World Journal of Biochemistry and Molecular Biology
2018; 3(2): 55-65
http://www.aascit.org/journal/wjbmb

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Circulating Genotypes and Drug Resistance Profiles of *Mycobacterium tuberculosis* in Brazilian Counties Bordering Paraguay and Bolivia

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Citation

Marli Marques, Eunice Atsuko Totumi Cunha, Adolfo C. Barreto Santos, Clarice Queico Fujimura Leite, Antonio Francisco de Souza Filho, Sonia Maria Oliveira de Andrade. Circulating Genotypes and Drug Resistance Profiles of *Mycobacterium tuberculosis* in Brazilian Counties Bordering Paraguay and Bolivia. *World Journal of Biochemistry and Molecular Biology.* Vol. 3, No. 2, 2018, pp. 55-65.

Received: February 20, 2018; **Accepted**: March 21, 2018; **Published**: May 16, 2018

Abstract: Along the southern part of Brazil's border with Paraguay and Bolivia, tuberculosis control is a challenging task. This scenario has prompted investigations to support the implementation of specific control measures for the region. This study examined spoligotyping of 60 isolates of *M. tuberculosis* from patients with pulmonary tuberculosis reported in the 2007-2010 period who resided in 12 Mato Grosso do Sul counties bordering Paraguay and Bolivia. Fifty-six strains with distinctive patterns and four orphan strains (6.7%) were identified. Family LAM predominated (30 cases; 50%), followed by families T (subfamily T1; 14 cases; 23.3%), Haarlem (8 cases; 13.3%), and families X (subfamily X1) and S (1 case, or 11.7%, each). Beijing genotypes were absent. A high rate of similarity of 75.1% (45) was found for 10 clusters, comprising subfamilies LAM9 (SIT42), T1 (SIT53), H1 (SIT47), LAM6 (SIT1610), LAM3 (SIT33), H3 (SIT50), and LAM5 (SIT1337) for both binational borders. Sensitivity testing for rifampicin, isoniazid, streptomycin, and ethambutol was performed in 80% (48) of isolates, with drug resistance detected in 35.4% (17). In the border areas investigated, pulmonary tuberculosis exhibited low genetic diversity and high drug resistance, calling for effective epidemiologic surveillance efforts and supplementary molecular studies.

Keywords: *Mycobacterium tuberculosis*, Genetic Lines, Spoligotyping, Resistance Patterns, Brazil–Paraguay–Bolivia Borders

1. Introduction

Worldwide, tuberculosis is the second-most-frequent cause of death by infectious disease, accounting for 6.3 million new cases and 1.3 million deaths in 2016. Such a high death rate is unacceptable, as most fatal cases can be avoided by patient access to healthcare services for diagnosis and appropriate treatment [1].

In Brazil, the disease follows a global pattern of decline, although in some areas the incidence of tuberculosis, ranging from 10.5/100 000 in the Federal District to 67.2/100 000 in Amazonas state, can be higher than the national average [2]. In Mato Grosso do Sul state, incidence and mortality rates for pulmonary tuberculosis are below the national average [2]. However, in the territory comprising the 12 Mato Grosso do Sul counties that share a border with Paraguay or Bolivia or both (counties in which 12.5% of the state population reside), an incidence rate of 49.1/100 000, a mortality rate of 4.0/100 000, and a treatment dropout rate of 11.3% were reported for the 2007-2010 period—1.6, 1.8, and 1.5 times higher than in non-border counties, respectively [3]. These high rates result from the poor living conditions of border populations, whether indigenous or non-indigenous; limited access to health services in some locations; and population mobility, which

hinders tuberculosis control [4-5].

In Corumbá, on the Bolivian border, daily economic activities, tourism (fishing and the Pantanal wetlands are highly popular attractions), and busy traffic between Andean countries and Brazil, translate to increased, permanent population mobility. Across the Paraguayan border, commuting is particularly intense in the twin cities of Ponta Porã–Pedro Juan Caballero and Coronel Sapucaia–Capitán Bado, owing to economic, social, and cultural activities, from shopping tourism to housing, work, and access to services and assistance. Comprising local residents from both sides of the border (including those with dual citizenship and the indigenous population) as well as tourists from other Brazilian states [6] this intensive flow facilitates the spread of tuberculosis.

In the two largest counties included in the study (Corumbá, bordering Bolivia, and Ponta Porã, bordering Paraguay), sputum seeding for *M. tuberculosis* culture has been performed in local laboratories since 2007, facilitating the collection of data on drug resistance and of further information on the disease in this area of risk. For this testing, Ogawa–Kudoh culture medium is supplied by the Mato Grosso do Sul Central Public Health Laboratory (LACEN-MS) [7].

Genotyping tools are used to elucidate the distribution of tuberculosis and its transmission dynamics in the population [8]. Clusters (strains with identical fingerprints) are usually associated with recent transmission, while samples with unique fingerprints suggest remote transmission [9]. Spoligotyping investigates the structure of a given *M. tuberculosis* population, identifying genotypical lines, spoligotype similarities, and geographical distributions [10] - highly relevant data in studies determining phylogeographical features of prevalent genotypical lines.

The present study employed spoligotyping to characterize *M. tuberculosis* strains isolated from Brazilian counties located along the border of Mato Grosso do Sul with Paraguay and Bolivia. The data obtained were compared against patterns found around the world.

2. Materials and Methods

2.1. Study Population

Sputum samples from patients with suspected disease undergoing treatment at public or private healthcare centers located in the border counties investigated in Mato Grosso do Sul state (Figure 1) were collected during routine service, seeded at local municipal laboratories, and sent to the Public Health Central Laboratory of Mato Grosso do Sul (LACEN-MS), in Campo Grande, the reference laboratory.

Figure 1. *International border counties in Mato Grosso do Sul state, Midwest Brazil.*

Samples from counties where these services were not available were seeded at the LACEN-MS using liquid culture media in a Bactec MGIT 960/BD apparatus or using Ogawa–Kudoh and Löwenstein–Jensen (LJ) solid media. Isolates identified as *M. tuberculosis* were tested for susceptibility to rifampicin (R), isoniazid (I), ethambutol (E), and streptomycin (S) using the proportion method [11]. The results were monitored by the Tuberculosis Center of the Instituto Adolfo Lutz in São Paulo and the Centro de Referência Professor Hélio Fraga in Rio de Janeiro. The specimens were stored at -20 °C until subculturing in LJ medium, performed during the first semester of 2013.

Of the 661 cases of pulmonary tuberculosis reported from the 12 counties situated on the border over the study period, 234 (35.4%) were culture-positive for *M. tuberculosis*.

Of 277 strains stored at the LACEN-MS after subculturing, growth was observed in 102 (36.8%). These strains were subsequently subjected to spoligotyping at the School of Pharmaceutical Sciences of the Universidade Estadual Paulista at Araraquara and classified as follows: 5 strains (4.9%) were discarded for being non-tuberculous mycobacteria, 5 (4.9%) showed conflicting results and were not included, 15 (14.7%) originated from non-residents in the counties investigated, and 17 (16.7%) showed similar results from the same patient, giving a total of 60 (58.8%) strains for inclusion in the study.

2.2. Spoligotyping and Database Comparison

Spoligotyping is a genotyping technique based on polymorphism of the direct repeat (DR) locus present in *M. tuberculosis*. The DR locus contains 36-bp well-conserved repeat sequences interspersed with 34-41-bp nonrepetitive spacers. The profile of presence/absence of the 43 spacers has been compared against more than 103 000 strains deposited in the SITVITWEB database (pasteur- guadaloupe.fr:8081/SITVITD) [8, 12].

Spoligotyping was performed according to a previously published protocol [12], using a commercial kit (Isogen Life Science BV, Utrecht, the Netherlands).

Phylogenetic assignment was based on SpolDB4 data, which defines 62 genetic lines or sub-lines [13], and compared with the SITVIT2 database of the Institut Pasteur de la Guadeloupe (France). In the SITVITWEB database, the "spoligotype international type" (SIT) designates any pattern shared by two or more patient isolates, whereas "orphan" designates patterns reported for a unique isolate [8]. The phylogenetic clades assigned included the Central-Asian (CAS), East-African-Indian (EAI), Haarlem (H), Latin-American and Mediterranean (LAM), and Manu families, the S clade, the IS*6110*-low-banding X clade, and an ill-defined T clade (defined "by default") [13]. Nominal data were expressed as absolute or relative numbers. A cluster was defined as two or more isolates with identical spoligotypes. Comparisons between categorical variables were performed using Microsoft Excel 7.0 software.

3. Results

The strains exhibiting visible growth were subjected to spoligotyping. Of these, 5 (4.9%) were excluded for being non-tuberculous mycobacteria (NTM), 5 (4.9%) for exhibiting conflicting results, 15 (14.7%) for originating from patients not residing in the counties investigated, and 17 (16.7%) for originating from the same patient. This gave a total of 60 strains, of which 58.3% and 41.7% were from regions bordering Bolivia and Paraguay, respectively (Table 1).

Table 1. *Distribution of subfamilies, based on spoligotyping, in the international border region of Mato Grosso do Sul state, Midwest Brazil.*

Region (number of counties)	Number of isolates (%)	Frequency	Subfamily	%
		12	LAM9	34.3
		7	T1	20.0
On the Bolivian border	35 (58.3%)	4	LAM5	11.4
(1)		3	Unknown	8.6
		3	LAM3	8.6
		3	H3	8.6
		2	H1	5.7
		1	T5	2.9
		7	LAM9	28.0
		7	T1	28.0
		3	H1	12.0
On the Paraguayan border	25 (41.7%)	3	LAM6	12.0
(2-12)		2	Unknown	8.0
		1	LAM2	4.0
		1	S1	4.0
		1	X1	4.0

The 60 isolates were distributed by year of isolation, sociodemographic characteristics, susceptibility (to streptomycin, isoniazid, rifampicin, and ethambutol), SIT, and family with subfamily (Table 2).

Table 2. *Distribution of 60 isolates, by sociodemographic features and spoligotyping results.*

#	Year	County	Race/skin color [a]	Gender	Age	Inst.[b]	DST SIRE [c]
1	2007	Corumbá	4	F	27	9	SSSS
2	2010	Corumbá	1	M	41	2	NR
3	2007	Corumbá	4	M	43	9	SSSS
4	2008	P. Murtinho	4	M	27	1	SRSS
5	2009	Aral Moreira	5	M	34	1	SSSS
6	2009	C. Sapucaia	5	F	19	1	SSSS
7	2009	C. Sapucaia	5	F	22	1	SSSS
8	2010	C. Sapucaia	5	M	24	1	SSSS
9	2008	C. Sapucaia	4	M	46	1	NR
10	2010	Corumbá	2	M	17	1	SSSS
11	2007	Corumbá	4	M	24	9	SSSS
12	2009	Corumbá	4	M	27	2	SSSS
13	2007	Corumbá	4	M	28	9	SSSS
14	2008	Corumbá	1	F	39	1	RSSS
15	2009	Corumbá	4	F	39	1	SSSS
16	2007	Corumbá	9	M	56	9	SSSS
17	2009	Corumbá	4	M	57	1	NR
18	2009	Corumbá	4	M	69	1	SSSS
19	2007	Corumbá	1	M	81	9	SSSS
20	2008	Paranhos	5	F	11	9	SRSS
21	2008	Ponta Porã	1	M	52	1	SRSS
22	2009	C. Sapucaia	5	F	28	1	SSSS
23	2008	Corumbá	9	M	30	2	RSSS
24	2010	Corumbá	9	M	40	2	RSSS
25	2009	Ponta Porã	1	M	25	9	NR
26	2009	Ponta Porã	4	M	64	1	SSSS
27	2010	Corumbá	1	F	21	1	RRSS
28	2009	Corumbá	4	M	22	2	NR
29	2009	Corumbá	2	F	27	1	RSSS
30	2009	Aral Moreira	4	M	61	1	SRRS
31	2007	Corumbá	4	M	35	9	SSRS
32	2010	Corumbá	4	M	40	1	RRSS
33	2007	Corumbá	9	M	48	6	SRSS
34	2008	Corumbá	4	F	55	9	NR
35	2010	Corumbá	4	F	65	1	SSSS
36	2007	Mundo Novo	1	M	19	6	SSSS
37	2009	Paranhos	5	F	4	6	NR
38	2009	Paranhos	5	M	11	1	NR
39	2009	Paranhos	5	F	28	6	NR
40	2010	Ponta Porã	1	M	27	2	SSSS
41	2010	Aral Moreira	1	M	41	1	SSSS
42	2010	Corumbá	1	F	29	1	NR
43	2009	Mundo Novo	1	M	19	1	SSSS
44	2008	Corumbá	4	M	76	1	SSSS
45	2009	Corumbá	1	M	23	1	SSSS
46	2007	Corumbá	4	M	36	9	SSSS
47	2010	Corumbá	1	M	36	2	SSSS
48	2008	Corumbá	4	M	49	1	RSSS
49	2010	Corumbá	2	M	48	1	SSSS
50	2010	P. Murtinho	4	F	22	1	NR
51	2009	P. Murtinho	1	M	48	1	RRSS
52	2010	Antônio João	2	M	33	1	SSSS
53	2007	Corumbá	4	M	27	2	SSSS
54	2008	Japorã	5	F	29	1	RSSS
55	2008	Corumbá	2	F	33	1	SSSS
56	2008	Corumbá	1	M	37	2	RRSS
57	2009	C. Sapucaia	5	F	67	1	NR
58	2009	Corumbá	4	M	24	2	RSSS
59	2008	Corumbá	4	F	27	1	SSSS
60	2009	Ponta Porã	4	M	69	1	SSSS

Table 2. *Continued.*

#	Year	SIT[d]	Strain	Octal	Spoligotyping spacers 1-43
1	2007	33	LAM3	776177607760771	■■■■■■■■□□□■■■■■■■■■□□□□■■■■■■■□□□□■■■■■■■
2	2010	33	LAM3	776177607760771	■■■■■■■■□□□■■■■■■■■□□□□■■■■■■■□□□□■■■■■■■
3	2007	33	LAM3	776177607760771	■■■■■■■■□□□■■■■■■■■□□□□■■■■■■■□□□□■■■■■■■
4	2008	34	S	776377777760771	■■■■■■■■□□■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■
5	2009	42	LAM9	777777607760771	■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■□□□□■■■■■■■
6	2009	42	LAM9	777777607760771	■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■□□□□■■■■■■■
7	2009	42	LAM9	777777607760771	■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■□□□□■■■■■■■
8	2010	42	LAM9	777777607760771	■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■□□□□■■■■■■■
9	2008	42	LAM9	777777607760771	■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■□□□□■■■■■■■
10	2010	42	LAM9	777777607760771	■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■□□□□■■■■■■■
11	2007	42	LAM9	777777607760771	■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■□□□□■■■■■■■
12	2009	42	LAM9	777777607760771	■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■□□□□■■■■■■■
13	2007	42	LAM9	777777607760771	■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■□□□□■■■■■■■
14	2008	42	LAM9	777777607760771	■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■□□□□■■■■■■■
15	2009	42	LAM9	777777607760771	■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■□□□□■■■■■■■
16	2007	42	LAM9	777777607760771	■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■□□□□■■■■■■■
17	2009	42	LAM9	777777607760771	■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■□□□□■■■■■■■
18	2009	42	LAM9	777777607760771	■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■□□□□■■■■■■■
19	2007	42	LAM9	777777607760771	■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■□□□□■■■■■■■
20	2008	42	LAM9	777777607760771	■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■□□□□■■■■■■■
21	2008	42	LAM9	777777607760771	■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■□□□□■■■■■■■
22	2009	47	H1	777777774020771	■■■■■■■■■■■■■■■■■■■■■■■□□□□□■□□□□■■■■■■■
23	2008	47	H1	777777774020771	■■■■■■■■■■■■■■■■■■■■■■■□□□□□■□□□□■■■■■■■
24	2010	47	H1	777777774020771	■■■■■■■■■■■■■■■■■■■■■■■□□□□□■□□□□■■■■■■■
25	2009	47	H1	777777774020771	■■■■■■■■■■■■■■■■■■■■■■■□□□□□■□□□□■■■■■■■
26	2009	47	H1	777777774020771	■■■■■■■■■■■■■■■■■■■■■■■□□□□□■□□□□■■■■■■■
27	2010	50	H3	777777777720771	■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■
28	2009	50	H3	777777777720771	■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■
29	2009	50	H3	777777777720771	■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■
30	2009	53	T1	777777777760771	■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■
31	2007	53	T1	777777777760771	■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■
32	2010	53	T1	777777777760771	■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■
33	2007	53	T1	777777777760771	■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■
34	2008	53	T1	777777777760771	■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■
35	2010	53	T1	777777777760771	■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■
36	2007	53	T1	777777777760771	■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■
37	2009	53	T1	777777777760771	■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■
38	2009	53	T1	777777777760771	■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■
39	2009	53	T1	777777777760771	■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■
40	2010	53	T1	777777777760771	■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■
41	2010	119	X1	777776777760771	■■■■■■■■■■■■■■■■□■■■■■■■■■■■■□□□□■■■■■■■
42	2010	172	T1	777777777740771	■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■
43	2009	353	T1	777777774760771	■■■■■■■■■■■■■■■■■■■■■■■■□□■■■■■□□□□■■■■■■■
44	2008	1214	T1	777617777760771	■■■■■■■■■■■■□□■■■■■■■■■■■■■■■■■□□□□■■■■■■■
45	2009	1337	LAM5	777700607760771	■■■■■■■■■■■■□□□□□□■■□□□□■■■■■■■□□□□■■■■■■■
46	2007	1337	LAM5	777700607760771	■■■■■■■■■■■■□□□□□□■■□□□□■■■■■■■□□□□■■■■■■■
47	2010	1337	LAM5	777700607760771	■■■■■■■■■■■■□□□□□□■■□□□□■■■■■■■□□□□■■■■■■■
48	2008	1337	LAM5	777700607760771	■■■■■■■■■■■■□□□□□□■■□□□□■■■■■■■□□□□■■■■■■■
49	2010	1528	LAM9	774377607760771	■■■■■■■■□□■■■■■■■■■□□□□■■■■■■■□□□□■■■■■■■
50	2010	1610	LAM6	763777607560771	■■■■■□□■■■■■■■■■■■■□□□□■■■■□■□■■□□□□■■■■■■■
51	2009	1610	LAM6	763777607560771	■■■■■□□■■■■■■■■■■■■□□□□■■■■□■□■■□□□□■■■■■■■
52	2010	1694	LAM2	277737607760771	□■□■■■■■■■■□■■■■■■■□□□□■■■■■■■□□□□■■■■■■■
53	2007	unknown	unknown	777710607760771	■■■■■■■■■■■■□□□□□□■■□□□□■■■■■■■□□□□■■■■■■■
54	2008	unknown	unknown	700176777760771	■■■□□□□□□□□■■■■■■□■■■■■■■■■■■■■□□□□■■■■■■■
55	2008	orphan	T5	637777557760771	■■□□■■■■■■■■■■■■■□■■□■■■■■■■■■□□□□■■■■■■■
56	2008	unknown	unknown	777577600020771	■■■■■■■■■■□■■■■■■■■□□□□□□□□□□□■□□□□■■■■■■■
57	2009	unknown	unknown	777777607340771	■■■■■■■■■■■■■■■■■■■■■□□□□■■■■□■■□□□□□■■■■■■■
58	2009	orphan	LAM9	777777607360771	■■■■■■■■■■■■■■■■■■■■■□□□□■■■■□■■■□□□□■■■■■■■
59	2008	orphan	unknown	677777470000771	■■□■■■■■■■■■■■■■■■■□□■■■□□□□□□□□□□□□■■■■■■■
60	2009	orphan	LAM6	777737606560771	■■■■■■■■■■■□■■■■■■■□□□□■■□■□■■□□□□□■■■■■■■

(a) Race/skin color: 1: white; 2: black; 3: Asian; 4: mixed black-white; 5: indigenous; 9: unknown.

(b) Institutionalized: 1: non-institutionalized; 2: incarcerated; 6: other; 9: unknown.

(c) DST: drug susceptibility test: R: rifampicin; I: isoniazid; S: streptomycin; E: ethambutol; S: sensitive: R: resistant; NR: not performed.

(d) SIT: Spoligotyping International Type

Eleven isolates were from patients diagnosed in 2007, 13 from 2008, 22 from 2009, and 14 from 2010. Twenty-five patients (41.7%) were mixed black-white, 15 (25.0%) white, 11 (18.3%) indigenous, five (8.3%) black, and six (6.7%) of unknown skin color. Male-to-female ratio was roughly 2:1, with 41 male patients (68.3%). Mean age was approximately 37 years, and age distribution was as follows: 4-19 years: seven patients (11.7%); 20-29 years: 21 (35.0%); 30-39 years: 10 (16.7%); 40-49 years: 10 (16.7%); 50-59 years: four (6.7%); 60-69 years: six (10.0%); 70 years and older: two patients (3.3%). One patient was 4 years old and one was 81 years of age; eight individuals (13.3%) were prison inmates. Fourteen previously described spoligotypes accounted for 52 isolates (86.7%). Of the remaining eight isolates, four had genotypes with undefined SITs and four had new genotypes, to be included in the SITVITWEB database. According to this database, LAM was the predominant family, with 30 genotypes (50.0%), followed by families T (subfamilies T1 and T5), with 15 (25.0%), Haarlem (H), with eight (13.3%), and one case (1.7%) each for families X (subfamily X1) and S. LAM9 (31.7%), T1 (23.3%), and H1 (8.3%) were the most frequent subfamilies. Families East-African–Indian (EAI), Central and Middle Eastern Asia (CAS), and Beijing were not identified (Table 3).

The 52 isolates with 14 pre-existing SITs were distributed by strain number and percentage, genotypic line, and county. Of these 52, seven (13.5%) had unique profiles and 45 (86.5%) were grouped into seven clusters consisting of two to 17 strains, comprising genotypes of subfamilies LAM9 (SIT42), T1 (SIT53), H1 (SIT47), LAM5 (SIT1337), LAM3 (SIT33), H3 (SIT50), and LAM6 (SIT1610). The clustered strains were from Corumbá, Porto Murtinho, Paranhos, Ponta Porã, and Coronel Sapucaia (Table 4).

Table 3. *Frequency of genotypic features in 60 isolates subjected to spoligotyping.*

Families (substrains and SIT)	Subfamilies			Shared types		
	Name	N	%	SIT	N	%
LAM (*n* = 28; 46.7%)	LAM2	1	1.7	1614	1	1.7
	LAM3	3	5.0	33	3	5.0
	LAM5	4	6.7	1337	4	6.7
	LAM6	2	3.3	1610	2	3.3
	LAM9	18	30.0	42	17	28.3
				1528	1	1.7
T (*n* = 14; 23.3%)	T1	14	23.3	53	11	18.3
				172	1	1.7
				353	1	1.7
				1214	1	1.7
H (*n* = 8; 13.3%)	H1	5	8.3	47	5	8.3
	H3	3	5.0	50	3	5.0
X (*n* = 1; 1.7%)	X1	1	1.7	119	1	1.7
S (*n* = 1; 1.7%)	S	1	1.7	34	1	1.7
Orphan (*n* = 4; 6.7%)	LAM6	1	1.7	U	1	1.7
	LAM9	1	1.7	U	1	1.7
	T5	1	1.7	U	1	1.7
	Unknown	1	1.7	U	1	1.7
Unknown (*n* = 4; 6.7%)	Unknown	4	6.7	U	4	6.7

SIT: Spoligotyping International Type.

Table 4. *Genotypic and epidemiological features of the 52 strains with pre-existing Spoligotyping International Type (SITs).*

SIT	Strains investigated		Families or subfamilies	Cluster *vs.* unique profile	Counties of provenance
	N	%			
33	3	5.0	LAM3	Cluster	Corumbá
34	1	1.7	S	Unique	P. Murtinho
42	17	28.3	LAM9	Cluster	Corumbá, C. Sapucaia
47	5	8.3	H1	Cluster	Ponta Porã, Corumbá
50	3	5.0	H3	Cluster	Corumbá
53	11	18.3	T1	Cluster	Paranhos, Corumbá
119	1	1.7	X1	Unique	Aral Moreira
172	1	1.7	T1	Unique	Corumbá
353	1	1.7	T1	Unique	Mundo Novo
1214	1	1.7	T1	Unique	Corumbá
1337	4	6.7	LAM5	Cluster	Corumbá
1528	1	1.7	LAM9	Unique	Corumbá
1610	2	3.3	LAM6	Cluster	P. Murtinho
1694	1	1.7	LAM2	Unique	Antonio João

Paraguay-bordering counties accounted for 41.7% (25/60) of isolates. Corumbá, which also borders Bolivia, accounted for 58.3% (35/60). Of the 42 clustered isolates with pre-existing SITs, spoligotypes SIT42, SIT53, and SIT47 were shared by both border regions. SIT1610 was found only on the Paraguayan border (in Porto Murtinho), while SIT33, SIT50, and SIT1337 were found only on the Bolivian border (Corumbá).

In Corumbá, all genotypes, except SIT53, were identified in at least one inmate, and an epidemiological link was detected between two inmates in the same prison, infected with the same genotype (SIT47).

Drug susceptibility testing, performed on 80% of isolates (48/60), revealed resistance in 35.4% (17/48), with a predominance of monoresistance (25.0%, or 12/48). SIT53 exhibited a variety of patterns: monoresistance (to isoniazid and rifampicin), polyresistance (to streptomycin + isoniazid), and multidrug resistance (to rifampicin + isoniazid). Two clusters showed resistance to streptomycin and isoniazid, but an epidemiologic link was found only for a patient infected with the SIT47 genotype (Table 5).

4. Discussion

This was the first study to identify the circulating genotypical strains of *M. tuberculosis* isolated from patients with pulmonary tuberculosis in the region where Mato Grosso do Sul borders Paraguay and Bolivia. A steady human flow in this region, whether for economic activities, tourism, or access to healthcare services not offered across the border, facilitates the spread of tuberculosis.

Obstacles to tuberculosis control are greater than in non-border areas, given the limited access of patients to healthcare services (resulting in poor clinical conditions and maintenance of the transmission chain) and a lack of binational efforts to implement control measures in the region [4]-[5]. Specifically on the border with Bolivia, the situation is aggravated by the
permanent human flow through Corumbá. In the Americas, Bolivia has one of the highest incidence rates for tuberculosis and ranks among the top ten countries with highest multidrug resistance rates [14].

Table 5. *Distribution of the 17 resistant strains, by profile and clustering.*

Resistant strains: 17/48 (35.4%)	%	Resistance pattern	SIT	Family
7	14.6	S	Orphan	LAM9
			50	H3
			Unknown	Unknown
			47[(2)]	H1[(2)]
			42	LAM9
			1337	LAM5
			42[(2)]	LAM9[(2)]
4	8.3	I	34	S
			53	T1
			53	T1
4	8.3	S+I	Unknown	Unknown
			50	H3
			1610	LAM6
1	2.1	R	53	T1
1	2.1	R+I	53	T1

On the border with Paraguay, pulmonary tuberculosis has the highest incidence and mortality rates among indigenous residents, whose treatment dropout rates were 2.5 times higher than for non-indigenous patients [3]. These elevated dropout rates result from the absence of geographical barriers, a feature that, while allowing local residents to seek healthcare services where these are more readily available, can weaken the establishment of ties with the service, particularly when notification services lose track of patients [4]-[5].

For its prominence as a risk area for drug resistance [15], the border region has been given technical priority since 2007 for diagnosing the disease and mapping drug resistance. These efforts have led to the confirmation of 35% of 661 cases of pulmonary tuberculosis by culture, as well as to the investigation of drug resistance in 30% of this universe—an initiative made possible by the decentralization of culture seeding in Ogawa–Kudoh medium, now performed locally [16], and by the investigation, using molecular biology procedures conducted elsewhere, of seeded biological materials from the region.

The concerted effort to apply molecular biology techniques to the isolates proved insufficient to preclude sample losses, because these were stored at -20 °C, as opposed to the recommended storage at -70 °C in a freezer or -196 °C in liquid
nitrogen [17]. The problem was further compounded by long storage times, of three to six years.

In the selected sample of patients, most were male (2:1) and of working age (20 to 50 years)—a population profile similar to those found elsewhere in Mato Grosso do Sul [18] and in the Brazil–Paraguay–Argentina border region [19].

In Mato Grosso do Sul, pulmonary tuberculosis was heterogeneously distributed across race/skin color categories, but the indigenous population was among the most affected [18], in whom children aged up to nine years accounted for 13.5% of cases *versus* less than 5% in other race/skin color categories, translating to a higher number of pediatric cases in the present study.

Spoligotyping revealed that families LAM, T, and H accounted for 86.6% of cases, mirroring other studies conducted in Latin America [13], [20]-[21]. A comprehensive study comprising 11 Brazilian states also found a predominance of families LAM (46.0%), T (18.6%), and H (12.1%) [22]. The predominant strains on both border regions (LAM, T and H) were similar to those circulating in Africa, the Caribbean, Oceania, the Americas, Asia (predominantly in Armenia), and Europe (particularly in Italy, Spain, France, Portugal, Poland, Germany, Russia, Austria, and Bulgaria), as well as in Middle Eastern countries [13]. In the present investigation, four new profiles, in addition to genotypes rare in Brazil and South America, were detected, which may have reached the region as a result of migratory flows from Europe, Asia, and Africa in the early 20th century. Corumbá was the port of entry for Arabs, Syrians, Lebanese, Turks, Armenians, and Greeks arriving by way of the Río de la Plata, as well as for Uruguayans, Paraguayans, Bolivians, and Argentinians [23]-[24].

In the present study, roughly 50% of genotypes belonged to family LAM, which is predominant in Latin America (39.5% in Chile [20], 52.3% in Paraguay [25], 38.3% in Argentina [26], 53.0% in Venezuela [27], 37.4% in Colombia [28], and 52.1% along the Brazilian border with Argentina and Paraguay [19]. Family T occurred in 23.3% of isolates—a rate akin to those found for Santa Catarina (23.4%) [29] and a group of 11 Brazilian states (18.6) [22]. Prospective studies conducted at a Brazilian referral hospital for tuberculosis [30]-[31] found a predominance of T strains. In the present study, SIT53 was the predominant spoligotype (78.6%) in family T, as also found in Rio de Janeiro, where this genotype accounted for two-thirds of the isolates assigned to this family [22]. Family T spoligotypes have a global distribution [13], [32].

The family Haarlem was detected in 13.3% of isolates in the present study, a rate similar to those obtained in Santa Catarina (12.8%) [29] and in a group of 11 Brazilian states (12.2%) [22], whereas in Bolivia was the most frequent family (39.4%) [33], and the second-most-frequent in Paraguay and Argentina [25, 26]. The family Haarlem is also widely distributed, having been detected in the Americas, Africa, Europe, and Asia [13, 32]. In these studies, the variability in LAM, T, and H rates may not only stem from differences in sampling methods, but also be shaped by historical factors, particularly migratory flows in each region, including a marked Spanish heritage in Paraguay and Bolivia.

In the present investigation, the SIT42 genotype, of global distribution, predominated (28.3%) at a higher rate than the averages found for Brazil (8.8%) and neighboring countries (11.8% in Venezuela and 5.6% in Peru) [13], [34]-[35]. Other LAM genotypes have been detected in Portugal, Belgium, France, Germany, Ireland, the Netherlands, Spain, and Switzerland, as well as in parts of Africa [36].

A common trait between the present findings and those of three surveys covering 13 of the 27 Brazilian states [22], [29], [37] was the presence of SITs 33, 34, 42, 47, 50, and 53, also detected in Venezuela [38], French Guiana [39], and Paraguay [25] and, except for SIT34 and SIT42, also in Bolivia [33]. By contrast, SIT172, SIT353, SIT1214, and SIT 1528 have been found to circulate exclusively in the international border areas of Mato Grosso do Sul state, in addition to new genotypes revealing the timeliness of the present findings and highlight the need to expand this study over a longer timeframe and include a higher number of strains.

A high rate of clustering, of 75.1% (45/60), was found, comprising seven groups—higher than the 67.5% rate obtained for Minas Gerais [35]. Of the clustered genotypes, SIT42, SIT53, and SIT47 were common to both border areas, while SIT1610 was found only on the Paraguayan border (Porto Murtinho county). SIT33, SIT50, and SIT1337 were found only on the Bolivian border (Corumbá county). SIT 42 was the most abundant clustered genotype. In Corumbá, this genotype had been found in 11 strains in 2007, but was not detected in subsequent years. This pattern warrants further investigation, since other spoligotypes have remained detectable.

A study that employed restriction fragment length polymorphism (RFLP) in Mato Grosso do Sul revealed persistence of a particular genotype for 12 years in the same community [7].

Another finding was a case of multidrug-resistant tuberculosis with SIT53, the second most frequent spoligotype, which has been implicated in cases of multidrug- resistant tuberculosis in Europe [40], South Africa [41], Argentina [26], Peru [42], Brazil, Chile, Colombia and Venezuela [43]. In Brazil this genotype has found in in São Paulo [37] and Santa Catarina [44], with similar patterns of resistance.

The data on household, place of abode, neighborhood of residence, and drug- resistance profile available from the Brazilian Information System for Notifiable Hazards (SINAN) enabled the establishment of an epidemiological link and evaluation of the persistence of circulating clustered spoligotypes in each period. On the border with Paraguay, a cluster comprising four strains with genotype SIT42 was found in the indigenous settlement of Taquapery (Coronel Sapucaia county), and a cluster comprising three strains with genotype SIT53, of unknown drug susceptibility, was detected in a woman and her children in the indigenous settlement of Arroio Corá (Paranhos county). A recent investigation [45] evidenced that differences in socioeconomic status contribute to the risk of tuberculosis transmission in indigenous communities, indicating that tuberculosis prevention should address the social factors that generate unequal health outcomes in this population. A substantial proportion of transmission in two indigenous communities has occurred between individuals who are not members of the same household. These findings underscore the importance of expanding contact investigations outside of households to improve control in indigenous communities in Mato Grosso do Sul state.

On the border with Bolivia (Corumbá county), a cluster comprising three SIT33 strains was found for two patients residing in the same neighborhood and a prison inmate, indicating possible transmission between community and

prison [46]. Another cluster, comprising two SIT47 strains, was detected for two inmates, aged 39 and 40 years. Both isolates were monoresistant to streptomycin, which may indicate endogenous reactivation of a long-established strain resistant to this drug [40], or show recent infection in one of these inmates within two years of contact with a source of resistant microorganism.

As shown by the present results, the establishment of an epidemiological link between some patients lends support to the hypothesis that cases caused by bacilli sharing the same genotype and resistance profile pertain to the same chain of transmission, possibly traceable to epidemiological surveillance measures that proved ineffective in interrupting this chain [8]. Confirming this hypothesis, however, would require additional molecular biology resources.

Limitations to the present study included the use of biological materials and delays in obtaining sensitivity test results. Nonetheless, the investigation paves the way for further research on a region not previously studied. Issues discussed in this report can be more thoroughly investigated using techniques such as mycobacterial interspersed repetitive units–variable number of tandem DNA repeats (MIRU-VNTR) and typing via restriction fragment length polymorphisms (RFLP) based on the insertion sequence IS*6110*, ultimately expanding the current knowledge of the pulmonary tuberculosis transmission chain in the border region investigated.

5. Conclusion

In conclusion, this study provided an overview, albeit limited, of the genetic diversity of *M. tuberculosis* isolated from patients with pulmonary tuberculosis in a region where Brazil borders Bolivia and Paraguay. It was also the first study to identify genotypes circulating in this area, revealing a high clustering rate and a high rate of resistance to first-line drugs. In addition, it provided evidence that control measures have proven insufficient to stop the circulation of certain genotypes of families LAM, T, and Haarlem in the region.

Ethical Approval

The study was approved by the Research Ethics Committee of the Universidade Federal de Mato Grosso do Sul and the National Committee for Research Ethics of the Brazilian Health Council (permits 141 949/2012 and 507-049/2013, respectively).

Conflict of Interests

The authors declare the absence of conflicts of interest regarding the publication of this paper.

Acknowledgements

The authors wish to express their thanks to the teams engaged in tuberculosis surveillance and laboratory services in the counties investigated; to the LACEN-MS team, who processed part of the material and made their strain bank available; to the laboratory team of the Universidade Estadual Paulista at Araraquara, who performed the exams requiring molecular biology techniques; and to Maria do Socorro Nantua Evangelista, for the support provided to the project under the terms of the Agreement for Technical Cooperation 120/2010, which partially covered the project's costs. Thanks are also given to the National Coordination of the Tuberculosis Control Program of the Brazilian Ministry of Health – National Fund for Healthcare, for the financial support provided.

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