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Mycobacterium tuberculosis Spoligotypes in Monterrey, Mexico▽

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Although tuberculosis is still a public health problem in Mexico, there is little information about the genetic characteristics of the isolates. In the present study, we analyzed by spoligotyping 180 *Mycobacterium tuberculosis* clinical isolates from the urban area of Monterrey, Mexico, including drug-susceptible and drug-resistant isolates. The spoligotype patterns were compared with those in the international SITVIT2 spoligotyping database. Four isolates presented spoligotype patterns not found in the database (orphan types); the rest were distributed among 44 spoligo international types (SITs). SIT53 (clade T1) and SIT119 (clade X1) were predominant and included 43 (23.8%) and 28 (15.5%) of the isolates, respectively. In order to determine if there was a dominant spoligotype in the group of multidrug-resistant isolates, 37 of them were analyzed by IS*6110*-based restriction fragment length polymorphism assays, and scarce clustering of strains with more than five bands was observed. Fourteen isolates of this multidrug-resistant group presented four bands or less and were distributed in four SITs: SIT53 ($n = 8$), SIT92 ($n = 3$), SIT70 ($n = 2$), and SIT3038 ($n = 1$). When the molecular detection of mutations in the *katG* and *rpoB* genes were analyzed in these isolates with low copy numbers of IS*6110*, only two isolates shared the same IS*6110*, spoligotyping, and mutations patterns. When the distribution of the spoligotypes was analyzed by age cohort, SIT119 was predominantly found in patients 0 to 20 years old, especially in males, accounting for up to 40% of the isolates. In contrast, SIT53 was more prevalent in older females. This analysis demonstrates the variability of *M. tuberculosis* isolates in Monterrey and the partial dominance of SIT53 and SIT119 in that area of Mexico.

Despite the efforts to control tuberculosis (TB), it is still one of the most important causes of morbidity and mortality in the world. About 9 million new TB cases and approximately 2 million TB deaths were reported in 2004 (26). Although Mexico is not considered a country with a high tuberculosis burden, the number of cases has remained unchanged in recent years. The city of Monterrey is located in northeast Mexico. In 2005, the population of the city was estimated to be 1,133,814 and its metropolitan area had a population of 3.8 million, making it the third largest city in the country. Every year about 1,000 new cases of tuberculosis are reported in the metropolitan area (Boletin Epidemiologico, Secretaria de Salud, Mexico, www.dgepi.salu.gob.mx), and about 250 cases per year are referred to the Jose E. Gonzalez Hospital. Despite the high number of new cases, the genetic diversity of the *Mycobacterium tuberculosis* isolates in that region is little known.

Diverse techniques have been developed to study the epidemiological distribution of the disease, from case-contact studies to the application of molecular techniques, such as for the determination of variations in specific loci (3, 9), the determination of conserved deletions of long stretches of DNA (14), and analysis of the distribution of single nucleotide polymorphisms (6). These methods have allowed the identification of specific outbreaks, the classification of isolates into families, and the spread to or within human populations.

One of the most simple methods for the subtyping of *M. tuberculosis* isolates is spoligotyping (3, 9). This method detects variations in the direct-repeat (DR) locus, which consists of a repeated 36-bp sequence interspersed with nonrepetitive 31- to 41-bp DNA segments called spacer sequences. The DR region is amplified by PCR and the amplicon is hybridized to probes that detect the specific sequences of the spacers. A specific pattern of recognition of the spacers is called a spoligotype. The identification of these allows us to study the phylogeographical distribution of isolates.

In the present work we studied the genetic diversity of *M. tuberculosis* clinical isolates from the Monterrey metropolitan area by analyzing their spoligotypes. A selected group of multidrug-resistant (MDR) isolates was studied by IS*6110*-based restriction fragment length polymorphism (IS*6110*-RFLP) assays, and mutations conferring rifampin and isoniazid resistance were characterized.

(This paper fulfills part of the requirements for a master of science in public health for E.M.-T.)

MATERIALS AND METHODS

Mycobacterial strains and DNA isolation. The *M. tuberculosis* isolates ($n = 180$) were obtained from the urban area of Monterrey, mostly from patients attending either the Laboratorio Estatal de Salud Pública or the tuberculosis

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TABLE 1. Descriptions of the four orphan strains

Isolate no.[a]	Yr of isolation	Strain	Spoligotype	Octal code	Clade[b]	Age (yr)
MEX011998200001	1998	00001	■■□■■■■■■■■■■■■■■■■□□□□□□□□□□□□□□□□□□□□□□□□□	677777600000000	Unk	23
MEX012005100009	2005	00009	■■□■■■■■■■■■■□□□□□□□□■■■■■■■■■□□□□■□■■■■■■■■■	677740077413771	EAI2-Manila	31
MEX012005100041	2005	00041	■■■■■■■□□□□□■■■■□■■■■■■■□□□□■■■■■■■■□□□□■■■■■■■	770167607760771	LAM3	58
MEX012005100052	2005	00052	■■■■■■■■■□□■■■■□■■■■■■■■■■■■■■■■■□□□□□□□■■■■■■	776357777700371	Unk	50

[a] All four isolates were from Mexican men.
[b] Clade designations are according to those used in the SITVIT2 database and use revised SpolDB4 rules. Unk, unknown patterns within any of the major clades described in the SITVIT2 database.

clinic at the Hospital Universitario, José E. Gonzalez U.A.N.L, in Monterrey, Mexico, from 1999 to 2005. Demographic data for the patients, including age, gender, and geographic origin, were obtained. We also determined the drug susceptibility patterns of the isolates by using the agar proportion method. The bacteria were grown on Lowenstein-Jensen medium, and DNA isolation was carried out by the method of Van Embden et al. (22).

Spoligotyping analysis. Spoligotyping was carried by the standard technique described previously (5, 9). The DR region was amplified with the oligonucleotides DRa (5′-GGTTTTGGGTCTGACGAC-3′; biotinylated 3′ end) and DRb (5′-CCGAGAGGGGACGGAAAC-3′). The biotinylated PCR products were hybridized to a membrane containing a set of 43 oligonucleotides corresponding to each spacer. DNAs from *M. tuberculosis* H37Rv and *M. bovis* BCG were used as controls. The hybridized PCR products were incubated with streptavidin-peroxidase conjugate, and the membrane was then exposed to the chemiluminescence system, followed by exposure to X-ray film, according to the manufacturer's instructions. The X-ray film was developed by standard photochemical procedures.

IS*6110*-RFLP analysis of multidrug-resistant strains. In order to determine the predominant strains present in the multidrug-resistant group, 37 isolates selected on the basis of the period of time when they were obtained (1998 to 2003) were analyzed by IS*6110*-RFLP analysis. IS*6110*-RFLP analysis was carried out by using Southern blot transfer-DNA hybridization with an IS*6110* probe, according to the international standard method (22). Briefly, chromosomal DNA was extracted by the cetyltrimethylammonium bromide method, digested with PvuII, and separated by electrophoresis in an agarose gel. After electrophoresis, DNA fragments were blotted onto a nylon membrane with a vacuum blotter and were hybridized with a peroxidase-labeled PCR product specific for the right arm of the IS*6110* element. As a control, we utilized DNA from *M. tuberculosis* strain 14323. An ECL direct labeling and detection system (Amersham Biosciences) was used for probe labeling and detection of the hybridization signals. The films (Hyperfilm ECL; Amersham) were scanned, and the patterns were compared as described below.

IS*6110* DNA fingerprint interpretation. The IS*6110* fingerprint analysis was performed with Bionumerics software, version 3.5 (Applied Maths, Kortrijk, Belgium). The comparison of the fingerprint patterns was calculated by using the Dice coefficient with a band tolerance of 1.5% and an optimization value of 0.75%. Isolates with clustered IS*6110*-RFLP banding patterns containing more than five bands were considered related.

Detection of genetic changes associated with resistance in low-copy-number isolates. The relationships among isolates with more than five copies were easily determined with the IS*6110* patterns and spoligotypes. However, for low-copy-number isolates the relationships were not conclusive, and we decided to detect point mutations associated with resistance in the *katG* and *rpoB* genes by using PCR, followed by nucleotide sequence analysis. For *katG*, we utilized primers TB86 (5′-GAAACAGCGGCGCTGGATCGT) and TB87 (5′-GTTGTCCCAT TTCGTCGGGG) (21). The *rpoB* gene was amplified with primers TR8 (5′-TG CACGTCGCGGGGACCTCCA) and TR9 (5′-TCGCCGCGATCAAGGAGT) (8, 21). The product sizes were 209 bp and 157 bp for *katG* and *rpoB*, respectively. After sequencing of the PCR products, BLASTn software was used for DNA sequence comparisons.

Database comparison of spoligotyping results. Spoligotypes in binary format were entered into the SITVIT2 database (Pasteur Institute of Guadeloupe), which is an updated version of the previously released SpolDB4 database (available online at http://www.pasteurguadeloupe.fr:8081/SITVITDemo). At the time of the present study, the SITVIT2 database contains data on 71,000 *M. tuberculosis* isolates from 160 countries of origin (and mycobacterial interspersed repetitive-unit–variable-number tandem repeats [MIRU-VNTRs] for about 11,000 isolates). In this database, the spoligo international type (SIT) numbers designate the spoligotypes shared by two or more patient isolates, whereas "orphan" designates patterns reported for a single isolate. Major phylogenetic clades were assigned according to the signatures provided in the SpolDB4 database, which defined 62 genetic lineages/sublineages (2). These include specific signatures for various *M. tuberculosis* complex members, as well as rules defining major lineages/sublineages for *M. tuberculosis sensu stricto*.

Statistical analysis. In order to determine potential differences in the prevalence of specific spoligotypes according to the ages of the patients, mean ages were compared by statistical analysis by the Student *t* test.

RESULTS

Characteristics of population studied. A total of 180 isolates from samples collected from 1998 to 2005 were included in this study; 11, 14, 9, 41, 104, and 1 samples were obtained in 1998, 1999, 2003, 2004, 2005, and 2006, respectively. They were from 53 women with ages ranging from 16 to 79 years and 127 males with ages ranging from 15 to 81 years. In the case of 4 women and 15 men, the age was unknown. Of all isolates, 86 isolates were susceptible to both isoniazid and rifampin, 34 were resistant to one of these drugs, and 60 were resistant to both drugs.

Comparison of the spoligotypes with those in the international database. When the spoligotypes of our isolates were compared with those in the international spoligotype database of the Institute Pasteur of Guadalupe, we found that four isolates were not identified in the SITVIT2 database: one corresponded to the EAI2-Manila family, one corresponded to the LAM3 (Latin American-Mediterranean) family, while two of them were of unknown origin (Table 1). The remaining 176 isolates were distributed in 50 shared types or SITs (Table 2). Twenty-four isolates presented unique SITs, while the rest were clustered in groups of 2 to 43 isolates each. SIT53 (T1 subclade) represented 23.89% of all isolates, followed in predominance by SIT119 (X1 subclade), which constituted 15.56% of all strains. We observed newly created shared types, which are SITs that matched another orphan in the database or that were due to two or more strains belonging to a new pattern in this study: SIT3034 matched an orphan from Poland, SIT3035 matched an orphan from Brazil, SIT3036 matched an orphan from The Netherlands, SIT3037 was created by two isolates in the present study, and SIT3038 matched an orphan from the United States. The orphan strains had octal numbers of 677777600000000 (unknown clade), 677740077413771 (EAI2-Manila), 770167607760771 (LAM3), and 776357777700371 (unknown family).

When the isolates were analyzed by family or clade (according to the SpolDB4 database (2), most of the isolates were distributed in the T superfamily, which included 41.6% of the isolates. The T families correspond to the modern *M. tuberculosis* strains with an ill-defined spoligotype signature in the SpolDB4 database (2), which contains more than 600 unclas-

TABLE 2. Descriptions of the 50 shared types from this study[a]

SIT[b]	Spoligotype description	Octal no.	Total no. of isolates in this study	% of the isolates in this study	% of isolates in this study compared to the no. in SITVIT2	Clade[c]	Clustered vs unique pattern[d]
3		000000007720771	2	1.11	2.22	H3	Clustered
19		677777477413771	2	1.11	0.26	EAI2-Manila	Clustered
20		677777607760771	5	2.78	0.74	LAM1	Clustered
33		776177607760771	1	0.56	0.11	LAM3	Unique
34		776377777760771	1	0.56	0.14	S	Unique
42		777777607760771	2	1.11	0.09	LAM9	Clustered
46		777777770000000	1	0.56	0.66	Unk	Unique
47		777777774020771	4	2.22	0.34	H1	Clustered
49		777777777720731	1	0.56	0.75	H3	Unique
50		777777777720771	2	1.11	0.08	H3	Clustered
52		777777777760731	5	2.78	0.82	T2	Clustered
53		777777777760771	43	23.89	0.91	T1	Clustered
64		777777607560771	2	1.11	0.71	LAM6	Clustered
70		700076777760671	3	1.67	2.68	X3	Clustered
73		777737777760731	4	2.22	2.16	T	Clustered
92		700076777760771	8	4.44	2.13	X3	Clustered
119		777776777760771	28	15.56	2.77	X1	Clustered
120		777777577760771	1	0.56	16.67	T1	Unique
137		777776777760601	1	0.56	0.10	X2	Unique
168		777777777720671	1	0.56	5.00	H3	Unique
176		777737607560771	2	1.11	5.88	LAM6	Clustered
207		767777777720771	3	1.67	16.67	H3	Clustered
211		776137607760771	6	3.33	8.00	LAM3	Clustered
217		777736777760771	3	1.67	10.00	T	Clustered
232		777777760000171	1	0.56	14.29	Unk	Unique
239		777777777760031	3	1.67	5.88	T2	Clustered
336		777776777760731	2	1.11	2.99	X1	Clustered
409		264073777777600	1	0.56	1.96	BOV_2	Unique
449		777776757760771	1	0.56	9.09	X1	Unique
450		777776770000000	1	0.56	1.20	Unk	Unique
478		617776777760601	4	2.22	12.90	X2	Clustered
519		777777777740371	1	0.56	5.56	T1	Unique
522		777777777760770	1	0.56	7.69	T1	Unique
573		777777663560771	1	0.56	12.50	T	Unique
1211		576377777760771	9	5.00	69.23	S	Clustered
1215		677735607760771	3	1.67	37.50	LAM	Clustered
1224		776177607760700	1	0.56	25.00	LAM3	Unique
1236		777777760000611	2	1.11	11.11	Unk	Clustered
1277		777777207760771	1	0.56	5.26	LAM9	Unique
1329		777776777560771	1	0.56	1.28	X1	Unique
1629		777777774020751	1	0.56	16.67	H1	Unique
2356		717776777760771	1	0.56	33.33	X1	Unique
2403		677737601760771	1	0.56	25.00	LAM	Unique
2914		677776777760771	3	1.67	60.00	X1	Clustered
3034*		776600037760771	1	0.56	50.00	Unk	Unique
3035*		777763607560771	1	0.56	50.00	LAM6	Unique
3036*		777777777760710	1	0.56	50.00	T2-Uganda	Unique
3037*		777777777560031	2	1.11	100.00	T2	Intra
3038*		617777777760601	1	0.56	50.00	Unk	Unique

[a] A total of 44 SITs containing 170 isolates matched a preexisting shared type in the SITVIT2 database, whereas 5 SITs (containing 6 isolates) were newly created either in our study or after a match with an orphan from the database.

[b] An SIT followed by an asterisk indicates a newly created shared type after a match with another orphan in the database or is due to two or more strains belonging to a new pattern within this study: SIT3034 matched an orphan from Poland, SIT3035 matched an orphan from Brazil, SIT3036 matched an orphan from The Netherlands, SIT3037 was created by two isolates in the present study, and SIT3038 matched an orphan from the United States.

[c] Clade designations according to the SITVIT2 databse and by use of the revised SpolDB4 rules. Unk, unknown patterns within any of the major clades described in SITVIT2 database.

[d] Clustered strains correspond to a similar spoligotype pattern shared by two or more strains in this study, whereas unique strains harbor a spoligotype pattern that does not match that of another strain from this study. Unique strains matching a preexisting pattern in the SITVIT2 database are classified as SITs, whereas in the case of no match, they are designated orphans.

sified SITs. On the basis of single-spacer differences, the T superfamily was previously divided into five subclades (subclades T1 to T5) and eight nested clades named after their presumed geographical specificities: T3-Ethiopia (ST149), T5-Russia/1 (ST254), T1-Russia/2 (ST280), T3-Osaka (ST627), T5-Madrid/2 (ST58), T4-Central Europe/1 (ST39), T2-Uganda (ST135), and Tuscany (ST1737). In our study, we found the T, T1, T2, and T2-Uganda patterns (Table 2). We found the X

TABLE 3. Descriptions of predominant shared types found in this study and their worldwide distribution in the SITVIT2 database

SIT (clade)[a]	Octal no.	Total no. (%) in this study	% in this study compared to no. in SITVIT2 database	Distribution (%) in regions with ≥5% of a given SIT[b]	Distribution (%) in countries with ≥5% of a given SIT[c]
20 (LAM1)	677777607760771	5 (2.78)	0.74	AMER-N, 25.48; AMER-S, 24.29; AFRI-S, 13.26; EURO-S, 11.77; EURO-W, 8.49; CARIB, 6.41	USA, 23.70; BRA, 14.75; NAM, 9.24; PRT, 7.30; VEN, 6.26
47 (H1)	777777774020771	4 (2.22)	0.34	AMER-N, 21.76; EURO-W, 20.91; EURO-S, 14.39; AMER-S, 10.67; EURO-E, 8.72	USA, 20.15; AUT, 10.67; ITA, 7.62; BRA, 6.10; CZE, 5.00
52 (T2)	777777777760731	5 (2.78)	0.82	EURO-W, 22.50; AMER-N, 19.70; EURO-S, 7.72; ASIA-W, 7.72; EURO-E, 6.57; EURO-N, 6.24; AFRI-M, 5.42	USA, 17.57; BEL, 7.22; FRA, 6.57; ITA, 5.09
53 (T1)	777777777760771	43 (23.89)	0.91	AMER-N, 19.58; AMER-S, 13.83; EURO-W, 12.75; EURO-S, 10.06; ASIA-W, 8.65; AFRI-S, 5.93	USA, 17.23; ZAF, 5.79; ITA, 5.18
73 (T)	777737777760731	4 (2.22)	2.16	AMER-N, 22.16; EURO-S, 20.54; AFRI-S, 14.05; EURO-W, 12.97; AMER-S, 10.81	USA, 19.46; ITA, 18.38; ZAF, 14.05
92 (X3)	700076777760771	8 (4.44)	2.13	AFRI-S, 50.27; AMER-N, 25.00; AMER-S, 9.84; EURO-N, 5.32	ZAF, 50.27; USA, 22.34; BRA, 5.85
119 (X1)	777776777760771	28 (15.56)	2.77	AMER-N, 70.26; AFRI-S, 14.13	USA, 62.25; ZAF, 14.13; MEX, 7.31
211 (LAM3)	776137607760771	6 (3.33)	8.00	AMER-N, 66.67; AMER-S, 14.67; EURO-S, 10.67	USA, 42.67; MEX, 24.00; BRA, 10.67; ESP, 6.67
478 (X2)	617776777760601	4 (2.22)	12.90	AMER-N, 80.65; CARIB, 9.68; EURO-N, 6.45	USA, 61.29; MEX, 19.35; TTO, 6.45
1211 (S)	576377777760771	9 (5.0)	69.23	AMER-N, 84.62; EURO-N, 7.69; EURO-S, 7.69	MEX, 76.92; ESP, 7.69; USA, 7.69; SWE, 7.69

[a] The predominant shared types (SITs) were defined as SITs representing 2% or more strains in a given data set (i.e., four or more strains in this study).
[b] The worldwide distribution is reported only for regions with ≥5% of a given SIT compared to their total number in the SITVIT2 database. The definition of macrogeographical regions and subregions is according to the United Nations (http://unstats.un.org/unsd/methods/m49/m49regin.htm); Regions: AFRI (Africa), AMER (Americas), ASIA (Asia), EURO (Europe), and OCE (Oceania) are subdivided into E (eastern), M (middle), C (central), N (northern), S (southern), SE (southeastern), and W (western). Furthermore, CARIB (Caribbean) belongs to the Americas, while Oceania is subdivided into four subregions: AUST (Australasia), MEL (Melanesia), MIC (Micronesia), and POLY (Polynesia). Note that in our classification scheme, Russia has been attributed a new subregion by itself (northern Asia) instead of being included among the rest of Eastern Europe. This reflects its geographical localization and is also due to the similarity of specific *M. tuberculosis* genotypes circulating in Russia (a majority of which are Beijing genotypes) to those prevalent in Central, Eastern, and Southeastern Asia.
[c] The three-letter country codes are according to http://en.wikipedia.org/wiki/ISO 3166-1 alpha-3; the countrywide distribution is shown only for SITs with ≥5% of a given SIT compared to their total number in the SITVIT2 database.

family of spoligotypes to be second in frequency, producing 28.8% of the cases: X1, 20%; X2, 2.77%; and X3, 6.1%. Within the LAM lineage we found 26 cases (14.4%). Among the families with more than 10 isolates was the Haarlem family, which comprised 7.7% of the isolates ($n = 14$). Unexpectedly, three isolates belonging to the Manila family (two SIT19 isolates and 1 orphan SIT isolate) were observed. In order to determine if the two SIT19 isolates were clonally related, we performed RFLP-IS*6110* analysis and observed identical spoligotypes and IS*6110* patterns; however, the patients from whom these isolates were recovered had no epidemiological links. We also detected the TBD1 gene fragment associated with this ancestral lineage by PCR. None of the patients with the Manila spoligotype were of Asian origin or had traveled to places where this clade is common. Isolates belonging to SIT1 or the Beijing family were not found among the 180 clinical isolates analyzed.

A description of the predominant spoligotypes in our study (patterns shared by 2% or more of the isolates) and their worldwide distribution in the SITVIT2 database (Table 3) showed that a total of 10 SITs predominated (representing 116/180 isolates, or 64.4% of all isolates); and corresponded to the following (in decreasing order): SIT53-T1 ($n = 43$, 23.8%), SIT119-X1 ($n = 28$, 15.56%), SIT1211-S ($n = 9$, 5.0%), SIT92-X3 ($n = 8$, 4.44%), SIT211-LAM3 ($n = 6$, 3.33%), SIT20-LAM1 ($n = 5$, 2.78%), SIT52-T2 ($n = 5$, 2.78%), SIT47-H1 ($n = 4$, 2.22%), SIT73-T ($n = 4$, 2.22%), and SIT478-X2 ($n = 4$, 2.22%). Interestingly, the bulk of these spoligotypes predominated in North America, including SIT119 and SIT53, the most predominant spoligotypes, which represented 70.3% and 19.6% of all reported cases in SITVIT2 database from North America, respectively (62.25% and 17.23% from the United States, respectively). Interestingly, the strains belonging to the LAM lineage (SIT20 and SIT211) were most commonly found in the Americas (North and South America), while the 3rd-highest proportion of predominant spoligotypes was from Europe. Lastly, four spoligotypes represented more than 5% of the worldwide recruitment of a given spoligotype from Mexico (Table 3) and corresponded to SIT119-X1 (7.3%), SIT211-LAM3 (24.0%), SIT478-X2 (19.35%), and SIT1211-S (76.92%).

IS*6110*-RFLP analysis of multidrug-resistant isolates and molecular characterization of mutations associated with resistance. In order to determine if any specific RFLP types predominated within our population of MDR strains, we performed IS*6110*-RFLP analysis with 37 multidrug-resistant isolates (Fig. 1) collected from 1998 to 2003 for an MDR characterization study in Monterrey. Only four of the isolates with four or more copies of IS*6110* were identical by both typing methods. When we checked their epidemiological data,

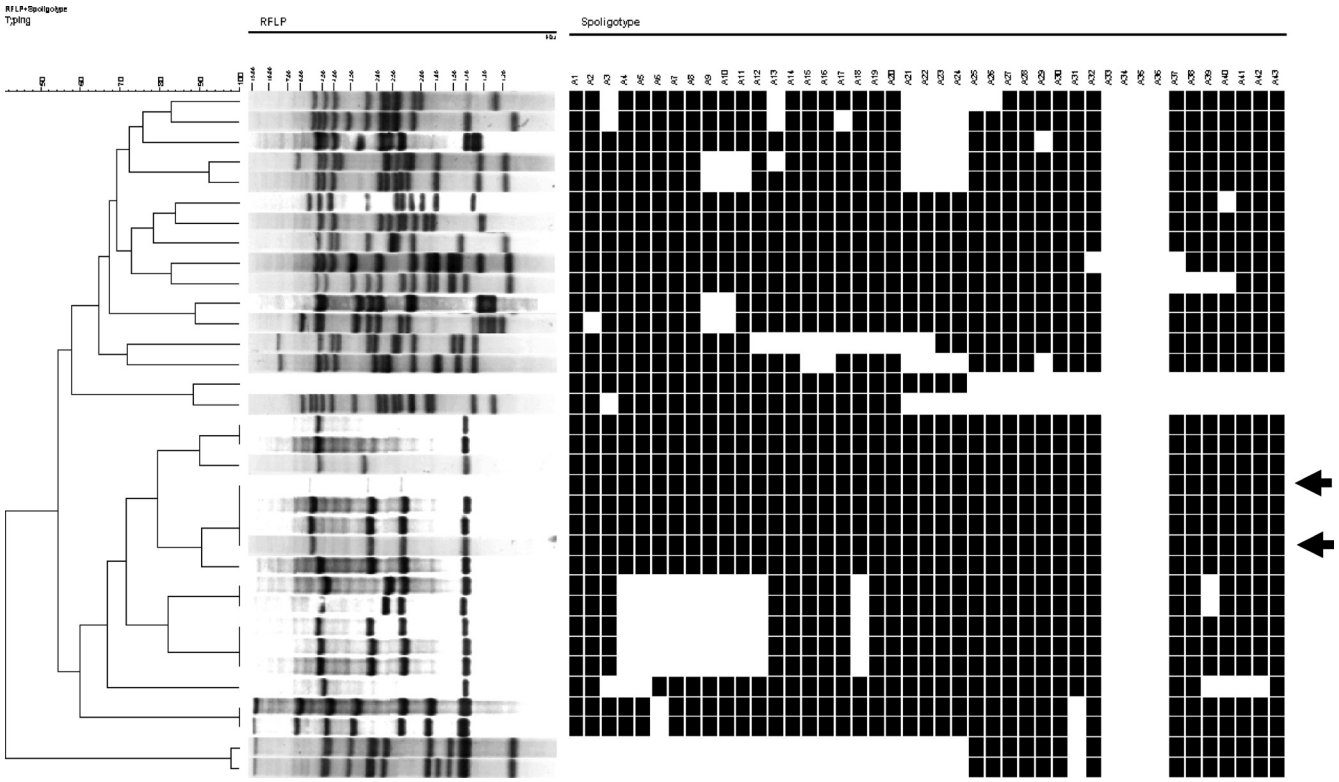

FIG. 1. IS*6110*-RFLP and spoligotyping analyses of 37 drug-resistant isolates from Monterrey, Mexico. Isolates are aligned according to their RFLP-IS*6110* patterns. Arrows on the right indicate MDR isolates 528-98 and 67-99, which share an IS*6110*-RFLP pattern, spoligotype, and mutations in the *katG* and *rpoB* genes.

we observed that they could not be considered close contacts. We observed the abundance of isolates with four or more copies in this group (14 of 37, 38%) (Fig. 1); they were distributed in four SITs: SIT53 (8/14, 57%, T1 subclade), SIT70 (2/14, 14.2%, X3 subclade), SIT92 (3/14, 21.4%, X3 subclade), and SIT3038 (1/14, 7.1%, unknown subclade). When these low-copy-number were analyzed for the presence of mutations in the *katG* and *rpoB* genes (Table 4), we found point mutations in the *rpoB* gene in 71% (10 of 14) of the isolates and point mutations in the *katG* gene in 50% (7 of 14) of the isolates. We found the Ser315Thr mutation in the *katG* gene in 5 (35%) of the 14 isolates. The S531L and H526Y mutations predominated in the *rpoB* gene (Table 4). Only two isolates shared the same IS*6110*-RFLP patterns, spoligotypes, and mutation patterns, suggesting a link between these cases.

Distribution of predominant spoligotypes by gender, age, and drug susceptibility. Table 5 shows the distributions of SIT53 and SIT119 by several ranges of ages and by gender. SIT53 was more prevalent than all other spoligotypes in women, being isolated from more than 30% of the patients

TABLE 4. Descriptions of the mutations associated with isoniazid and rifampin resistance in the IS*6110* low-copy-number isolates

Isolate identification no.	SIT	Octal no.	Catalase gene		RNA polymerase beta subunit	
			Codon	Amino acid change	Codon	Amino acid change
291-98	53	777777777760771	315	S → T	526	H → Y
528-98	53	777777777760771	315	S → T	526	H → Y
67–99	53	777777777760771	315	S → T	526	H → Y
238–99	53	777777777760771	315	S → T	531	S → L
242–99	53	777777777760771	315	S → T	531	S → L
35–03	53	777777777760771	None	None	None	None
192–03	53	777777777760771	None	None	None	None
227–03	53	777777777760771	None	None	None	None
511–98	3038	617777777760601	None	None	516	D →V
340–98	92	700076777760771	271	T → P	531	S → L
02–99	92	700076777760771	271	T → S	531	S → L
208–99	92	700076777760771	None	None	522	S → Q
434–98	70	700076777760671	None	None	516	D → X
55–99	70	700076777760671	None	None	None	None

TABLE 5. Distributions of SIT53 and SIT119 according to gender and age[a]

Age range (yr)	Female patients			Male patients			Both genders		
	No. of patients	% of isolates		No. of patients	% of isolates		No. of patients	% of isolates	
		SIT53	SIT119		SIT53	SIT119		SIT53	SIT119
0–20	6	66.60	16.60	15	0	40	21	20	35
21–40	24	8.30	8.30	43	18.60	16.20	67	15.30	12.30
41–60	12	33.30	16.60	41	24.30	12.19	53	26.40	13.20
>60	7	42.00	0	14	21.40	14.20	21	28.55	9.50

[a] We had data for 162 isolates from the 180 patients.

over 40 years old. When the SIT prevalence was classified by the mean age of the patients with that SIT, we observed that SIT53 was more prevalent (mean age, 43.82 years) than the rest of the spoligotypes (mean age, 36.69 years) ($P = 0.012$). SIT119 was more commonly seen in younger patients (mean age, 35.44 years) than the rest of the spoligotypes (41.4 years old) ($P = 0.041$).

In the analysis of the spoligotype distribution by drug susceptibility, SIT53 and SIT119 were also predominant. The prevalence rates of SIT53 isolates susceptible, MDR, and resistant to one drug were 27.9, 15, and 29.4%, respectively. The values for SIT119 were 19.7, 11.6, and 11.7%, respectively. The male/female sex ratio for the total study population ($n = 180$) was 2.4, but for the pansusceptible group it decreased to 2. For the MDR group, the sex ratio increased to 2.75, and for the group resistant to one drug, it was 2.77. Spoligotype SIT1211 was highly prevalent in the multidrug-resistant group, comprising 13.3% of all MDR cases. Of a total of nine SIT1211 isolates, eight were MDR.

DISCUSSION

The most predominant spoligotypes found in our study, SIT53 and SIT119, are not the same previously reported for Mexico in the SITVIT2 database (Table 3), although when we analyzed reports from regions neighboring Monterrey, we observed that SIT53 and SIT119 are quite commonly found. In a study conducted in Houston, TX, SIT1 (S1, Beijing family) was the most common spoligotype found (25% of isolates) (16). However, among a total of 1,429 isolates, isolates of SIT119 (X1 sublineage) were recovered from 110 (7.69%) cases and isolates of SIT53 were recovered from 46 (3.2%) cases. Houston has a very large Mexican population, and that would explain in part the abundance of these spoligotypes.

Quitugua et al. (12), studying isolates from the Mexican border with the United States, analyzed the IS*6110*-RFLP patterns and spoligotypes of isolates from 313 patients from border cities in Mexico in the states of Tamaulipas (located beside Nuevo Leon and Texas) and Chihuahua, as well as 606 cases from Texas. They predominantly found SIT119 in the border region of Mexico and the United States, as well as in the interior of Texas, among both susceptible and drug-resistant isolates. SIT53 was scarcely found. In that study, 51% of the patients from Texas were Hispanic, and of those individuals, 57% were born in Mexico.

Soini et al. found in Houston that 20.3% of the total cases studied had from zero to four IS*6110* copies (17). When they analyzed 377 isolates with equal to or less than four copies, a total of 72 spoligotypes were found. Of those spoligotypes detected, SIT119 (S3 subclade) was found in 39 cases and SIT53 (S29 subclade) and SIT92 (S27 subclade) were found in 8 cases each. Ramaswamy et al. (13) found that 10 of 50 isolates from Monterrey had less than six copies (13), although they did not perform spoligotyping with those isolates. In our work, we found that 14 (50%) of the 37 drug-resistant isolates had four copies of IS*6110* or less; 8 belonged to SIT53, 3 belonged to SIT92, 2 belonged to SIT70, and 1 belonged to SIT3038. Although we did not perform IS*6110*-RFLP analysis with all 180 isolates, it seems that low-copy-number isolates are abundant in this region. Warren et al. (24) found that many low-copy-number isolates share identical IS*6110* insertion points and spoligotypes; in contrast, they failed to demonstrate clustering when they analyzed the isolates by MIRU-VNTR on the basis of 12 loci, suggesting that spoligotype determination and IS*6110* positioning are earlier events (24). It is possible that other changes, such as the acquisition of point mutations related to drug resistance, also appear in a later period of time. This was observed in our low-copy-number isolates. When we analyzed the isolates for point mutations in the *katG* and *rpoB* genes, we observed that only two SIT53 isolates (Table 3, isolates 528-98 and 67-99) had identical, IS*6110* fingerprinting, spoligotype, and mutation patterns. The point mutations detected were quite similar to those reported previously in drug-resistant isolates in Monterrey (23). Five of the SIT53 low-number-copy isolates shared the same spoligotype and IS*6110* pattern; it is possible that these SITs were predominant before the introduction of isoniazid and rifampin and that the isolates acquired point mutations after exposure to these drugs in the 1960s and 1970s.

Beijing strains (SIT1) are very common in many parts of the world, but they are rarely reported in Mexico or in the rest of the Latin American region (10, 15). Although our state (Nuevo Leon) is beside Texas, where a high incidence of Beijing isolates have been reported in cities like Houston (25% of total isolates studied), we did not find any. That may be explained by the small Asian population in Monterrey.

In this study we found three isolates that belonged to the EAI2-Manila family. These ancestral isolates are more commonly found in Asian countries, such as Indonesia or the Philippines, where they account for high percentages of the *M. tuberculosis* isolates (4). In Mexico, a few Manila isolates were previously reported (10). The Philippines was a Spanish colony, governed as a territory of the Viceroyalty of New Spain (Mexico) from 1565 to 1821, that was part of the Spanish East Indies. A galleon transporting spices and materials from the

Far East navigated between the Philippines and Acapulco, Mexico, two times a year. It is thus possible that during this period some cases of tuberculosis were imported from the Philippine islands to Mexico.

In conclusion, SIT53 and SIT119 seem to be very predominant in Texas and northern Mexico. The predominance of SIT53 in Monterrey is consistent with that observed throughout the world, where this SIT is the most abundant, representing 17.85% of all *M. tuberculosis* isolates, and it is predominant in places distant from Mexico, such as Madrid and South Africa (2, 7, 20). SIT119, the second most predominant pattern in Monterrey, belongs to the X family (which includes sublineages X1 to X3), a well-characterized IS*6110* family with low band copy numbers prevalent in the United Kingdom, Australia, the United States, South Africa, and former British colonies (2, 20). Historically, until the 1800s, when Texas became part of the United States, Texas and Monterrey belonged to the same region. It is possible that SIT53 and SIT119 were prevalent in that region in past centuries and that the spoligotypes in distant central Mexico may differ, although the spoligotypes from the highly populated region of Mexico City are not available at this time for comparison.

Modern molecular tools have demonstrated the evolution of microorganisms and their association with human migrations. Studies of single nucleotide polymorphisms of *Mycobacterium leprae* have suggested that leprosy originated in Africa and that the Hansen disease cases in the Americas are from European and African descendants as a result of emigration and the slave trade (11). Even though molecular evidence of the presence of *M. tuberculosis* in the pre-Colombian age has been reported (19, 25), controversy over this issue remains. If tuberculosis existed in native populations, the prevalence of specific spoligotypes would shrink significantly, since the population of about 22 million people living in Mesoamerica in 1520 was reduced by 95% by 1600, mainly because of infectious diseases (1). Therefore, the *M. tuberculosis* genetic pool was also reduced by the same proportion. It is possible that isolates with low levels of representation (e.g., those belonging to orphan SITs not found in other places) were predominant at some time, although this deserves further study, perhaps by using spoligotyping of pre-Colombian human remains.

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REFERENCES

1. **Acuna-Soto, R., D. W. Stahle, M. K. Cleaveland, and M. D. Therrell.** 2002. Megadrought and megadeath in 16th century Mexico. Emerg. Infect. Dis. **8:**360–362.
2. **Brudey, K., J. R. Driscoll, L. Rigouts, W. M. Prodinger, A. Gori, S. A. Al-Hajoj, C. Allix, L. Aristimuno, J. Arora, V. Baumanis, L. Binder, P. Cafrune, A. Cataldi, S. Cheong, R. Diel, C. Ellermeier, J. T. Evans, M. Fauville-Dufaux, S. Ferdinand, D. Garcia de Viedma, C. Garzelli, L. Gazzola, H. M. Gomes, M. C. Gutierrez, P. M. Hawkey, P. D. van Helden, G. V. Kadival, B. N. Kreiswirth, K. Kremer, M. Kubin, S. P. Kulkarni, B. Liens, T. Lillebaek, H. M. Ly, C. Martin, I. Mokrousov, O. Narvskaya, Y. F. Ngeow, L. Naumann, S. Niemann, I. Parwati, M. Z. Rahim, V. Rasolofo-Razanamparany, T. Rasolonavalona, M. L. Rossetti, S. Rusch-Gerdes, A. Sajduda, S. Samper, I. Shemyakin, U. B. Singh, A. Somoskov, R. Skuce, D. van Soolingen, E. M. Streicher, P. N. Suffys, E. Tortoli, T. Tracevska, V. T. Vincent, T. C. Victor, R. Warren, S. F. Yap, K. Zaman, F. Portaels, N. Rastogi, and C. Sola.** 2006. *Mycobacterium tuberculosis* complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. BMC Microbiol. **6:**23.
3. **Dale, J. W., D. Brittain, A. A. Cataldi, D. Cousins, J. T. Crawford, J. Driscoll, H. Heersma, T. Lillebaek, T. Quitugua, N. Rastogi, R. A. Skuce, C. Sola, D. Van Soolingen, and V. Vincent.** 2001. Spacer oligonucleotide typing of bacteria of the Mycobacterium tuberculosis complex: recommendations for standardised nomenclature. Int. J. Tuber. Lung Dis. **5:**216–219.
4. **Douglas, J. T., L. Qian, J. C. Montoya, J. M. Musser, J. D. A. Van Embden, D. Van Soolingen, and K. Kremer.** 2003. Characterization of the Manila family of *Mycobacterium tuberculosis*. J. Clin. Microbiol. **41:**2723–2726.
5. **Driscoll, J. R.** 2009. Spoligotyping for molecular epidemiology of the *Mycobacterium tuberculosis* complex. Methods Mol. Biol. **551:**117–128.
6. **Filliol, I., A. S. Motiwala, M. Cavatore, W. Qi, M. H. Hazbón, M. Bobadilla del Valle, J. Fyfe, L. García-García, N. Rastogi, C. Sola, T. Zozio, M. I. Guerrero, C. I. León, J. Crabtree, S. Angiuoli, K. D. Eisenach, R. Durmaz, M. L. Joloba, A. Rendón, J. Sifuentes-Osornio, A. Ponce de León, M. D. Cave, R. Fleischmann, T. S. Whittam, and D. Alland.** 2006. Global phylogeny of *Mycobacterium tuberculosis* based on single nucleotide polymorphism (SNP) analysis: insights into tuberculosis evolution, phylogenetic accuracy of other DNA fingerprinting systems, and recommendations for a minimal standard SNP set. J. Bacteriol. **188:**759–772.
7. **Garcia de Viedma, D., E. Bouza, N. Rastogi, and C. Sola.** 2005. Analysis of *Mycobacterium tuberculosis* genotypes in Madrid: description of two new families specific to Spain-related settings. J. Clin. Microbiol. **43:** 1797–1806.
8. **García de Viedma, D., M. S. Díaz Infantes, F. Lasala, F. Chaves, L. Alcalá, and E. Bouza.** 2002. New real-time PCR able to detect in a single tube multiple rifampin resistance mutations and high-level isoniazid resistance mutations in *Mycobacterium tuberculosis*. J. Clin. Microbiol. **40:** 988–995.
9. **Kamerbeek, J., L. Schouls, A. Kolk, M. van Agterveld, D. van Soolingen, S. Kuijper, A. Bunschoten, H. Molhuizen, R. Shaw, M. Goyal, and J. van Embden.** 1997. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J. Clin. Microbiol. **35:**907–914.
10. **Martinez-Gamboa, A., A. Ponce-de-Leon, A. Galindo-Fraga, M. Bobadilla-del-Valle, M. Kato-Maeda, B. D. Robertson, D. B. Young, P. M. Small, and J. Sifuentes-Osornio.** 2008. Molecular analysis of *Mycobacterium tuberculosis* strains with an intact pks15/1 gene in a rural community of Mexico. Arch. Med. Res. **39:**809–814.
11. **Monot, M., N. Honoré, T. Garnier, R. Araoz, J. Y. Coppée, C. Lacroix, S. Sow, J. S. Spencer, R. W. Truman, D. L. Williams, R. Gelber, M. Virmond, B. Flageul, S. N. Cho, B. Ji, A. Paniz-Mondolfi, J. Convit, S. Young, P. E. Fine, V. Rasolofo, P. J. Brennan, and S. T. Cole.** 2005. On the origin of leprosy. Science **13:**1040–1042.
12. **Quitugua, T. N., B. J. Seaworth, S. E. Weis, J. P. Taylor, J. S. Gillette, I. I. Rosas, K. C. Jost, Jr., D. M. Magee, and R. A. Cox.** 2002. Transmission of drug-resistant tuberculosis in Texas and Mexico. J. Clin. Microbiol. **40:**2716–2724.
13. **Ramaswamy, S. V., S. J. Dou, A. Rendon, Z. Yang, M. D. Cave, and E. A. Graviss.** 2004. Genotypic analysis of multidrug-resistant *Mycobacterium tuberculosis* isolates from Monterrey, Mexico. J. Med. Microbiol. **53:**107–113.
14. **Reed, M. B., V. K. Pichler, F. McIntosh, A. Mattia, A. Fallow, S. Masala, P. Domenech, A. Zwerling, L. Thibert, D. Menzies, K. Schwartzman, and M. A. Behr.** 2009. Major *Mycobacterium tuberculosis* lineages associate with patient country of origin. J. Clin. Microbiol. **47:**1119–1128.
15. **Ritacco, V., B. López, P. I. Cafrune, L. Ferrazoli, P. N. Suffys, N. Candia, L. Vásquez, T. Realpe, J. Fernández, K. V. Lima, J. Zurita, J. Robledo, M. L. Rossetti, A. L. Kritski, M. A. Telles, J. C. Palomino, H. Heersma, D. van Soolingen, K. Kremer, and L. Barrera.** 2008. *Mycobacterium tuberculosis* strains of the Beijing genotype are rarely observed in tuberculosis patients in South America. Mem. Inst. Oswaldo Cruz **103:**489–492.
16. **Soini, H., X. Pan, A. Amin, E. A. Graviss, A. Siddiqui, and J. M. Musser.** 2000. Characterization of *Mycobacterium tuberculosis* isolates from patients in Houston, Texas, by spoligotyping. J. Clin. Microbiol. **38:**669–676.
17. **Soini, H., X. Pan, L. Teeter, J. M. Musser, and E. A. Graviss.** 2001. Transmission dynamics and molecular characterization of *Mycobacterium tuberculosis* isolates with low copy numbers of IS*6110*. J. Clin. Microbiol. **39:**217–221.
18. Reference deleted.
19. **Sotomayor, H., J. Burgos, and M. Arango.** 2004. Demonstration of tuberculosis by DNA ribotyping of *Mycobacterium tuberculosis* in a Colombian prehispanic mummy. Biomedica **24:**18–26.
20. **Stavrum, R., M. Mphahlele, K. Ovreås, T. Muthivhi, P. B. Fourie, K. Weyer, and H. M. Grewal.** 2009. High diversity of *Mycobacterium tuberculosis* genotypes in South Africa and preponderance of mixed infections among ST53 isolates. J. Clin. Microbiol. **47:**1848–1856.

21. **Telenti, A., N. Honore, C. Bernasconi, J. March, A. Ortega, B. Heym, H. E. Takiff, and S. T. Cole.** 1997. Genotypic assessment of isoniazid and rifampin resistance in *Mycobacterium tuberculosis*: a blind study at reference laboratory level. J. Clin. Microbiol. **35:**719–723.
22. **Van Embden, J. D. A., E. Cave, J. T. Crawford, J. W. Dale, K. D. Eisenach, B. Gicquel, P. Hermans, C. Martin, R. McAdam, T. M. Shinnick, and P. M. Small.** 1993. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendation for a standardized methodology. J. Clin. Microbiol. **31:**406–409.
23. **Viader-Salvadó, J. M., C. M. Luna-Aguirre, J. M. Reyes-Ruiz, R. Valdez-Leal, L. del Bosque-Moncayo Mde, R. Tijerina-Menchaca, and M. Guerrero-Olazarán.** 2003. Frequency of mutations in *rpoB* and codons 315 and 463 of *katG* in rifampin- and/or isoniazid-resistant *Mycobacterium tuberculosis* isolates from northeast Mexico. Microb. Drug Resist. **9:**33–38.
24. **Warren, R. M., T. C. Victor, E. M. Streicher, M. Richardson, G. D. van der Spuy, R. Johnson, V. N. Chihota, C. Locht, P. Supply, and P. D. van Helden.** 2004. Clonal expansion of a globally disseminated lineage of *Mycobacterium tuberculosis* with low IS*6110* copy numbers. J. Clin. Microbiol. **42:**5774–5782.
25. **Wilbur, A. K., and J. E. Buikstra.** 2006. Patterns of tuberculosis in the Americas: how can modern biomedicine inform the ancient past? Mem. Inst. Oswaldo Cruz **5:**59–66.
26. **World Health Organization.** 2006. Global tuberculosis control: surveillance, planning, financing. WHO report WHO/HTM/TB/2006.362. World Health Organization, Geneva, Switzerland.