

Research Article

Mutations in *Mycobacterium tuberculosis* Isolates with Discordant Results for Drug-Susceptibility Testing in Peru

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Evaluation of resistance to antituberculosis drugs is routinely performed with genotypic or phenotypic methods; however, discordance can be seen between these different methodologies. Our objective was to identify mutations that could explain discordant results in the evaluation of susceptibility to rifampicin and isoniazid between molecular and phenotypic methods, using whole genome sequencing (WGS). Peruvian strains showing sensitive results in the GenoType MTBDRplus v2.0 test and resistant results in the proportions in the agar-plaque test for isoniazid or rifampin were selected. Discordance was confirmed by repeating both tests, and WGS was performed, using the Next Generation Sequencing methodology. Obtained sequences were aligned “through reference” (genomic mapping) using the program BWA with the algorithm “mem”, using as a reference the genome of the *M. tuberculosis* H37Rv strain. Discordance was confirmed in 14 strains for rifampicin and 21 for isoniazid, with 1 strain in common for both antibiotics, for a total of 34 unique strains. The most frequent mutation in the *rpoB* gene in the discordant strains for rifampicin was V170F. The most frequent mutations in the discordant strains for isoniazid were *katG* R463L, *kasA* G269S, and *Rv1592c* I322V. Several other mutations are reported. This is the first study in Latin America addressing mutations present in strains with discordant results between genotypic and phenotypic methods to rifampicin and isoniazid. These mutations could be considered as future potential targets for genotypic tests for evaluation of susceptibility to these drugs.

1. Introduction

Peru is one of the 30 countries in the world with the highest burden of multidrug-resistant tuberculosis (MDR-TB). The public health system provides free diagnosis and drug-susceptibility testing with phenotypic (proportions in 7H10) [1] and molecular methods (Genotype, Hain Lifesciences (Genotype™)) [2] for first- and second-line drugs. Molecular diagnosis of drug resistance has been shown to significantly reduce the time to diagnosis, most of all to isoniazid and rifampicin, the pillars of antituberculosis regimens, and thus contribute to improve the clinical outcomes [3]. However, the reference standard continues to be phenotypic methods, such as MGIT 960 and proportions in agar plaque, both currently used at the National Reference Laboratory of Mycobacteria of Peru.

The Genotype method is based on the identification of mutations of the mutations of the *rpoB* gene in the Rifampicin-resistance determining region (RRDR), an 81 base-pair segment of the gene. Mutations in the *katG* and *inhA* genes are identified to diagnose resistance to isoniazid. However, this assay identifies the most frequent mutations but not every possible mutation conferring resistance to these drugs. There could be mutations unidentified by these tests (“noncanonical” mutations), and so the test would be reported as susceptible [4]. Noteworthy, if phenotypic methods were used, a resistant pattern would be detected. There are reports postulating novel mutations to explain discrepancies between molecular and phenotypic methods [5, 6], as these could explain the discordant results. Whole Genome Sequencing (WGS) is a technology that can identify new sequences that could explain certain traits related to

resistance, and could currently be used to investigate the genome of *Mycobacterium tuberculosis* strains showing this kind of discordant results [7].

However, not all existing mutations determine resistance, so it is important to consider existing evidence about their association with drug resistance. The World Health Organization has issued recommendations on their interpretation [8]. Knowledge on this topic continues to evolve, and it is very important to generate additional information about the presence of these novel mutations. Our aim was to identify mutations that could explain discordant results in the evaluation of susceptibility to rifampicin and isoniazid between molecular and phenotypic methods, using whole genome sequencing. This information could contribute in the future to develop more comprehensive diagnostic devices for evaluation of drug resistance in our country.

2. Materials and Methods

2.1. Strains. The National Reference Laboratory for Mycobacteria of the National Institute of Health of Peru performs evaluation of first-line drug-resistance with GenoType™ since 2011. Only samples showing any kind of resistance undergo phenotypic drug resistance evaluation with the proportions method in Middlebrook 7H10 Agar for 11 drugs, including isoniazid and rifampicin. However, between 2013 and 2015, a national survey was performed, and samples were analysed with both methods in parallel. We selected strains collected in that period showing a discordant result between both methods (sensitive in the GenoType MTBDRplus v2.0 test and resistant in the proportions in agar-plaque test) for isoniazid or rifampicin.

2.2. Laboratory Procedures. *Mycobacterium tuberculosis* strains kept at -80°C available at the biobank of this laboratory were reactivated in Middelbrook 7H9 broth for 7 days. An aliquot of 0.5 mL was then transferred to Löwenstein–Jensen media for further growth.

According to Hofmann-Thiel et al.’s recommendations [9], we repeated the GenoType® MTBDRplus, Hain LifeScience, Nehren, Germany v2.0 test and additionally the proportions in the agar-plaque test to confirm the results. GenoType MTBDRplus v2.0 (<http://www.hain-lifescience.com>) was performed following the manufacturer’s indications. The GenoLyse v1.0 kit was used for DNA extraction, and the DNA amplification and hybridization was performed using the GenoType MTBDRplus v2.0 probes for identification of mutations in the *rpoB*, *katG*, and *inhA* genes.

For the proportions in the agar-plaque test, the colonies were transferred from the Löwenstein–Jensen media to Middlebrook 7H9 for 7 days and subsequently to Middlebrook 7H10 for 21 days. The plaques contained rifampicin at a minimum inhibitory concentration of $1.0\ \mu\text{g}/\text{mL}$ and isoniazid at 2 minimum inhibitory concentrations: $0.2\ \mu\text{g}/\text{mL}$ and $1.0\ \mu\text{g}/\text{mL}$. For the interpretation, if the critical proportion was higher than 1%, the strain was considered resistant, and if it was less than 1%, it was

TABLE 1: Characteristics of included patients.

Characteristic	Number (percentage) ($n = 36$)
Sex, male	29 (80.6%)
Age (median, interquartile range)	28.9 (24–38)
Treatment history	
No previous treatment	17 (47.2%)
Previously treated	8 (22.2%)
No information	11 (30.5%)
Area of residence	
Lima (capital)	30 (83.3%)
Province	6 (16.7%)

considered sensible [10, 11]. Only strains showing persisting discordance after repeating the tests were included for analysis of WGS. Genomic DNA was extracted from these strains using the “GenJet Genomic DNA purification” (<http://www.thermofisher.com>) kit, according to the manufacturer’s recommendations. Then, the double-stranded DNA concentration was quantified, by fluorescence, using the Qubit 2.0 fluorometer kit (Invitrogen, Carlsbad, CA, USA). Whole Genome Sequencing was performed using paired-end of 2×250 and 2×300 pb of the Nextera XT kit with the system NGS illumina MiSeq (Illumina Inc., San Diego, CA, USA). The MiSeq Reagent Kit v3 (600 Cycles) was used as recommended (<https://www.illumina.com>).

2.3. Bioinformatic Analysis. Quality control was performed to the obtained reads using the FastQC program v0.11.8 [12]. The estimation of the purity of the reads and of the purity of the sequencing was determined with the programs Kraken2 v2.0.7 [13] and Bracken v2.2 [14]. The reads were then cleaned using the program Trimmomatic v0.38 [15].

The sequences obtained were aligned “through reference” (genomic mapping) using the program BWA [16], with the algorithm “mem”, using as a reference the genome of the strain *M. tuberculosis* H37Rv (GenBank, Access number: NC_000962.3). Identification of duplicates and the final ordering was performed with the tool Picard v2.18.25 (<http://broadinstitute.github.io/picard>).

Finally, we report the sequences of the *rpoA*, *rpoB*, and *rpoC* genes for isolates with discordant results for rifampicin and the sequences of the *katG* and *inhA* genes for isolates with discordant results for isoniazid. In order to classify the mutations as having low, median, or high probability of conferring drug resistance (as not all mutations are necessarily associated with resistance), we followed the recommendations of the World Health Organization [9] and complemented with information found in other publications. For rifampicin resistance, we report mutations both according to the consensus proposed by Telenti et al. [17], as well as to Andre et al.’s notation [18]. For the descriptive statistical analysis, Stata ver. 15 was used.

3. Results

3.1. Strains. From 2013 to 2015, we were able to confirm discordant results (reported as sensitive for rifampicin or isoniazid with the GenoType MTBDRplus v2.0 and

		<i>rpoB</i>										<i>rpoA</i>		<i>rpoC</i>							
N° MTB ¹⁶		D103D	V170F	H194R	E250G	F424V	L430R	L452P	I491F	V695L	A960E	R263W	V564A	V546G	L597P	E599Q	A623A	G652R	G675E	E1114K	E1113D
N° E. coli ¹⁷		D184D	V251F	H275R	E331G	F505V	L511R	L533P	I572F	V776L	A1041E	R182W	V483A	V483G	L516P	E518Q	A542A	G571R	G594E	E1033K	E1113D
Changed amino-acid		Asp 103 Asp	Val 170 Phe	His 194 Arg	Glu 250 Gly	Phe 424 Val	Leu 430 Arg	Leu 452 Pro	Ile 491 Phe	Val 695 Leu	A 960 Glu	Arg 182 Trp	Val 483 A	Val 483 Gly	Leu 516 Pro	Glu 518 Gln	A 542 A	Gly 571 Arg	Gly 594 Glu	Glu 1033 Lys	Glu 1113 Asp
Strain	Type of mutation	SYN	MIS	MIS	MIS	MIS	MIS	MIS	MIS	MIS	MIS	MIS	MIS	MIS	MIS	MIS	SYN	MIS	MIS	MIS	MIS
1		X		X																	X
2		X			X																X
3		X						X													X
4			X															X	X		
5			X									X									X
6			X								X		X								
7			X								X		X								
8			X												X		X				
9			X										X				X				
10						X	X									X	X				
11									X								X			X	
12									X										X		X
13*										X											
14																	X				

SYN: synonymous variant MIS: missense variant
 *Strain found to be discordant both for rifampicin and isoniazid

FIGURE 1: Mutations present in genes *rpoB*, *rpoA*, and *rpoC* in strains with discordant results between genotypic and phenotypic methods for rifampicin. SYN: synonymous variant. MIS: missense variant. *Strain found to be discordant both for rifampicin and isoniazid.

N° MTB		c.-7G>T	c.-8G>C	Q4H		H97L	N133fs	N138S	L141F	Y155S	W191G	G234E	F272L	G358fs	R463L	A551fs	L587P	R595L	N596H	T690P	
Changed amino-acid		c.-7G>T	c.-8G>C	Gln 4 His	Gln 4*	His 97 Leu	Asn 133 fs	Asn 138 Ser	Leu 141 Phe	Tyr 155 Ser	Trp 191 Gly	Gly 234 Glu	Phe 272 Leu	Gly 358 fs	Arg 463 Leu	A 551 fs	Leu 587 Pro	Arg 595 Leu	Asn 596 His	Thr 690 Pro	
Strain	Type of mutation	UPS	UPS	MIS	MIS	MIS	FS	MIS	MIS	MIS	MIS	MIS	MIS	FS	MIS	FS	MIS	MIS	MIS	MIS	
1																					X
2																X					
3*																					
4																					
5																					
6																					
7																				X	
8									X												
9				X	X			X													
10							X														
11															X						
12						X									X			X			
13											X										
14			X																		
15																					
16										X											
17		X															X				
18			X																		
19														X	X						
20													X								
21												X									

UPS: upstream gene variant MIS: missense variant FS: frameshift variant
 *Strain found to be discordant both for rifampicin and isoniazid

FIGURE 2: Mutations present in the *katG* gene in strains with discordant results between genotypic and phenotypic methods for isoniazid. UPS: upstream gene variant. MIS: missense variant. FS: frameshift variant. *Strain found to be discordant both for rifampicin and isoniazid.

resistant to the same drug with the proportions method in Middlebrook 710 Agar) in 36 strains, 16 for rifampicin and 21 for isoniazid (1 sample was discordant for both drugs), which were processed for whole genome sequencing. Table 1 shows the main characteristics of the corresponding patients. Noteworthy, this accounts for approximately 0.5% of the 7194 valid results for this period.

3.2. *Rifampicin*. Sixteen strains were found to have discordant results for rifampicin in the phenotypic and genotypic methods. Of these, we were able to recover and perform WGS in 14. All strains had missense mutations in *rpoB*. The most frequent mutation was V170F, found in 6 samples. Figure 1 shows all mutations present in the genes *rpoB* (probably the ones determining resistance), *rpoA*, and *rpoC* (probably compensatory mutations) in these 14 strains.

Gene		<i>inhA</i>			<i>fabG1</i>			<i>oxyR</i>	<i>p-ahpC</i>			<i>kasA</i>		<i>ndh</i>	<i>furA</i>	<i>efpA</i>	<i>nat</i>	<i>fbpC</i>	<i>Rv0340</i>	<i>iniB</i>			<i>iniA</i>		<i>Rv1592c</i>		<i>Rv1772</i>	<i>srnR</i>	<i>accD6</i>			<i>fadE24</i>								
N° MTB	Changed amino-acid	c.-17G>T	S94A	I194T	S142S	L203L	V221V	n.501C>T	c.-48G>A	c.-52C>T	c.-54C>T	A209T	G269S	V181A	V251L	V435V	D27N	G207R	E103G	I301L	A70T	A193A	G386D	G178G	S284A	E321E	I322V	C80C	S296S	Q113R	D200D	D229G	G151D	K98K	P130L					
Strain	Type of mutation	UPS	MIS	MIS	SYN	SYN	SYN	NC	UPS	UPS	UPS	MIS	MIS	MIS	MIS	SYN	MIS	MIS	SYN	SYN	MIS	SYN	MIS	SYN	MIS	SYN	MIS	SYN	SYN	MIS	SYN	MIS	MIS	MIS	SYN	MIS				
1			X																																					
2										X																														
3*		X														X	X				X								X								X	X		
4																		X	X																					
5																																								
6				X															X	X																				
7													X						X	X																				
8													X						X	X																				
9													X						X	X																				
10								X				X																												
11																																								
12																												X	X									X	X	
13														X						X																				
14					X										X													X	X											
15																																								
16						X																																		
17			X																			X																		
18						X										X																								
19													X																											
20					X	X					X												X																	
21								X	X																															

UPS: upstream gene variant

MIS: missense variant

SYN: synonymous variant

NC: noncoding transcript exon variant

*Strain found to be discordant both for rifampicin and isoniazid

FIGURE 3: Mutations present in diverse genes in strains with discordant results between genotypic and phenotypic methods for isoniazid. UPS: upstream gene variant. MIS: missense variant. SYN: synonymous variant. NC: noncoding transcript exon variant. *Strain found to be discordant both for rifampicin and isoniazid.

3.3. Isoniazid. Twenty-one strains were found to have discordant results for isoniazid between the phenotypic and genotypic methods. We were able to recover and perform WGS in all of them. The mutations found were very diverse in gene *katG*, (Figure 2) being the most frequent missense mutation R463L, present in 3 samples. We did not find the following mutations in the *katG* gene: c.-10A>C, P92S, A109T, K154T, P235S, T271A, G307V, S315T, S315N, P365P, T380I, R396L, D419, P432L, A478A, A551A, N660T, N660D, E709G, and G177S. Figure 3 shows all mutations present in the other genes (*inhA*, *fabG1*, *oxyR*, *p-ahpC*, *kasA*, *ndh*, *furA*, *efpA*, *nat*, *fbpC*, *Rv0340*, *iniB*, *iniA*, *Rv1592*, *Rv1772*, *srnR*, *accD6*, and *fadE24*) that could be related to isoniazid resistance, the most frequent mutations being missense: *kasA* G269S in 3 strains and *Rv1592c* I322V in 9 samples. Interestingly, we were not able to find mutations other than synonymous in strains 4 and 15, and in strain 5, we were not able to find any mutation.

4. Discussion

This is the first study in Latin America addressing mutations present in a considerable group of strains of *Mycobacterium tuberculosis* that show sensitivity to the most important first-line antituberculosis drugs (rifampicin and isoniazid) in genotypic tests and resistance to the same drugs (discordance) in phenotypic tests, using Whole Genome Sequencing to find “noncanonical” mutations. Indeed, we were able to find missense mutations present in these discordant strains that could explain the resistance to rifampicin (*rpoB* V170F, I491F and others) and to isoniazid (*katG* R463L, *kasA* G269S, *Rv1592c* I322V, and others).

This is a relevant finding, because these could be considered potential targets for the development of diagnostic devices for drug resistance in the future [19]. Noteworthy, some of the mutations, such as R463L in *katG*, have also been signaled by other groups, highlighting their potential relevance to confer resistance [20]. Others are reported for the first time in this publication, some of which could be incidental findings, not necessarily conferring resistance to antituberculosis drugs, and others could be confirmed through future research. Although we have focused on missense and frame shifting mutations, we have reported upstream and synonymous mutations as well, to provide a complete oversight of the detected changes. All this will contribute to the available information about the genomic variants of the *Mycobacterium tuberculosis* and could have other potential applications, such as in the study of epidemiological dynamics and spreading of drug-resistant strains.

We have to acknowledge some issues that could limit the external validity of our results. In the first place, the samples were taken in the years 2013–2015, so we would have to be cautious before considering these strains are representative of the strains currently circulating in our territory. In the second place, we have to consider that, although there are some initiatives for approaching consensus in the mutation conferring resistance, this is a new field and the information is still preliminary. Hence, we cannot ensure that the mutations we have reported are really associated with resistance. The ideal approach, though resource-consuming, would be to produce these mutations in sensitive organisms to evaluate if they actually become resistant [21].

5. Conclusions

In *Mycobacterium tuberculosis* strains showing discordant results between phenotypic and genotypic diagnostic tests for evaluation of drug resistance to rifampicin and isoniazid, we were able to report several mutations identified through WGS. Some of these, in particular *rpoB* V170F and I491F for rifampicin and *katG* R463L, *kasA* G269S, and Rv1592c I322V for isoniazid, could be considered in the future for development of local genotypic diagnostic tests for diagnosis of drug resistance.

Data Availability

The data on Whole Genome Sequencing used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflicts of interest.

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