

Molecular characterization of mutations associated with resistance to second line drugs in *Mycobacterium tuberculosis* patients from Casablanca, Morocco

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ABSTRACT

The emergence and spread of extensively drug-resistant tuberculosis (XDR-TB) is a serious threat to global health. Therefore, its rapid diagnosis is crucial. The present study aimed to characterize mutations conferring resistance to second line drugs (SLDs) within multidrug *Mycobacterium tuberculosis* (MDR-MTB) isolates and to estimate the occurrence of XDR-TB in Casablanca, Morocco. A panel of 200 MDR-TB isolates was collected at the Pasteur Institute between 2015-2018. Samples were subjected to drug susceptibility testing to Ofloxacin (OFX), Kanamycin (KAN) and Amikacin (AMK). The mutational status of *gyrA*, *gyrB*, *rrs*, *tlyA* and *eis* was assessed by sequencing these target genes. Drug susceptibility testing for SLDs showed that among the 200 MDR strains, 20% were resistant to OFX, 2.5% to KAN and 1.5% to AMK. Overall, 14.5% of MDR strains harbored mutations in *gyrA*, *gyrB*, *rrs* and *tlyA* genes. From the 40 OFX^R isolates, 67.5% had mutations in QRDR of *gyrA* and *gyrB* genes, the most frequent one being Ala90Val in *gyrA* gene. Of note, none of the isolates harbored simultaneously mutations in *gyrA* and *gyrB* genes. In eight out of the 200 MDR-TB isolates resistant either to KAN or AMK, only 25% had A1401G or Lys89Glu change in *rrs* and *tlyA* genes respectively. This study is very informative and provides data on the alarming rate of fluoroquinolone resistance which warrants the need to implement appropriate drug regimens to prevent the emergence and spread of more severe forms of *Mycobacterium tuberculosis* drug resistance.

KEYWORDS: *Mycobacterium tuberculosis*. XDR-TB. DNA sequencing. Drug susceptibility testing. Morocco.

INTRODUCTION

Worldwide, the emergence and spread of multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) is a real threat to global health^{1,2}. MDR-TB is defined as a TB strain resistant to the two first-line anti-TB drugs, namely Rifampicin (RIF) and Isoniazid (INH). XDR-TB is an MDR-TB resistant to one of the fluoroquinolones (FQs) and one of the three injectable second-line drugs (SLDs). XDR-TB emerges mainly because of mismanagement of MDR-TB and the erratic use of SLDs³.

Molecular diagnosis of MTB drug resistance has been extensively used during the three last decades⁴. These molecular tools are based on the detection of mutations in specific genes of MTB that are associated with resistance to anti TB drugs⁵.

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Treatment of MDR-TB requires the use of FQs and injectable drugs. FQs are broad-spectrum antibacterial agents against *Mycobacterium tuberculosis* exerting their bactericidal effects by inhibiting the mycobacterial DNA gyrase activity, which prevents bacterial DNA from unwinding and replicating⁶. Mutations in genes encoding DNA gyrase subunits *gyrA* and *gyrB* are the most common mechanisms conveying FQ resistance in TB. The most frequent resistance-associated mutations occur in a conserved region of the *gyrA* gene (codons 74 to 113) and, less frequently, the *gyrB* gene (codons 461 to 499), known as the quinolone resistance-determining region (QRDR)^{7,8}.

Injectable drugs, namely Kanamycin (KAN), Amikacin (AMK) and Capreomycin (CAP) are antibiotics that inhibit protein synthesis⁷. Mutations at positions 1401, 1402, and 1484 in the *rrs* gene encoding 16S rRNA confer cross-resistance to second-line injectable drugs (AMK, KAN, and CAP). Mutations in the *tlyA* gene, which encodes a 2'-O-methyltransferase that modifies nucleotides in 16S rRNA and 23S rRNA, have been suggested to confer isolated resistance of *M. tuberculosis* to CAP. Finally, resistance to KAN is due to substitutions G-37T, C-14T, C-12T and G-10A affecting the promoter region of the *eis* gene encoding an aminoglycoside acetyltransferase^{7,9}.

In Morocco, as it is the case in resources limited countries, drug resistant testing is mainly based on conventional methods, including culture and drug susceptibility testing (DST). The latter is widely known to be slow and laborious, requiring sequential procedures for MTB diagnosis and drug resistance profile analysis¹⁰. During this time, patients may be treated inappropriately, drug resistant strains may emerge or may continue to spread, and amplification of resistance is likely to occur¹¹. Therefore, rapid diagnosis and identification of MDR-TB or XDR-TB strains are prerequisites to the fight against TB¹².

In Morocco, TB affects more than 28.000 people every year. The global incidence of all clinical forms of tuberculosis is very high, reaching 107 new cases per 100 thousand inhabitants yearly, Casablanca being the area with the highest incidence of TB (139.8 per 100 thousand inhabitants annually). According to the last resistance survey, the prevalence of DR strains was 1% among new cases and 8.7% among patients with a previous TB history¹¹.

In the absence of national studies for assessing drug resistance and genotyping of resistant isolates in Morocco, data are retrieved from sporadic studies on a limited number of MTB strains. To date, three reports are available on mutations associated with resistance of MTB to SLDs.

A first preliminary study on mutations within *gyrA* and *gyrB* genes on MTB isolates from a laboratory collection (2003-2007) with a small panel of MDR-TB isolates from Morocco was conducted by Chaoui *et al.*¹³. Later on, Oudghiri *et al.*¹¹ screened for mutations in *gyrA*, *gyrB*, *rrs*, *tlyA* genes and the *eis* promoter region, and their frequencies within MDR-MTB clinical isolates associated with resistance to SLDs, in a large panel of clinical isolates from TB patients recruited from 2009 to 2012 in Casablanca, the highest TB incidence region in Morocco. Finally, another study was performed using MTBDRs/ commercial genotyping tests on a very restricted number of pre-XDR MTB isolates (n=21) mainly from Rabat¹⁴. The present study was conducted (i) to characterize mutations associated with resistance to SLDs in a larger panel of MDR MTB isolates, (ii) to detect the possible disagreements between genotypic and phenotypic DST results and (iii) to estimate the occurrence of pre-XDR and XDR strains in one of the five hot spot areas of TB in Morocco.

MATERIALS AND METHODS

Study design

This study was conducted at the Pasteur Institute Mycobacteria Laboratory in Casablanca. From 2015 to 2018, a total of 200 MDR isolates were assessed for resistance to RIF and INH by DST. All strains were considered unique, *i.e.*, each strain was collected from a different TB patient. All isolates were subjected to DST for SLDs, DNA genotyping by PCR and DNA sequencing.

The study protocol was approved by the Ethics Committee of Morocco's Institut Pasteur (IPM2013-P3), and written informed consent was obtained from each study participant.

Drug susceptibility testing

MTB isolates were obtained from Lowenstein-Jensen (L/J) medium and tested for drug susceptibility to OFX, KAN and AMK. DST was performed using the proportional method. The critical drug concentrations were 2 µg/mL for OFX, 30 µg/mL for KAN and 40 µg/mL for AMK¹⁴. The critical proportion of resistant bacilli necessary to define a resistant strain is 1% for the three tested drugs¹⁵.

Mycobacterium tuberculosis crude DNA isolation

Scraped bacterial colonies from L/J medium were recovered in 400 µL of distilled water and boiled at

100 °C for 10 min. to inactivate bacteria and release the mycobacterial DNA¹⁶. The latter was immediately used for PCR amplification or stored at -20 °C until use.

PCR amplification of *gyrA*, *gyrB*, *rrs*, *eis* and *tlyA* genes

Target sequences of *gyrA*, *gyrB*, *rrs*, and *tlyA* genes as well as the promoter region of the *eis* gene were amplified by PCR using their corresponding primers (Table 1). Amplification reactions were performed in a total volume of 25 µL containing 0.5 mM of each primer, 2.5 mM of each dNTP, 25 mM MgCl₂, 1 unit of Hotstar Taq DNA polymerase (Invitrogen, SaintAubin, France) and 2 µL of crude DNA sample in 1X Taq polymerase buffer.

The reagents mixtures were first denatured at 94 °C for 7 min. Thirty-five cycles of PCR were then performed, with denaturation at 94 °C for 1 min, annealing at the corresponding T_m for 30 s and extension at 72 °C for 30 s. At the end of the last cycle, mixtures were incubated at 72 °C for further 7 min. For each reaction, a positive control containing DNA from H37Rv strain, and a negative control containing sterile H₂O instead of template DNA were included. Amplicons were visualized after electrophoretic fractionation in 1 to 2% agarose gels in 0.5 X TBE buffer and staining with ethidium bromide.

DNA sequencing

Amplified fragments were firstly purified using the illustra ExoProStar 1-Step (GE Healthcare Life Sciences). Direct sequencing of amplicons was performed using the Big Dye Terminator Kit (version 3.1, Applied Biosystem, Foster City, CA, USA) that includes dideoxynucleotides

labeled with four different fluorochromes. For each PCR product, both strands were sequenced in independent reactions, using the mentioned primers. The resulting chromatograms were manually edited to ensure sequence accuracy and analyzed using the Molecular Evolutionary Genetics Analysis (MEGA) software (version 5, Center for Evolutionary Functional Genomics, Tempe, AZ, USA).

RESULTS

According to demographic data, the mean age of patients was 35 (SD: 11.7), with extreme ages of 14 and 80 years old. Of note, 90% of MDR-TB patients were in the age group of 20-50 years old, with a male to female sex ratio of 3.3:1.

Patients were clinically categorized according to WHO guidelines; 7.5% of MDR-TB patients were new cases (15/200), 10.5% had treatment failure (21/200), 64.5% relapsed (129/200) and 17.5% of patients were under treatment after loss to follow-up (35/200).

Surprisingly, MDR TB occurred in 7.5% of newly diagnosed cases meaning that the corresponding patients had a primary resistance. In Morocco, the last national surveillance study reported that drug resistance occurred rarely among new cases (1%)¹¹. Hence, the difference could be due to a sampling bias.

Drug susceptibility testing

Data of DST for SLDs showed that among the 200 MDR studied strains, 20% were resistant to OFX (40/200), 2.5% to KAN (5/200) and 1.5% to AMK (3/200). Of particular interest, five isolates were XDR-MTB, three were resistant to OFX and KAN (1.5%) and two were resistant to OFX and AMK (1%).

Table 1 - Primers for PCR amplifications.

Gene	Primer	Sequence 5' to 3'	Fragment length (bp)	Annealing temperature (°C)
<i>gyrA</i>	<i>gyrA</i> For	5'-TGACATCGAGCAGGAGATGC-3'	320	59
	<i>gyrA</i> Rev	5'-GGGCTTCGGTGTACCTCATC-3'		
<i>gyrB</i>	<i>gyrB</i> For	5'-GTGGAAATATGTTGGCCGTC-3'	413	58
	<i>gyrA</i> Rev	5'-GTCGTTGTGAACAACGCTGTG-3		
<i>rrs</i>	<i>rrs</i> For	5'-GTAATCGCAGATCAGCAACG-3'	216	58
	<i>rrs</i> Rev	5'-GTGATCCAGCCGCACCTT-3'		
Eis promoter	<i>eis</i> p For	5'-AAATTCGTCGCTGATTCTCG-3'	387	56
	<i>eis</i> p Rev	5'-CGCGACGAAACTGAGACC-3'		
<i>tlyA</i>	<i>tlyA</i> For	5'-GTCTCTGGCCGAACCTCGAAG-3'	1,000	52
	<i>tlyA</i> Rev	5'-ATTGTCGCCAATACTTTTCTAC-3'		

bp = base pairs.

The correlation between DST results and clinical features showed that almost all resistant isolates to SLDs belonged to relapsed, chronicle or treatment failure cases. It is also noteworthy that among the 200 MDR-TB strains, two new cases had pre-XDR (MDR and OFX^R) phenotype.

Genotypic results

Genotypic analysis of *gyrA*, *gyrB*, *rrs*, *tlyA* genes and the *eis* gene promoter was performed on all MDR strains and results are reported in Table 2. Overall, 15.5% of MDR-TB strains harbored mutations in *gyrA*, *gyrB*, *rrs* and/or *tlyA* genes conferring resistance to SLDs (31/200). Mutations in QRDR regions of *gyrA* or *gyrB* genes, conferring resistance to FQs, were found in 29 cases, with the most frequent one being Ala90Val in *gyrA* gene observed in 48.8% of isolates (14/29). Other point mutations were also found in *gyrA* gene: Asp94Gly in 10.3% (3/29) strains, Asp94Ala and Asp94His in four strains each (13.8%). Mutations in *gyrB* gene were reported in four cases and affected exclusively the codon 472, with two types of substitutions occurring in codon 472 of *gyrB* gene, namely the Asp/His (3 cases) or Asp/Asn (one case). Of note, none of the isolates harbored simultaneously mutations in *gyrA* and *gyrB* genes.

The correlation between conventional DST and molecular genotyping to detect FQs resistance was performed and results are reported in Table 3. Accordingly,

among the 40 phenotypically resistant strains, only 27 had mutations in QRDR regions of *gyrA* or *gyrB* genes. Moreover, two strains phenotypically FQ-sensitive harbored mutations in *gyrA* gene.

Depending on these results, specificity and sensitivity of the molecular resistance genotyping of FQs to the conventional DST were calculated for the recruited MDR strains, giving a sensitivity of 67.5% and a specificity of 86.7 %.

The molecular analysis showed that among the 200 MDR strains, two strains had mutations in genes conferring resistance to injectable drugs; one strain had an A/G point mutation in *tlyA* gene (Lys89Glu) and the other had A1401G polymorphism in the *rrs* gene (Table 2), these two strains are phenotypically resistant to KAN and AMK, respectively.

Other mutations considered as genetic polymorphisms and known not to be associated with drug resistance were also reported, these SNPs occurred at the codon 95 (Ser95Thr) of *gyrA* gene and at position 33 (A/G) of *tlyA* gene (Table 4).

DISCUSSION

The emergence of pre-extensively and extensively drug-resistant tuberculosis (Pre-XDR/XDR-TB) among MDR-TB isolates represents a serious hurdle for TB

Table 2 - Frequency of mutations associated with SLDs resistance within MDR MTB isolates.

ATB drug	Gene	Codon/Nucleotide Position	Substitution	Amino acid change	Number of isolates	Sub-total
FQs	<i>gyrA</i>	Codon 90	GCG → GTG	Ala / Val	14	25
		Codon 94	GAC → GCC	Asp / Ala	4	
			GAC → GGC	Asp / Gly	3	
	<i>gyrB</i>	Codon 472	GAC → CAC	Asp / His	3	4
		Codon 472	GAC → AAC	Asp / Asn	1	
		<i>rrs</i>	N1401	SNP A/G	–	1
Aminosides /cyclic peptides	<i>tlyA</i>	Codon 89	AAA → GAA	Lys / Glu	1	1
	<i>eis</i>	–	–	–	–	–

Table 3 - Correlation between phenotypic and genotypic results for OFX resistance.

Techniques for detection of MTB	Phenotypic DST		Total	
	FQ ^R	FQ ^S		
Molecular drug resistance genotyping	Presence of resistance associated mutations	27	2	29
	Absence of resistance associated mutations	13	158	171
Total	40	160	200	

Table 4 - Frequency of mutations associated with gene polymorphism in MDR TB isolates.

ATB drug	Gene	Position	Substitution	Amino acid change	Number of isolates
FQs	<i>gyrA</i>	Codon 95	AGC→ACC	Ser / Thr	3
Aminosides	<i>tlyA</i>	Position 33	A/G	–	10

control programs especially in developing countries². Rapid and efficient methods for the timely detection of drug resistance are therefore highly in demand. Within this context, we investigated 200 MDR MTB isolates to evaluate resistance to SLDs and to identify the main genetic mutations associated with drug resistance in Casablanca, which includes almost one-fifth of the total TB cases recorded in Morocco.

Mutations in DNA gyrase conferring resistance to quinolones have been extensively studied^{7,8,13}. Several reports mentioned that most FQs-resistant MTB isolates had mutations in the QRDR of DNA gyrase, which is the case of the present study, as 67.5% of phenotypically OFX-resistant isolates were found to harbor mutations in *gyrA* or *gyrB* genes. Our results showed that Ala90Val was the most predominant substitution (52.2%; 12/23) in contrast to previous studies reporting the predominance of Asp94Gly (25%)¹⁴, Ala90Thr (50%)¹³ and Asp94Ala (67.6%) substitutions¹¹.

Likewise, 27.5% of phenotypically FQs-resistant isolates harbored different substitutions in codon 94 of the *gyrA* gene, with substitutions Asp/Ala found in 10%, Asp/Gly in 7.5% and Asp/His in 10% of phenotypically FQs-resistant isolates. Supporting these data, a systematic review by Avalos *et al.*¹⁷ on FQ-resistance mutations reported that Asp94Gly and Ala90Val substitutions in the *gyrA* gene were found in 21–32% and 13–20% of FQ^R isolates, respectively. Point mutations at codons 90, 91, and 94 in the *gyrA* gene were also found in 54% of FQ-resistant MTB strains in a previous study¹⁸. Of note, our data have further shown that Ser95Thr substitution in the *gyrA* gene was found in 1.5% of MDR-TB isolates, indicating that the corresponding strains belong to the Principal Genetic Group 1/2 of the *M. tuberculosis* complex¹⁹.

In *Mycobacterium tuberculosis*, *gyrB* mutations confer resistance to FQs both individually and through interactions with *gyrA* mutations. In the present investigation, mutations in the *gyrB* gene were less commonly found in OFX-resistant isolates as they occurred in only 10% of OFX-resistant isolates with mutations detected in codon 472 of the *gyrB* gene. Indeed, *gyrA* substitutions, are much more common and generally confer higher levels of resistance than those in *gyrB*. As a result, the dose and duration of treatment are impacted by the difference of FQs-resistance

levels²⁰. However, the inclusion of *gyrB* mutations increased the sensitivity of genotypic FQ resistance from 67.5% to 77.14% in the present study; 32.5% of FQ-resistant strains (13/40) lack mutations in both *gyrA* and *gyrB* genes. It was reported that the percentage of strains lacking known mutations in the QRDR of *gyrA* or *gyrB* or both, varied from 0 to 60%, which unlikely compromises the sensitivity and specificity of molecular testing methods²¹. Molecular techniques to detect resistance mutations lack sensitivity as they do not allow to detect all phenotypically FQs-resistant strains due to the hetero resistance of mycobacteria²². Also, alternative FQs resistance mechanisms such as efflux pumps may occur^{8,11}.

Although the sensitivity of molecular diagnosis based on *gyrA* mutations reaches 95%, it may suffer from the geographic variability of *gyrA* mutations distribution. In fact, geographic differences in *gyrA* mutations across the globe have been well documented, varying from 3% in Iran to 95% in Morocco^{13,17,23-25}.

Furthermore, disagreements between genotypic and phenotypic DST were observed in 1% of phenotypically OFX^S (2/200) harboring mutations in codon 90 of the *gyrA* gene probably due to MIC values close to the critical concentration used to define routine resistance to FQs with DST and thus not detected by phenotypic testing.

There is a growing evidence that the quite high prevalence of FQ resistance in Morocco within MDR MTB isolates can be attributed to its previous use for treating other infectious diseases¹¹. Indeed, FQs have been extensively used to treat a range of infections especially pneumonia²⁶. As such, FQ resistance became a major problem worldwide. Hence, in a setting of undiagnosed TB, drug-resistant MTB mutants may arise and are likely to be selected during FQ-based treatment of other infections^{27,28}.

In contrast to FQs resistance, resistance to injectable drugs has occurred less frequently, since only few strains exhibited a resistant status in our study (4%; 8/200). The target gene sequencing of *rrs*, *tlyA* and *eis* genes showed that only two substitutions were detected: A1401G and lys89Glu in *rrs* and *tlyA* genes, respectively, each found in one case. In the present study, A1401G SNP in *rrs* gene was associated with resistance to AMK. This SNP is common in *rrs* gene and was reported in several studies to be associated with resistance to AMK (56% to 100%), KAN (44% to 84%) and CAP (51% to 96%)^{8,29-32}. Cross-resistance to second-

line injectable drugs (AMK, KAN and CAP) is now well documented. In fact, mutations at positions 1401 (A/G) displays CAP resistance along with high-level resistance to AMK and KAN, 1402 (C/T) substitution displays low-level resistance to KAN and high-level resistance to CAP, whereas 1484 (G/T) change displays high-level resistance to all three drugs^{33,34}.

CAP resistance may arise as a result of the lack of 2'-O-methyltransferase, encoded by the *tlyA* gene. Previous reports revealed that *tlyA* substitutions were found only in CAP^R isolates and the corresponding strains were reported to exhibit a high level of resistance to this antibiotic³⁵. In the present investigation, the *tlyA* substitution Lys89Glu was observed in one MDR OFX^R isolate but its sensitivity/resistance towards CAP was unknown because the latter was not available for DST. The Lys89Glu is an infrequent mutation that is associated with CAP resistance and was described in one previous report³⁵. Overall, mutations in the *tlyA* gene associated with CAP resistance were reported to be scarce (~ 0 to 3% of resistant strains)³⁶. However, when reported, mutations in the *tlyA* gene were not found in any CAP^S strains, making them highly specific markers of CAP resistance³⁰.

It is noteworthy that 5% of tested strains had the A33G substitution without any amino acid change, a SNP known not to be associated with a resistant genotype¹³.

Mutations in the *eis* promoter region were reported to be largely associated with KAN resistance^{30,37}. In the present study, none of the SNP known to be associated with KAN resistance in the *eis* promoter region was found. Therefore, no conclusion could be drawn regarding the frequencies of SNPs within the *eis* promoter gene, due to the small number of strains resistant to KAN in our sample. However, previous studies reported the occurrence of several SNPs, namely, G-10A, C-12T, C-14T in both KAN and AMK sensitive and resistant strains^{5,37}. Being very common, the SNPs within the *eis* promoter region are non-specific markers of KAN and/or AMK resistance³⁸.

From a molecular point of view, resistance to injectable drugs is very tricky to interpret. Indeed, a single mutation, or even a set of mutations in a single gene is insufficient to predict resistance to AMK, KAN and CAP. It is likely that a combination of different gene mutations for each of the injectable drugs could better predict the phenotypic resistance and promptly guide drug regimens³⁰.

In large scale, early detection of second line anti-TB drugs resistance is crucial to timely adjust the treatment regimen of MDR-TB and to reduce Pre-XDR and XDR-TB strains transmission. The inability to perform DST for SLDs is partly responsible for the misuse of anti-tuberculous drugs in several countries³⁹. This is not the case in Morocco, as

routine second-line anti-TB drug susceptibility testing of MDR isolates has been included in the diagnostic algorithm since 2015¹¹. Previous reports along with results from this study have clearly demonstrated that resistance to SLDs in Morocco is mostly driven by mutations within the *gyrA* gene (codons 90-94) linked to FQ resistance, generating thus pre-XDR TB.

CONCLUSION

This study is informative and provides data on genetic mutations associated with SLDs resistance in Casablanca, a high incidence area for TB. To the best of our knowledge, this is the first report describing mutations associated with SLDs resistance in a large panel of MDR-MTB isolates from Casablanca, the region with the highest incidence of TB in Morocco. The findings corroborate preexisting data on an alarming rate of FQs resistance, which warrants the need to timely detect FQ resistant strains and to implement appropriate drug regimens through the prescription of alternative and/or additional ATBs. Also, the introduction of newer generation of FQs is crucial for successful treatment of drug resistant TB to improve treatment outcomes for patients with MDR-TB and to prevent the emergence, as well as the spread of more severe forms of drug resistance.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

ETHICAL APPROVAL

The study protocol was approved by the Ethics Committee of the Pasteur Institute of Morocco (IPM2013-P3), and written informed consent was obtained from each study participant.

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ERRATUM

Page 1, where it reads:

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Should be read:

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