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 katG, inhA, rpoB, embB, rrs, rpsL, and pncA genes. Mutations in katG and the promoter of the mabA-inhA operon were found in isoniazid resistant isolates. Sequence analysis of Rifampin Resistance-Determining Region in the rpoB gene showed novel mutations along this region besides mutations at codons 516, 526 and 531. Polymorphisms at codon 306 embB gene were found in ethambutol resistant isolates. Frequent mutations associated to resistance to streptomycin were characterized in rrs and rpsL genes. pncA 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 rpoB, rrs and rpsL 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. canetti, M. caprae, M. pinnipedi, 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-Córdoa 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: katG and inhA (INH), rpoB (RIF), embB (EMB), rrs and rpsL (STR) and pncA (PZA) (Abbadi et al., 2009; Bakula et al., 2013; Cuevas-Córdoa et al., 2013b; Pang et al., 2017).
Reports from different regions around the world have coincided in the occurrence of specific and recurring mutations in those genes. Thus, mutations at codon 315 in the katG gene or within the rifampin Resistance-Determining Region (RRDR) in the rpoB gene or in codon 306 of embB gene have been used to identify resistant strains. In contrast, mutations in rrs, rpsL and pncA 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 \[ a = \frac{2a}{2a + b + c} \]

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

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

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

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
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 1X Buffer, 25 mM MgCl₂, 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, Thermoe 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 gyrB gene employing 50 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 rpoB 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 embB 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, rpsL and pncA genes were obtained using specific primers (Table 1) in independent PCR reaction mixes including 50 µM PR13F/PR30R (rrs) or 50 µM STR52R/STR43F (rpsL) (Cuevas-Córdoba et al., 2002, 2003).

| Gene | Primer | Sequence (5’ - 3’) | Amplicon size |
|------|--------|-------------------|--------------|
| gyrB | MTUBf  | teggacgcgtatgcgatctc | 1020 pb |
|      | MTUBr  | acatacagtggacgctttgcc | |
| katG | KatGOF | gcagatgagggtcagtacgc | 296 pb |
|      | R315mut| tccatagcgctgggtgcagcg | |
| inhA | mabAF  | cgaagtgtgtgagccacaccgc | 146 pb |
|      | inhARMut | atgcacctgcagaacactatta | |
| rpoB | ARMS516 | cagctgagccaaatcagggga | 261 pb |
|      | ARMS526 | cgtgtggctgggttcctcccc | 230 pb |
|      | ARMS531 | acaccagaagcgccagatctc | 216 pb |
|      | CtrlFw | cgaatatgctgccttgcc | |
|      | ComRv | gtcgacacccctcgcttgacc | 537 pb |
| embB | Emb1F  | ggcgccccgctcaattgcgcc | 324 pb |
|      | Emb2R  | gcgcctccacagactggctc | 160 pb |
|      | Emb306A | gcagacgctcatctgctgggga | 210 pb |
|      | Emb306B | gctgcgccagctgggccc | 552 pb |
| rrs  | PR13F  | aacacctttcaccatgcagc | 272 pb |
|      | PR30R  | cagcttgtgctctcggttg | |
| rpsL | STR52R | gtcgccgccgctctggcagta | |
|      | STR43F | tggcttcgccctctcct | |
| pncA | pncA-F | aacagcttacccgcggtc | 668 pb |
|      | pncA-R | gcgtctagcggccattcacta | |

| Primer sequences | Table 1 | Sequence (5’ - 3’) | Amplicon size |
|------------------|---------|-------------------|--------------|
| gyrB             | MTUBf  | teggacgcgtatgcgatctc | 1020 pb |
|                  | MTUBr  | acatacagtggacgctttgcc | |
| katG             | KatGOF | gcagatgagggtcagtacgc | 296 pb |
|                  | R315mut| tccatagcgctgggtgcagcg | |
| inhA             | mabAF  | cgaagtgtgtgagccacaccgc | 146 pb |
|                  | inhARMut | atgcacctgcagaacactatta | |
| rpoB             | ARMS516 | cagctgagccaaatcagggga | 261 pb |
|                  | ARMS526 | cgtgtggctgggttcctcccc | 230 pb |
|                  | ARMS531 | acaccagaagcgccagatctc | 216 pb |
|      | CtrlFw | cgaatatgctgccttgcc | |
|      | ComRv | gtcgacacccctcgcttgacc | 537 pb |
| embB             | Emb1F  | ggcgccccgctcaattgcgcc | 324 pb |
|                  | Emb2R  | gcgcctccacagactggctc | 160 pb |
|                  | Emb306A | gcagacgctcatctgctgggga | 210 pb |
|                  | Emb306B | gctgcgccagctgggccc | 552 pb |
| rrs               | PR13F  | aacacctttcaccatgcagc | 272 pb |
|                  | PR30R  | cagcttgtgctctcggttg | |
| rpsL              | STR52R | gtcgccgccgctctggcagta | |
|                  | STR43F | tggcttcgccctctcctc | |
| pncA              | pncA-F | aacagcttacccgcggtc | 668 pb |
|                  | pncA-R | gcgtctagcggccattcacta | |
2013a) or 50 μM pncA-F/pncA-R (pncA) (Pang et al., 2017) primers. PCR products were analyzed on 1.5% agarose gel electrophoresis.

Sequencing and Mutation Characterization

Amplification products for rpoB, embB, rrs, rpsL and pncA 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: rpoB (ID: 888164), embB (ID: 886126), rrs (ID: 2700429), rpsL (ID: 888259) and pncA (ID: 888260).

Results

*Mycobacterium tuberculosis* Isolates Drug Resistance Profile

MTBc isolates were identified in 15.2% (38) of the 250 clinical samples by gyrB 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 0.18±0.059 and that of culture with PCR was 0.04±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 katG 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 katG mutation and three showed C-15T *mabA*-inhA 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 katG were resistant to all first line drugs and two isolates that showed mutation C-15T *mabA*-inhA were MDR (Table 2).

*rpoB* 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 (TGG→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 *rpoB* 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 *embB* 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 *rpsL* 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 *rpsL* 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 *rpsL* 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-rpsL*) simultaneously, 11 of them were resistant to STR according to phenotypic analysis (Table 2). Most frequent mutations identified in *rrs* and *rpsL* genes are summarized in Table 4.
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 pncA 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 *rpsL*, identified in 10 different isolates (Table 2). Mutations in *inhA*, *katG*, *rpoB* and *embB* were registered in one isolate each, resistant to INH, RIF and EMB, respectively. *pncA* 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 *rpoB* and *embB* genes were found, while both showed mutations in *rrs* and/or *rpsL* 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 *katG*, *embB*, *rrs* and *rpsL* genes were found only in one of them, while in the second, only mutations at *rpsL* gene were identified. A third isolate showed mutations in *rrs* related to STR resistance. Last isolate was pansusceptible, although mutations at *pncA* gene were found (Table 2).

| Isolate | Comorbidity | I   | R   | S   | Z   | katG            | inhA     | rpoB     | embB     | rrs       | rpsL       | pncA     |
|---------|-------------|-----|-----|-----|-----|-----------------|----------|----------|----------|-----------|-----------|----------|
| 18-ex   | 0           | R   | S   | R   | R   | No mutations detected | No mutations detected | No mutations detected | No mutations detected | No mutations detected | 88 AAG→GTG  | Ni 315 G→C   |
|         |             |     |     |     |     |                  |          |          |          |           |           | Ni 315 G→C   |
|         |             |     |     |     |     |                  |          |          |          |           |           | Ni 316 C→G   |
|         |             |     |     |     |     |                  |          |          |          |           |           | Ni 323 G→C   |
|         |             |     |     |     |     |                  |          |          |          |           |           | Ni 337 G→A   |
|         |             |     |     |     |     |                  |          |          |          |           |           | Ni 380 G→C   |
|         |             |     |     |     |     |                  |          |          |          |           |           | Ni 385 G→A   |
|         |             |     |     |     |     |                  |          |          |          |           |           | Ni 523 G→A   |
|         |             |     |     |     |     |                  |          |          |          |           |           | Ni 541 G→C   |
|         |             |     |     |     |     |                  |          |          |          |           |           | Ni 562 T→C   |
|         |             |     |     |     |     |                  |          |          |          |           |           | Ni 84 deletion |
|         |             |     |     |     |     |                  |          |          |          |           |           | Ni 305 A→G   |
|         |             |     |     |     |     |                  |          |          |          |           |           | Ni 307 C→A   |
|         |             |     |     |     |     |                  |          |          |          |           |           | Ni 309 T→G   |
|         |             |     |     |     |     |                  |          |          |          |           |           | Ni 310 in T  |
|         |             |     |     |     |     |                  |          |          |          |           |           | Ni 311 in T  |
|         |             |     |     |     |     |                  |          |          |          |           |           | Ni 312 A→C   |
|         |             |     |     |     |     |                  |          |          |          |           |           | Ni 313 C→A   |
|         |             |     |     |     |     |                  |          |          |          |           |           | Ni 314 A→G   |
| 19-ex   | DM/Mn       | S   | S   | R   | R   | No mutations detected | No mutations detected | No mutations detected | 306 ATG→ATA | No mutations detected | 88 AAG→GTG  | Ni 306 G→A   |
|         |             |     |     |     |     |      |          |          |          |           |           | Ni 306 G→A   |
|         |             |     |     |     |     |      |          |          |          |           |           | Ni 307 C→A   |
|         |             |     |     |     |     |      |          |          |          |           |           | Ni 309 T→G   |
|         |             |     |     |     |     |      |          |          |          |           |           | Ni 310 in T  |
|         |             |     |     |     |     |      |          |          |          |           |           | Ni 311 in T  |
|         |             |     |     |     |     |      |          |          |          |           |           | Ni 312 A→C   |
|         |             |     |     |     |     |      |          |          |          |           |           | Ni 313 C→A   |
|         |             |     |     |     |     |      |          |          |          |           |           | Ni 314 A→G   |
| 20-ex   | N           | R   | R   | R   | R   | No mutations detected | No mutations detected | No mutations detected | 531 TCG→TTG | No mutations detected | 87 GTG→TGA  | Ni 89 G→A    |
|         |             |     |     |     |     |      |          |          |          |           |           | Ni 89 G→A    |
|         |             |     |     |     |     |      |          |          |          |           |           | Ni 90 T→C    |
|         |             |     |     |     |     |      |          |          |          |           |           | Ni 94 C→T    |
|         |             |     |     |     |     |      |          |          |          |           |           | Ni 95 C→T    |
| 23-ex   | A/HIV       | R   | R   | R   | R   | No mutations detected | No mutations detected | No mutations detected | 306 ATG→GTG | No mutations detected | 88 AAG→AGG  | Ni 84 deletion |
|         |             |     |     |     |     |      |          |          |          |           |           | Ni 84 deletion |
|         |             |     |     |     |     |      |          |          |          |           |           | Ni 85 deletion |
| 24-ex   | HIV         | R   | R   | R   | R   | No mutations detected | No mutations detected | No mutations detected | 531 TCG→TTG | No mutations detected | 87 GTG→TGA  | Ni 87 G→A    |
|         |             |     |     |     |     |      |          |          |          |           |           | Ni 87 G→A    |
|         |             |     |     |     |     |      |          |          |          |           |           | Ni 88 T→C    |
|         |             |     |     |     |     |      |          |          |          |           |           | Ni 89 C→T    |
|         |             |     |     |     |     |      |          |          |          |           |           | Ni 90 T→C    |
| 26-ex   | Mn          | R   | R   | R   | R   | No mutations detected | No mutations detected | No mutations detected | 306 ATG→ATA | No mutations detected | 87 GTG→TGA  | Ni 87 G→A    |
|         |             |     |     |     |     |      |          |          |          |           |           | Ni 87 G→A    |
|         |             |     |     |     |     |      |          |          |          |           |           | Ni 88 T→C    |
|         |             |     |     |     |     |      |          |          |          |           |           | Ni 89 C→T    |

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

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

| Isolate | Comorbidity | Drug resistance | Analyzed genes |
|---------|-------------|-----------------|----------------|
|         |             |                 | kasG inhA rpoB  
|         |             |                 | embB rrs rplL  
|         |             |                 | pmeA           |
| 27-ex   | N R S R S   | No mutations    | No mutations   |
|         |             | detected        | detected       |
|         |             | 306 ATG→GTG    | 88 AAG→AGG    |
|         |             |                 | Ni 357 A→T    |
|         |             |                 | Ni 363 C→G    |
|         |             |                 | Ni 97 G→A     |
|         |             |                 | Ni 103 C→T    |
|         |             |                 | Ni 128 A→C    |
|         |             |                 | Ni 130 G→T    |
|         |             |                 | Ni 131 T→C    |
|         |             |                 | Ni 134 T→G    |
|         |             |                 | Ni 139 A→C    |
|         |             |                 | Ni 162 C→G    |
|         |             |                 | Ni 185 C→G    |
|         |             |                 | Ni 187 G→A    |
|         |             |                 | Ni 190 T→A    |
|         |             |                 | Ni 199 T→C    |
|         |             |                 | Ni 209 C→G    |
|         |             |                 | Ni 221 G→C    |
|         |             |                 | Ni 223 T→G    |
|         |             |                 | Ni 227 C→T    |
|         |             |                 | Ni 238 A→G    |
|         |             |                 | Ni 242 T→C    |
|         |             |                 | Ni 245 A→T    |
|         |             |                 | Ni 251 G→A    |
|         |             |                 | Ni 266 C→A    |
|         |             |                 | Ni 280 T→G    |
|         |             |                 | Ni 296 C→T    |
|         |             |                 | Ni 323 G→A    |

| 28-ex   | DM R R R R R | No mutations detected | No mutations detected |
|         |             | 47 TCG→TGG         | No mutations detected |
| 30-ex   | DM/Mu/Ch/Ph | No mutations detected | No mutations detected |
|         |             | Ni 453 A→G         | No mutations detected |
|         |             | Ni 795 C→T         | No mutations detected |
|         |             | Ni 870 C→A         | Ni 908 A→T |
|         |             | Ni 47 TCG→TGG      | Ni 87 G→TGG |
| 31-ex   | DM/Sm R R R R R | No mutations detected | No mutations detected |
|         |             | Ni 453 A→G         | No mutations detected |
|         |             | Ni 795 C→T         | No mutations detected |
|         |             | Ni 870 C→A         | Ni 908 A→T |
|         |             | Ni 47 TCG→TGG      | Ni 87 G→TGG |
| 33-ex   | N R S S S S | No mutations detected | No mutations detected |
|         |             | 516 GAC→GAA       | No mutations detected |
|         |             | Ni 795 C→T         | Ni 908 A→T |
|         |             | Ni 907 A→G         | No mutations detected |
| 34-ex   | DM R S R R R | No mutations detected | C-15 T         |
|         |             | 72 CAC→CAT         | No mutations detected |
|         |             | Ni 795 C→T         | Ni 906 A→T |
|         |             | Ni 907 A→G         | No mutations detected |
| 36-ex   | DM R R R S R | No mutations detected | No mutations detected |
|         |             | No mutations detected | No mutations detected |
| 37-ex   | HIV/Mu/Sm S S S S | No mutations detected | No mutations detected |
|         |             | No mutations detected | No mutations detected |
| 40-lb   | Og/Ep S S S R R | No mutations detected | No mutations detected |
|         |             | No mutations detected | No mutations detected |
|         |             | Ni 845 C→T         | No mutations detected |
|         |             | Ni 862 A→G         | No mutations detected |
|         |             | Ni 87 G→A          | No mutations detected |
|         |             | Ni 877 T→C         | No mutations detected |
|         |             | Ni 886 C→T         | No mutations detected |
|         |             | Ni 887 G→C         | No mutations detected |
|         |             | Ni 926 G→C         | No mutations detected |
|         |             | Ni 924 A→C         | No mutations detected |
|         |             | Ni 930 A→C         | No mutations detected |
|         |             | Ni 932 G→A         | No mutations detected |
|         |             | Ni 936 C→G         | No mutations detected |
|         |             | Ni 938 G→C         | No mutations detected |
| 42-ex   | DM R R R R R | 315 S→T           | No mutations detected |
|         |             | 526 CAC→TAC       | 306 ATG→ATA     |
|         |             | Ni 453 A→G         | No mutations detected |
|         |             | Ni 496 G→C         | Ni 906 A→C     |
|         |             | Ni 907 A→T         | No mutations detected |

**Table 2: Continue**
### Table 2: Molecular and phenotypic pattern of 38 MTB isolates (cont.)

| Isolate | Genotype | Analyzed genes |
|---------|----------|----------------|
| 49-ex   | N        | 81 CGT—>CAA    |
| 50-ex   | N        | 82 GTG—>GAC    |
| 51-ex   | Mu/Sm    | 84 GCC—>GGG    |
| 52-ex   | Mu/Sm    | 85 GGG—>GGT    |
| 53-ex   | N        | 86 CGG—>GCT    |
| 54-ex   | N        | 87 AAG—>AGA    |
| 55-ex   | DM       | 89 GAC—>GAC    |
| 56-ex   | DM       | 90 GTC—>GTC    |
| 60-bi   | HIV/Sm/Ac/CSm | 91 GCC—>GC    |
| 62-ex   | Mn/Sm/Ac | 92 CGG—>GGT    |
| 63-ex   | N        | 93 GTT—>GTG    |
| 64-ex   | N        | 94 GCC—>GGC    |
| 65-ex   | N        | 95 TAC—>ACA    |
| 66-ex   | N        | 96 AAG—>AGA    |
| 67-ex   | N        | 97 N209delA    |
| 68-ex   | DM/Mn    | 98 GCC—>GAC    |
| 69-ex   | Uk       | 99 GGC—>GGC    |
| 100-lp  | Uk       | 101 TAC—>ACA   |
| 101-lp  | Uk       | 102 AAG—>AGA   |
| 102-lp  | Uk       | 103 N209delA   |
| 103-lp  | Uk       | 104 GCC—>GAC   |

**Note:** 10.3844/ajidsp.2021.138.149

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**Table 2:**

| 43-ex | HIV/Sm | R | R | R | R | 315 S→T | No mutations detected | No mutations detected | No mutations detected | 306 ATG→GTG | Nt 485 A→G | Nt 514 A→C | Nt 795 C→G | Nt 906 A→T | Nt 907 A→G | 82 GTG→GTG | Nt 453 A→G | Nt 494 C→T | Nt 495 C→G | Nt 496 A→G | Nt 499 C→A | No mutations detected |

| 44-ex | DM/Om/AH | S | S | R | R | S | No mutations detected | No mutations detected | No mutations detected | No mutations detected | Nt 485 A→G | Nt 495 C→A | Nt 495 C→T | Nt 496 A→G | No mutations detected |

| 45-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 906 A→T | Nt 907 A→G | No mutations detected |

| 46-ex | Dm/AH/Sm | S | R | R | R | No mutations detected | No mutations detected | No mutations detected | No mutations detected | Nt 485 A→G | Nt 495 C→A | Nt 495 C→T | No mutations detected |

| 47-ex | N | S | R | S | R | S | No mutations detected | No mutations detected | No mutations detected | No mutations detected | Nt 485 A→G | Nt 907 A→G | No mutations detected |

| 48-ex | N | S | R | S | R | No mutations detected | No mutations detected | No mutations detected | No mutations detected | Nt 485 A→G | Nt 907 A→G | Ni 39 InsC |

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

| Isolate | Comorbidity | Drug resistance | Analyzed genes | Mutations along RRDR | Phenotypic rifampin resistance |
|---------|-------------|-----------------|-----------------|----------------------|-------------------------------|
| 199-bi  | Uk          | R R S S S       | kanG            | No mutations detected | C -15 T                       | S                             |
| 228-ex  | Uk          | R R S S S       | inhA            | No mutations detected | C -15 T                       | S                             |
|         |             |                 | rpoB            | No mutations detected |                               |                               |
|         |             |                 | embB            | No mutations detected |                               |                               |
|         |             |                 | rrs             | No mutations detected |                               |                               |
|         |             |                 | pncA            | No mutations detected |                               |                               |

Isolates: ex (sputum); lb (broncoal lavage); lh (lavage); ly (pleural liquid); Comorbidity: AH (arterial hypertension), AI (alcoholism), An (Anemia), As (asthma), Ch (Chronic), Cr (carnitine smoker), DM (diabetes mellitus), Ep (epilepsy), HIV (human immunodeficiency virus), Ht (hypothyroidism), Mn (malnutrition), N (none), Og (obesity), Ov (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 rpoB RRDR

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

Table 4: Most frequent mutations characterized in rrs and rpsL genes

| Gene | Polymorphism | Aminoacid change | Isolates with polymorphism | Number of STR resistant isolates |
|------|--------------|------------------|-----------------------------|----------------------------------|
| rrs  | 162 GAA>GGA  | Glu>Gly          | 18                          | 15                               |
|      | 265 GTC>GTT  | Val/Val          | 11                          | 8                                |
|      | 290 TAC>TAA  | Tyr/Stop         | 5                           | 3                                |
|      | 302 TCA>TCC  | Ser/Ser          | 2                           | 2                                |
|      | 302 TCA>TCT  | Ser/Ser          | 10                          | 7                                |
|      | 303 AAG>GAC  | Lys/Asp          | 7                           | 6                                |
|      | 303 AAG>TAG  | Lys/Stop         | 2                           | 2                                |
|      | 31 CGT>ATT   | Arg/Leu          | 2                           | 2                                |
|      | 31 CGT>ATT   | Arg/Leu          | 2                           | 2                                |
|      | 39 G>T       | Arg/Ile          | 4                           | 4                                |
|      | 43 AAG>ACG   | Lys/Thr          | 1                           | 1                                |
|      | 43 AAG>AGG   | Lys/Arg          | 2                           | 2                                |
|      | 47 TCG>TGG   | Ser/Trp          | 8                           | 7                                |
|      | 87 GTG>TGA   |                  |                             |                                  |
In this study no mutation TCG→CTG was identified, as previously reported in RIF resistant isolates. It is worth mentioning that none of new mutations with previous evidence from Mexico about INH resistant isolates were characterized in those with TCG531TTG mutation. This supports that TCG531TTG 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 (Bakula 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 rpsL in STR resistant isolates (Cuevas-Cordoba et al., 2013a; Jagielski et al., 2014). In the present report, 28 phenotypically STR resistant isolates were identified, 4/28 with mutations in rpsL, 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 rpsL genes are reported here for the first time: A→T in nucleotide 907 in rrs and codon 88 AAG→GTT/GAA/AGA (Lys→Val/Glu/Arg) in rpsL. Mutation TCG→TG (Ser→Trp) at codon 47 in rpsL 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 rpsL gene and (c) alone, being responsible itself for STR resistance. Also, polymorphisms found at rpsL 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 resistance to STR resistance.

### 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 katG and inhA 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 (Ramasawamy 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 oxyR-ahpC, kasA, furA, fabG1, efpA, fabE24, innA, innB, innC, kasA, nat, ndh, Rv1772, Rv1592c, Rv0340, or smrR 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 rpoB 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 rpoB 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 TCG531TTG 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 TCG531TTG mutation. This supports that TCG531TTG polymorphism itself is enough to cause RIF resistance.

| Codon | Original | Novel Mutations | Frequency |
|-------|----------|----------------|-----------|
| 87 GTOGTG | 261 G→A | Val/Stop | 4/4 |
| 87 GTOGTC | 259 G→T | Val/Leu | 1/0 |
| 87 GTOCTG | 259 G→C | Val/Cys | 1/1 |
| 87 GTGCGT | 260 T→G | Val/Gly | 2/1 |
| 87 GTGCTA | 261 G→A | Val/Val | 1/1 |
| 88 AAGGAGT | 262 A→G | Lys/Val | 1/1 |
| 88 AAGAGAG | 263 A→T | Lys/Arg | 5/5 |
| 88 AAGAGAG | 263 A→G | Lys/Arg | 1/1 |
| 88 AAGAGAG | 264 G→A | Lys/Glu | 2/1 |

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

Numerous mutations in pncA have been reported worldwide (Ramírez-Busby and Valafar, 2015) in both, PZA resistant and susceptible MTBc isolates. In this study, two out of seven isolates with mutations in pncA 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 double mutations (Cuevas-Córdoba et al., 2013). Our findings support the proposal of PZA resistance being due to cellular and/or molecular mechanisms such as mutations in pnsA 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 pncA (Cuevas-Córdoba et al., 2013) or resistance to STR and mutations in rrs/rpsL (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 rpsL genes in STR resistant isolates and to establish a correlation between comorbidities 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 embB or the sequence out of RRDR in rpoB.

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 rpoB, rrs and rpsL genes it is important to study a larger number of MTBc isolates in order to stabilize 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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