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Mycobacterium tuberculosis Complex Mutations in Drug Resistant Clinical Isolates from Southwest Mexico

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Abstract: Mutations in target genes have been described in *Mycobacterium* tuberculosis Complex (MTBc) drug resistant isolates worldwide. In Mexico, not enough information has been reported in this concern. The aim of this study was to characterize mutations related to resistance to first line drugs in MTBc isolates from Oaxaca, Mexico. MTBc isolates were identified in clinical samples from Tuberculosis (TB) patients. Susceptibility to isoniazid, rifampin, ethambutol, streptomycin and pyrazinamide was tested through nitrate reductase assay. PCR based analysis and sequencing were employed to characterize mutations in *kat*G, *inh*A, *rpo*B, *emb*B, *rrs*, *rps*L and *pnc*A genes. Mutations in *kat*G and the promoter of the *mab*A-*inh*A operon were found in isoniazid resistant isolates. Sequence analysis of Rifampin Resistance-Determining Region in the *rpo*B gene showed novel mutations along this region besides mutations at codons 516, 526 and 531. Polymorphisms at codon 306 *emb*B gene were found in ethambutol resistant isolates. Frequent mutations associated to resistance to streptomycin were characterized in *rrs* and/or *rps*L genes. *pnc*A analysis showed variable number of mutations in resistant and susceptible pyrazinamide isolates. Most frequent mutations related to resistance to first line antituberculous drugs were identified in phenotypically resistant MTBc isolates. New mutations were characterized in *rpo*B, *rrs* and *rps*L genes.

Keywords: Tuberculosis, First-Line-Drugs, PCR, Sequencing

Introduction

According to World Health Organization (WHO) tuberculosis (TB) is one of the top ten causes of death and the leading cause from a single infectious agent, causing 1.2 million deaths around the world. Mexico ranks third in the Americas region, just below Brazil and Peru, with 23 cases per 100,000 persons (WHO, 2019). TB is caused by nine mycobacterial species clustered as *Mycobacterium* tuberculosis Complex (MTBc), namely: *M.* tuberculosis, *M. bovis*, *M. africanum*, *M. microti*, *M. canettii*, *M. caprae*, *M. pinnipedii*, *M. mungi* and *M. orygis*.

Once TB is diagnosed, first line antituberculous drugs are administered: isoniazid (INH), rifampin (RIF), ethambutol (EMB), streptomycin (STR) and pyrazinamide (PZA). Antimycobacterial drugs may inhibit cell wall synthesis (INH, EMB), interfere with DNA replication and protein synthesis (RIF, STR) or acidify cytoplasmic environment altering metabolic pathways (PZA) (Fig. 1) (Cuevas-Córdoba *et al*., 2013a; Malone *et al*., 2016). Increasing number of TB cases is partly due to the transmission of drug resistant strains.

Drug Resistance (DR) in MTBc strains has been explained by mutations occurring in the different genes that encode for target proteins for each first line antituberculous drug: *kat*G and *inh*A (INH), *rpo*B (RIF), *emb*B (EMB), *rrs* and *rps*L (STR) and *pnc*A (PZA) (Abbadi *et al*., 2009; Bakuła *et al*., 2013; Cuevas-Córdoba *et al*., 2013b; Pang *et al*., 2017).

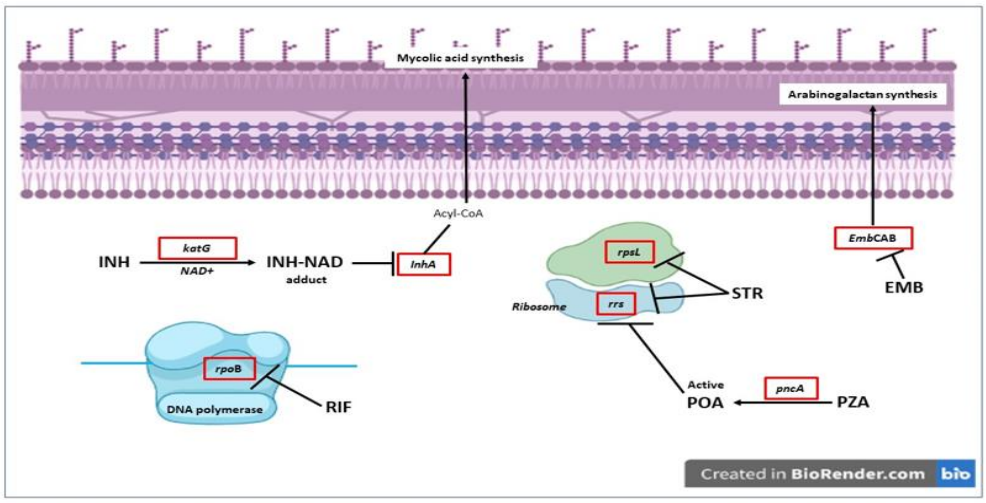

Fig. 1: Metabolic pathways disrupted by first line antimycobacterial drugs. Abbreviations: INH, isoniazid; RIF, rifampin; EMB, ethambutol; STR, streptomycin; PZA, pyrazinamide; POA, pirazinoic acid; NAD, nicotinamide adenine dinucleotide. Adapted from Malone *et al*. (2016)

Reports from different regions around the world have coincide in the occurrence of specific and recurring mutations in those genes. Thus, mutations at codon 315 in the *kat*G gene or within the rifampin Resistance-Determining Region (RRDR) in the *rpo*B gene or in codon 306 of *emb*B gene have been used to identify resistant strains. In contrast, mutations in *rrs*, *rps*L and *pnc*A genes related to DR vary between reports although there have been few coincidences.

In Mexico, molecular data about DR is scarce and restricted to the high TB incidence states of the country. In Oaxaca, located in the southwest of Mexico, TB incidence is above national rate and molecular drug resistance information has not been reported. Therefore, the aim of this study was to characterize mutations related to resistance to first line drugs in MTBc isolates from Oaxaca, Mexico.

Materials and Methods

Sample Collection and Decontamination

Two hundred fifty clinical samples from newly diagnosed TB patients were collected between September 2016 and September 2018 through ten different public health institutions throughout Oaxaca State. Some 199 (79%) samples were pulmonary (sputum) and 51 (21%) were extra-pulmonary (pleural liquid, cerebrospinal fluid, bronchial lavage, biopsy, gastric fluid, pericardial fluid, urine, peritoneal fluid, feces and blood). Pulmonary samples were decontaminated following Petroff's modified method as previously described (Peres *et al*., 2009). Decontaminated samples were used for DNA extraction and nitrate reductase assay.

Ziehl-Neelsen Stain

Clinical samples were smeared on slides and stained by the conventional Ziehl-Neelsen method for the presence of Acid Fast Bacilli (AFB). Slides were covered with 3% basic fuchsin, heated gently until it produced fumes and gently washed with flowing tap water. Slides were then decolorized with acid-alcohol solution (35% chlorhydric acid/95% ethanol) and counterstained with methylene blue dye. Finally, they were observed under a light microscope.

Calculation of positive and negative Agreement

The proportion of agreements between two tests (X and Y) and standard error were calculated using the formulae described by Nagarajan *et al*., 2012:

$$Positive\ agreement = \frac{2a}{2a+b+c}$$

$$Negative\ agreement = \frac{2d}{2d+b+c}$$

$$Standard\ error\ for\ positive\ agreement = \frac{\sqrt{\left[4a(c+b)(a+c+b)\right]}}{(2a+b+c)^2}$$

$$Standard\ error\ for\ negative\ agreement = \frac{\sqrt{\left[4d(c+b)(d+c+b)\right]}}{(2d+b+c)^2}$$

Where:

- a - Number of samples positive by both X and Y tests
- b - Number of samples positive by X test and negative by Y test
- c - Number of samples negative by X test and positive by Y test
- d - Number of samples negative by both X and Y tests

Table 1: Primer sequences

Gene	Primer	Sequence (5'- 3')	Amplicon size
*gyr*B	MTUBf	tcggacgcgtatgcgatatc	1020 pb
	MTUBr	acatacagttcggacttgcg	
katG	KatgOF	gcagatggggctgatctacg	296 pb
	R315mut	tccatacgacctcgatgccag	
inhA	mabAF	cgaagtgtgctgagtcacaccg	146 pb
	inhARmut	agtcaccccgacaacctatta	
*rpo*B	ARMS516	cagctgagccaattcacgga	261 pb
	ARMS526	cgctgtcggggttgtccc	230 pb
	ARMS531	acccacaagcgccgacagtc	216 pb
	CtrlFw	cgaatatctggtccgcttgc	
	ComRv	gtcgaccaccttgcggtacg	537 pb
*emb*B	Emb1F	gggcggggctcaattgcc	324 pb
	Emb2R	gcgcatccacagactggcgtc	
	Emb306A	gacgacggctacatcctgggca	160 pb
	Emb306B	ggtcggcgactcgggcc	210 pb
rrs	PR13F	aaacctctttcaccatcgac	552 pb
	PR30R	caggtaaggttcttcgcgttg	
*rps*L	STR52R	gtcaagaccgcggctctgaa	272 pb
	STR43F	ttcttgacaccctgcgtatc	
*pnc*A	pncA-F	aacagttcatcccggttc	668 pb
	pncA-R	gcgtcatggaccctatatc	

PCR was taken as standard test for comparison of the agreements between Ziehl-Neelsen stain and culture.

Nitrate Reductase Assay

The assay was conducted as previously described (Abilleira *et al*., 2014) using first line drugs concentrations as recommended (0.2 µg/mL isoniazid, 1 µg/mL rifampicin, 100 µg/mL pyrazinamide, 2 µg/mL streptomycin and 7.5 µg/mL ethambutol) (OMS, 2012).

Molecular Assays

All PCR assays described below were conducted in 25 µL volume reaction containing 1*X* Buffer, 25 mM $MgCl_2$, 10 mM dNTPs mix, 1.25 U *taq* polymerase (GoTaq Flexi DNA Polymerase, Promega. Madison, WI, USA), 1.0 µL DNA and the corresponding primers.

Mycobacterium tuberculosis *Complex (MTBc) Identification*

Genomic DNA was extracted using the phenol-chloroform method as described elsewhere (De Almeida *et al*., 2013) and quantified by UV spectrophotometry (Nanodrop Lite, Thermo Scientific. Waltham, MA, USA). DNA was observed by 0.8% agarose gel electrophoresis. MTBc isolates were identified by PCR amplification of a 1020 bp fragment of the *gyr*B gene employing 50 µM MTUBf and MTUBr primers (Table 1) (Abass *et al*., 2010; Chimara *et al*., 2004) and verified by 1% agarose gel electrophoresis. *Mycobacterium* species were identified using Huard's panel as previously described (Huard *et al*., 2003).

Multiplex PCR

Evaluation of mutations associated to isoniazid resistance in *kat*G gene and promoter of the *mab*A-*inh*A operon was conducted *via* multiplex PCR assay as previously described (Herrera-León *et al*., 2005). PCR mix contained 200 µM MTUBf, MTUBr, KatGOF and R315mut primers plus 400 µM mabAF and InhARmut primers (Table 1). Products were analyzed on 1% agarose gel electrophoresis.

Amplification Refractory Mutation System (ARMS)

Mutations in codons 516, 526 and 531 in *rpo*B gene were assessed by ARMS (Fan *et al*., 2003). Three independent PCR reactions were conducted using 50 µM CtrlFw, ComRv and ARMS516 or ARMS526 or ARMS531 primers (Table 1). PCR products were analyzed on 1.5% agarose gel electrophoresis. When mutations were detected in any of the studied codons, a 537 bp fragment was amplified employing 50 µM CtrlFw and ComRv primers and sequenced for further analysis.

Multiplex Allele-Specific PCR Assay (MAS-PCR)

Mutations in the first and third nucleotides of codon 306 *emb*B gene were assessed simultaneously by MAS-PCR (Mokrousov *et al*., 2002). 50 µM Emb1F, Emb2R, Emb306A and Emb306B primers were included in the same PCR reaction (Table 1). PCR products were analyzed on 3% agarose gel electrophoresis. For those isolates in which mutations were detected, a 324 bp fragment was amplified employing Emb1F and Emb2R primers which was sequenced for further analysis.

rrs, rpsL and pncA Amplification

PCR products for *rrs*, *rps*L and *pnc*A genes were obtained using specific primers (Table 1) in independent PCR reaction mixes including 50 µM PR13F/PR30R (*rrs*) or 50 µM STR52R/STR43F (*rps*L) (Cuevas-Córdoba *et al*.,

2013a) or 50 µM pncA-F/pncA-R (*pnc*A) (Pang *et al*., 2017) primers. PCR products were analyzed on 1.5% agarose gel electrophoresis.

Sequencing and Mutation Characterization

Amplification products for *rpo*B, *emb*B, *rrs*, *rps*L and *pnc*A genes were purified (Wizard SV Gel and PCR Clean-up System, Promega. Madison, WI, USA) and sequenced using Sanger sequencing performed at Macrogen Inc. (Seoul, South Korea). Sequences were analyzed and mutations characterized by the multiple sequence alignment program Clustal Omega (EMBL-EBI) (Madeira *et al*., 2019). GenBank sequences for *M.* tuberculosis H37Rv strain were used as reference for each gene: *rpo*B (ID: 888164), *emb*B (ID: 886126), *rrs* (ID: 2700429), *rps*L (ID: 888259) and *pnc*A (ID: 888260).

Results

Mycobacterium tuberculosis *Isolates Drug Resistance Profile*

MTBc isolates were identified in 15.2% (38) of the 250 clinical samples by *gyr*B gene 1020 bp fragment amplification, all belonged to TB patients. Ziehl-Neelsen stain showed that 18/38 isolates were acid-fast positive while 7/38 were acid-fast negative. The remaining 13 isolates were directly cultured. *Mycobacterium* species identification showed that in most isolates (37/38) *M.* tuberculosis was the infective species while *M. bovis* was present in one isolate (56-ex).

Proportion of agreement was used to compare Ziehl-Neelsen stain and culture with PCR as standard in the identification of MTBc in clinical isolates. The positive agreement of Ziehl-Neelsen stain with PCR was 0.64±0.042 and that of culture with PCR was found to be 0.66±0.036. The negative agreement of Ziehl-Neelsen stain with PCR results was 0.181±0.059 and that of culture with PCR was 0.0±0.0.

Drug Resistance (DR) profile as determined by nitrate reductase assay on the 38 MTBc isolates showed 17 (44.7%) were multidrug resistant (MDR), 15 of which were resistant to at least another drug besides INH and RIF. Five (13.1%) isolates were monoresistant; 13 (34.2%) polyresistant and 2 (5.2%) were pansusceptible. No DR profile could be obtained for one isolate as growth was not registered in the control wells along the phenotypic assay (Table 2).

Drug Resistance Related Mutations

According to molecular data, mutations S315T *kat*G and C-15T in the promoter of the *mabA-inhA* operon are most frequently related to INH resistance. The presence of those mutations in the 38 MTBc isolates was evaluated employing a PCR multiplex assay. Three isolates showed S315T *kat*G mutation and three showed C-15T *mabA- inh*A mutation; five of those isolates were phenotypically resistant to INH. In 18 isolates, resistant to INH according to phenotypic analysis, no mutations were identified at the analyzed positions. All INH susceptible isolates showed neither mutation. Two isolates with mutation S315T *kat*G were resistant to all first line drugs and two isolates that showed mutation C-15T *mabA-inh*A were MDR (Table 2).

*rpo*B gene analysis through ARMS revealed mutations at codons 516, 526 or 531 in eleven isolates. Nevertheless, sequence analysis showed mutations just in five of them: 526 (CAC→TAC), one isolate; 531 (TCG→TTG), three isolates and 516 (GAC→GAA)/526 (CAG→CAA), one isolate. Nitrate reductase assay showed that the isolate containing mutations at codons 516/526 was susceptible to RIF, while the rest, were resistant to the drug. Those with mutations at codon 531 were MDR. Sequence analysis of the whole RRDR (81 bp, codons 507-533) contained in the amplified 537 bp *rpo*B fragment for the above mentioned five isolates, revealed several mutations along this region in two isolates, none of them reported on the TB Drug Resistance Mutation Data Base (Sandgren *et al*., 2009) (Table 3). Interestingly, isolates with mutations at codon 531 contained no additional mutations along the RRDR.

Most EMB resistant cases have been explained by mutations in *emb*B gene, specifically at the first and third nucleotides in codon 306. Multiplex PCR and sequence results revealed mutations in six isolates (6/38), three with ATG→GTG substitution and three with ATG→ATA polymorphism. Except for one, all isolates were phenotypically resistant to EMB and at least to another first line drug as well (Table 2).

Drug resistance to STR has been related with mutations at *rrs* and *rps*L genes, which encode for 16S rRNA and ribosomal protein S12, respectively. Sequence analysis of 552 pb *rrs* fragment, revealed that 23 of the 38 isolates had mutations at nucleotides 485 (A→G) and/or 906 (A→C/T) and/or 907 (A→T/G); 18 of those isolates were phenotypically resistant to STR (Table 2). Concerning *rps*L gene, mutations in codons 43 and 88 have been most frequently associated to resistance to STR. In the present study, mutations in those positions were identified in eleven isolates: 43 (AAG→ACG/AGG) and 88 (AAG→GTG/AGG/AGA/GAA). Ten of those isolates were phenotypically resistant to STR.

Interestingly, some previously unreported mutations in STR resistant isolates were characterized in *rrs* and *rps*L genes, identifying one of those mutations, or a combination of them, in phenotypically STR resistant isolates (Table 4). It is worth mentioning that in 12 isolates, we found mutations in both genes (*rrs-rps*L) simultaneously, 11 of them were resistant to STR according to phenotypic analysis (Table 2). Most frequent mutations identified in *rrs* and *rps*L genes are summarized in Table 4.

*pnc*A sequence analysis revealed mutations in 7 of the 38 MTBc isolates. Only three of them possessed mutations at the so called hot spots (nucleotides 3-17, 61-85 and 132-142), however, all were susceptible to PZA. Two isolates (2/7), phenotypically resistant to PZA, showed mutations along *pnc*A gene, none of them at the hot spots (Table 2).

Comorbidities, Mutations and Drug Resistance

Among the 38 cases included in the study, most frequent comorbidities were Diabetes Mellitus (DM) (12), malnutrition (4) and HIV (4). Within DM patient isolates, 50% were phenotypically MDR and resistant at least to another drug, 16% were monoresistant and 34% were polyresistant. Among these isolates, genes related to STR resistance showed the highest polymorphism diversity, being the most frequent at nucleotides 485 and 795 in *rrs* and at codons 47 and 87 in *rps*L, identified in 10 different isolates (Table 2). Mutations in *inh*A, *kat*G, *rpo*B and *emb*B were registered in one isolate each, resistant to INH, RIF and EMB, respectively. *pnc*A mutations were found only in one isolate susceptible to PZA (Table 2).

In the malnutrition group, two isolates were MDR. In one of them, mutations at *rpo*B and *emb*B genes were found, while both showed mutations in *rrs* and/or *rps*L genes. In the last two isolates only mutations at *rrs* gene were characterized (Table 2).

Two isolates from TB-HIV coinfected patients were resistant to all first line drugs, but mutations in *kat*G, *emb*B, *rrs* and *rps*L genes were found only in one of them, while in the second, only mutations at *rps*L gene were identified. A third isolate showed mutations in *rrs* related to STR resistance. Last isolate was pansusceptible, although mutations at *pnc*A gene were found (Table 2).

Table 2: Molecular and phenotypic pattern of 38 MTBc isolates.

		Drug resistance					Analyzed genes						
Isolate	Comorbidity	I	R	E	S	Z	*kat*G	*inh*A	*rpo*B	*emb*B	*rrs*	*rps*L	*pnc*A
18-ex	An/Pn	R	S	R	R	R	No mutations detected	No mutations detected	No mutations detected	No mutations detected	No mutations detected	88 AAG-->GTG	Nt 315 G-->C Nt 316 C-->G Nt 323 G-->C Nt 337 G-->A Nt 380 G-->C Nt 385 G-->A Nt 523 G-->A Nt 541 G-->C Nt 562 T-->C Nt 84 deletion C Nt 305 A-->G Nt 306 G-->A Nt 307 C-->A Nt 309 T-->G Nt 310 ins T Nt 311 ins A Nt 312 A-->C Nt 313 C-->A Nt 314 A-->G
19-ex	DM/Mn	S	S	R	R	S	No mutations detected	No mutations detected	No mutations detected	306 ATG --> ATA	Nt 485 A-->G Nt 906 A-->C	30 CGT--> AGT 31 CGT-->CTT 47 TCG-->TGG 87 GTG-->TGA 88 AAG-->AGG 89 GAC-->ACC	Nt 85 deleción C
20-ex	N	R	R	R	R	R	No mutations detected	No mutations detected	531 TCG-->TTG	No mutations detected	Nt 485 A-->G Nt 906 A-->T Nt 907 A-->T	30 CGT--> AGT 31 CGT--> ATT 47 TCG-->TGG 87 GTG-->TGA 88 AAG-->AGG	No mutations detected
23-ex	AH/Pn	R	R	R	R	R	No mutations detected	No mutations detected	No mutations detected	306 ATG --> GTG	Nt 485 A-->G Nt 795 C-->T	No mutations detected	No mutations detected
24-ex	HIV	R	R	R	R	R	No mutations detected	No mutations detected	No mutations detected	No mutations detected	No mutations detected	52 GTT-->TTT 69 GGC-->GTC 78 TCG-->TGG 87 GTG-->TGA 88 AAG-->AGG 30 GT-->AGT 31 CGT-->ATT 40 ACC-->ATC 47 TCG-->TGG 49 CTT-->ATT 50 CGG-->GGG	No mutations detected
26-ex	Mn	R	R	R	R	R	No mutations detected	No mutations detected	531TCG-->TTG	306 ATG -->ATA	No mutations detected Nt 907 A-->G	43 AAG-->AGG 30 CGT-->AGT 47 TCG-->TGG 69 GGC-->GTC 87 GTG-->TGA 88 AAG-->AGG 78 TCG--> TGG 81 CTG-->GAG 84 GGC-->GTC 87 GTC-->GTA	No mutations detected Nt 266 C--<A Nt 280 T-->G Nt 296 C-->T Nt 323 G-->A Nt 335 G-->A Nt 337 G-->A Nt 340 C-->A Nt 343 C-->A

Table 2: Continue

											88 AAG->AGG	Nt 357 A-->T Nt 365 C-->G
27-ex	N	R	S	S	R	S	No mutations detected	No mutations detected	No mutations detected	306 ATG --> GTG	Nt 491 C-->T Nt 496 G-->C Nt 516 C-->T Nt 907 A→G	30 CGT-->AGT 31 CGT-->ATT 47 TCG-->TGG 49 CTT-->ATT 50 CGG-->GAG 52 GTT-->TTT 57 TTG-->CCG 58 ACG -->TCG 60 CAG-->CAA 69 GGC→GTC 70 GAG-->GTG 74 CTG-->CGG 78 TCG→TGG 81 CTG→GAG 84 GGC→GTC 87 GTC→GTA 88 AAG→AGG	Nt 97 G-->A Nt 103 C-->T Nt 128 A-->C Nt 130 G--> T Nt 131 T-->C Nt 134 T-->G Nt 139 A-->C Nt 162 C-->G Nt 185 C-->G Nt 187 G-->A Nt 190 T-->A Nt 199 T-->C Nt 209 C-->G Nt 221 G-->C Nt 225 T-->G Nt 227 C-->T Nt 238 G-->A Nt 242 T-->C Nt 245 A-->T Nt 251 G-->A Nt 266 C→A Nt 280 T→G Nt 296 C→T Nt 323 G→A

Table 2: Molecular and phenotypic pattern of 38 MTBc isolates (cont.)

		Drug resistance					Analyzed genes						
Isolate	Comorbidity	I	R	E	S	Z	*kat*G	*inh*A	*rpo*B	*emb*B	*rrs*	*rps*L	*pnc*A
28-ex	DM	R	R	R	R	R	No mutations detected	No mutations detected	No mutations detected	No mutations detected	No mutations detected	47 TCG-->TGG	No mutations detected
30-ex	DM/Mn/ Ch/Ht/Pht	R	S	S	S	S	No mutations detected	No mutations detected	No mutations detected	No mutations detected	Nt 485 A-->G Nt 795 C-->T Nt 870 C-->A Nt 906 A-->T	47 TCG-->TGG 87 GTG-->TTG	No mutations detected
31-ex	DM/Sm	R	R	R	R	R	No mutations detected	No mutations detected	No mutations detected	No mutations detected	Nt 485 A-->G Nt 795 C-->T Nt 870 C-->A Nt 906 A-->T Nt 907 A-->G	30 CGT-->AGT 31 CGT-->ATT 47 TCG-->TGG 87 GTG-->TGC	No mutations detected
33-ex	N	R	S	S	S	S	No mutations detected	No mutations detected	516 GAC-->GAA 526 CAC-->CAA	No mutations detected	Nt 795 C-->T Nt 870 C-->A Nt 906 A-->T Nt 907 A-->G	No mutations detected	No mutations detected
34-ex	DM	R	S	R	R	R	No mutations detected	C -15 T	No mutations detected	No mutations detected	Nt 795 C-->T Nt 870 C-->A Nt 906 A-->T Nt 907 A-->G	72 CAC-->CAT 80 GTG-->TTG 82 GTG-->GTT 86 CGG-->CTG 87 GTG-->CTG	No mutations detected
36-ex	DM	R	R	R	S	R	No mutations detected	No mutations detected	No mutations detected	No mutations detected	No mutations detected	No mutations detected	No mutations detected
37-ex	HIV/Mn/Sm	S	S	S	S	S	No mutations detected	No mutations detected	No mutations detected	No mutations detected	No mutations detected	No mutations detected	Nt 26 G-->A Nt 27 G-->A Nt 174 C-->T Nt 218 T->G Nt 262 T-->G Nt 407 A-->T Nt 440 G-->C Nt 474 T-->G Nt 482 A-->G Nt 539 G-->C
40-lb	Og/Ep	S	S	S	R	R	No mutations detected	No mutations detected	No mutations detected	No mutations detected	Nt 845 C-->T Nt 862 A-->G Nt 867 G-->A Nt 877 T-->C Nt 886 C-->T Nt 887 G-->A Nt 926 G-->C Nt 928 A-->C Nt 930 A-->C Nt 932 G-->A Nt 936 C-->G Nt 938 G-->C	No mutations detected	No mutations detected
42-ex	DM	R	R	R	R	R	315 S→T	No mutations detected	526 CAC-->TAC	306 ATG --> ATA	Nt 485 A-->G Nt 496 G-->C Nt 906 A-->C Nt 907 A-->T	43 AAG -->AGG	No mutations detected

Table 2: Continue

43-ex	HIV/CSm	R	R	R	R	R	315 S→T	No mutations detected	No mutations detected	306 ATG --> GTG	Nt 485 A-->G Nt 514 A-->C Nt 795 C-->A Nt 906 A-->T Nt 907 A-->G	82 GTG-->GTT 84 GGC-->TGC 86 CGG-->CTG	No mutations detected
45-ex	DM/Ow/AH	S	S	R	R	S	No mutations detected	No mutations detected	No mutations detected	No mutations detected	Nt 485 G-->A Nt 491 G-->A Nt 492 C-->T Nt 493 A-->C Nt 494 C-->G Nt 495 C-->T	No mutations detected	No mutations detected
46-ex	AH/Al	S	S	S	S	S	No mutations detected	No mutations detected	No mutations detected	No mutations detected	Nt 485 A-->G Nt 795 C-->T Nt 906 A-->T	No mutations detected	No mutations detected
47-ex	Dm/AH/Sm	S	R	R	R	R	No mutations detected	No mutations detected	No mutations detected	No mutations detected	Nt 485 A-->G Nt 496 G-->C	No mutations detected	No mutations detected
48-ex	N	S	R	S	R	S	No mutations detected	No mutations detected	No mutations detected	No mutations detected	Nt 485 A-->G Nt 906 A-->T	No mutations detected	Nt 39 Ins C

Table 2: Molecular and phenotypic pattern of 38 MTBc isolates (cont.)

		Drug resistance					Analyzed genes						
Isolate	Comorbidity	I	R	E	S	Z	*kat*G	*inh*A	*rpo*B	*emb*B	*rrs*	*rps*L	*pnc*A
49-ex	N	S	R	R	R	S	No mutations detected	No mutations detected	No mutations detected	No mutations detected	Nt 493 A-->G Nt 494 C-->T Nt 495 C-->G Nt 496 A-->G Nt 499 C-->A	No mutations detected	No mutations detected
50-ex	N	R	S	S	R	R	No mutations detected	No mutations detected	No mutations detected	No mutations detected	Nt 485 A-->G Nt 795 C-->T Nt 906 A-->T Nt 907 A-->G	No mutations detected	No mutations detected
51-ex	Mn/Sm	R	R	R	R	R	No mutations detected	No mutations detected	No mutations detected	No mutations detected	Nt 795 C-->T Nt 870 C-->A Nt 906 A-->T Nt 907 A-->G	81 CTG-->CAA 82 GTG-->GAC 84 GGC-->GGG 85 GGC-->CGT 86 CGG-->GCT 88 AAG-->AGA 89 GAC-->GCC	No mutations detected
52-ex	Mn/Sm	S	S	S	S	R	No mutations detected	No mutations detected	No mutations detected	No mutations detected	Nt 485 A-->G Nt 514 A-->C	No mutations detected	No mutations detected
53-ex	N	R	R	R	R	R	No mutations detected	No mutations detected	No mutations detected	No mutations detected	Nt 485 A-->G Nt 514 A-->C	43 AAG-->ACG	No mutations detected
54-ex	N	S	S	S	S	R	No mutations detected	No mutations detected	No mutations detected	No mutations detected	Nt 491 C-->A	No mutations detected	No mutations detected
55-ex	DM	S	S	S	R	S	No mutations detected	No mutations detected	No mutations detected	No mutations detected	Nt 795 C-->T	No mutations detected	No mutations detected
56-ex	DM	R	R	R	R	R	No mutations detected	No mutations detected	No mutations detected	No mutations detected	Nt 485 A-->G	No mutations detected	57 CAC→GAC
60-bi	HIV/ Sm/Al/CSm	S	S	S	R	R	No mutations detected	No mutations detected	No mutations detected	No mutations detected	Nt 795 C-->T	No mutations detected	No mutations detected
62-ex	Mn /Sm/As	S	S	S	R	R	No mutations detected	No mutations detected	No mutations detected	No mutations detected	Nt 485 A-->G Nt 795 C-->T	No mutations detected	No mutations detected
65-ex	N	Uk	Uk	Uk	Uk	Uk	315 S→T	No mutations Detected	531 TCG-->TTG	No mutations detected	Nt 485 A-->G Nt 906 A-->T Nt 907 A-->T	No mutations detected	No mutations detected
66-ex	N	S	R	R	R	R	No mutations detected	No mutations detected	No mutations detected	No mutations detected	Nt 769 G→C Nt 785 A→T Nt 786 C→T Nt 808 C→A Nt 816 A→T Nt 817 C→A	No mutations detected	No mutations detected
68-ex	DM/Mn	R	R	R	R	R	No mutations detected	No mutations detected	No mutations detected	No mutations detected	Nt 485 A-->G	No mutations detected	No mutations detected
186-ex	Uk	R	R	R	R	S	No mutations detected	No mutations detected	No mutations detected	No mutations detected	Nt 720 G→T Nt 761 C→T Nt 807 C→T Nt 830 C→A Nt 852 T→C Nt 898 G→C	31 CGT-->CTT 93 GTG-->GGC 94 CGC-->GAT 95 TAC -->ACA 96 AAG-->TGA Nt289 del A 99 CGC-->GAC	No mutations detected
198-lp	Uk	R	R	R	R	R	No mutations detected	No mutations detected	No mutations detected	No mutations detected	Nt 485 G-->A Nt 491 G-->C	80 GTG--> TTG 84 GGC-->TCG	Nt 225 T-->G Nt 421 G-->A

Table 2: Continue

											Nt 492 C-->A Nt 493 A-->C Nt 495 C-->G Nt 496 G-->T	86 CGG-->CTG 87 GTG-->GGT 88 AAG-->GAA 89 GAC-->GGA 90 CTG-->TCC	Nt 521 G-->A

Table 2: Molecular and phenotypic pattern of 38 MTBc isolates (cont.)

		Drug resistance					Analyzed genes						
Isolate	Comorbidity	I	R	E	S	Z	*kat*G	*inh*A	*rpo*B	*emb*B	*rrs*	*rps*L	*pnc*A
199-bi	Uk	R	R	S	S	S	No mutations detected	C -15 T	No mutations detected	No mutations detected	No mutations detected	86 CGG-->CTG 87 GTG-->GGT 88 AAG-->GAA 89 GAC-->GGA 90 CTG-->CCT 91 CCT-->GCC 92 GGT-->TGG 94 CGC-->ACG 95 TAC-->AAT 96 AAG-->CCT	Nt 51 C-->G Nt 136 G-->A Nt 225 T-->G Nt 265 G-->A Nt 266 C-->G Nt 267 A-->C Nt 269 T-->A Nt 270 C-->T Nt 270 Ins CG Nt 271 G-->A Nt 272 A-->G Nt 273 G-->C Nt 295 C-->A Nt 314 C-->A Nt 317 C-->A Nt 340 G-->A Nt 359 G-->T Nt 421 G-->A Nt 451 G-->A Nt 456 G-->C
228-ex	Uk	R	R	S	S	S	No mutations detected	C -15 T	No mutations detected	No mutations detected	Nt 483 G-->A Nt 491 G-->T Nt 493 A-->C Nt 495 C-->T Nt 498 C-->G Nt 499 C-->A Nt 512 G-->A	86 CGG-->CGT 90 CTG-->GGA 91 CCT-->CAT 92 GGT-->GAC 93 GTG-->CTG 94 CGC-->GTG 95 TAC-->ACA 96 AAG-->GTC 97 ATC-->CCT 98 ATC-->CCC	No mutations detected

Isolates: ex (sputum); lb (bronquial lavage); bi (biopsy); lp (pleural liquid) Comorbidities: AH (arterial hypertension), Al (alcoholismo), An (Anemia), As (asthma), Ch (Cirrhosis), CSm (cannabis smoker), DM (diabetes mellitus), Ep (epilepsy), HIV (human immunodeficiency virus), Ht (hypothyroidism), Mn (malnutrition), N (none), Og (oligoclonal gamapathy), Ow (overweight), Pht (portal hypertension), Pn (pneumonia), Sm (smoker), Uk (unknown), Drug resistance: I (isoniazid); R (rifampicin); E (ethambutol); Z (pyrazinamide); S (streptomycin); R (resistant); S (sensitive); Uk (unknown)

Table 3: Mutations characterized in *rpo*B RRDR

Isolate	Mutations along RRDR	Phenotypic rifampin resistance
33ex	521 CTG-GTG (Leu-Val) 524 TTG-TTT (Leu-Phe) 525 ACC-GAC (Thr-Asp) 528 CGC-AGT (Arg-Ser) 529 CGA-CAC (Arg-His) 530 CTG-TGG (Leu-Trp) 532 GCG-TGG (Ala-Trp)	S
42ex	529 CGA-CGC (Arg-Arg) 530 CTG-GTG (Leu-Val)	R

Table 4: Most frequent mutations characterized in *rrs* and *rps*L genes

	Polymorphism				
Gene	codon	nucleotide	Aminoacid change	Isolates with polymorphism	Number of STR resistant isolates
rrs	162 GAA→GGA	485 A→G	Glu/Gly	18	15
	265 GTC→GTT	795 C→T	Val/Val	11	8
	290 TAC→TAA	870 C→A	Tyr/Stop	5	3
	302 TCA→TCC	906 A→C	Ser/Ser	2	2
	302 TCA→TCT	906 A→T	Ser/Ser	10	7
	303 AAG→GAC	907 A→G	Lys/Asp	7	6
	303 AAG→TAG	907 A→T[a]	Lys/Stop	2	2
*rps*L	30 CGT→AGT	88 C→A	Arg/Ser	6	6
	31 CGT→CTT	92 G→T	Arg/Leu	2	2
	31 CGT→ATT	92 C→T 93 G→T	Arg/Ile	4	4
	43 AAG→ACG	128 A→C	Lys/Thr	1	1
	43 AAG→AGG	128 A→G	Lys/Arg	2	2
	47 TCG→TGG	140 C→G	Ser/Trp	8	7
	87 GTG→TGA	259 G→T 260 T→G			

	261 G→A	Val/Stop	4	4
87 GTG→TTG	259 G→T	Val/Leu	1	0
87 GTG→TGC	259 G→T			
	260 T→G			
	261 G→C	Val/Cys	1	1
87 GTG→CTG	259 G→C	Val/Leu	1	1
87 GTG→GGT	260 T→G			
	261 G→T	Val/Gly	2	1
87 GTG→GTA	261 G→A	Val/Val	1	1
88 AAG→GTG[a]	262 A→G			
	263 A→T	Lys/Val	1	1
88 AAG→AGG	263 A→G	Lys/Arg	5	5
88 AAG→AGA[a]	263 A→G			
	264 G→A	Lys/Arg	1	1
88 AAG→GAA[a]	262 A→G			
	264 G→A	Lys/Glu	2	1

a: Novel mutations

Discussion

DR is a major cause of increasing TB incidence mainly due to mutations in target genes. Mutations in MTBc isolates have only been reported for high TB incidence states in Mexico. This report is the first insight into polymorphisms in target genes to INH, RIF, EMB, STR and PZA in southwest Mexico.

Mutations in *kat*G and *inh*A are the main cause of resistance to INH. It was reported that simultaneous mutations in both genes were responsible for DR to INH in other regions of the world (Mathuria *et al*., 2009; Gonçalves *et al.*, 2012); nevertheless, our data coincide with previous evidence from Mexico about INH resistant MTBc isolates that hold mutations in either *kat* G or *inh*A genes (Ramaswamy *et al*., 2004; Molina-Torres *et al*., 2010; Zenteno-Cuevas *et al*., 2015). In this study no mutations in the analyzed regions were found in 18 phenotypically INH resistant isolates, suggesting that polymorphisms in different genes such as *oxy*R-*ahp*C, *kas*A, *fur*A, *fab*G1, *efp*A, *fad*E24, *ini*A, *ini*B, *ini*C, *kas*A, *nat*, *ndh*, Rv1772, Rv1592c, Rv0340, or *srm*R may be responsible for this behavior (Herrera-León *et al*., 2005; Seifert *et al*., 2015).

Twenty-one isolates were RIF resistant according to nitrate reductase assay, but just in six of them mutations in codons 516, 526 or 531 at *rpo*B gene were found. RIF resistance of the remaining isolates may be explained by mutations in codons different to 516, 526 or 531 or in other regions of *rpo*B out of RRDR (Zaw *et al*., 2018), this highlights the importance of sequencing the whole RRDR in order to associate new mutations to RIF resistance.

Polymorphisms in codon 531 are responsible for RIF resistance in over 60% of the cases around the world (Agapito et al., 2002; Bolotin et al., 2009; Gonçalves et al., 2012). In Mexico, mutation TCG**531**TTG has been previously reported in RIF resistant isolates (Cuevas-Cordoba *et al.*, 2010; Zenteno-Cuevas *et al*., 2015; Lopez-Avalos *et al*., 2017); here, we report this same mutation which explains only 14.2% of RIF resistant isolates. It is worth mentioning that none of new mutations within RRDR found in this study in RIF resistant isolates were characterized in those with TCG**531**TTG mutation. This supports that TCG**531**TTG polymorphism itself is enough to cause RIF resistance.

Mutations in codon *emb* B306 are responsible for EMB resistance according to reports from Mexico, Cuba, Poland, China and Iran, among other countries (Guerrero *et al*., 2013; Cuevas-Cordoba *et al*., 2013; Li *et al*., 2016; Ramazanzadeh and Mohammadi, 2016), despite being also reported in susceptible isolates. Our findings confirm both facts, as one of the six isolates with mutation in *emb* B306 was susceptible while the five others were resistant to EMB. Other eighteen isolates were phenotypically resistant to EMB and at least to another drug, however no mutation in *emb* B306 was identified, probably they hold mutations in codons *emb* B406 or *emb* B497, described in resistant isolates (Bakuła *et al*., 2013), which were not included in the 324 pb fragment analyzed here.

Studies conducted in Poland, Cameroon and Mexico reported mutually exclusive mutations in *rrs* and *rps*L in STR resistant isolates (Cuevas-Córdoba *et al*., 2013a; Jagielski *et al*., 2014). In the present report, 28 phenotypically STR resistant isolates were identified, 4/28 with mutations in *rps*L, 13/28 in *rrs* and 11/28 in both genes. Among those with *rrs* mutations, polymorphisms at nucleotides 485 A→G, 906 A→T/C and 907 A→T/G were the most frequent. In fact, in two isolates, phenotypically resistant to STR, A→G mutation in nucleotide 485 was the only one characterized, suggesting its importance in STR resistance. Additionally, mutations at *rrs* and *rps*L genes are reported here for the first time: A→T in nucleotide 907 in *rrs* and codon 88 AAG→GTG/GAA/AGA (Lys→Val/Glu/Arg) in *rps*L. Mutation TCG→TGG (Ser→Trp) at codon 47 in *rps*L was found in STR resistant isolates under three different circumstances: (a) simultaneously with mutations at nucleotides 485, 906 and/or 907 in *rrs* gene; (b) with a mutation at codon 88 in *rps*L gene and (c) alone, being responsible itself for STR resistance. Also, polymorphisms found at *rps*L gene in codon 87 seem to be important for resistance to the drug as they appeared in 8 resistant isolates. The high incidence of these polymorphisms suggests an important

role in STR resistance which deserves further study. To our knowledge, this is the first report about mutations at codons 37 and 87 in *rps*L related to STR resistance and the occurrence of simultaneous mutations in *rrs* and *rps*L genes in STR resistant isolates.

Numerous mutations in *pnc*A have been reported worldwide (Ramirez-Busby and Valafar, 2015) in both, PZA resistant and susceptible MTBc isolates. In this study, two out of seven isolates with mutations in *pnc*A were phenotypically resistant to PZA, with 3 and 19 mutations respectively, none of them within any hot spot. This contrasts with a previous mexican report, in which several polymorphisms were characterized in PZA resistant isolates, including doble mutations (Cuevas-Córdoba *et al*., 2013). Our findings support the proposal of PZA resistance being due to altern cellular or molecular mechanisms such as mutations in *rps*A gene (Barco *et al.*, 2006; Akhmetova *et al*., 2015; Khan *et al*., 2018).

International studies report that DM is a major risk factor for tuberculosis infection and triples the risk of developing active TB (Jeon and Murray, 2008; Lutfiana *et al*., 2019). Studies conducted along Mexico indicate that between 19-40% of TB cases are associated to DM (Ponce de Leon *et al.*, 2004; Jiménez-Corona *et al*., 2013; Delgado-Sanchez *et al.*, 2015; Restrepo, 2016); in the present report, 31.5% cases showed TB-DM association. Moreover, 50% of total TB-DM cases were MDR close to the 42.2% reported by in San Luis Potosí, a state located in Central Mexico (Gómez-Gómez *et al*., 2015). High incidence of TB among diabetic patients has been explained by metabolic and immunological alterations due to DM, although this has not been fully understood. Our data, confirm that more TB-DM patients are infected with MDR isolates than those without any comorbidity (50% *Vs*. 25%) which complicates their treatment. This highlights the importance of providing an acute TB diagnosis which includes resistance profile to first line antituberculous drugs, in order to provide an efficient and effective treatment to diabetic patients.

Most studies conducted in Mexico describe drug resistance based in phenotypic assays, only a few have described a correlation between drug resistance and mutations in target genes e.g., resistance to PZA and mutations in *pnc*A (Cuevas-Córdoba *et al*., 2013) or resistance to STR and mutations in *rrs*/*rps*L (Cuevas-Córdoba *et al*., 2013). Our study reports the presence of mutations in seven different genes associated to resistance to five first line antimycobacterial drugs and the phenotypic drug resistance pattern observed in clinical isolates. Furthermore, to our knowledge, this is the first study to report simultaneous mutations in *rrs* and *rsp*L genes in STR resistant isolates and to stablish a correlation between comorbidites and mutations in target genes of first line antituberculous drugs in southwest Mexico.

In order to get a wider perspective of DR in this mexican region, it would be of great interest to study a larger number of clinical samples. Its molecular analysis should include search of mutations in regions related to DR besides those considered in this report such as codons 406 or 497 in *emb*B or the sequence out of RRDR in *rpo*B.

Our findings highlight that even punctual mutations have been useful in molecular diagnostic techniques to determine DR, it is time to analyze longer fragments of target genes as new DR mutations are being reported continuously.

Conclusion

This is the first report about phenotypic drug resistance and molecular data in MTBc isolates from Oaxaca, Mexico, a region with scarce TB information. Most frequent mutations related to resistance to first line antituberculous drugs were identified in phenotypically resistant MTBc isolates. As also new mutations were found in *rpo*B, *rrs* and *rps*L genes it is important to study a larger number of MTBc isolates in order to stablish the occurrence of mutations associated to drug resistance in this region as our findings differ from previous reports in this country. Of special concern is the urgency of an accurate TB diagnosis in diabetic patients as they seem to be good targets for drug resistant mycobacteria strains.

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Author's contributions

Martínez-Cruz, Perla Mónica: Conducted molecular and phenotypic experiments, data analysis and manuscript writing.

Nakamura-López, Yuko: Contributed to data analysis and manuscript writing.

Quintero-Hernández, Verónica: Academic advisor on molecular data and manuscript writing reviewer.

Pérez-Campos Mayoral, Laura: Academic advisor on molecular data and manuscript writing reviewer.

Martínez-Martínez, Lucía Lourdes: Designed and supervised the study and contributed to data analysis.

Ethics

All individuals included in this study, signed a written informed consent and answered a questionnaire to obtain socio-demographic and clinical data. The Ethical Committee on Investigation of the Consejo Estatal para la Prevencion y Control del Sida, Oaxaca-Mexico, approved the protocol.

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