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MUTACIONES PUNTUALES RELACIONADAS CON LA RESISTENCIA EN *Mycobacterium tuberculosis* AISLADAS DE UN HOSPITAL DE TERCER NIVEL EN ECUADOR: PRIMER REPORTE DEL GENOTIPO BEIJING

Disertación previa a la obtención del título de Licenciada en Ciencias Biológicas

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Certifico que la Disertación de Licenciatura en Ciencias Biológicas de la Srta. Nathaly Monserrate Espinel Díaz ha sido concluida de conformidad con las normas establecidas; por lo tanto, puede ser presentada para la calificación correspondiente.

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A mis padres y hermanas

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TABLA DE CONTENIDOS

LISTA DE ABREVIATURAS

Abreviatura	Significado
Ala	Alanine
AMK	Amikacin
Arg	Arginine
Asn	Asparagine
Asp	Aspartate
°C	Degrees celsius
C	Cytosine
CAP	Capreomycin
CC	Clonal complex
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
FLQ	Fluoroquinolone
Gly	Glycine
HGDI	The Hunter-Gaston index
His	Histidine
Ile	Isoleucine
INH	Isoniazid
KAN	Kanamycin
LAM	Latin American Mediterranean

LISTA DE ABREVIATURAS (CONTINUACIÓN)

Abreviatura	Significado
Leu	Leucine
MDR	Multi drug-resistant
Met	Methionine
MIRU-VNTR	Mycobacterial Interspersed Repetitive-Unit–Variable-Number Tandem-Repeat
MTBC	*Mycobacterium tuberculosis* complex
PCR	Polymerase chain reaction
Pro	Proline
QRDRs	Quinolone resistance-determining regions
RIF	Rifampicin
RNA	Ribonucleic acid
RRDR	RIF resistance determining region
Ser	Serine
T	Thymine
TB	Tuberculosis
Thr	Threonine
Tyr	Tyrosine
µl	Microliter
Val	Valine
WHO	World Health Organization
X^2	Chi-square test
XDR	Extremely drug-resistant

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1. RESUMEN

La tuberculosis es una de las enfermedades más prevalentes a nivel mundial. Es ocasionada por un complejo bacteriano conocido como complejo *Mycobacterium tuberculosis* (MTBC). A pesar de ser una enfermedad curable, con el desarrollo y dispersión de cepas resistentes a drogas se ha limitado la alterativa terapéutica. La identificación rápida y correcta de la resistencia a través del análisis de mutaciones puntuales implicadas en la resistencia a anti-tuberculosis es fundamental para prevenir el desarrollo y dispersión de cepas multirresistentes. Nuestro objetivo es caracterizar las mutaciones más frecuentes en genes relacionados con la resistencia a anti-tuberculosis y determinar el genotipo de MTBC que circula en nuestra región, apartando información para el control y prevención del desarrollo de cepas de MTBC resistentes a drogas. **Métodos:** Se analizaron 82 aislados de MTBC colectados de un hospital de tercer nivel en Quito-Ecuador. Se secuenció las regiones determinantes de la resistencia en los genes *rpoB*, *katG*, *gyrA* y *rrs* y las regiones promotoras *inhA* y *eis*. La identificación de sublinajes se realizó utilizando Repeticiones en Tándem de Número Variable de Unidades Repetitivas Intercaladas Micobacterianas (MIRU-VNTR). **Resultados:** Se registraron 48 aislados MDR, 14 aislados INH-monoresistentes, 5 aislados RIF-monoresistentes y 15 aislados sensibles a todas las drogas analizadas. 48 aislamientos RIF-resistentes muestran al menos una mutación en *rpoB*. La mutación Ser531Leu fue el más prevalente. 47 aislamientos resistentes a INH tenían una mutación en el gen *katG* y/o en el promotor *inhA*; la substitución Ser315Asn y [C (-15) T] fueron las mutaciones más prevalentes en *katG* y promotor *inhA*, respectivamente. Tres mutaciones encontradas en este estudio no se han registrado en la base de datos TB Drug Resistance Mutation:

Met515Ile en *rpoB* [GenBank: KP732541]; Thr314Ala [GenBank: KP732540] y Thr324Ser en *katG* [GenBank: KP732539]. El análisis MIRU-VNTR reveló la presencia de LAM (17,1%), Ghana (15,9%), Haarlem (8,5%), tipo S (4,9%), Camerún (3,7%) y Beijing (1,2%). **Conclusiones:** Las mutaciones de resistencia más frecuentes fueron consistentes con los reportados para la región. Encontramos tres nuevas mutaciones no reportados en los genes *rpoB* y *katG*, que podrían estar relacionados con la resistencia a RIF e INH. Registramos el primer informe del genotipo Beijing en Ecuador.

Palabras clave: *Mycobacterium tuberculosis*, resistencia, mutaciones, sub-linajes.

2. ABSTRACT

Background: Tuberculosis is one of the world's deadliest bacterial infectious diseases. *Mycobacterium tuberculosis* complex (MTBC) genotypes have been related to acquire more virulent determinants and have an innate predisposition for developing multi-resistance. Rapid and correct identification of the antimicrobial susceptibility and analyses of punctual mutations involved in antimicrobial resistance are critical to prevent the development and dissemination of new MTBC multi-resistant strains. Due to the lack of information about the lineages of MTBC circulating in Ecuador and the drug resistance molecular genes associated to them, we aim to determine the most common resistance related mutations and genotypes present in our region, contributing with information for control and prevention of new MTBC multi-resistant strains. **Methods:** We analysed 82 MTBC isolates in an 11 year period from a tertiary hospital in Quito, Ecuador. Resistance determining regions of *rpoB*, *katG*, *gyrA* and *rrs* genes, and promoter regions of *inhA* and *eis*, were sequenced. Identification and clustering of sub-lineages was done using mycobacterial interspersed repetitive-unit-variable-number tandem-repeat (MIRU-VNTR) analysis. **Results:** We registered 48 MDR, 14 INH-monoresistant, 5 RIF-monoresistant and 15 susceptible isolates. 48 RIF-resistant isolates showed at least one mutation in *rpoB*. Mutation Ser531Leu was the most prevalent. 47 INH-resistant isolates (47/62) had a mutation in the *katG* gene and/or *inhA* promoter; the substitution Ser315Asn and [C(-15)T] (13/62) were the most prevalent mutations in *katG* and *inhA* promoter, respectively. Three mutations found in this study have not been registered in the TB Drug Resistance Mutation database: Met515Ile in *rpoB* [GenBank:KP732541]; Thr314Ala [GenBank:KP732540] and Thr324Ser in *katG* [GenBank:KP732539]. The MIRU-

VNTR analysis revealed the presence of LAM (17.1%), Ghana (15.9%), Haarlem (8.5%), S-type (4.9%), Cameroon (3.7%), and Beijing (1.2 %). **Conclusions:** The most frequent resistance mutations were consistent with those reported in the region. We found three new unreported mutations in *rpoB* and *katG* genes, which could be related to rifampicin and isoniazid resistance. We registered the first report of Beijing genotype in Ecuador. The sequencing of genes' hot-spot regions involved in resistance used in this study can be a rapid method to determine new mutations associated with clinically important resistance in MTBC.

Key Words: *Mycobacterium tuberculosis*, resistance, mutations, sub-lineages

3. MANUSCRITO PARA PUBLICACIÓN

REVISTA

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TÍTULO

Punctual mutations related to resistance in *Mycobacterium tuberculosis* isolated in a tertiary hospital in Ecuador: First report of Beijing genotype.

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Introduction

Tuberculosis (TB) continues to be one of the main causes of morbidity and mortality burden worldwide. It's estimated that 2 billion people have a latent infection [1]. Nine million new cases of TB and 1.5 million deaths were reported in 2013. A great percentage of these cases were registered in poor or developing countries [1, 2].

TB is spread from person to person through the air for the disease mainly affects the lungs [3]. The pathogenic agent of TB is a bacteria from the *Mycobacterium tuberculosis* complex (MTBC). This complex includes two human pathogens, *M. tuberculosis* (main etiological agent of TB in humans) and *M. africanum.* It also includes *M. bovis* a clade of mycobacteria that can infect humans but manly infects animals [3].

Despite being a curable disease, with the emergence and spread of drug resistant MTBC strains, mainly multi drug-resistant (MDR) and extremely drug-resistant (XDR), TB possess a challenge for therapeutic alternatives compromising the success of disease control programs [1, 4]. The World Health Organization (WHO) reported around 480 000 new cases of MDR tuberculosis in 2013 [1], 10% of these XDR [5]. Moreover, in 2011 in India was reported first case of Totally drug-resistant TB (TDR) [1].

MDR is defined as rifampicin (RIF) and isoniazid (INH) resistant isolates, the first line most effective anti-tuberculosis drugs. XDR isolates are resistant to RIF, INH, fluoroquinolone (FLQ) and at least one of the injectable agents:

amikacin (AMK), kanamycin (KAN) or capreomycin (CAP) [2, 5]. TDR is defined as resistant to all available first-line and second-line TB drugs [2].

Resistance in TB originates by accumulation of specific mutations in genes which encode antibiotic target sites [6]. About 95% of RIF-resistant clinical MTBC isolates have mutations in the RIF resistance-determining region (RRDR); an 81-bp region in the *rpoB* gene that encodes RNA polymerase, the RIF target [5, 6, 7, 8].

Mutations in *katG* gene and *inhA* promotor are mainly involved in INH-resistance [6]. Nearly 50% of INH-resistant MTBC isolates have mutations in *katG* gene, which encodes the catalase-peroxidase KatG enzyme that activates INH [9]. Another 25% of INH-resistant isolates have mutations in *inhA* promoter that is associated with the over expression of enoyl-acyl carrier InhA. InhA is involved in the synthesis of micolitic acid, the main target of the active INH [5].

The FLQ-resistance in MTBC is mainly caused by mutations in conserved regions called quinolone resistance-determining regions (QRDRs) in the *gyrA* and *gyrB* genes. These genes code for DNA-gyrase subunits and the mutations inhibit DNA supercoiling and DNA replication. The mutations in QRDR of *gyrA* are mainly involved in FLQ-resistance [10, 11].

The injectable second-line drugs inhibit protein synthesis in bacteria by binding to the 30S subunit of the ribosomal 16S rRNA. The mutations in the *rrs* gene producing KAN, AMK and CAP resistance are mainly found at nucleotide positions 1401, 1402 and 1484. A mutation at positions 1401 or 1484 is associated with high resistance to all injectable agents, while mutations at position 1402

confer high resistance to CAP and resistance to KAN in a lower level. Mutations in *eis* promoter gene cause overexpression of aminoglycoside-acetyl transferase, generating low level KAN resistance [6, 12].

Epistatic interactions between mutations conferring drug resistance and compensatory mutations that increase the fitness of the strains also play an important role in the emergence of MDR-TB and XDR-TB [13]. In addition, several studies have shown that certain strains of MTBC, from different lineages, are more prone to develop drug resistance, due to their specific genetic background [14]. Of the six lineages of MTBC (1, Indo-Oceanic; 2, East-Asian; 3, East-African-Indian; 4, Euro-American; 5, West I and 6, West Africa II), the East-Asian lineage is most commonly associated with multi-drug resistance, including Beijing family strains [3, 14, 15].

In order to understand the dynamics of TB transmission, regional knowledge of MTBC diversity, population structure and main MTBC lineages is needed.

In Ecuador, the prevalence of pulmonary TB is approximately 60 cases per 100 000 inhabitants, 26% of all cases treated and 4.9% of all new cases were identified as MDR [1]. Unfortunately, there are no studies regarding genetic mutations in clinical isolates of MTBC in Ecuador, reported in indexed literature. Several studies have shown the distribution of lineages and sub-lineages in Latin America, confirming the Euro-American lineage as predominant in the region [17]. However, to our knowledge, no studies have been conducted in order to determine predominant lineages and sub-lineages in Ecuador.

In this study, we determined the most common mutations associated with drug resistance in MTBC isolates and identified the diversity of lineages and sub-lineages present in the population. Mycobacterial Interspersed Repetitive-Unit–Variable-Number Tandem-Repeat (MIRU-VNTR) analysis offers the solution to this issue as a discriminative and powerful genotyping tool [16].

Methods

Bacteria Strains

To study the antimicrobial resistance in MTBC we selected eighty two clinical isolates of MTBC; forty eight phenotypically resistant to RIF and INH, fourteen resistant to INH and five resistant to RIF. Additionally, we randomly selected fifteen susceptible isolates. MTBC clinical isolates were donated by the Departamento de Microbiología y Tuberculosis del Hospital Vozandes, Quito. The isolates were cultivated in the Lowenstein-Jensen solid medium (35° C, 5% CO_2). The species identification was confirmed by sequencing the *rrs* rRNA gene.

PCR amplification and sequencing

Genomic DNA was extracted using the commercial kit High Pure PCR template preparation kit (Roche, Switzerland), following the manufacture's specifications.

To characterize the drug resistant related mutations, we analyzed the genes *rpoB*, *katG*, *gyrA* and *rrs*, and the promoter region of *inhA* and *eis*. PCR amplifications were achieved with primers listed in Table 1.

The PCR protocol was followed as described elsewhere with modifications [7, 18, 19, 20]. Briefly, the reactions were prepared in a final volume of 25 µl, including 12.5 µl of GoTaq Green Master Mix (Promega, USA), 10 µM of each primer (Invitrogen, USA) and 5 µl of genomic DNA.

PCR products were resolved in 2% agarose gels. The amplification products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA), following manufacture's specifications. Sequencing was performed at Macrogen, South Korea. The sequences were aligned and analyzed in Geneious Pro 5.6.4 software. The strain H37Rv was used as a control in all the analysis.

Determination of the main *M. tuberculosis* lineages

MIRU-VNTR genotyping was achieved through PCR amplification of 15 loci, following the procedure described by Supply *et al*. [16]. PCR products were sized on 2% agarose gels using the Image Lab 4.0.1 Rev G (BioRad, USA). The allelic profile of each locus was calculated using the corresponding conversion table [16]. To determine the MTB strain lineages, MIRU-VNTR 15-loci profiles were analyzed in the open access database MIRU-VNTR*plus* (http://www.miru-vntrplus.org). The genotypic profile was used to construct an UPGMA tree.

Statistical analyses

The statistical analysis was implemented using the IBM® SPSS® Statistics software 22.0. Categorical data analysis was achieved applying X^2 test. The

Hunter-Gaston index (HGDI) of MIRU-VNTR types was calculated as described previously [21]. A cluster was defined as two or more isolates sharing the same MIRU-VNTR genotypic profile. The clustering rate was defined as (nc–C)/N, where nc is the total number of clustered cases, C is the number of clusters, and N is the total number of cases in the sample [22]. A *P* value < 0.05 was considered to be statistically significant.

Results

Bacteria strains

A total of eighty two *Mycobacterium tuberculosis* isolates, collected from 2002 to 2013, were tested. Of these, sixty seven were drug resistant: including forty eight (58.5%) MDR isolates, fourteen (17.1%) INH-monoresistant isolates and five (6.1%) RIF-monoresistant isolates. The remaining fifteen isolates (18.3%) exhibited susceptibility to all drugs (Table 3).

Drug resistance associated with genetic mutations in MTBC

Mutations associated with resistance to RIF were located mostly in the 81-bp hot-spot region of the *rpoB* gene. Fifty-three RIF-resistant isolates (53/82) were analyzed, of which forty-eight (90.6%) had at least one mutation in this gene, whereas five (9.4%) resistant isolates lacked these mutations (*P* = 0).

Eight types of missense mutations were identified in *rpoB*. These mutations involved codons 511, 516, 526 and 531. The most prevalent mutation was the substitution Ser531Leu (31/53; 58.5%); followed by the transitions His526Asp

(5/53; 9.4%); Asp516Val (4/53; 7.5%) and His526Leu (3/53; 5.7%). The remaining mutations were reported less frequently: the change His526Tyr was observed in two isolates (3.8%), whereas the variations His526Leu (1.9%) and Leu511Arg (1.9%) were found in one isolate. Finally one (1.9%) isolate showed a double mutation: the changes Met515Ile and Asp516Tyr (P< 0.00001). We found one mutation that is not described in the TB Drug Resistance Mutation data base (https://tbdreamdb.ki.se/Info/); codon 515 [GenBank:KP732541] (Table 4).

Among the 29 RIF-sensitive isolates, seven had a mutation at *rpoB* gene, five isolates (17.2%) were reported with the change Ser531Leu, one (3.4%) isolate with a change the His526Arg and one (3.4%) isolate with transition His526Tyr (*P*< 0.00001). (Table 3).

To determine the genotypic resistance to INH, *katG* gene and *inhA* promoters were sequenced. Sixty-two INH-resistant isolates were analyzed, of which forty-seven (75.8%) had a mutation in the *katG* gene and/or *inhA* promoter. Of the forty-seven isolates, two (3.3%) had a mutation in both loci; while thirty-four (54.8%) presented mutations only in *katG,* and eleven (17.7%) showed mutations only in *ihnA* promoter. Fifteen (24.2%) of the sixty-two INH-resistant isolates lacked the mutation in both loci (*P* = 0.000195).

We found seven mutations in the *katG*, five of which are known to correlate with INH resistance. We found two mutations not previously reported in the TB Drug Resistance Mutation database,Thr314Ala (1.6%) [GenBank:KP732540] and Thr324Ser (1.6%) [GenBank:KP732539]. Also, the genotypic profile of one if the isolates could not be established (1.6%).

The most prevalent change in *katG* was the replacement Ser315Asn (31/62; 50.0%). The mutation Ser315Thr was found in two isolates (3.2%) (*P*< 0.00001). The twenty INH-susceptible isolates did not show mutations in *katG*.

Of the sixty two INH-resistant isolates thirteen (21.0%) presented a mutation at *inhA* promoter. In the study population, only the change [C (-15) T] was found 15 nucleotides upstream of the start codon. Six (6/20; 30.0%) of the twenty INH-susceptible isolates had a mutation [C (-15) T] in the *inhA* promoter (*P* = 0.405145).

Furthermore, in the study we analyzed the loci mainly involved with second line tuberculosis drug resistance. FQ genotypic resistance was determined analyzing the RDRQ sequence of the *gyrA* gene, while, resistance to aminoglycosides (AMK, KAN, CAP) was determined by the analysis of *rrs* gene and *eis* promoter (Table 5).

Of the eighty-two isolates studied, six (7.3%) presented a point mutation in the *gyrA*, these mutations were registered in codons 90, 91 and 94. The most common mutation was the change Asp94Try (2/82; 2.4%), followed the changes Asp94Gly (1/82; 1.2%), Asp94Asn (1/82; 1.2%), Ala90Val (1/82; 1.2%) and Ser91Pro (1/82; 1.2%).

Only four (4.9%) isolates of the eighty two tested showed a change of nucleotide adenine to guanine in position 1401 of the *rrs*. No mutations in *eis* promoter were registered.

In total, we identified sequence changes in eight INH-monoresistant isolates, eleven RIF-monoresistant isolates, thirty eight MDR isolates, three pre-

XDR isolates and three XDR isolates. Eighteen isolates showed no mutation in the analyzed loci, and the genotypic profile of one isolate could not be determined.

MIRU-VNTR genotype profiles of the MTBC isolates

The eighty-two isolates were genotyped with MIRU-VNTR using a 15 loci panel. Six different genotypes were determined: Cameroon, Ghana, Haarlem, LAM (Latin American Mediterranean), S-type and Beijing. Forty (48.8%) genotypes could not be identified in the database; these strains were classified as orphan types. The most prevalent genotype was LAM (14; 17.1%), followed by Ghana (13; 15.9%), Haarlem (7; 8.5%), S-type (4/82; 4.9%), Cameroon (3; 3.7%), and Beijing (1/82; 1.2 %).

The UPGMA tree, based on the MIRU-VNTR 15-loci, showed a total of 73 different patterns, which included six clusters, one with five isolates and the remaining clusters with two isolates each (Fig 1). In total, fifteen strains grouped into clusters and sixty seven strains with unique pattern were reported (11% clustering rate). Genotyping showed a high diversity in the study population; the Hunter & Gaston discriminatory index (HDGI) results in a value of 0.995.

The minimum spanning tree analysis indicated that the eighty-two isolates were divided into ten clonal complexes (CC1-CC10) and thirty-six singletons. The CC3, CC7, CC8, CC9 and CC10 were conformed of strains of the LAM sub-lineage; the CC1 had mostly strains of the Ghana sub-lineage, the CC5 was conformed of strains of Haarlem sub-lineage, strains of sub-lineages Cameroon belonged to CC6 and the orphans strains belonged to CC2 and CC4. Also, the Beijing sub-lineage strain is one of the thirty-six singletons (Fig 2).

Genotyping and mutational analysis

We were unable to correlate a particular mutation with a specific sub-lineage as the mutations found were distributed randomly in the study population and were not associated with a sub-lineage in this study

Discussion

Early detection of resistance in MTBC is important to achieve a successful TB treatment. Conventional methods to detect resistance to most anti-TB drugs are very laborious and time consuming [8] compared to molecular diagnosis. However, molecular analysis has limitations because it requires complete information about the type and frequency of resistant related mutations linked to the region [23], also most of the commercial systems available have a narrow panel of mutations which is not specific for any region

Recent studies have shown that 10.0-40.0% of mutations causing resistance in clinical isolates have not been identified, so it is important to find new mutations that could lead to the emergence of resistance in MTBC [24]. Three mutations found in this study have not been previously reported in the TB Drug Resistance Mutation database (https://tbdreamdb.ki.se/Info/). The new mutations Met515Ile in *rpoB*; Thr314Ala in *katG* and Thr324Ser in *katG,* were only identified in drug resistant isolates, however, more studies are necessary to confirm their role in resistance.

Studies have shown that mutations in the RDRR of *rpoB* region are found in 95.0% of RIF-resistant isolates [18, 25]. In our study, a significant correlation

between phenotypic and genotypic profile of resistance was observed, 90.6% (P = 0.00).

Mutations at codons 531, 526 and 516 were recorded in forty-seven of the fifty-three resistant isolates. The change Ser531Leu was the most frequent (P = 0.00032) as reported in other studies [8, 26, 27]. However, the frequency (of the mutations His526Asp and the Asp516Val differ with the values reported for other geographic regions [28].

Similar to other studies [8, 27], we found that 9.4% of RIF-resistant isolates did not show mutations in RDRR. Furthermore, seven of the twenty-two RIF-sensitive isolates were cataloged as resistant to RIF by sequencing of the *rpoB* gene. . This is because not all of the *rpoB* gene genotypic changes affect the phenotypic resistance to RIF, the position and nature of the mutation (amino acid substitution) is strongly correlated with the MIC of RIF resistance [28]. It is known that mutations at codons 516, 518, 522, 529 and 533 give low resistance to RIF [26]. Several studies have shown that conventional diagnostic methods, mainly broth cultures based, where the average range of MIC for the critical concentration of resistance is at least 4 mg/mL, may categorize some isolates as sensitive RIF, although these present a mutation in the *rpoB* gene [29, 30, 31].

It is known that the *katG*, *inhA*, *ahpC*, *ndh* and *oxyR* genes are involved with resistance to INH. However, many studies found that resistance to INH in TBMC is mainly correlated to mutations in the *katG* gene and the *inhA* promoter [8, 26]. We also found a strong correlation (P = 0.000195) between INH resistance and mutations in katG and/or the inhA promoter; 75.8% of INH-resistant isolates presented a mutation in these loci.

Our sample had a high prevalence (53.2%) of mutations at codon 315 of *katG*. Other studies, have reported this mutation in up to 95% of INH-resistant isolates, which is why this region is considered a molecular target for resistance to INH [8, 14, 26, 32].

Among the registered mutations at *katG* codon 315, Ser315Asn was reported as the most frequent (P <0.00001), unlike what has been found in other studies, where the change Ser315Thr has significant clinical importance [18]. This contrast shows that the frequency and the type of mutation in *katG* can vary with the geographical regions [8, 24, 29]. No INH-sensitive isolates had mutations in *katG*. We only found the change [C (-15) T] in the *inhA* promoter. In this study 21% of INH-resistant isolates showed this mutation, indicating that INH resistance is largely associated with a *katG*. Six of the twenty INH-sensitive isolates showed mutation in the *inhA* promoter, this is possibly because mutations in the *inhA* promoter are associated with a low level of INH-resistance [8].

Finally, fifteen (24.2%) INH-resistant isolates showed no mutation in the two analyzed loci. This discrepancy may be attributed to the presence of mutations outside the analyzed region or other genes involved in INH-resistance [8].

FQ are the base treatment for MDR-TB [33]. *gyrA* and *gyrB* genes have mainly been associated with FQ resistance. It has been shown that mutations in *gyrB*, are less common than mutations in *gyrA* [34In this study only the QRDR of *gyrA* gene was analyzed. It was observed that 7.3% of the analyzed isolates had a mutation in this region, so they can be categorized as FQ resistant. Mutations in *gyrA* were found only in MDR isolates. In our study, the change Asp94Tyr was the most prevalent, however, other studies have reported changes of Asp94Gly as the

most common [26, 10]. The finding of FQ genotypically resistant isolates suggests the need of implementing FQ in the susceptibility tests for MDR isolates.

The second-line injectable agents (AMK, KAN and CAP) are used in the treatment of MDR-TB [35]. Although AMK and KAN are aminoglycoside antibiotics and CAP is a macrocyclic peptide, cross resistance between these anti-TB has been described widely [26, 29]. Mutations in the *rrs* gene encoding for 16sRNA are mainly involved to a high level of resistance to these antibiotics [35, 36]. However, recent studies have reported that low levels of resistance to these agents, mainly KAN, are due to the presence of mutations in *eis* promoter, related to overexpression of aminoglycoside acetyltransferase [37]. Because of its relationship with resistance to second-line injectable agents the *rrs* gene and the *eis* promoter were analyzed in this study. 4.9% of the studied isolates had a mutation associated with resistance to second-line injectable agents. The change of adenine for thymine in the 1401 position of the gene was reported. This is consistent with previous descriptions, where 30 to 90% of the isolates resistant to this anti-TB present this change [37]. No changes were registered in the *eis* promoter. These mutations were found in MDR isolates, indicating that it is important to test susceptibility to second line drugs in all MDR isolates to provide appropriate treatment.

The typing method MIRU-VNTR allows epidemiologist to investigate the dynamics of TB transmission, to find possible focal infection and, to identify outbreaks. This typing method can be also used for phylogenetic characterization of TB infections, allowing us to know the lineage / sub lineage and clonal

complexes of a MTBC population [23, 38, 39]. Using MIRU-VNTR 15-loci technique, it was possible to classify within a lineage to 51.2%.

We observed a great deal of genetic diversity in the population. Six different genotypes were found, with the LAM sub-lineage being the most predominant (17.1%), followed by Ghana (15.9%), Haarlem (8.5%), S-type (4.9%), Cameroon (3.7%) and Beijing (1/82; 1.2 %). Our results are similar to previous reports from Colombia and Peru, where LAM or Haarlem are the predominant sub-lineages [17, 40].

The sub-lineages Ghana, LAM, Haarlem, Cameroon, and S-type belong to the Euro-American (lineage 4), one of the six lineages mainly reported in Europe, America and Middle East [17, 41].

One isolate was classified in the Beijing sub-lineage, belonging to the East-Asian (lineage 2). While seven countries in South America, including Colombia and Peru, have reported the Beijing genotype [42], this is the first report of this sub-lineage in Ecuador. Identifying the Beijing genotype is important due to their ability to acquire resistance to drugs, their hyper mutability, transmission capacity and virulence [43].

Grouping rate is a marker used to determine the recent transmission of TBM [39]. In this study the presence of six clusters is recorded. However, clustering index was 11%, indicating that the isolates do not have an epidemiological link.

UPGMA dendrogram analysis showed that each isolate from a cluster had a different mutational profile (Figure 1).This is consistent with that described by

Wang *et al*. (2014) suggesting that the resistance in MTBC isolates is not conserved among the defined strains of a cluster but individual isolates of each cluster evolve unique characteristics during TB treatment [23].

No significant association between the lineage / sub lineage and mutational profile was found but it has been shown that each strain generic background plays a major role in the development of drug resistance, meaning a single mutation in different lineage isolates also present a different resistance levels [14].

Even though, the sample size was limited, our results establish a basic understanding of the molecular epidemiology of MBTC in Ecuador. Additionally, we show that the sequencing of hot-spot genetic regions is a useful in screening for MBTC. However, to determine new mutations related to resistance it is likely necessary to sequence the complete open reading frame of each gene [29]. It's important to note that for a better correlation between the genotypic patterns and phenotypic susceptibility profiles, the Minimum Inhibitory Concentration (MIC) of the isolates to anti-TB drugs is required, unfortunately in our countries the MIC in TB techniques are yet to be established. Finally, despite the high discrimination obtained with 15 MIRU-VNTR panel, in order to create better grouping analysis we proposed the 24 MIRU-VNTR panel [22].

Conclusion

The accurate and rapid detection of MTBC resistant isolates is critical for proper treatment of TB. Molecular detection of resistance by analyzing mutations in related genes is an excellent choice for proper treatment of this disease. We found a high correlation between genotypic and phenotypic resistance in our

Ecuadorian samples, demonstrating the reliability of this detection method. Our results suggest a high utility of the identification of mutations in *rpoB* and *katG* to infer resistance to RIF and INH.

Furthermore, although the frequency of some mutations associated with resistance is similar to what reported in other countries (Ser531Leu in *rpoB*), others mutations frequencies are unique (Ser315Asn in *katG*), suggesting that the type and frequency of each mutation varies depending on geographic region.

This highlighting the importance of having good data local populations rather than relying on data from populations in distinct region.

Three new mutations were found, one in the *rpoB* and two in the *katG*, which could be involved with RIF and INH resistance respectively; demonstrating the need to look for mutations not yet identified as potential causes of resistance. Regarding the genomic diversity of MTBC, the most predominant sub-lineage in this study was LAM, similar to those reported in neighboring countries. Furthermore, Beijing genotype was recorded for the first time in Ecuador. These results provide invaluable information for supplementing the epidemiological map of MTBC for the region, and contribute to the understanding of the TB resistance dynamics in Ecuador.

Availability of supporting data

The data set supporting the results of this article is included as an additional file.

Competing interests

All authors report no conflicts of interest relevant to this article.

Authors' contributions

NED performed all experiments and writing the manuscript (bachelor thesis). DOP project design, data collect, laboratory supervision, results analysis, literature search, manuscript supervision. PB data collected laboratory supervision, results analysis, literature search, manuscript supervision. MM project designer (He worked in Unidad de Investigaciones en Biomedicina. Zurita&Zurita Laboratorios, Quito. Now he is working at University of British Columbia, Vancouver). IA assisted in project management, laboratory facilities. JZ project designer, project manager and project supervisor, provided TB clinical strains, involved in analysis of clinical aspects, analysis and control of article. All authors read and approved the final manuscript.

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References

1. World Health Organization. Guidelines for Global Tuberculosis reported 2014. http://apps.who.int/iris/bitstream/10665/137094/1/9789241564809_eng.pdf.

2. Zumla A, Nahid P, Cole ST. Advances in the development of new tuberculosis drugs and treatment regimens. Nature Reviews Drug Discovery. 2013;12(5):388-404.
3. Galagan JE. Genomic insights into tuberculosis. Nature Reviews Genetics. 2014;15(5):307-20.
4. Martinez-Guarneros A, Rastogi N, Couvin D, Escobar-Gutierrez A, Rossi LMG, *et al.* Genetic diversity among multidrug-resistant *Mycobacterium tuberculosis* strains in Mexico. Infection, Genetics and Evolution 2013;14:434-443.
5. Koch A, Mizrahi V, Warner DF. The impact of drug resistance on *Mycobacterium tuberculosis* physiology: what can we learn from rifampicin?. Emerging Microbes & Infections. 2014;3(3)e17.
6. Engström A, Morcillo N, Imperiale B, Hoffner SE, Juréen P. Detection of first- and second-line drug resistance in *Mycobacterium tuberculosis* Clinical Isolates using Pyrosequencing. Journal of Clinical Microbiology. 2012;50(6):2026-33.
7. Zakham F, Chaoui I, Echchaoui AH, Chetioui F, Elmessaoudi MD, Ennaji MM, Abid M, Mzibri ME. Direct sequencing for rapid detection of multidrug resistant *Mycobacterium tuberculosis* strains in Morocco. Infection and Drug Resistance. 2013;28(6):207-13.
8. Tekwu EM, Sidze LK, Assam JP, Tedom JC, Tchatchouang S, Makafe GG, et al. Sequence analysis for detection of drug resistance in *Mycobacterium tuberculosis* complex isolates from the Central Region of Cameroon. BMC Microbiology. 2014;14(1):113.

9. Lee JH, Ammerman NC, Nolan S, Geiman DE, Lun S, Guo H, et al. Isoniazid resistance without a loss of fitness in *Mycobacterium tuberculosis*. Nature Communications. 2012;20(3):753.
10. Li J, Gao X, Luo T, Wu J, Sun G, Liu Q, Jiang Y, Zhang Y, Mei J, Gao Q. Association of *gyrA/B* mutations and resistance levels to fluoroquinolones in clinical isolates of *Mycobacterium tuberculosis*. Emerging Microbes & Infections. 2014;3(3):e19.
11. Singh P, Jain A, Dixit P, Prakash S, Jaiswal I, Venkatesh V, Singh M. Prevalence of *gyrA* and *B* gene mutations in fluoroquinolone-resistant and sensitive clinical isolates of *Mycobacterium tuberculosis* and their relationship with MIC of ofloxacin. The Journal of Antibiotics. 2014;68(1):63-6.
12. Lin SYG, Desmond EP. Molecular Diagnosis of Tuberculosis and Drug Resistance. Clinics in Laboratory Medicine. 2014;34(2):297-314.
13. Borrell S, Gagneux S. Strain diversity, epistasis and the evolution of drug resistance in *Mycobacterium tuberculosis*. Clinical Microbiology and Infection. 2012;17(6):815-820.
14. Müller B, Borrell S, Rose G, Gagneux S. The heterogeneous evolution of multidrug-resistant *Mycobacterium tuberculosis*. Trends in Genetics. 2013;29(3):160-169.
15. Coll F, McNerney R, Guerra-Assunção JA, Glynn JR, Perdigão J, Viveiros M, et al. A robust SNP barcode for typing *Mycobacterium tuberculosis* complex strains. Nature Communications. 2014;1(5):4812.
16. Supply P, Allix C, Lesjean S, Cardoso-Oelemann M, Rüsch-Gerdes S, Willery E, et al. Proposal for standardization of optimized mycobacterial interspersed

repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. Journal of Clinical Microbiology. 2006;44(12):4498-4510.

17. Realpe T, Correa N, Rozo JC, Ferro BE, Gomez V, Zapata E, et al. Population Structure among *Mycobacterium tuberculosis* Isolates from Pulmonary Tuberculosis Patients in Colombia. PloS one. 2014;9(4):e93848.
18. Cho EH, Bae HK, Kang SK, Lee EH. Detection of isoniazid and rifampicin resistance by sequencing of *katG*, *inhA*, and *rpoB* genes in The Korean. Journal of Laboratory Medicine. 2009; 29:455–460.
19. Lau RW, Ho PL, Kao RY, Yew WW, Lau TC, Cheng VC, et al. Molecular characterization of fluoroquinolone resistance in *Mycobacterium tuberculosis*: functional analysis of *gyrA* mutation at position 74. Antimicrobial Agents and Chemotherpy. 2011;55(2):608-14.
20. Roberts EA, Clark A, McBeth S, Friedman RL. Molecular characterization of the *eis* promoter of *Mycobacterium tuberculosis*. Journal of Bacteriology. 2004;186(16):5410.
21. Hunter PR, Gaston MA. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. Journal Of Clinical Microbiology.1988; 26(11):2465-2466.
22. Wang J, Liu Y, Zhang CL, Ji BY, Zhang LZ, Shao YZ, et al. Genotypes and characteristics of clustering and drug-susceptibility of *Mycobacterium tuberculosis* isolates in Heilongjiang Province, China. Journal of Clinical Microbiology. 2011;49(4):1354-62.
23. Wang Q, Lau SK, Liu F, Zhao Y, Li HM, Li BX, et al. Molecular Epidemiology and Clinical Characteristics of Drug-Resistant *Mycobacterium tuberculosis* in a Tuberculosis Referral Hospital in China. PloS one. 2014;9(10):e110209.

24. Farhat MR, Shapiro BJ, Kieser KJ, Sultana R, Jacobson KR, Victor TC, et al. Genomic analysis identifies targets of convergent positive selection in drug-resistant *Mycobacterium tuberculosis*. Nature Genetics. 2013;45(10):1183-1189.
25. Comas I, Borrell S, Roetzer A, Rose G, Malla B, Kato-Maeda M, et al. Whole-genome sequencing of rifampicin-resistant *Mycobacterium tuberculosis* strains identifies compensatory mutations in RNA polymerase genes. Nature Genetics. 2012;44(1):106-110.
26. Almeida Da Silva PE, Palomino JC. Molecular basis and mechanisms of drug resistance in *Mycobacterium tuberculosis*: classical and new drugs. Journal of Antimicrobial Chemotherapy. 2011;66(7):1417-1430.
27. Campbell PJ, Morlock GP, Sikes RD, Dalton TL, Metchock B, Starks AM, et al. Molecular detection of mutations associated with first-and second-line drug resistance compared with conventional drug susceptibility testing of *Mycobacterium tuberculosis*. Antimicrobial Agents and Chemotherapy. 2011;55(5):2032-2041.
28. Ocheretina O, Escuyer VE, Mabou MM, Royal-Mardi G, Collins S, Vilbrun SC, et al. Correlation between genotypic and phenotypic testing for resistance to rifampin in *Mycobacterium tuberculosis* clinical isolates in Haiti: investigation of cases with discrepant susceptibility results. PloS one. 2014;9(3):e90569.
29. Williamson DA, Roberts SA, Bower JE, Vaughan R, Newton S, Lowe O, et al. Clinical failures associated with *rpoB* mutations in phenotypically occult multidrug-resistant *Mycobacterium tuberculosis*. The International Journal of Tuberculosis and Lung Disease. 2012;16(2):216-220.

30. Van Deun A, Barrera L, Bastian I, Fattorini L, Hoffmann H, Kam KM, et al. *Mycobacterium tuberculosis* strains with highly discordant rifampin susceptibility test results. Journal of Clinical Microbiology. 2009;47(11):3501-3506.

31. Andres S, Hillemann D, Rüsch-Gerdes S, Richter E. Occurrence of *rpoB* Mutations in Isoniazid-Resistant but Rifampin-Susceptible *Mycobacterium tuberculosis* Isolates from Germany. Antimicrobial Agents and Chemotherapy. 2014;58(1):590-592.

32. Gonzalo X, Ambroggi M, Cordova E, Brown T, Poggi S, Drobniewski F. Molecular epidemiology of *Mycobacterium tuberculosis*, Buenos Aires, Argentina. Emerging Infectious Diseases Infectious Diseases. 2011;17(3):528-31.

33. Yuan X, Zhang T, Kawakami K, Zhu J, Li H, Lei J, et al. Molecular characterization of multidrug and extensively drug-resistant *Mycobacterium tuberculosis* strains in Jiangxi, China. Journal of Clinical Microbiology. 2012;50(7):2404-13.

34. Eilertson B, Maruri F, Blackman A, Herrera M, Samuels DC, Sterling TR. High proportion of heteroresistance in *gyrA* and *gyrB* in fluoroquinolone-resistant *Mycobacterium tuberculosis* clinical isolates. Antimicrobial Agents and Chemotherapy. 2014;58(6):3270-3275.

35. Gikalo MB, Nosova EY, Krylova LY, Moroz AM. The role of *eis* mutations in the development of kanamycin resistance in *Mycobacterium tuberculosis* isolates from the Moscow region. Journal of Antimicrobial Chemotherapy. 2012;67(9):2107-9.

36. Sowajassatakul A, Prammananan T, Chaiprasert A, Phunpruch S. Molecular characterization of amikacin, kanamycin and capreomycin resistance in M/XDR-TB strains isolated in Thailand. BMC Microbiology. 2014;14(1):165.
37. Georghiou SB, Magana M, Garfein RS, Catanzaro DG, Catanzaro A, Rodwell TC. Evaluation of genetic mutations associated with *Mycobacterium tuberculosis* resistance to amikacin, kanamycin and capreomycin: a systematic review. PloS one. 2012;7(3):e33275.
38. Barnes PF, Cave M.D. Molecular epidemiology of tuberculosis. New England Journal of Medicine. 2003;349(12):1149-1156.
39. Tessema B, Beer J, Merker M, Emmrich F, Sack U, Rodloff AC, et al. Molecular epidemiology and transmission dynamics of *Mycobacterium tuberculosis* in Northwest Ethiopia: new phylogenetic lineages found in Northwest Ethiopia. BMC infectious Diseases. 2013;13(1):131.
40. Sheen P, Couvin D, Grandjean L, Zimic M, Dominguez M, Luna G, et al. Genetic diversity *of Mycobacterium tuberculosis* in Peru and exploration of phylogenetic associations with drug resistance. PloS one. 2013;8(6):e65873.
41. Rindi L, Medici C, Bimbi N, Buzzigoli A, Lari N, Garzelli C. Genomic Variability of *Mycobacterium tuberculosis* Strains of the Euro-American Lineage Based on Large Sequence Deletions and 15-Locus MIRU-VNTR Polymorphism. PloS one. 2014;9(9):e107150.
42. Ritacco V, López B, Cafrune PI, Ferrazoli L, Suffys PN, Candia N, et al. *Mycobacterium tuberculosis* strains of the Beijing genotype are rarely observed in tuberculosis patients in South America. Memórias do Instituto Oswaldo Cruz. 2008;103(5):489-492.

43. Parwati I, van Crevel R, van Soolingen D. Possible underlying mechanisms for successful emergence of the *Mycobacterium tuberculosis* Beijing genotype strains. The Lancet Infectious Diseases. 2010;10(2):103-111.

Figure 1. UPGMA dendrogram based on the 15-loci MIRU-VNTR data for eighty-two clinical isolates of MTBC. Six different genotypes were determined, labeled with different color. The MIRU-VNTR genotypic profile is included. A total of 73 different patterns were found, which included six clusters with a maximum of five and minimum two isolates; clustering rate was 11,0%. The phenotypic resistance to RIF and INH is observed, as well as the mutations present in rpoB, katG, inhA promoter, *gyrA*, *rrs* and *eis* promoter.

Figure 2. Minimum spanning tree based on MIRU-VNTR 15-loci profiles. Each circle represents a different strain, the frequency (number of strains) is determined by the color of each circle (white: 1 isolate, purple: 2 isolates and blue: 5 isolates) Each CC is marked by a different color.

Table 1. Primers used in resistance identification

Gene	Sequences (5′-3′)	size (bp)	Annealing temperature	Resistance[a]	Reference
rpoB	rpoB-F: AGGACGTGGAGGCGATCA	245	58 °C	RIF	7, 18
	rpoB-R: GGTTTCGATCGGGCACAT				
katG	katG-F: TGGCCGCGGCGGTCGACATT	420	62 °C	INH	7, 18
	katG-R: GGTCAGTGGCCAGCATCGTC				
inhA **promoter**	inhA-P-F: CCTCGCTGCCCAGAAAGGGA	249	60 °C	INH	7, 18
	inhA-P-R: ATCCCCCGGTTTCCTCCGGT				
gyrA	gyrA-F: CAGCTACATCGACTATGCGA	320	55 °C	FQ	19
	gyrA-R: GGCTTCGGTGTACCTCAT				
rrs	27F: AGAGTTTGATCMTGGCTCAG	1200	72 °C	AMK, KAN, CAP	20
	1492R:TACGGYTACCTTGTTACGACTT				
eis **promoter**	Eis-P-F: AATTCGTCGCTGATTCTCGC	210	55 °C	AMK, KAN, CAP	20
	Eis-P-R: TGAAATCGGTGAAACTGGCC				

[a] RIF, rifampicin; INH, isoniazid; FQ, fluoroquinolone; AMK, amikacin; KAN, kanamycin; CAP, Capreomycin.

Table 2. Locus and primers used for amplification of MURU-VNTR

Locus	Alias	Repeat unit length (bp)	PCR primers pair (5´-3´)
580	MIRU 4	77	MIRU 4F GCGCGAGAGCCCGAACTGC
			MIRU 4 R GCGCAGCAGAAACGCCAGC
2996	MIRU 26	51	MIRU 26F TAGGTCTACCGTCGAAATCTGTGAC
			MIRU 26R CATAGGCGACCAGGCGAATAG
802	MIRU 40	54	MIRU 40 F GGGTTGCTGGATGACAACGTGT
			MIRU 40 R GGGTGATCTCGGCGAAATCAGATA
960	MIRU 10	53	MIRU 10F GTTCTTGACCAACTGCAGTCGTCC
			MIRU 10R GCCACCTTGGTGATCAGCTACCT
1644	MIRU 16	53	MIRU 16F TCGGTGATCGGGTCCAGTCCAAGTA
			MIRU16R CCCGTCGTGCAGCCCTGGTAC
3192	MIRU 31	53	MIRU 31F ACTGATTGGCTTCATACGGCTTTA
			MIRU 31R GTGCCGACGTGGTCTTGAT
424	Mtub04	51	Mtub04F CTTGGCCGGCATCAAGCGCATTATT
			Mtub04R GGCAGCAGAGCCCGGGATTCTTC
577	ETR C	58	ETR C F CGAGAGTGGCAGTGGCGGTTATCT
			ETR C R AATGACTTGAACGCGCAAATTGTGA
2165	ETRA	75	ETR A F AAATCGGTCCCATCACCTTCTTAT
			ETR A R CGAAGCCTGGGGTGCCCGCGATTT
2401	Mtub30	58	Mtub30 F CTTGAAGCCCCGGTCTCATCTGT
			Mtub30 R ACTTGAACCCCCACGCCCATTAGTA
3690	Mtub39	58	Mtub39 F CGGTGGAGGCGATGAACGTCTTC
			Mtub39 R TAGAGCGGCACGGGGGAAAGCTTAG
4156	QUB-4156	59	QUB-4156 F TGACCACGGATTGCTCTAGT
			QUB-4156 R GCCGGCGTCCATGTT
2163b	QUB-11b	69	QUB-11b F CGTAAGGGGGATGCGGGAAATAGG
			QUB-11b R CGAAGTGAATGGTGGCAT
1955	Mtub21	57	Mtub21 F AGATCCCAGTTGTCGTCGTC
			Mtub21 R CAACATCGCCTGGTTCTGTA
4052	QUB-26	111	QUB-26 F AACGCTCAGCTGTCGGAT
			QUB-26 R CGGCCGTGCCGGCCAGGTCCTTCCCGAT

Table 3. Resistance profiles of MTBC isolates collected between 2002 -2013

Profile	N° of isolates	% of isolates
Sensitive	15	18.3
INH	14	17.1
RIF	5	6.1
INH+RIF	48	58.5
Total	82	100.0

INH, Isoniazid; RIF, Rifampicin

Table 4 Mutations identified in drug resistance-associated loci in MTBC

Gene	Codon	Base mutation	Amino acid change[g]	Drug[a]	N° of genotypic resistant isolates/ N° of phenotypic resistant isolates	N° of genotypic resistant isolates/ N° of phenotypic susceptible isolates	*P* value
rpoB	None	None	None	RIF	5/53	22/29	0.00121
rpoB	511	CTG→CCG	Leu→Arg	RIF	1/53	0/29	0.45671
rpoB	516	GAC→GTC	Asp→Val	RIF	4/53	0/29	0.12929
rpoB	516/515[d]	GAC→TAC ATG→ATC[f]	Asp→Tyr Met→Ile[b]	RIF	1/53	0/29	0.45671
rpoB	526	CAC → CTC	His→Leu	RIF	3/53	0/29	0.19178
rpoB	526	CAC→CGC	His→Arg	RIF	1/53	1/29	0.66120
rpoB	526	CAC→GAC	His→Asp	RIF	5/53	0/29	0.11874
rpoB	526	CAC→TAC	His→Tyr	RIF	2/53	1/29	0.94020
rpoB	531	TCG→TTG	Ser→Leu	RIF	31/53	5/29	0.00032
katG	None	None	None	INH	26/62	0/20	0.00045
katG	314	ACC→GCC[b]	Thr→Ser	INH	1/62	0/20	0.56769
katG	315	AGC→ACA	Ser→Thr	INH	2/62	0/20	0.41610
katG	315	AGC→ACC	Ser→Asn	INH	31/62	0/20	>0.00001
katG	324	AAC→TCC[b]	Thr→Ser	INH	1/62	0/20	0.56769
katG	ND[e]	ND	ND	INH	1/62	1/20	0.39318
inhA **promoter**	None	None	None	INH	49/62	14/20	>0.00001
inhA **promoter**		C(-15)T		INH	13/62	6/20	0.405145

[a] RIF, Rifampicin; INH, Isoniazid
[b] Mutations not previously reported.
[c] None, no mutation
[d] Multiple mutations in one isolate.
[e] Not determined
[f] A, adenine; G, guanine; C, cytosine; T, thymine
[g] His, Histidine; Asp, Aspartate; Val, Valine; Leo, Leucine; Tyr, Tyrosine; Arg, Arginine; Met, Methionine; Ile, Isoleucine; Ser, Serine; Ala, Alanine, Thr, Threonine, Asn, Asparagine; Gly, Glycine; Pro, Proline.

Table 5 Mutations associated second line drug resistance

Gene	Codon	Base mutation	Amino acid change	Drug[a]	N° (%) of isolates
gyrA	94	GAC→GGC	Asp→Gln	FQ	1 (1.2%)
gyrA	94	GAC→TAC	Asp→Try	FQ	2 (2.4%)
gyrA	94	GAC→AAC	Asp→Asn	FQ	1 (1.2%)
gyrA	90	GCG→CTG	Ala→Val	FQ	1 (1.2%)
gyrA	91	TCG→CCG	Ser→Pro	FQ	1 (1.2%)
gyrA	None	None	None	FQ	77 (92.8%)
rrs	None	1401A→T	None	AMK, KAN,CAP	4 (4.9%)
rrs	None	None	None	AMK, KAN,CAP	78 (95.1%)
***eis* promoter**	None	None	None	AMK, KAN,CAP	82 (100.0%)

[a] FLQ, fluoroquinolone; AMK, amikacin; KAN, kanamycin; CAP capreomycin, A, adenine; G, guanine; C, cytosine; T, thymine; Asp, Aspartate; Gln, Glutamine, Val, Valine; Tyr, Tyrosine; Ser, Serine; Asn, Asparagine; Pro, Proline.

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5. ANEXO

Anexo 1. Perfil fenotípico y genotípico de susceptibilidad a anti-TB en aislados clínicos de *Mycobacterium tuberculosis*

#	Aislado	Fecha de aislamiento	Susceptibilidad a anti-TB		Mutaciones relacionadas con la resistencia a anti-TB de primera y segunda línea					
			RIF	INH	Gen *rpoB*	Gen *katG*	Promotor *inhA*	Gen *gyrA*	Gen *rrs*	Promotor *eis*
1	3	18-1-02	S	S	A	A	C →T (-15)	A	A	A
2	9	13-12-03	S	S	A	A	C →T (-15)	A	A	A
3	44	16-5-02	S	S	A	A	A	A	A	A
4	53	20-6-02	R	S	531TCG→TTG	A	C →T (-15)	94 GAC→GGC	1401 A→G	A
5	55	22-7-02	S	S	A	A	A	A	A	A
6	58	22-4-02	R	R	526 CAC→TAC	315 AGC→ACC	A	A	A	A
7	63	9-5-02	S	R	531TCG→TTG	A	C →T (-15)	A	A	A
8	80	22-7-02	S	S	A	A	A	A	A	A
9	98	5-8-02	S	R	531TCG→TTG	315 AGC→ACC	A	A	A	A
10	140	20-11-02	R	R	531TCG→TTG	315 AGC→ACC	A	A	A	A
11	150	3-1-03	R	R	531TCG→TTG	315 AGC→ACC	A	A	A	A
12	151	9-9-03	R	R	A	A	A	A	A	A
13	159	30-1-00	R	R	526 CAC→GAC	A	A	A	A	A
14	166	6-3-03	S	S	A	A	A	A	A	A
15	174	6-3-03	S	R	A	A	A	A	A	A
16	180	5-5-03	R	R	531TCG→TTG	315 AGC→ACC	A	A	A	A
17	181	6-5-03	S	S	A	A	A	A	A	A
18	185	28-5-03	S	R	A	A	A	A	A	A
19	204	25-7-03	R	R	531TCG→TTG	315 AGC→ACC	A	A	A	A
20	208	23-8-03	R	R	531TCG→TTG	315 AGC→ACC	A	A	A	A

Anexo 1. Perfil fenotípico y genotípico de susceptibilidad a anti-TB en aislados clínicos de *Mycobacterium tuberculosis* (Continuación)

#	Aislado	Fecha de aislamiento	Susceptibilidad a anti-TB		Mutaciones relacionadas con la resistencia a anti-TB de primera y segunda línea					
			RIF	INH	Gen *rpoB*	Gen *katG*	Promotor *inhA*	Gen *gyrA*	Gen *rrs*	Promotor *eis*
21	214		R	R	526 CAC→GAC	315 AGC→ACC	A	A	A	A
22	221	27-7-03	S	R	A	A	C →T (-15)	A	A	A
23	225	12-8-03	R	R	516 GAC→GTC	315 AGC→ACC	A	A	A	A
24	238	16-9-03	S	R	A	A	A	A	A	A
25	239	17-9-03	S	R	A	ND	A	A	A	A
26	244	30-9-03	R	R	526 CAC→CGC	A	A	A	A	A
27	246	5-9-03	S	R	A	315 AGC→ACC	A	A	A	A
28	247	19-9-03	S	R	A	A	A	A	A	A
29	258	14-10-03	S	S	A	A	A	A	A	A
30	276	19-12-03	S	R	A	315 AGC→ACC	A	A	A	A
31	287	20-1-04	R	R	531 TCG→TTG	324 ACC→TCC	A	A	A	A
32	303	10-2-04	R	R	526 CAC→GAC	315 AGC→ACC	A	A	A	A
33	309	2-3-04	R	R	531 TCG→TTG	315 AGC→ACA	A	A	A	A
34	315	3-3-04	S	R	A	315 AGC→ACC	A	A	A	A
35	369	4-8-04	S	S	531 TCG→TTG	A	C →T (-15)	A	A	A
36	389	30-8-09	R	R	516 GAC→TAC 515 ATG→ATC	315 AGC→ACC	A	A	A	A
37	395	6-10-04	R	R	526 CAC→ CTC	A	A	A	A	A
38	398	21-10-04	S	S	A	A	A	A	A	A
39	405	17-9-04	R	R	531 TCG→TTG	315 AGC→ACC	A	A	A	A

Anexo 1. Perfil fenotípico y genotípico de susceptibilidad a anti-TB en aislados clínicos de *Mycobacterium tuberculosis* (Continuación)

#	Aislado	Fecha de aislamiento	Susceptibilidad a anti-TB		Mutaciones relacionadas con la resistencia a anti-TB de primera y segunda línea					
			RIF	INH	Gen *rpoB*	Gen *katG*	Promotor *inhA*	Gen *gyrA*	Gen *rrs*	Promotor *eis*
40	406	16-9-04	S	R	526 CAC→TAC	315 AGC→ACC	A	A	A	A
41	419	24-12-04	R	R	A	A	C →T (-15)	A	A	A
42	421	12-1-05	R	R	531 TCG→TTG	A	A	A	A	A
43	445	28-3-05	R	R	526 CAC→TAC	315 AGC→ACC	A	A	A	A
44	447	16-4-05	R	R	531 TCG→TTG	A	A	A	A	A
45	459	27-4-05	R	R	A	A	A	A	A	A
46	467	26-5-05	S	R	526 CAC→CGC	315 AGC→ACC	A	A	A	A
47	478	5-8-05	R	R	531 TCG→TTG	315 AGC→ACC	A	A	A	A
48	479	18-6-05	S	S	A	A	A	A	A	A
49	485	30-6-05	R	R	531 TCG→TTG	A	C →T (-15)	A	A	A
50	492	13-7-05	R	S	A	A	A	A	A	A
51	535	26-9-05	S	R	A	A	A	A	A	A
52	538	10-1-05	R	R	531 TCG→TTG	A	C →T (-15)	94 GAC→TAC	1401 A → G	A
53	547	25-10-05	R	S	531 TCG→TTG	A	A	A	A	A
54	550	23-11-05	R	R	531 TCG→TTG	315 AGC→ACC	A	A	A	A
55	559	24-1-06	S	S	A	A	A	A	A	A
56	570	12-7-05	R	R	526 CAC → GAC	315 AGC→ACC	A	90 GCG→CTG	A	A
57	572	20-1-06	R	R	526 CAC → GAC	A	C →T (-15)	A	A	A
58	608	21-5-06	R	R	531 TCG→TTG	A	A	A	A	A

Anexo 1. Perfil fenotípico y genotípico de susceptibilidad a anti-TB en aislados clínicos de *Mycobacterium tuberculosis* (Continuación)

#	Aislado	Fecha de aislamiento	Susceptibilidad a anti-TB		Mutaciones relacionadas con la resistencia a anti-TB de primera y segunda línea					
			RIF	INH	Gen *rpoB*	Gen *katG*	Promotor *inhA*	Gen *gyrA*	Gen *rrs*	Promotor *eis*
59	612	25-5-06	R	R	531 TCG→TTG	A	C →T (-15)	94 GAC→TAC	1401 A → G	A
60	617	6-9-06	R	R	531 TCG→TTG	314 ACC→GCC	C →T (-15)	A	A	A
61	623	27-9-06	R	R	531 TCG→TTG	A	A	A	A	A
62	625	14-9-06	R	R	531 TCG→TTG	315 AGC→ACC	A	A	A	A
63	630	15-9-06	R	R	531 TCG→TTG	315 AGC→ACC	A	A	A	A
64	634	12-10-06	R	S	531 TCG→TTG	A	C →T (-15)	A	A	A
65	648	18-10-06	R	R	516 GAC→GTC	A	A	A	A	A
66	658	23-1-07	R	R	511 CTG→CCG	315 AGC→ACC	A	A	A	A
67	666	13-2-07	R	R	526 CAC→ CTC	315 AGC→ACC	A	91 TCG → CCG	A	A
68	668	14-2-07	R	R	A	315 AGC→ACC	A	A	A	A
69	682	3-5-07	S	S	531 TCG→TTG	A	A	A	A	A
70	686	23-10-09	R	R	531 TCG→TTG	A	C →T (-15)	A	A	A
71	691	15-6-07	S	S	A	A	A	A	A	A
72	697	7-8-07	R	R	531 TCG→TTG	315 AGC→ACC	A	94 GAC → AAC	A	A
73	706	5-10-07	S	S	531 TCG→TTG	A	A	A	A	A
74	760	8-9-08	R	S	516 GAC→GTC	A	C →T (-15)	A	A	A
75	765	9-10-08	R	R	531 TCG→TTG	315 AGC→ACA	A	A	A	A

Anexo 1. Perfil fenotípico y genotípico de susceptibilidad a anti-TB en aislados clínicos de *Mycobacterium tuberculosis* (Continuación)

#	Aislado	Fecha de aislamiento	Susceptibilidad a anti-TB		Mutaciones relacionadas con la resistencia a anti-TB de primera y segunda línea					
			RIF	INH	Gen *rpoB*	Gen *katG*	Promotor *inhA*	Gen *gyrA*	Gen *rrs*	Promotor *eis*
76	766	17-11-08	R	R	531 TCG→TTG	315 AGC→ACC	A	A	A	A
77	770	16-11-09	R	R	531 TCG→TTG	315 AGC→ACC	A	A	A	A
78	844	23-10-09	R	R	531 TCG→TTG	A	C →T (-15)	A	A	A
79	849	24-2-10	R	R	531 TCG→TTG	A	C →T (-15)	A	A	A
80	899	30-11-10	R	R	516 GAC→GTC	A	C →T (-15)	A	A	A
81	932	9-12-11	R	R	526 GAC→CTC	315→ACC	A	A	A	A
82	977	14-1-13	R	R	531 TCG→TTG	315→ACC	C →T (-15)	A	1401 A→G	A
78	844	23-10-09	R	R	531 TCG→TTG	A	C →T (-15)	A	A	A
79	849	24-2-10	R	R	531 TCG→TTG	A	C →T (-15)	A	A	A
80	899	30-11-10	R	R	516 GAC→GTC	A	C →T (-15)	A	A	A
81	932	9-12-11	R	R	526 GAC→CTC	315→ACC	A	A	A	A

RIF, Rifampicina; INH, Isoniacida; R, resistente; S, sensible; anti-TB, anti-tuberculosis; A, ausente, ND, no determinado

Anexo 2. Perfil MIRU-VNTR 15-Loci en aislados clínicos de *Mycobacterium tuberculosis*

#	Aislado	MIRU-VNTR 15 loci															Linaje
		MIRU 4	MIRU 26	MIRU 40	MIRU 10	MIRU 16	MIRU 31	Mtub04	ETRC	ETRA	Mtub30	Mtub39	Qub4156	Qub11b	Mtub21	Qub26	
1	3	2	4	2	4	3	3	3	4	2	1	2	2	2	3	7	LAM
2	9	2	5	4	2	4	3	2	4	2	2	4	2	5	2;0	6	ND
3	44	2	6	2	2	3	3	5	3	2	4	3	1	4	5; 3	7	ND
4	53	2	4	2	4	3	3	3	3	3	4	3	3	6	4	6	Ghana
5	55	2	5	3	4	3	3	2	3	3	4	3	4	4	3	7	Haarlem
6	58	2	4	2	4	3	3	2	3	3	4	3	3	7	4	6	Ghana
7	63	2	3	1	1	3	3	5	4	2	2	1	2	4	3	6	ND
8	80	3	5	1	3	3	3	4	4	2	1	2	2	4	3	7	ND
9	98	1	4	2	4	2	3	2	3	3	4	3	3	7	4	6	ND
10	140	2	4	2	4	2	3	2	3	3	4	3	3	7	4	6	ND
11	150	1	4	2	4	3	3	2	3	3	4	3	3	7	5	6	ND
12	151	2	5	2	3	3	3	2	4	2	2	3	2	4	3	8	Cameroon
13	159	2	8	5	4	3	2	3	4	3	1	2	2	2	3	6	LAM
14	166	2	5	4	5	3	3	2	3	3	4	3	4	4	3	7	Haarlem
15	174	2	3	5	4	3	3	4	4	2	2	1	2	4	3	7	ND
16	180	4	3	1	2	3	3	4	4	3	2	1	2	7	1	4	ND
17	181	2	6	2	3	3	3	4	3	2	4	3	1	4	3	7	ND
18	185	2	4	3	3	3	3	3	4	2	1	3	3	2	4	7	ND
19	204	2	5	0	3	3	2	3	4	2	1	3	2	2	3	6	LAM
20	208	2	5	0	4	3	2	3	4	2	1	3	2	2	3	6	LAM
21	214	1	4	2	3	3	3	4	3	3	4	2	3	7	4	6	ND
22	221	2	4	2	4	3	3	2	3	3	4	3	3	7	4	6	Ghana
23	225	2	1	0	4	3	3	3	4	2	1	1	2	2	1	6	ND
24	238	2	5	3	5	3	3	1	3	3	4	4	3	4	2	6	Haarlem
25	239	2	5	2	4	4	3	3	3	3	4	3	3	7	4	6	ND

Anexo 2. Perfil MIRU-VNTR 15-Loci en aislados clínicos de *Mycobacterium tuberculosis* (Continuación)

#	Aislado	MIRU-VNTR 15 loci															Linaje
		MIRU 4	MIRU 26	MIRU 40	MIRU 10	MIRU 16	MIRU 31	Mtub04	ETRC	ETRA	Mtub30	Mtub39	Qub4156	Qub11b	Mtub21	Qub26	
26	244	2	5	2	3	5	2	2	3	2	2	3	2	4	2	5	ND
27	246	2	5	4	3	3	3	2	4	4	2	3	2	3	3	2	Cameroon
28	247	2	5	3	5	3	3	2	3	2	4	3	3	5	2	7	Haarlem
29	258	2	8	4	4	3	2	3	4	2	1	2	2	2	3	6	LAM
30	276	2	5	2	3	3	3	2	4	2	2	3	2	3	2	8	ND
31	287	2	4	2	4	3	3	2	3	3	4	3	3	7	4	6	Ghana
32	303	1	4	2	4	4	4	2	3	3	4	3	3	7	4	6	ND
33	309	2	5	1	4	3	4	4	4	2	1	2	2	4	3	8	ND
34	315	2	6	3	3	3	3	4	3	2	4	3	1	4	4	4	ND
35	369	2	5	3	5	3	4	2	3	3	4	3	3	5	2	4	Haarlem
36	389	1	4	2	4	3	3	2	3	3	4	3	3	7	5	6	ND
37	395	2	4	2	4	3	3	2	3	3	4	3	3	7	5	6	ND
38	398	2	5	4	4	3	3	4	4	2	2	1	3	3	3	4	LAM
39	405	1	4	2	4	3	4	2	3	3	4	3	4	7	4	6	ND
40	406	2	5	0	3	2	3	4	4	2	1	2	2	4	3	6	LAM
41	419	2	5	3	4	3	3	1	3	3	4	3	2	5	2	7	Haarlem
42	421	3	5	2	3	2	2	2	4	4	2	3	1	4	1	9	ND
43	445	2	5	1	4	2	3	4	4	2	1	2	2	4	3	6	LAM
44	447	2	5	6	4	3	2	3	4	4	1	2	0	2	3	7	LAM
45	459	3	4	4	3	3	3	3	4	4	2	3	1	3	1	8	S
46	467	2	5	5	2	3	3	3	4	2	1	2	2	2	3	6	LAM
47	478	2	4	2	4	3	3	2	3	3	4	3	3	7	4	6	Ghana
48	479	2	5	5	3	2	3	3	4	3	2	3	2	4	2	5	ND
49	485	2	3	1	2	3	3	4	4	2	2	1	1	4	3	6	ND

Anexo 2. Perfil MIRU-VNTR 15-Loci en aislados clínicos de *Mycobacterium tuberculosis* (Continuación)

#	Aislado	MIRU-VNTR 15 loci															Linaje
		MIRU 4	MIRU 26	MIRU 40	MIRU 10	MIRU 16	MIRU 31	Mtub04	ETRC	ETRA	Mtub30	Mtub39	Qub4156	Qub11b	Mtub21	Qub26	
50	492	3	5	4	3	2	3	1	3	4	2	3	2	3	1	10	S
51	535	3	4	4	3	2	3	2	4	4	2	3	1	4	1	8	S
52	538	2	4	1	4	3	3	2	3	3	4	3	3	6	4	6	Ghana
53	547	2	5	2	3	4	3	2	4	2	2	3	0	4	2	6	ND
54	550	2	5	0	4	4	3	4	4	2	1	2	2	2	3	6	ND
55	559	3	5	4	3	3	3	3	4	4	2	3	2	3	1	10	S
56	570	1	4	2	4	3	3	2	3	3	4	3	2	7	4	6	ND
57	572	2	5	6	3	4	2	2	4	2	2	3	1	4	2	5	ND
58	608	2	5	2	4	2	2	3	4	2	1	2	2	2	3	7	LAM
59	612	2	4	2	4	3	3	2	3	3	4	3	3	6	4	6	Ghana
60	617	2	4	2	4	3	3	2	3	3	4	3	3	6	4	6	Ghana
61	623	2	4	3	4	3	3	2	3	3	4	3	3	5	4	6	Ghana
62	625	2	4	2	6	3	3	3	3	3	4	3	2	7	3	6	ND
63	630	2	4	2	6	3	3	3	3	3	4	3	5	7	3	6	ND
64	634	2	3	5	4	3	3	4	4	2	2	1	2	4	3	7	ND
65	648	2	4	2	4	3	3	2	3	3	4	3	3	10	4	6	Ghana
66	658	1	4	2	4	3	3	2	3	3	4	3	3	7	4	6	ND
67	666	2	5	2	4	3	3	4	4	2	2	1	3	3	3	4	LAM
68	668	2	4	2	4	3	3	2	3	3	4	3	2	6	4	5	ND
69	682	3	5	5	2	3	3	3	4	4	2	3	1	3	1	5	ND
70	686	2	5	2	3	4	3	2	4	2	2	3	2	4	2	5	ND
71	691	2	6	1	4	3	3	1	3	3	4	2	3	4	2	8	ND
72	697	2	4	2	4	3	3	2	3	3	4	3	6	7	4	6	Ghana
73	706	2	4	3	4	3	3	2	3	3	4	3	5	5	4	6	Ghana

Anexo 2. Perfil MIRU-VNTR 15-Loci en aislados clínicos de *Mycobacterium tuberculosis* (Continuación)

#	Aislado	MIRU-VNTR 15 loci															Linaje
		MIRU 4	MIRU 26	MIRU 40	MIRU 10	MIRU 16	MIRU 31	Mtub04	ETRC	ETRA	Mtub30	Mtub39	Qub4156	Qub11b	Mtub21	Qub26	
74	760	2	4	2	4	3	3	2	3	3	4	3	3	7	4	6	Ghana
75	765	2	5	7	4	3	3	3	4	2	1	2	2	2	3	7	LAM
76	766	1	4	2	4	3	3	2	3	3	4	3	5	7	4	6	ND
77	770	2	4	2	4	3	3	2	3	3	4	3	3	7	4	5	ND
78	844	2	5	2	3	4	3	2	4	2	2	3	2	4	2	5	ND
79	849	2	4	2	4	3	3	2	3	3	4	3	4	6	4	5	ND
80	899	2	5	2	2	3	3	2	4	2	2	4	2	4	2	5	Cameroon
81	932	2	5	0	4	3	2	3	4	2	1	3	2	2	3	6	LAM
82	977	2	5	3	3	3	5	4	4	4	4	3	2	6	5	8	Beijing
74	760	2	4	2	4	3	3	2	3	3	4	3	3	7	4	6	Ghana
75	765	2	5	7	4	3	3	3	4	2	1	2	2	2	3	7	LAM
76	766	1	4	2	4	3	3	2	3	3	4	3	5	7	4	6	ND
77	770	2	4	2	4	3	3	2	3	3	4	3	3	7	4	5	ND
78	844	2	5	2	3	4	3	2	4	2	2	3	2	4	2	5	ND
79	849	2	4	2	4	3	3	2	3	3	4	3	4	6	4	5	ND
80	899	2	5	2	2	3	3	2	4	2	2	4	2	4	2	5	Cameroon
81	932	2	5	0	4	3	2	3	4	2	1	3	2	2	3	6	LAM
82	977	2	5	3	3	3	5	4	4	4	4	3	2	6	5	8	Beijing

ND, no determinado; MIRU-VNTR, Repeticiones en Tándem de Número Variable de Unidades Repetitivas Intercaladas Micobacterianas

Anexo 3. Ejemplo de una publicación en la revista seleccionada