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Original article

Characterization of phenotypic and genotypic drug resistance patterns of *Mycobacterium tuberculosis* isolates from a city in Mexico

Samantha Flores-Treviño[a], Rayo Morfín-Otero[b], Eduardo Rodríguez-Noriega[b], Esteban González-Díaz[b], Héctor Raúl Pérez-Gómez[b], Soraya Mendoza-Olazarán[a], Isaías Balderas-Rentería[c], Gloria María González[d], Elvira Garza-González[a,e,*]

[a] *Servicio de Gastroenterología, Hospital Universitario Dr. José Eleuterio González, Universidad Autónoma de Nuevo León, Monterrey, Nuevo León, Mexico*
[b] *Hospital Civil de Guadalajara, Fray Antonio Alcalde, y el Instituto de Patología Infecciosa y Experimental, Centro Universitario de Ciencias de la Salud, Universidad de Guadalajara, Guadalajara, Jalisco, Mexico*
[c] *Laboratorio de Ingeniería Genética y Genómica, Facultad de Ciencias Químicas, Universidad Autónoma de Nuevo León, Monterrey, Nuevo León, Mexico*
[d] *Departamento de Microbiología, Facultad de Medicina, Universidad Autónoma de Nuevo León, Monterrey, Nuevo León, Mexico*
[e] *Departamento de Patología Clínica, Hospital Universitario Dr. José Eleuterio González, Universidad Autónoma de Nuevo León, Monterrey, Nuevo León, Mexico*

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ABSTRACT

Introduction: The emergence of multidrug-resistant (MDR) *Mycobacterium tuberculosis* strains has become a worldwide health care problem, making treatment of tuberculosis difficult. The aim of this study was to determine phenotypic resistance and gene mutations associated with MDR of clinical isolates of *Mycobacterium tuberculosis* from Guadalajara, Mexico.
Methods: One hundred and five isolates were subjected to drug susceptibility testing to first line drugs using the proportion and Mycobacteria Growth Indicator Tube (MGIT) methods. Genes associated with isoniazid (*inhA*, *katG*, *ahpC*) and rifampicin (*rpoB*) resistance were analyzed by either pyrosequencing or PCR-RFLP.
Results: Resistance to any drug was detected in 48.6% of isolates, of which 40% were isoniazid-resistant, 20% were rifampicin-resistant and 19% were MDR. Drug-resistant isolates had the following frequency of mutations in *rpoB* (48%), *katG* (14%), *inhA* (26%), *ahpC* (26%). Susceptible isolates also had a mutation in ahpC (29%).
Conclusions: This is the first analysis of mutations associated with MDR of *M. tuberculosis* in Guadalajara. Commonly reported mutations worldwide were found in *rpoB*, *katG* and *inhA* genes. Substitution C to T in position -15 of the *ahpC* gene may possibly be a polymorphism.

Caracterización de los patrones de farmacorresistencia fenotípica y genotípica de aislamientos de *Mycobacterium tuberculosis* de una ciudad de México

Palabras clave:
Multifarmacorresistente
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RESUMEN

Introducción: La emergencia de cepas de *Mycobacterium tuberculosis* multifarmacorresistentes (MFR) se ha convertido en un problema de salud mundial, dificultando el tratamiento de la tuberculosis. El objetivo de este estudio fue determinar la resistencia fenotípica y las mutaciones en genes asociados a MFR en aislamientos clínicos de *M. tuberculosis* de Guadalajara, México.
Métodos: Se determinó la susceptibilidad a fármacos de primera línea de 105 aislamientos, usando los métodos de proporciones y MGIT. Los genes asociados a resistencia a isoniazida *(inhA, katG, ahpC)* y a rifampicina *(rpoB)*, se analizaron por pirosecuenciación o por PCR-RFLP.
Resultados: Se detectó la resistencia a cualquier fármaco en 48.6% de los aislamientos, 40% fueron resistentes a isoniazida, 20% fueron resistentes a rifampicina y 19% fueron MFR. Los aislamientos farmacorresistentes presentaron la siguiente frecuencia de mutaciones en *rpoB* (48%), *katG* (14%), *inhA* (26%), *ahpC* (26%). Además, los aislamientos susceptibles también mostraron una mutación en *ahpC* (29%).

* Corresponding author.
E-mail address: elvira_garza_gzz@yahoo.com (E. Garza-González).

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Conclusiones: Este es el primer análisis de mutaciones asociadas con MFR de *M. tuberculosis* en Guadalajara. Se detectaron mutaciones comúnmente reportadas a nivel mundial en los genes *rpoB, katG* e *inhA*. La sustitución de C por T en la posición -15 del gen *ahpC* posiblemente puede ser un polimorfismo.

Introduction

Tuberculosis (TB) is an important death-causing disease, considered to be the second leading cause of death from an infectious disease worldwide. Despite the availability of highly efficacious treatment for decades, TB remains a major global health problem. The World Health Organization (WHO) estimates that about one-third of the global population is infected with *Mycobacterium tuberculosis*.[1] In Mexico, more than two thousand people die of this disease every year and the TB rate per 100,000 population was 16.8 in 2010. In Jalisco, the TB rate was 13 per 100,000 population.[2] These data render tuberculosis an important public health problem in this state that deserves special attention.

One of the most alarming trends concerning TB is the emergence of multidrug-resistant strains of *M. tuberculosis* (MDR-TB), defined as resistant to at least isoniazid (INH) and rifampicin (RIF), which has been steadily increasing over the years, making treatment of TB difficult. Thus, it is important to identify resistant strains as soon as possible to allow adjustments in treatment and minimize transmission. Drug resistance (DR) in *M. tuberculosis* is caused by mutations in conserved regions of the genome. RIF-resistance is mainly caused by mutations in the β subunit of the RNA polymerase, which is encoded by the *rpoB* gene. More than 95% of resistant strains harbor mutations within the rifampicin resistance determining region (RRDR), an 81-bp hot-spot (codons 507–533) of *rpoB*, the most common of which occur at codons 526 and 531. Mutations in the catalase peroxidase gene (*katG*) and in a gene encoding the enoyl acyl carrier protein reductase (*inhA*) have been found to account for 40–60% and 20–34% of INH-resistant *M. tuberculosis* strains, respectively. Similarly, mutations in the intergenic region *oxyR-ahpC* can reduce the level of expression of *inhA* and have been associated with INH-resistance (10–15%).[3,4]

DR tuberculosis is a serious public health issue, especially in developing countries. To solve this problem it is necessary to carry out surveillance of resistance patterns and the mutations associated to this resistance. Some studies in Mexico have analyzed the frequency of gene mutations associated with drug resistance of clinical isolates of *M. tuberculosis*.[5–15] Guadalajara, Jalisco is one region that has been not studied. The aim of this study is to determine phenotypic resistance to first-line drugs and gene mutations associated with INH and RIF resistance of clinical isolates of *M. tuberculosis* in Guadalajara, Mexico.

Materials and methods

Clinical specimens and isolates

The Hospital Civil de Guadalajara, Fray Antonio Alcalde, admits >240 patients suspected of having tuberculosis per year (2005–2011). Beginning in September 2010 to November 2011, three hundred and fifty one specimens were collected. Samples were decontaminated by modified Petroff's method and cultured on Löwestein-Jensen slants at 37 °C. Smears were made and stained by the Ziehl Neelsen staining method and examined for the presence of acid-fast bacilli (AFB). The resulting isolates were identified by traditional biochemical tests, niacin production and nitrate reduction. The local ethics committee approved this study (MB11-005) and informed consent was obtained from patients.

DNA isolation

A loopful of bacterial colony suspended in 1 mL of sterile water was first inactivated at 80 °C for 1 h. The suspensions were then centrifuged at 8000 rpm for 5 min. Supernatants were discarded and pellets resuspended in 200 μL of 100 mM Tris–HCl and incubated with lysozyme (1 mg/mL) at 37 °C overnight followed by incubation with 1% SDS and proteinase K (10 mg/L) at 55 °C for 2 h. DNA was then extracted using a phenol extraction, ethanol precipitation method.[16]

Molecular identification of M. tuberculosis strains

Species identification was performed using multiplex PCR amplification of *cfp32*, RD9 and RD12.[17,18] Primer pairs for *cfp32* (5′-ATGCCCAAGAGAAGCGAATACAGGCAA-3′ and 5′-CTATTGCTGCGGTGCGGGCTTCAA-3′), RD9 (5′-TCGCCGCTGCCAGATGAGTC-3′ and 5′-TTTGGGAGCCGCCGGTGGTGATGA-3′) and RD12 (5′-GTCGGCGATAGACCATGAGTCCGTCTCCAT-3′ and 5′-GCGAAAAGTGGGCGGATGCCAG-3′) were used as a primer mixture for three simultaneous PCRs in one tube. The reaction mixture contained 1X PCR buffer, 2 mM MgCl2, 0.2 mM of each dNTP, 2.5 μL of dimethyl sulfoxide, 750 nM each of *cfp32* primers (Rv0577F and Rv0577R), 250 nM each of RD primers (Rv2073cF, Rv2073cR, Rv3120F and Rv3120R), 1 U of AmpliTaq polymerase (Bioline USA Inc., Randolph, MA, USA) and 200 ng of DNA. PCR was initiated by denaturation for 1 min at 96 °C, followed by 35 cycles of 10 s at 96 °C, 20 s at 60 °C and 1 min at 72 °C with final extension for 5 min at 72 °C. With this PCR, an isolate possessing all three regions can be identified as *M. tuberculosis*.

Phenotypic drug susceptibility testing

Drug susceptibility testing (DST) of all *M. tuberculosis* isolates was performed using two methods. The indirect proportion method on Löwestein-Jensen slants was performed as described previously with the following critical concentrations: STR 4.0 μg/mL, INH 0.2 μg/mL, RIF 40 μg/mL, EMB 2.0 μg/mL.[19] Drug resistance was expressed as the proportion of colonies that grow on drug containing medium to drug-free medium and the critical proportion for resistance was 1% for all drugs. The manual MGIT method was performed using the following critical concentrations: STR 0.8 μg/mL, INH 0.1 μg/mL, RIF 1.0 μg/mL, EMB 3.5 μg/mL, according to the protocol provided by the manufacturers (Becton Dickinson, Sparks, MD).[20] Fluorescence indicating microbial growth was detected with a 365-nm UV light from a transiluminator. When discordant results were found in both methods, testing was repeated. Strain H37Ra was used as control. Physicians in charge of TB patients were informed of all the DST results as soon as they were available. These results were then used to modify drug management of the patients accordingly.

Amplification of rpoB, inhA, ahpC and katG genes

Genes associated to INH and RIF resistance were amplified as described previously with the following primers: *rpoB*: 5′-GCGATCAAGGAGTTCTT and biotin labeled 5′-CGATCAGACCGATGTTGG;