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Mutation at *embB* Codon 306, a Potential Marker for the Identification of Multidrug Resistance Associated with Ethambutol in *Mycobacterium tuberculosis*

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Ethambutol inhibits arabinogalactan and lipoarabinomannan biosynthesis in mycobacteria. The occurrence of mutations in *embB* codon 306 in ethambutol-susceptible isolates and their absence in resistant isolates has raised questions regarding the utility of this codon as a potential marker for resistance against ethambutol. The characterization of mutations on *embB* 306 will contribute to a better understanding of the mechanisms of resistance to this drug; therefore, the purpose of this study was to investigate the association between *embB* 306 mutations and first-line drug resistance profiles in tuberculosis isolates. We sequenced the region surrounding the *embB* 306 codon in 175 tuberculosis clinical isolates, divided according to drug sensitivity, in three groups: 110 were resistant to at least one first-line drug, of which 61 were resistant to ethambutol (EMB^r), 49 were sensitive to ethambutol (EMB^s) but were resistant to another drug, and 65 were pansensitive isolates (P^s). The associations between *embB* 306 mutations and phenotypic resistance to all first-line drugs were determined, and their validity and safety as a diagnostic marker were assessed. One of the P^s isolates (1/65), one of the EMB^s isolates (1/49), and 20 of the EMB^r isolates (20/61) presented with an *embB* 306 mutation. Four different single-nucleotide polymorphisms (SNPs) at *embB* 306 were associated with simultaneous resistance to ethambutol, isoniazid, and rifampin (odds ratio [OR], 17.7; confidence interval [CI], 5.6 to 56.1) and showed a positive predictive value of 82%, with a specificity of 97% for diagnosing multidrug resistance associated with ethambutol, indicating its potential as a molecular marker for several drugs.

Tuberculosis (TB) is an infectious disease caused mainly by *Mycobacterium tuberculosis*. The global 2014 TB report of the World Health Organization (WHO) estimated there to be 9 million new cases of TB in 2013, with 1.5 million deaths attributed to the disease (1).

The mismanagement of patients and inadequate administration of antimicrobial therapy are the most important factors contributing to the development of drug-resistant TB (DR-TB). According to the WHO, DR-TB has become a major public health problem in several settings and, depending on the particular country, 5% of TB new cases and 20% of previously treated cases exhibit simultaneous resistance to isoniazid (INH) and rifampin (RIF) and an aggravated form of DR-TB known as multidrug-resistant TB (MDR-TB) (2).

Ethambutol (EMB) was introduced in 1961 as a bacteriostatic agent effective against actively growing mycobacteria. It is currently used in combination with other first-line drugs, such as INH and RIF, for the treatment of new TB cases and, depending on the resistance profile, retreatment cases (3). Resistance to EMB has been associated with acquired mutations in the *emb* cluster of three contiguous genes named *embC*, *embA*, and *embB* (4, 5). The *embA* and *embB* genes encode arabinosyl transferases involved in the arabinosylation of arabinogalactan, while *embC* is implicated in the arabinosylation of lipoarabinomannan (6–8).

Several studies have identified mutations at codon 306 of *embB* as the most common alterations in EMB-resistant (EMB^r) isolates (9–20). However, the occurrence of *embB* codon 306 mutations in EMB-susceptible isolates (EMB^s) and the absence of mutations in EMB^r isolates have raised questions regarding the utility of this codon as a potential marker for resistance (11, 19, 21–24). In addition, a significant increase in the proportion of MDR-TB strains bearing *embB* 306 mutations has recently been observed (25, 26), and 10 mutations from codons 306 to 508 of *embB* have been suggested as candidate markers for the prediction of quadruple resistance to INH, RIF, streptomycin (STR), and EMB (27).

The identification of mutations on *embB* 306 from several geographical settings will contribute to a better understanding of the mechanisms of resistance to this antibiotic, help assess the real value of the mutations considered to be potential diagnostic markers of drug resistance against ethambutol and MDR-TB, and finally help understand the mechanisms associated with such resistance. The purpose of this study was therefore to investigate the association between *embB* 306 mutations and first-line drug resistance profiles and to assess the utility of this mutation as a poten-

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tial molecular marker for the diagnosis of resistance to ethambutol and MDR-TB strains of *M. tuberculosis* isolates from Mexico.

MATERIALS AND METHODS

Collection of clinical samples, *Mycobacterium* isolation, and drug susceptibility test. During the period of 2007 to 2014, the public health laboratory of the state of Veracruz and the tuberculosis laboratory of the Tijuana General Hospital, Baja California, Mexico, referred 110 clinical isolates from patients with a positive result for resistance to at least one first-line drug (97 from Veracruz and 13 from Baja California). The control group consisted of 65 isolates from individuals with pansensitive TB. These samples were provided by both institutions from the same locations and during the same period of time as those of the drug-resistant isolates. No outbreaks of tuberculosis occurred during that period in the regions from which the samples were obtained, and the isolates do not have an epidemiological link. The states of Veracruz and Baja California are two of the regions with the highest prevalence of DR-TB in Mexico, and the general hospital and public health laboratory are reference centers that receive samples from their respective state.

Sputum decontamination, primary isolation, and susceptibility testing were performed by the respective laboratories using *N*-acetyl-L-cysteine-NaOH, Lowenstein-Jensen medium, and fluorometric methods (Bactec-MGIT 960; Becton-Dickinson) with the following MICs of first-line drugs: STR, $>$1.0 μg/ml; INH, $>$0.1 μg/ml; RIF, $>$1.0 μg/ml; pyrazinamide (PZA), $>$100 μg/ml; and EMB, $>$5.0 μg/ml.

The susceptibility testing results allowed the creation of three groups: (i) isolates with resistance to any of the first-line drugs, including ethambutol (EMB^r) ($n = 61$); (ii) isolates with resistance to any of the first-line drugs except ethambutol (EMB^s) ($n = 49$); and (iii) a control group with pansensitive isolates (P^s) ($n = 65$).

Patient epidemiological characteristics and ethical concerns. Patient variables, such as age, gender, place of residence, treatment type, cooccurrence of diabetes, cancer, malnutrition, anemia, coinfection by human immunodeficiency virus (HIV), and tobacco, drug, and alcohol consumption were recovered from clinical charts.

No physical interventions were performed on the patients, ethical considerations were strictly observed, and all information collected was confidential, with prior written consent obtained from each patient. Ethical issues derived from this study were overseen by the respective committee of the Public Health Institute of the University of Veracruz.

DNA purification and PCR amplification of *embB* fragment. Extraction of DNA from the clinical isolates was conducted with one loop of cultured mycobacteria, according to a procedure described by van Soolingen (28). DNA was resuspended in nuclease-free water and the concentration determined by spectrophotometry in a NanoDrop 1000 (Thermo Scientific, USA). The DNA solution was stored at −20°C until use.

The 260-bp fragment of the gene *embB*, including codon 306 and the ethambutol resistance-determining region (ERDR) (codons 264 to 349), was amplified by PCR using the primers *embB*F (5′-CGGCATGCGCCG GCTGATTC-3′) and *embB*R (5′-TCCACAGACTGGCGTCGCTG-3′) (29). The PCR mix consisted of 10 mM Tris (pH 8), 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate, 10 pmol of PF and PR primers, 1.25 U of *Taq* polymerase (Promega, USA), 5% glycerol, and 100 ng of DNA template, which was brought with nuclease-free water to a final volume of 25 μl. Amplification was performed in a Veriti thermocycler (Applied Biosystems, USA) using the following cycling parameters: 95°C for 3 min, 35 cycles of 95°C for 40 s, 57°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 3 min.

The products were electrophoretically separated in 2% agarose gels and further purified using Amicon Ultra centrifugal filters (Millipore, Ireland). The final DNA concentration was determined by electrophoresis using the MassRuler low-range DNA ladder (Fermentas, USA).

***embB* gene sequencing of ethambutol-resistant and -sensitive isolates.** The sequencing reactions were performed in forward and reverse directions using 6 μl of BigDye Terminator cycle sequencing kit version 3.1 (Applied Biosystems, USA), 3.2 pM PF or PR primer, and 20 ng of purified PCR product in a final volume of 20 μl. The amplification parameters were 25 cycles of 95°C for 30 s, 50°C for 15 s, and 60°C for 4 min.

Amplified products were purified using the ZR DNA sequencing cleanup kit (Zymo Research, USA), resuspended in Hi-Di formamide (Applied Biosystems, USA), heated to 95°C for 5 min, cooled on ice, and finally loaded onto a 96-well MicroAmp reaction plate (Applied Biosystems).

Sequencing of the DNA products was performed by capillary electrophoresis in a 3500 genetic analyzer (Applied Biosystems, USA). Fluorescence spectra were analyzed with the software Data Collection version 1.01 (Applied Biosystems). Analysis of sequences and the identification of mutations were performed using the Sequencing Analysis version 5.4 and SeqScape version 2.7 programs (Applied Biosystems). The wild-type *embB* gene from *M. tuberculosis* strain H37Rv (GenBank accession no. 886126) was used as the reference sequence. It is important to highlight that sequences were included in the analysis only if they showed quality values of individual mutation and a sample score of $>$20, ensuring that base assignment error was $<$1%.

Genotype characterization. Spoligotyping was performed according to the standard technique (30, 31). As controls, DNA from *M. tuberculosis* H37Rv, *M. tuberculosis* strain CDC1551, and *Mycobacterium bovis* BCG were used. Assignment of spoligotype international type (SIT) and lineage were performed using the database SITVITWEB (32).

Statistics and association with patient epidemiological characteristics. Data analysis for patients and isolates included in the study was performed using descriptive and inferential statistics. Statistical associations were determined by the chi-square test, Fisher's exact test, analysis of variance (ANOVA), and Kruskal-Wallis test. Odds ratios (OR) were calculated like a risk measure. A *P* value of $<$0.05 was considered significant.

RESULTS

Phenotypic characteristics of isolates. A total of 198 isolates were obtained from 2007 to 2014; however, 23 were eliminated because some patients provided double or triple samples. Therefore, 175 isolates from 175 different patients were considered in this study. One hundred ten isolates were resistant to at least one first-line drug, of which 61 were resistant to EMB (EMB^r) and 49 sensitive to EMB (EMB^s) but with resistance to other drugs, and 65 were pansensitive (P^s).

The mean age of the study individuals was 44 years (±15), and 67% were male. The most frequent comorbidity was diabetes mellitus (DM), which occurred in 31% of cases; malnutrition and anemia were found in 6% and 3%, respectively; 11% and 3% of the patients reported the consumption of alcohol and drugs; and retreatment was found in 50% of the individuals. The P^s group showed the lowest percentage of patients with retreatment (8%), and patients with resistance to at least one first-line drug had a strong association with retreatment (odds ratio [OR], 39). No significant differences were observed between the groups in the other host variables analyzed (Table 1).

The drug sensitivity of the group of isolates with resistance to at least one first-line drug most frequently involved resistance to INH and RIF, shown in 82% and 68% of the isolates, respectively, while resistances to STR and PZA were observed in 63% and 39% of the isolates, respectively. Finally, MDR-TB was observed in 65% of the DR-TB strains. Bivariate analysis showed significant associations between resistance to EMB and to RIF (OR, 3.6), MDR (isoniazid plus rifampin [INH+RIF]) (OR, 4.6), PZA (OR, 5.6), and a combination of three or more drugs (OR, 12.8) (Table 2).

Changes in *embB* gene sequences. The *embB* sequence analysis of 175 isolates revealed that 22 had an *embB* 306 mutation. In the EMB^r group, 62% (38/61) of the isolates had no mutation,

TABLE 1 Host variable bivariate analysis in isolates of *M. tuberculosis* by groups

Host variable	Total		Resistance results by group[a]						
			Resistant to at least one first-line drug (EMB^r+EMB^s)		P^s				
	No. (n = 175)	%	No. (n = 110)	%	No. (n = 65)	%	Odds ratio[b]	95% confidence interval	P value
Male gender	118	67	70	64	48	74	0.6	0.3–1.2	0.16[c]
Diabetes mellitus	55	31	34	31	21	32	0.9	0.5–1.8	0.79[c]
Malnutrition	10	6	8	7	2	3	2.5	0.5–12.0	0.32[d]
Anemia	5	3	5	5	0	0	NA[e]	NA	0.16[d]
Drug	5	3	5	5	0	0	NA	NA	0.15[d]
Alcohol addiction	20	11	16	15	4	6	2.6	0.8–8.1	0.13[d]
Tobacco addiction	5	3	3	3	2	3	0.9	0.1–5.3	1.00[d]
Retreatment	87	50	82	75	5	8	39.4	14.2–108.7	<0.001[d]

[a] EMB^r, ethambutol resistant; EMB^s, ethambutol sensitive; P^s, pansensitive.
[b] The OR was calculated by associating the risk of different host variables with drug resistance. The opposite event of each variable was used like a comparison group (male/female; with/without diabetes mellitus; with/without malnutrition; with/without anemia; with/without drugs; with/without alcohol addiction; with/without tobacco addiction; retreatment/new treatment.
[c] By chi-square test. P values of <0.05 are significant.
[d] By Fisher's exact test. P values of <0.05 are significant.
[e] NA, not applicable, as cells with values of 0 do not permit the calculation of OR or confidence interval (CI).

while 38% (23/61) presented changes in three codons: *embB* 306 in 20 isolates, *embB* 328 in two isolates, and *embB* 330 in one isolate. The *embB* 306 codon showed four mutations in two nucleotides: the A→G single-nucleotide polymorphism (SNP) at nucleotide 916, resulting in the amino acid change M→V in 8 isolates; and three SNPs of G→C, G→A, and G→T at nucleotide 918, with the first substitution resulting in the amino acid change M→V and the last two SNPs resulting in the M→I amino acid change, identified in five, five, and two isolates, respectively. The codon *embB* 328 showed the A→G SNP at nucleotide 983, causing the amino acid change D→G; this mutation was found in two isolates. Finally, codon *embB* 330 showed the T→C SNP at nucleotide 988, causing the amino acid change F→L in a single isolate (Table 3).

The *embB* characterization of EMB^s isolates showed only one mutation at codon *embB* 306, and 22% (11/49) of them showed changes in three other codons: (i) the codon *embB* 320 showed the C→G SNP at nucleotide 960, producing the amino acid change F→L, found in seven isolates; (ii) the codon *embB* 326 showed the deletion of C at nucleotide 977 and a frameshift in three isolates; and (iii) codon *embB* 328 showed the A→G SNP, which caused the mutation D→G, found in one isolate (Table 3).

The sequencing of P^s isolates showed only one mutation at codon *embB* 306 at nucleotide 918, with the SNP G→A, resulting in the M→I amino acid change; the remaining 64 isolates showed no mutation (Table 3).

***embB* 306 and phenotypic association to first-line antituberculosis drugs.** The mean number of resistances in the 175 isolates analyzed was 1.9 (standard deviation [SD], 1.8); however, a significant difference from this mean was observed in the isolates that lacked (mean ± SD, 1.6 ± 1.8) and carried (mean ± SD, 3.8 ± 1.2) a mutation at codon *embB* 306 (Kruskal-Wallis, 23.2; P = <0.001). No mutations at codon *embB* 306 were observed in isolates with mono- and biresistance; these were found only in one P^s isolate and in 21 isolates with resistance to three or more drugs. The estimated risk for a strain being resistant to three or more drugs was 52 times higher in patients with a mutation at *embB* 306 (Table 4).

A high percentage of isolates bearing the mutation at codon *embB* 306 also had resistance to INH (95%), EMB (91%), RIF

TABLE 2 Bivariate analysis of resistance to ethambutol and *M. tuberculosis* drug sensitivity profiles

Drug resistance of *M. tuberculosis*[b]	Resistant to at least one drug		Resistance results by group[a]						
			EMB^r		EMB^s				
	No. (n = 110)	%	No. (n = 61)	%	No. (n = 49)	%	Odds ratio[c]	95% confidence interval	P value[d]
INH^r	90	82	53	87	37	76	2.2	0.8–5.8	0.12
RIF^r	75	68	49	80	26	53	3.6	1.6–8.4	<0.01
STR^r	69	63	40	66	29	59	1.3	0.6–2.9	0.49
PZA^r	43	39	34	56	9	18	5.6	2.3–13.6	<0.001
MDR-TB (INH+RIF)	72	65	49	80	23	47	4.6	2.0–11.0	<0.001
≥3 drugs	65	59	51	84	14	29	12.8	5.0–32.0	<0.001

[a] EMB^r, ethambutol resistant; EMB^s, ethambutol sensitive.
[b] INH^r, isoniazid resistant; RIF^r, rifampin resistant; PZA^r, pyrazinamide resistant; EMB^r, ethambutol resistant; STR^r, streptomycin resistant.
[c] The OR was calculated by associating the risk of different drug resistances with ethambutol resistance. The opposite event of each variable was used like a comparison group (INH^r/INH^s; RIF^r/RIF^s; STR^r/STR^s; PZA^r/PZA^s; MDR-TB (INH+RIF)/INH^s+RIF^s; ≥3 drugs/resistant to 1 or 2 drug).
[d] By chi-square test. P values of <0.05 are significant.

TABLE 3 Analysis of *embB* mutations in ethambutol-resistant, ethambutol-sensitive, and pansensitive *Mycobacterium* isolates

Group[a]	Codon	Nucleotide	Polymorphism	aa change	Frequency: No. of isolates	Frequency: %
EMB[r]	306	916	ATG→GTG	M→V	8	13
		918	ATG→ATC	M→V	5	20
			ATG→ATA	M→I	5	
			ATG→ATT	M→I	2	
	328	983	GAT→GGT	D→G	2	3
	330	988	TTC→CTC	F→L	1	2
Isolates without mutations					38	62
Total					61	100
EMB[s]	306	918	ATG→ATA	M→I	1	2
	320	960	TTC→TTG	F→L	7	14
	326	977	Deletion of base C	Frameshift	3	6
	328	983	GAT→GGT	D→G	1	2
Isolates without mutations					37	76
Total					49	100
P[s]	306	918	ATG→ATA	M→I	1	2
Isolates without mutations					64	98
Total					65	100

[a] EMB[r], ethambutol resistant; EMB[s], ethambutol sensitive; P[s], pansensitive.

(86%), MDR-TB (86%), and EMB+INH+RIF (82%). There was a significant association between this mutation and phenotypic resistance against these drugs: i.e., INH OR, 25.6; EMB OR, 27.3; RIF OR, 11; MDR OR, 11.9; and EMB+INH+RIF OR, 17.7. In isolates without drug resistance, mutation at codon *embB* 306 was observed as a protection factor (OR, 0.06) (Table 5).

The potential use of mutations at *embB* 306 as a diagnostic marker led us to calculate specificity with results of >97% and positive predictive values of >82% for the drugs INH, RIF, EMB, combinations INH+RIF (MDR) and EMB+INH+RIF, and for more than three drugs (Table 6).

Genotyping characterization. With the aim of identifying the clonality of the isolates, a sample of the 175 isolates recovered was analyzed by spoligotyping. We calculated a sample size of 93 isolates (expected proportion, 50%; precision, 7%); a stratified random sample was estimated to ensure the representativeness of the isolates with and without *embB* 306 mutations, obtaining 12 samples from the group with *embB* mutations and 81 from the wild-type group (Table 7).

In total, 40 (43%) of the isolates analyzed were singletons, and 53 (57%) were grouped in 14 clusters. From the singletons, 34 were orphans, among which 13 were EMB[r] isolates and four showed an *embB* 306 mutation; six were located at clades Manu2, SIT58 (T5-Madrid), SIT67 (H3), SIT 450, SIT51 (T1), and SIT180 (H3). With the exception of SIT450, the remaining isolates were *embB* 306 wild type.

TABLE 4 Mutations at *embB* 306 and association with the number and combinations of drug resistance in *M. tuberculosis* isolates

Number of resistances (type)[a]	Total no. (%) (n = 175)	No. of isolates (%) in *embB* 306 group: Mutant (n = 22)	No. of isolates (%) in *embB* 306 group: Wild type (n = 153)	Odds ratio[b]	95% confidence interval	P value
P[s]	65 (37)	1 (4)	64 (42)	0.06	0.01–0.50	<0.001[c]
One (EMB, INH, RIF, STR, PZA)	21 (12)	0 (0)	21 (14)	NA[d]	NA	0.08[c]
Two (INH+RIF, INH+EMB, INH+STR, EMB+STR, STR+PZA)	24 (14)	0 (0)	24 (15)	NA	NA	0.04[c]
Three (**INH+RIF+EMB**, **INH+EMB+STR**, INH+RIF+STR, INH+STR+PZA)	17 (10)	7 (32)	10 (7)	6.7	2.2–20.1	<0.001[e]
Four (**INH+RIF+EMB+STR**, INH+RIF+PZA+STR, **INH+RIF+EMB+PZA**)	22 (12)	7 (32)	15 (10)	4.3	1.5–12.2	<0.01[e]
Five (**INH+RIF+EMB+PZA+STR**)	26 (15)	7 (32)	19 (12)	3.3	1.2–9.1	<0.01[e]
≥3 drugs	65 (37)	21 (95)	44 (29)	52	6.8–398.7	<0.001[c]

[a] INH[r], isoniazid resistant; RIF[r], rifampin resistant; PZA[r], pyrazinamide resistant; EMB[r], ethambutol resistant; STR[r], streptomycin resistant. Drug resistances and combinations in bold were found exclusively in isolates with *embB* 306 mutations.
[b] The OR was calculated by associating the risk of having a determinate number of resistances with the presence of an *embB* 306 mutation. The opposite event of each variable was used like a comparison group (none, yes/no; one, yes/no; two, yes/no; three, yes/no; four, yes/no; five, yes/no; ≥3, yes/no).
[c] By Fisher's exact test. P values of <0.05 are significant.
[d] NA, not applicable, as cells with values of 0 do not permit the calculation of OR or confidence interval (CI).
[e] By chi-square test. P values of <0.05 are significant.

TABLE 5 Bivariate analysis of mutations at *embB* 306 and phenotypic drug resistance in *M. tuberculosis* isolates

	Isolates in *embB* 306 group						
	Mutant ($n = 22$)		Wild type ($n = 153$)				
Drug resistance[a]	No.	%	No.	%	Odds ratio[b]	95% confidence interval	*P* value
None	1	5	64	42	0.06	0.0–0.5	<0.01[c]
EMB	20	91	41	27	27.3	6.1–122.0	<0.01[d]
INH	21	95	69	45	25.6	3.4–194.9	<0.01[c]
RIF	19	86	56	37	11	3.1–38.7	<0.01[c]
PZA	9	41	34	22	2.4	1.0–6.2	0.06[d]
STR	15	68	54	35	3.9	1.5–10.2	<0.01[d]
MDR (INH+RIF)	19	86	53	35	11.9	3.4–42.2	<0.01[c]
EMB+INH+RIF	18	82	31	20	17.7	5.6–56.1	<0.01[c]

[a] EMB, ethambutol; INH, isoniazid; RIF, rifampin; PZA, pyrazinamide; STR, streptomycin; multidrug resistance (MDR).
[b] The OR was calculated by associating the risk of having determined drug resistance with the presence of *embB* 306 mutation. The opposite event of each variable was used like a comparison group (no. resistant/resistant; EMB resistant/EMB sensitive; INH resistant/INH sensitive; RIF resistant/RIF sensitive; PZA resistant/PZA sensitive; STR resistant/STR sensitive; MDR (INH+RIF) resistant/MDR sensitive; EMB+INH+RIF resistant/EMB+INH+RIF sensitive).
[c] By Fisher's exact test. *P* values of <0.05 are significant.
[d] By chi-square test. *P* values of <0.05 are significant.

The remaining 53 isolates were grouped in 14 clusters. Five clusters, 1 to 5, had two isolates each, four were orphans, and one was included at SIT20 (LAM1); genotypic and phenotypic characterizations showed that only one isolate was EMB^r, and all were wild type.

Four clusters, 6 to 9, were located with three isolates each, with exception of cluster 9 SIT1246 (H3); the rest of the groups had one strain with an *embB* 306 mutation and included from two to three EMB^r isolates. Cluster 10 with the SIT92 (X3) clade grouped four isolates; only one was EMB^r and had an *embB* 306 mutation.

Two orphan clusters, 11 and 12, included five isolates each. Cluster 11 had one EMB^r isolate with a mutation at *embB* 306. Cluster 12 included two EMB^r isolates, both without any mutation.

Cluster 13 included seven isolates sharing the SIT8 (EAI5) clade, from which five were EMB^r. Only two isolates had an *embB* 306 mutation, and four isolates were from individuals with retreatment. Finally, cluster 14 included 10 isolates sharing the SIT50 (H3) clade, 7 were P^s, three EMB^s, and none had a mutation at *embB* 306 (Table 7).

DISCUSSION

Mexico ranks third in the incidence of TB and accounted for a considerable number of the MDR-TB cases in Latin America in 2013 (33). According to the 2014 national report on mycobacteriosis, of the 16,000 cases of pulmonary TB diagnosed each year in Mexico, close to 200 (1.3%) were DR-TB. Veracruz and Baja California are among the top four states with more DR-TB cases in the country (34, 35). Thus, the samples analyzed could serve as an excellent model to explain the mechanisms that produce resistance against EMB in isolates from Mexico.

According to several authors (11, 19, 21–24), *embB* 306 is the most frequent mutation associated with resistance to EMB; however, only 33% (20/61) of the EMB^r isolates analyzed in this study showed an *embB* 306 mutation, while 62% (38/61) presented no mutation in the region sequenced. These data support the hypothesis that this codon is not the main factor responsible for resistance to ethambutol and indicate the participation of other mechanisms, including mutations outside the ERDR of *embB*, other genes, such as *embC*, *embA*, and the recently described *Rv3806c* and *Rv3792* (22, 27, 38–40). These have important implications for the further characterization of isolates with resistance to ethambutol and for the objective assessment of *embB* 306 mutations as a molecular marker for the diagnosis of resistance to EMB.

Only one of the isolates with resistance to any of the first-line drugs except ethambutol (EMB^s, $n = 49$) had a mutation at *embB* 306; this result agrees with other reports (11, 21, 27) but is con-

TABLE 6 Analysis of mutation in codon 306 of *embB* as a diagnosis test for drug resistance

	Validity (%)				Security (%)[b]			
	Sensitivity		Specificity		PPV		NPV	
Drug resistance[a]	Value	95% CI[c]	Value	95% CI	Value	95% CI	Value	95% CI
INH	23	14–33	99	96–100	95	84–100	55	47–63
RIF	25	15–36	97	93–100	86	70–100	63	55–71
EMB	33	20–45	98	95–100	91	77–100	35	28–42
PZA	21	8–34	90	85–96	73	66–80	78	71–85
STR	22	11–32	93	88–99	68	46–90	65	57–73
MDR (INH+RIF)	26	16–37	97	93–100	86	70–100	65	57–73
EMB+INH+RIF	37	22–51	97	93–100	82	63–100	80	73–86
≥3 drugs	32	20–44	99	97–100	95	84–100	71	64–79

[a] INH, isoniazid; RIF, rifampin; EMB, ethambutol; PZA, pyrazinamide; STR, streptomycin; multidrug resistance (MDR).
[b] PPV, positive predictive value; NPV, negative predictive value.
[c] 95% CI, 95% confidence interval.

TABLE 7 Genotyping characterization of isolates of *M. tuberculosis*

Cluster	No. (%) of isolates	Octal	SIT (clade)	No. by *embB* 306 genotype		No. with drug resistance profile[a]		
				Mutant (n = 12)	Wild type (n = 81)	EMB^r (n = 32)	EMB^s (n = 34)	P^s (n = 27)
Singleton	34 (37)	—[b]	Orphan	4	30	13	10	11
	1 (1)	777777777423771	(Manu2)		1	1		
	1 (1)	777777557760771	58 (T5-Madrid)		1		1	
	1 (1)	777777037720771	67 (H3)		1		1	
	1 (1)	777776770000000	450	1		1		
	1 (1)	777777777760700	51 (T1)		1			1
	1 (1)	677777777720771	180 (H3)		1		1	
Total	40 (43)			5	35	15	13	12
1	2 (2)	575537607600471	Orphan		2		2	
2	2 (2)	775737607560771	Orphan		2		2	
3	2 (2)	775777764020771	Orphan		2			2
4	2 (2)	777747607560771	Orphan		2		2	
5	2 (2)	677776666760771	20 (LAM1)		2	1	1	
6	3 (3)	000000000003771	1 (Beijing)	1	2	2		1
7	3 (3)	000000007720771	3 (H3)	1	2	2	1	
8	3 (3)	777777607760771	42 (LAM9)	1	2	3		
9	3 (3)	777776777720771	1246 (H3)		3		1	2
10	4 (4)	700076777760771	92 (X3)	1	3	1	3	
11	5 (5)	770037777720771	Orphan	1	4	1	3	1
12	5 (5)	700076717760771	Orphan		5	2	1	2
13	7 (8)	400037777413771	8 (EAI5)	2	5	5	2	0
14	10 (11)	777777777720771	50 (H3)		10	0	3	7
Total	53 (57)			7	46	17	21	15

[a] EMR^r, resistant to ethambutol; EMB^s, sensitive to ethambutol; P^s, pansensitive.

[b] —, 000007776070771, 007737507707771, 007737607777771, 007737607760771, 100006034550410, 100006034540410, 175577607540450, 177775601760771, 374177677560431, 555766630000000, 575477627500671, 577666627570671, 575377077600671, 575577677700771, 575577677720471, 575777777720771, 675577477403671, 675567477403671, 676377477417771, 676377666620771, 737767777760771, 757737677540430, 770037777720771, 770037777620771, 770037777307771, 775477677550671, 775477677540671, 775575667700030, 775547666600030, 777767777760771, 775577777760771, 775776777600171, 777737607260771, 777767776000371, 777777774020171, 777767777720770.

trary to that of Plinke et al. (14), who did not find this mutation in ethambutol-sensitive isolates. Moreover, 11 EMB^s isolates showed mutations on three codons: *embB* 320 (7 isolates), which was not associated with ethambutol resistance, and *embB* 326 (3 isolates) and *embB* 328 (1 isolate), which were previously identified in EMB^r isolates (27, 40, 41), including some analyzed here. Last, only one of the 65 P^s isolates had a mutation at *embB* 306 (M→I); this absence of mutations was in agreement with previous reports (14, 21, 26).

In order to explain these ambiguities, it has been reported that resistance to this drug is produced through the acquisition of mutations that interact to produce a range of MICs, from those falling below breakpoint values to those representing high-level resistance (39). It is likely that mutations found in EMB^s isolates fit into the group of polymorphisms below the threshold of the Bactec-MGIT diagnostic system used here. In support of this, it has been reported that the concordance rate for ethambutol resistance determined by nucleotide sequencing is dependent on the particular phenotypic susceptibility test employed (42). These findings have important implications for molecular diagnostic techniques that aim to identify resistance to ethambutol and should encourage the parallel use of phenotypic diagnostic methods.

Ethambutol resistance has been described as an important risk factor in the development of resistance to RIF and MDR (21, 22, 26, 27). In the population analyzed here, phenotypic resistance to ethambutol increased the risk of developing resistance to RIF and MDR almost 4- and 5-fold, respectively. At the molecular level, an *embB* 306 mutation was found in 86% of the MDR isolates and increased the risk of developing MDR almost 12-fold. This represents one of the highest risks observed to date (21, 22, 26, 43). In addition, we found that *embB* 306 mutations increased the risk of combined EMB resistance plus MDR by 17-fold, reflecting a close relationship between *embB* 306 mutations, MDR-TB, and resistance to EMB. One possible explanation for this high observed risk can be found in the work of Safi et al. (41), who demonstrated that experimental transfer of *embB* 306 mutations promotes the appearance of resistance to RIF, INH, and consequently of MDR, and it suggests that these mutations might be involved in changes in cell wall permeability and inhibition of the synergistic effect of anti-TB drugs (43). All this evidence indicates that phenotypic resistance to EMB, and the *embB* 306 mutation in particular, are risk factors that might be relevant in the diagnosis of potential MDR-TB patients, supporting preliminary reports (26, 27, 40).

Other studies have found susceptible isolates with the *embB* 306 mutation (11, 21, 26, 27), and therefore, the identification of this polymorphism may not be a good marker for ethambutol resistance diagnosis. Moreover, given the values reported here and in other studies, it is possible that the clinical use of *embB* 306 mutations is more appropriate as a marker for resistance to MDR.

Regarding its validity as a biomarker, the test identifying *embB* 306 mutations does not have the ability to identify all patients with MDR or with resistance to several drugs, since these conditions are

ruled by mutations on other loci (low sensitivity, 26%). However, the specificity value found indicates that the test might have the ability to identify 97% of patients without MDR and 99% of patients without resistance to three or more drugs. Thus, from an epidemiological point of view, this could be used as a confirmatory test.

From a clinical point of view, the security values suggest the possibility of using this mutation as a potential molecular biomarker; the positive predictive values indicate that 86% of patients with an *embB* 306 mutation (positive result) really have an MDR strain, and 95% are resistant to three or more drugs, which is important for avoiding false positives leading to drug treatment in patients and their adverse effects. However, in order to confirm this hypothesis, it is necessary to analyze the behavior of *embB* 306 mutations in populations with different prevalences of drug resistance.

Spoligotyping analysis showed an important number of orphan strains, nine clades, 12 SITs, and 14 clusters. The clusters SIT42 (LAM9) and SIT8 (EAI5) comprised three and seven strains, respectively, and were the only ones that showed some degree of clonality for EMB^r isolates, but an *embB* 306 mutation was present in only one and two isolates, respectively. These data confirm preliminary information related to the high diversity and increasing presence of East-African Indian (EAI) and Latin-American Mediterranean (LAM) lineages circulating in Mexico and that are frequently associated with MDR-TB (36, 37).

The main limitation of our study was the inability to perform sequencing analysis for other genes related to ethambutol resistance and additional phenotypic tests, such as broth dilution or the proportion method, to correlate the different concentrations of antibiotic with the mutations identified in EMB^s and EMB^r isolates. Experiments that incorporate these analyses are warranted and would help identify the mutations and understand the mechanisms associated with resistance to EMB and MDR.

In conclusion, our results present one of the most detailed characterizations of the *embB* gene in one of the largest collections of isolates of ethambutol-resistant TB from a Latin American country. We report an important absence of *embB* 306 mutations in EMB^r isolates and an array of different mutations in other codons. All of our data support the idea that *embB* 306 mutations are associated with ethambutol resistance but are not the causative marker of this resistance. Moreover, *embB* 306 SNPs seem to be an attractive biomarker for the screening of resistance to several drugs, even MDR itself. More studies are necessary in order to confirm its utility as a potential biomarker.

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