thus, this exchange inactivates the RNAP site. Interestingly, in this study, the double mutations observed in two of the isolates include one amino acid within the group that surrounds the RMP binding pocket and another amino acid that does not belong to this group. We believe that these mutations could be compensatory mutations, improving the fitness cost imposed by the primary mutation that is responsible for RMP resistance.

Molecular tools reduce the time in which it takes to detect drug resistance and, consequently, improve treat-

ment and prevent transmission of MDR-TB (Jacobson et al. 2013). In this study, only two isolates did not have mutations in *rpoB*. Additionally, although two mutations (F505V and I572V) outside of the 81-bp hotspot of the *rpoB* gene were identified in two of the isolates, one of these isolates had a second mutation in *rpoB* (F505V/D516F). As reported in previous studies from other Brazilian regions and other countries (Spindola de Miranda et al. 2001, Hofling et al. 2005, Santos et al. 2010, Alves et al. 2011, Perizzolo et al. 2012), these results confirm that the 81-bp hotspot region can be useful for molecular diagnosis of RMP resistance in TB isolates from SC.

Spoligotyping patterns revealed that the LAM family was the most prevalent (47.2% of isolates), followed by H (9.5%) and T (7.5%). Together, these three families cover 64.2% of the isolates included in this study, which is less than that expected for South America (more than 90% expected) (Demay et al. 2012). This finding could be explained by the high number of SIT106 isolates, corresponding to 26.4% of all MDR isolates included in this study. SIT106 is related to the subfamily U (LAM3) in SpolDB4 (Brudey et al. 2006), but recently, in the SIT-VITWEB database, was reclassified as "unknown" because it did not belong strictly to any lineage (Demay et al. 2012). This SIT was not reported in a study containing 1,991 isolates from 11 states within Brazil (not including SC) (Gomes et al. 2012). In our study, all isolates typed by MIRU-12 (5) from SIT106 belonged to MIT251 (224326133323). Although it was not possible to analyse nine of the remaining isolates, this group probably represents a big large cluster formed by 14 isolates. Indeed, 10 out of the 14 isolates had the S531L mutation, which represented 18.9% of all isolates in this study. Furthermore, five other different mutations were found in SIT106 isolates. Thus, resistant bacteria from this SIT may generate strains with different mutations due to antibiotic pressure. Isolates within SIT106 and MIT251 were only reported in the SITVITWEB database between 2003-2004 in Spain, Belgium and the United States of America, but they were not related to any spoligotype family (Demay et al. 2012). Interestingly, Gavin et al. (2012), in a study conducted in Spain, found a cluster formed by 14 SIT106 isolates with the S531L mutation that were classified as belonging to the LAM family. Although they performed MIRU-15, overlapping repetitive regions (MIRU04, MIRU10, MIRU16, MIRU26, MIRU31, MIRU39 and MIRU40) had the same number of copies as our SIT106 isolates typed by MIRU-12.

In this study, we demonstrate an association between the *rpoB* S531W mutation and the LAM family because all SIT2263 isolates (7) had this mutation. Because spoligotyping alone is not sufficient to confirm an epidemiological link, a second method with higher discriminatory power was utilised to highlight potential epidemiological clusters (Jagielski et al. 2014). MIRU-12 was performed on nine isolates with the S531W mutation (isolates 1-9) (Table IV) and a cluster formed by four isolates with SIT2263/MIT163 was confirmed (isolates 5, 6, 8 and 9) (Table IV). Two isolates were not analysed by MIRU-12 (isolates 10 and 11) (Table IV) because there was not enough DNA for analysis. Interestingly,

TABLE IV
Characteristics of *rpoB* S531W isolates

Isolate ID	SIT	Subfamily	MIRU	MIT
1	106	-	224326133323	251
2	42	LAM9	224126152321	163
3	42	LAM9	225126152321	Orphan
4	150	LAM9	224126152321	163
5	2263	LAM9	224126152321	163
6	2263	LAM9	224126152321	163
7	2263	LAM9	224226112321	Unknown
8	2263	LAM9	224126152321	163
9	2263	LAM9	224126152321	163
10	2263	LAM9	Not performed MIRU	-
11	2263	LAM9	Not performed MIRU	-

bold indicate different number of copy of majority. LAM: Latin American Mediterranean; MIT: mycobacterial interspersed repeat unit (MIRU) international type; SIT: spoligotype international type.

among the four isolates with S531W mutations (those that were not SIT2263; isolates 1-4) (Table IV), two had the same profile as the four initial isolates and only one had a single different allele. The final isolate with the S531W mutation belonged to SIT106 and was not related to the other isolates (isolate 1) (Table IV). Only a single spacer, as determined by spoligotyping of 43 spacers, distinguished SIT42 from SIT150, as well as SIT42 from SIT2263. Our MIRU-12 results indicate a recent spread by infectious MDR-TB containing the S531W mutation. Mariam et al. (2004), in a study with Harlingen strains (T family), demonstrated that the S531W mutation reduces the fitness of *M. tuberculosis* in vitro both in single cultures and in macrophage cultures, imposing a biological cost to tubercle bacillus. However, fitness cost may be dependent not only on the *rpoB* mutation, but also on a strain's genotype (Gagneaux et al. 2006). Therefore, it is possible that strains from the LAM9 subfamily may be better adapted than T family strains.

This study sought to define the molecular basis behind RMP resistance in clinical TB isolates from SC. Molecular characterisation of resistant strains is important to suggest nucleic acid amplification methods that can reduce the time required to detect resistance. We found an association between the *rpoB* S531W mutation and LAM family strains, which highlights a successful strategy by S531W-containing strains to resist antibiotics. These results may lead to a way in which to improve the control and treatment of MDR-TB within SC.

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