Molecular Assessment, Drug-Resistant Profile, and Spacer Oligonucleotide Typing (Spoligotyping) of *Mycobacterium tuberculosis* Strains From Tamaulipas, México

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Background: Tuberculosis remains a serious global health problem involving one-third of the world population. A wide diversity of *Mycobacterium tuberculosis* strains cause about 1.5 million deaths/year worldwide, but in developing countries, the genetic diversity of *M. tuberculosis* strains remains largely unknown. We conducted a first insight into the population diversity of *M. tuberculosis* strains from Tamaulipas, Mexico. **Methods:** Seventy-two *M. tuberculosis* strains were identified and genetic diversity determined by spoligotyping. Drug sensibility testing and punctual mutations in *inhA*, *ahpC*, *rpoB*, and *katG* genes were assessed. **Results:** Spoligotyping analysis showed a higher prevalence of LAM9 > T1 > Haarlem3 subfamilies among 53 spoligotype patterns.

Key words: tuberculosis; Beijing; spoligotyping; *katG*; *rpoB*

Unexpectedly, five Beijing strains conforming four unique spoligopatterns were recovered. The more frequently isolated strains (LAM9 and T1), but none of the Beijing strains, were found resistant to INH or RIF. Also, no drug resistance was found among Haarlem3 isolates. The *katG*³¹⁵ gene mutation was found in 83% of INH-resistant strains, whereas *rpoB*⁵²⁶ were associated in only 43% of RIF *M. tuberculosis* drug-resistant strains. **Conclusions:** This and other studies report a high rate of orphan spoligotypes, which highlights the need for genotyping implementation as a routine technique for better understanding of *M. tuberculosis* strains in developing countries such as Mexico. J. Clin. Lab. Anal. 28:97–103, 2014. © 2014 Wiley Periodicals, Inc.

INTRODUCTION

Pulmonary tuberculosis (TB) is one of the most serious global health problems. One-third of the world population is infected with *Mycobacterium tuberculosis*, and 10% of these individuals progress to the clinical form of TB during their lifetime. According to the WHO Global TB control report 2011, there are about 8.8 million new cases annually causing 1.1 million deaths worldwide among HIV-negative individuals and 0.35 million deaths from HIV-associated TB (1). In developing countries, a more complex scenario emerges since primary care

centers frequently lack standardized molecular technologies for microbiological identification or epidemiological

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follow-up studies. Furthermore, control of pandemic TB is increasingly complicated because of the emergence of multidrug-resistant TB (MDR-TB) and even extensively drug-resistant TB strains (2).

In Mexico, a developing country with about 113 million people, a total of 15,384 new TB cases were reported to the Public Health Care System in 2010, and 47% of all TB cases originated in only 6 (Baja California Norte, Guerrero, Tamaulipas, Nuevo León, Veracruz y Chiapas) of the 32 Mexican Federal States (3). With the exception of Baja California Norte and Guerrero, the other four states are contiguous and constitute an established route for Mexican and Central American immigrants to the United States. In this scenario, modern molecular approaches to trace transmission or *M. tuberculosis* sub-strain identification are necessary to highlight aspects of TB epidemiology in Mexico.

In addition to the IS6110 DNA fingerprinting technique, further molecular approaches such as spoligotyping, identification of chromosomal deletions in regions of difference (RD) from the *M. tuberculosis* genome, and the use of mycobacterial interspersed repetitive unit-variable number tandem repeats have been used to analyze diversity in clinical *M. tuberculosis* strains (4–6). Since it has been established that the transmissibility rate or pathophysiological aspects of the disease can be influenced by strain genomic background (7), the use of molecular genotyping techniques is mandatory in epidemiological TB studies, in particular in Latin-American countries, where information about the population structure of *M. tuberculosis* isolates is still scarce.

In this article, we summarize data for molecular genotyping of *M. tuberculosis* clinical strains isolated in northern Tamaulipas. In addition to molecular typing, drug susceptibility testing was carried out by conventional microbiologic techniques and their correlation with more frequent DNA mutations in *inhA*, *ahpC*, *rpoB*, and *katG* genes is reported.

MATERIALS AND METHODS

Study Population

Sputum specimens from 72 AFB smear-positive patients with pulmonary TB attending the Hospital General de Reynosa “Dr. Jose Maria Cantú Garza,” Unidad de Medicina Familiar No. 33 (IMSS) and Sanitary Jurisdiction No. IV from 2007 to 2009 were collected. The three medical institutions cover about 600,000 inhabitants and are located in Reynosa, Tamaulipas (26°04'N; 98°17'W), in northern Mexico. As TB is a notifiable disease in Mexico, all diagnosed cases are reported to the local public health authorities. The median time of residence of TB patients was 30 years, and about 70% reported hav-

ing at least 10 years of residence in the region involved in this study. Since the geographic location of Reynosa, Tamaulipas at the Mexican/American border constitutes a required pathway for a high rate of illegal migration to the United States, nonpermanent Mexican or Central American residents and immigrants were excluded from this study.

Specimen Collection and Processing

The sputum samples were decontaminated with a modified Petroff's method, and 200 µl of the processed sediment was inoculated onto two Löwenstein–Jensen (LJ) solid agar tubes and incubated for 8 weeks at 37°C. LJ slants were inspected weekly for growth, and niacin(+) and nitrite production was assessed for AFB(+) colonies.

Mycobacterial DNA Extraction

As previously reported (8), mycobacterial cells were harvested from LJ medium resuspending them in 1 ml of 50 mM Tris-HCl (pH 8.0) and then inactivating them by heating at 85°C for 60 min. Inactivated bacilli were subjected to enzymatic lysis [20 mM Tris-HCl (pH 8.0), 2 mM EDTA, 1.2% Triton X-100, 20 mg/ml lysozyme] incubating for 2 h at 37°C in agitation, and then proteinase K digestion was allowed for 1 h at 55°C. Samples were treated in high-salt Cetyl Trimethyl Ammonium Bromide (CTAB) buffer to dissociate DNA from polysaccharides, and proteins were phenol/chloroform extracted to organic phase. Finally, mycobacterial DNA was ethanol precipitated and resuspended in Tris-Ethylenediaminetetraacetic acid (EDTA) buffer. Samples were stored at –20°C until use.

Molecular Identification of *Mycobacterium*

Species identification among *M. tuberculosis*-complex (MTC) members was done by a previously reported method based on Polymerase Chain Reaction (PCR) amplification of seven genomic RD through the mycobacterial genome (6). Primer pairs were used to amplify 16S rRNA, Rv0577, IS1561', Rv1510, Rv1970, Rv3877/8, and Rv3120 conforming a PCR-typing panel for differentiation of *M. tuberculosis*, *M. africanum* type I, *M. bovis*, *M. bovis* Bacillus Calmette–Guérin (BCG), *M. caprae*, *M. microti*, and even the *M. tuberculosis* “variant” *M. canettii* from *Mycobacterium* other than tuberculosis strains.

Spoligotyping of *M. tuberculosis* Isolates

Spoligotyping was performed as described previously (9). Briefly, the extracted DNA (20 ng)

was subjected to standard PCR reaction including primers to amplify the DR region: DRa (5'-GGTTTGGGTCTGACGAC-3' biotinylated 5' end) and DRb (5'-CCGAGAGGGGACGGAAAC-3' by Iso-gen Bioscience, Maarssen, The Netherlands). PCR products were hybridized with a set of 43 spacer oligonucleotides covalently linked to the Biotin C membranes. Bound fragments were incubated with streptavidin-peroxidase conjugate and then detected by chemiluminescence (ECL detection kit, Amersham, Buckinghamshire, UK). Autoradiograms were developed using standard Kodak photochemical products. Hybridization patterns were converted into binary by visual inspection in two independent readings, and then binary to octal conversion was done as described (5). *Mycobacterium tuberculosis* H37Rv strain and *M. bovis* genomic DNA were used as controls for spoligotyping.

Family Assignment

Spoligopatterns obtained were first matched to the international SpolDB4 database and assigned to corresponding families (10), then unmatched records were analyzed with SPOTCLUST software, using an unsupervised mixture model. This algorithm takes into account recent knowledge of the evolution of the mycobacterial DR region and assigns spoligopatterns following rules from the SpolDB3 database (11).

Drug Susceptibility Testing

For all AFB(+), niacin(+), and nitrite(+) clinical isolates, the resistance to isoniazid (INH) and rifampicin (RIF) was determined by using the indirect proportion method on LJ medium at a critical concentration of 0.2 and 40 µg/ml for INH and RIF, respectively. The pyrosequencing method used for monitoring punctual mutations in regions in the *inhA*, *ahpC*, and *rpoB* genes of *M. tuberculosis* was carried out as previously reported (12), and *katG* mutations at codon 315 by PCR-RFLP were assessed essentially as described in Viader-Salvadó et al. (13).

RESULTS

For this study, we recruited 72 pulmonary TB patients, 48 men (66.65%) and 24 women (33.35%) with a mean age of 42 years (range 19–78 years). HIV/AIDS co-morbidity was found in two cases (2.7%). Niacin(+) and nitrite(+) colonies recovered from LJ slants were unambiguously identified as *M. tuberculosis* by the seven-band pattern of PCR genotyping panel.

Spoligotyping analysis revealed that only 28 strains (38.9%) from 72 isolates have a match to the in-

ternational SpolDB4 database (<http://www.pasteur-guadeloupe.fr:8081/SITVITDemo/>) corresponding to 16 unique Spoligotype International Types (SITs): six strains belonging to the T1 subfamily, four strains for Beijing and H3, three strains for H3-LAM and H1, two strains for X1 and LAM9, and only one for LAM4, LAM1-LAM4, LAM2, and X2 (Table 1).

The remaining 44 isolates (61.1%) belonging to 37 different spoligotype patterns absent in the SpolDB4 database (orphans) were analyzed with SPOTCLUST software to assign them to the most probable subfamilies. In order to analyze data under the same criteria, the 28 spoligotype data matched to the SpolDB4 database were re-assigned to subfamilies according to DB3 rules in SPOTCLUST software. Table 2 summarizes results for full data analysis. Seventy-two isolated *M. tuberculosis* strains conforming 53 different spoligotype patterns were assigned as follows: 14 strains for LAM9, 13 for T1, 9 for Haarlem3, 6 for H37Rv, 6 for X1, 5 were clustered as Beijing strains, 4 for Family34, 3 for Haarlem1, 2 for EAI1, 2 for LAM2, and only one isolate was assigned to each of Family33, Family36, EAI2, LAM1, LAM3, LAM8, T2, and X2 subfamilies.

Isoniazid resistance (INH) was found in 12 strains (16.6%) whereas 7 (9.7%) were resistant to rifampicin (RIF). Only three strains (4.1%) were simultaneously resistant to INH and RIF, belonging to Family34, LAM9, and T1 subfamilies. Of the 12 strains resistant to isoniazid, 4 (33.3%) were LAM9, 2 (16.1%) were T1, and 2 (16.1%) were X1 subfamilies. For rifampicin resistance, three strains (42.8%) were LAM9 and two (28.5%) were T1. No clusters were identified because all of the drug-resistance isolates corresponded to a different spoligotype. LAM9 and T1 subfamilies were at the same time the most frequent strains infecting people (37.5% of isolates) and the strains with the highest rate of drug resistance observed (Table 3). No drug resistance was found in *M. tuberculosis* Beijing ($n = 5$) or Harlem1/3 ($n = 12$) strains in this study.

For the 12 isoniazid-resistant strains, we detected *katG* mutation at codon 315 in ten isolates (83.3%), but not in two cases; also six detected mutations in *katG*³¹⁵ by PCR-RFLP were not associated to isoniazid resistance. We also failed to detect mutations at *inhA* and *ahpC* genes from INH-resistant isolates.

On the other hand, from seven rifampicin-resistant isolates, we detected a double mutation in one LAM9 strain at *rpoB*⁵¹⁶ and *rpoB*⁵²⁶, and two more *rpoB*⁵²⁶ mutations were found in one T1 and one X1 *M. tuberculosis* strains (Table 3). The remaining four RIF-resistant isolates were found “wild type” for *rpoB* 516, 517, 526, 531, and 533 codons.

TABLE 1. *Mycobacterium tuberculosis* Spoligotypes Matched to the SpolDB4 Database

Spoligotype (Octal)	SpolDB4 (SIT)	Lineage	Number of isolates
000000000003661	1,651	BEIJING	1
000000000003761	1,674	BEIJING	1
000000000003771	1	BEIJING	2
757777777760771	154	T1	4
777777777760771	53	T1	2
000000007720771	3	H3	1
757777777720771	99	H3	1
777777777720771	50	H3	2
777777037720771	67	H3-LAM	3
777777774020771	47	H1	3
777777607760771	42	LAM9	2
777776777760771	119	X1	2
777777607760731	60	LAM4	1
677777607760731	1,321	LAM1/4	1
657737607760771	377	LAM2	1
757776777760601	1,270	X2	1

DISCUSSION

Mycobacterium tuberculosis causes about 1.5 million deaths around the world every year (1). BCG vaccination at birth is mandatory for Mexican citizens throughout public health care institutions integrating the Ministry of Health. Despite this, in Mexico, 15,384 new cases of pulmonary TB were reported in 2010, which results in a median incidence of 14.19 (cases/100,000); however, a first insight into the distribution of cases by age makes it clear that in the productive years (>25 years of age) incidence increases gradually, and at age 50–59 years the reported incidence is 28.27, and even 40.59 at 65+ years (3). With a global trend to senescence of the world population, and the accepted concept that tuberculosis is mostly (although not exclusively) an age-related disease, an important increase in prevalence would be expected, and the epidemiological description of *M. tuberculosis* strains/lineages is necessary to contribute to the knowledge of the population structure of *M. tuberculosis* bacilli.

In this line, we conducted an epidemiological study involving people living in the state of Tamaulipas, in northern Mexico. Seventy-two pulmonary TB confirmed cases were recruited for this study, showing a 2:1 ratio between male/female patients, which despite not having a clear explanation, is a consistent finding in similar studies in the world (14, 15).

In this study, only 38.9% of *M. tuberculosis* isolates have a match to the SpolDB4 database, which contains spoligotypes from 122 countries, and 39,295 clinical isolates classified into 1,939 spoligotypes (SITs; 10); however, at the time of this study, the SpolDB4 database had a low number of Mexican isolates ($n = 386$ or 0.98%), which in part is explained by the high rate of unassigned SITs to Mexican samples. In agreement with these data, two

recent studies conducted in Acapulco, Mexico (2011) and in Veracruz, Mexico (2012) also reported a high rate of orphan spoligotypes, 21.7% and 48%, respectively (16, 17).

Despite this low matching to SpolDB4, we identified four *M. tuberculosis* Beijing strains as corresponding to SIT1 ($n = 2$), SIT1674 ($n = 1$), and SIT1651 ($n = 1$). The importance of such Beijing strains relies on hypervirulence, fast spreading, and a high rate of associated drug resistance in other countries (18, 19), but this strain is rarely or not found in Latin-American patients (20).

Since 61.1% (44/72) of *M. tuberculosis* strains involved in this study were absent in the SpolDB4 database, we used SPOTCLUST software to assign the most probable family to all 72-set isolates. SPOTCLUST software uses an unsupervised algorithm to identify potential MTC strains and spoligotype classification following SpolDB3 rules to assign subfamilies (11). From this analysis, 53 unique spoligotype patterns emerged (Table 2) with a high prevalence for LAM9 (19.4%), T1 (18.1%), and Haarlem3 (12.5%), which accounts for 50% of all isolates. Also, five (6.9%) isolates were identified as belonging to the Beijing family, one more data retrieved from the SpolDB4 database. Recently, the first report of a Beijing-type isolate being transmitted in a Mexican rural setting (Huauchinango, Puebla, Mexico) was obtained through analysis of clustered family TB outbreaks (21). Authors believe that the most plausible explanation is that some members of these communities migrated to the eastern United States and became infected with these clones and then returned to their homes and infected their families. It is important to note that a high frequency of Beijing (SIT1) genotype in *M. tuberculosis* isolates has been reported in Houston, Texas (up to 25% of isolates), and in general in the south-eastern United States (22).

TABLE 2. SPOTCLUST Analysis of *M. tuberculosis* Strains^a

Spoligotype (Octal)	Most probable family	<i>P</i>	Number of isolates
557747637743771	Family33	1.000	1
557747770000000	Family34	1.000	1
757767730000000	Family34	1.000	1
757767770000000	Family34	1.000	1
770036770000000	Family34	1.000	1
000000007720771	Family36	1.000	1
000000000002661	Beijing	1.000	1
000000000003661	Beijing	1.000	1
000000000003761	Beijing	1.000	1
000000000003771	Beijing	1.000	2
700046720000001	EAI1	1.000	1
757767636000021	EAI1	1.000	1
657767437412661	EAI2	0.998	1
515347436300241	H37Rv	0.680	2
557347437740661	H37Rv	0.865	1
757346437540661	H37Rv	0.902	1
777777037720771	H37Rv	0.931	2
757767734000661	Haarlem1	1.000	1
777777774020771	Haarlem1	1.000	2
517767637500661	Haarlem3	0.752	1
557747537700751	Haarlem3	0.772	2
757767777720771	Haarlem3	0.773	3
757777777720771	Haarlem3	0.773	1
777777777720771	Haarlem3	0.773	2
677777607760731	LAM1	0.663	1
637737607760771	LAM2	0.817	1
657737607760771	LAM2	0.817	1
556167607740661	LAM3	0.996	1
677777600060771	LAM8	1.000	1
515347206740261	LAM9	1.000	1
557347507740261	LAM9	0.688	3
577777607760751	LAM9	1.000	2
715347606140621	LAM9	1.000	1
757767607760771	LAM9	1.000	1
757777607760731	LAM9	1.000	3
777777607760731	LAM9	1.000	1
777777607760771	LAM9	1.000	2
457307637740261	T1	0.999	1
555347736740261	T1	0.999	2
557747623540261	T1	0.639	1
557767661740021	T1	0.801	1
717767777760661	T1	1.000	1
757767623540661	T1	0.639	1
757767777761371	T1	0.992	1
757777777760771	T1	1.000	3
777777777760771	T1	1.000	2
415307034440001	T2	0.776	1
555346737740261	X1	0.653	1
557766637740261	X1	0.653	1
717746337540261	X1	0.652	1
757766777740661	X1	0.653	1
777776777760771	X1	0.653	2
757767777760601	X2	1.000	1

^aData from 72 *M. tuberculosis* strains belonging to 53 unique spoligopatterns.

TABLE 3. Gene Mutations Associated With Drug-Resistant *M. tuberculosis* Strains

Subfamily ^a (number of isolates)	INH ^b resistance	Gene mutations	RIF ^b resistance	Gene mutations
Family34 (4)	1 (8.33%) ^c	<i>katG</i> (1/1)	1 (14.28%) ^d	None detected
Family36 (1)	1 (8.33%) ^c	<i>katG</i> (1/1)	0	—
H37Rv (6)	1 (8.33%) ^c	<i>katG</i> (1/1)	0	—
LAM9 (14)	4 (33.33%) ^c	<i>katG</i> (4/4)	3 (42.85%) ^d	<i>rpoB</i> ^{516 y 526} (1/3)
T1 (13)	2 (16.66%) ^c	<i>katG</i> (1/2)	2 (28.57%) ^d	<i>rpoB</i> ⁵²⁶ (1/2)
T2 (1)	1 (8.33%) ^c	<i>katG</i> (1/1)	0	—
X1 (6)	2 (16.66%) ^c	<i>katG</i> (1/2)	1 (14.28%) ^d	<i>rpoB</i> ⁵²⁶ (1/1)

^aSPOTCLUST assigned lineage to *M. tuberculosis* strains.

^bIsoniazid- or rifampicin-resistant isolates/total of isolates in the *M. tuberculosis* subfamily.

^cINH relative resistance = number of resistant strains in subfamilies/total of resistant strains (12).

^dRIF relative resistance = number of resistant strains in subfamilies/total of resistant strains (7).

From a recent study in Monterrey, Mexico, it has been described that T1 (SIT53) and X1 (SIT119) were the most frequently isolated strains of *M. tuberculosis* in TB patients, but in contrast, this study failed to identify Beijing strains (SIT1) (23). From this study, we found two *M. tuberculosis* Beijing strains with an identical spoligotype (Octal: 3,771) from sputum samples corresponding to unrelated female patients who were attending two different health institutions; one of the patients refers living for 1 year (for work) in Veracruz, a Mexican federal state with a high rate of TB burden and legal/illegal migration. *Mycobacterium tuberculosis* Beijing spoligotype 000000000003771 has been reported as ubiquitous and epidemic, and accounts for 3,758 entries to the SpolDB4 database (10). Four major spoligopatterns obtained in this study (75776777720771, 557347507740261, 75777607760731, and 75777777760771), each one integrated with three *M. tuberculosis* isolates corresponding to Haarlem3, LAM9, and T1 families, were found as orphans in SpolDB4. Low representation of such EuroAmerican lineages in international spoligotyping databases highlights the relevance of carrying out *M. tuberculosis* genotyping in Latin-American countries.

On the other hand, isoniazid (INH) resistance was found in 16.6% of clinical isolates, and 9.7% were resistant to rifampicin (RIF). Only three strains belonging to Family34, LAM9, and T1 subfamilies were resistant to both drugs, and hence were multidrug-resistant strains (MDR-TB). The MDR-TB prevalence of 4.1% is in accord with recently published data from Veracruz, Mexico (16), but remains below levels found in high-endemic countries such as China, where an MDR-TB prevalence of 12.8% is reported (24). Beijing strains isolated in this study were found not resistant to INH or RIF, and recently it has been reported that in Latin-American countries such as Argentina, or in low-prevalence countries such as the United States, the Beijing strains infecting people are drug sensitive, in contrast with Beijing strains isolated in the former Soviet Union, Vietnam, and South

Africa (25). In China, where Beijing strains account for about 85% of TB cases, a similar rate of drug resistance between Beijing and non-Beijing strains is reported (24).

In this study, LAM9 strains account for 33.3% of RIF resistance and 42.8% of INH resistance. Also, 16.6% of INH and 28.5% of RIF resistance were associated to T1 strains. In some studies, specific spoligotypes were found not associated to drug resistance (26), although in others, MDR-TB was significantly higher in Beijing strains, such as in India (27), Russia (28), and Estonia (29). In a study involving MDR-TB from Poland, the strains of the T1 family (13%), LAM9/LAM9_var (13.1%) were reported as the most frequent isolates (30). Since LAM9/T1 strains are the most frequent *M. tuberculosis* strains causing pulmonary disease in Latin-American countries, the emergence of a high rate of mono/multidrug resistance in these strains must be seriously considered by health authorities.

Finally, molecular methods for drug-resistance detection are still in development and are not recommended to replace conventional susceptibility testing.

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