Original Article

Analysis of Isoniazid, Streptomycin and Ethambutol resistance in *Mycobacterium tuberculosis* isolates from Morocco

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Abstract

Background: Drug-resistant tuberculosis is a major problem worldwide. Based on the knowledge of specific mutations occurring in *Mycobacterium tuberculosis* genome, drug resistance can be detected earlier. The aim of this study was to determine the prevalence of the most common mutations associated with resistance to Isoniazid (INH), Streptomycin (SM) and Ethambutol (EMB) in *Mycobacterium tuberculosis* isolates from Morocco in order to select target mutations to develop tests for rapid detection of drug-resistant *Mycobacterium tuberculosis* Moroccan isolates.

Methodology: A total of 199 *M. tuberculosis* isolates collected from the National Tuberculosis Reference Laboratory in Morocco were subject to katG, inhA, rrs, rpsL and emb mutation analysis by PCR probe-based assay. The genotypic results were then compared to drug susceptibility testing results for the corresponding drugs.

Results: Among 66 phenotypically INH resistant isolates, 80.3% (53/66) were found to be genotypically INH resistant from which 77.3% (51/66) and 3% (2/66) had respective mutations in katG315 and inhp-15 codons. Of the 58 phenotypically SM resistant isolates, genotypic SM resistance was confirmed in 17.2% (10/58) cases. Nucleotide mutations at codons 43 and 88 of *rpsL* gene and at codon 512 of *rrs* gene were found respectively in 12.1% (7/58); 1.7% (1/58) and 3.4% (2/58) of the phenotypically SM resistant *Mycobacterium tuberculosis* isolates. Finally, mutations at codon 306 of *embB* gene were identified in 42.3% (11/26) of *Mycobacterium tuberculosis* isolates phenotypically EMB resistant.

Conclusion: This study showed that a large proportion of *Mycobacterium tuberculosis* resistant isolates from Morocco carry a large number of mutations in different codons (especially katG315, embB306 and rpsL43) of the corresponding genes associated with drug resistance. Thus, molecular analysis based on the identification of such mutations is useful but not fully sufficient to predict all drug resistance cases. Based on these results, rapid drug resistance genotyping can be used as an adjunct to the traditional culture based methods in reference laboratories.

Key Words: genotyping, drug resistance, *Mycobacterium tuberculosis*, isoniazid, streptomycin, ethambutol

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Introduction

Mycobacterium tuberculosis is the causative agent of tuberculosis (TB). The disease has affected humans for at least several millennia [1]. It is believed that about one third of the world's population, according to the World Health Organization, is infected by *M. tuberculosis* [2]. Each year, 3 million people globally die of TB and 8 million new individuals become infected. Thus it constitutes a major public health problem, especially in developing countries where almost 98% of all TB deaths occur and where surveillance for resistance of *M. tuberculosis* isolates to anti-TB drugs is uncommon [3].

Further contributing to the increased death rate of TB is the emergence of drug-resistant (DR) strains to one or more drugs commonly used for TB treatment; this poses a real threat to the success of national TB control programs.

DR-TB is usually treated with first- and second-line antituberculosis drugs. In addition to rifampicin (RIF), which is the main drug used for TB treatment, isoniazid (INH), streptomycin (SM), and ethambutol (EMB) are also critical components of first-line

multidrug therapy for TB [4]. Mutations in several genes in genomic regions of *M. tuberculosis* are involved in the occurrence of resistance to various first- and second-line antituberculosis drugs [4]. Several studies were conducted worldwide for genetic analysis of drug resistance, and great interest has been focused on resistance to RIF [5]. The majority (85-98%) of RIF resistant strains harbour mutations in the 81 Bp Rifampin Resistance Determining region (RRDR) of the *rpoB* gene encoding DNA-dependant RNA polymerase β-subunit. The most common mutations occur at codon ser531 (42%) and at codon His526 (23%) [5].

INH resistance is apparently controlled by a complex genetic system involving several genes, and mutations in the *katG*, *inhA*, *kasA*, and *ahpC* and *ndh* genes result in resistance. Such mutations have been found to be associated to INH resistance in approximately 60 to 70% of INH resistant strains [5]. The substitution AGC to ACC (Ser/Thr) in codon 315 of the *katG* gene is reported to be the most prevalent worldwide [5-8].

Mutations in *rpsL* and *rrs* genes, which are involved in the synthesis of ribosomal proteins, have been shown to be responsible for about 70% of SM resistant strains [5,7,9]. Mutations in position 43 of *rpsL* gene are reported to be the most common [10].

EMB is recommended for use in combination with INH, RIF, SM and/or Pyrazinamide (PZA) [11] in the first line of antituberculosis treatment. EMB resistance is due to mutations in particular codons of the *embB* gene, whose product, arabinosyl transferase, is involved in mycolic acid metabolism [12]. Aminoacid replacement at position 306 of the *emb* gene is found to be most frequent [13,14] and is represented in approximately 90% of EMB resistant isolates [15,16].

M. tuberculosis is a slow-growing organism. Its isolation, identification and drug susceptibility testing (DST) can therefore take several weeks or longer [4]. In recent years, many molecular methods based on the knowledge of genomic mutations have been developed for rapid detection, species identification, and DST of mycobacteria [4,17].

In Morocco, a previous work conducted by Soualhine *et al*. aimed to identify mutations conferring resistance to Rifampicin [18]. It was shown that the most prominent mutations occurred at codons 531, 526 and 516 of the *rpoB* gene. Thus, in the present study, we focused our interest on the analysis of prominent mutations in the most commonly targeted genes (*katG* and *inhA*, *rpsL and rrs*, and *embB*) associated respectively with INH, SM and EMB resistances. The aim of this study was to determine the prevalence of these mutations in *M. tuberculosis* resistant isolates from Morocco. This will allow us to select target mutations to develop tests for rapid detection of drug-resistant *M. tuberculosis* Moroccan isolates.

Materials and Method

Mycobacterial isolates

One hundred and ninety nine *M. tuberculosis* isolates collected at the National Tuberculosis Reference Laboratory were included in this study. These strains were obtained from sputa of patients from various geographic regions of Morocco who were diagnosed with active pulmonary TB. Demographic and clinical data was not available for all patients, but the majority were males (73%), and most were between 14 and 87 years old (the average age was 38). Most of the patients had experienced either treatment failure using the first-line drugs or were TB relapse cases, some of whom were chronic cases.

All the sputum samples were decontaminated and cultured on Lowenstein-Jensen medium. The cultures were then tested for drug susceptibility to RIF, INH, SM and EMB by the proportional method [19]. The critical concentrations of RIF, INH, SM and EMB in the medium are respectively: 40µg/ml; 0.2 µg/ml; 4 µg/ml and 2 µg/ml.

Sample preparation for molecular analysis

DNA templates for drug resistance genotyping were prepared from scraped colonies in 400 µl of distilled water and boiled at 100°C for 10 minutes to inactivate bacteria and release DNA.

Genotypic drug resistance testing was performed by mutation analysis according to the PCR probe-based assay described previously [20].

Amplification of target genes

A 5 µl aliquot of the resulting crude DNA as well as a well-characterized mutant and wild type controls were amplified by PCR for different genes: *katG*, *rpsL*, *rrs*, *embB* and *inhA* promoter gene. The information about the primers used for amplification of the target sequence in each gene, the fragment length, and the annealing temperature are summarised in Table 1.

Dot Blot Hybridisation

The most common mutations leading to INH, SM and EMB resistance were identified by Dot blot hybridisation assay. Wild-type and mutant-specific probes to screen for the presence or absence of specific mutations in selected regions of the five genes *katG*, *inhp*, *rrs*; *rpsL* and *embB* were designed (Table 2) [23].

PCR products were then loaded onto nitrocellulose membrane for Dot Blot analysis. The specific probes for different genes were radioactively labelled and hybridized under stringent conditions to the amplicons. The presence or the absence of specific mutations was confirmed by autoradiography.

Results

Susceptibility of *M. Tuberculosis* isolates to INH, SM and EMB determined by the proportional method revealed that 33% (66/199) of the isolates are INH resistant (INH^R), from which 92.4% (61/66) are multidrug resistant (MDR), whereas 29.1% (58/199) and 13.1% (26/199) *M. tuberculosis* isolates are respectively resistant to SM (SM^R) and EMB (EMB^R) (Table 3).

All *M. tuberculosis* isolates as well as mutant and wild type controls were successfully amplified and were hybridised with suitable probes. Two examples of typical results obtained with KatG315 wild type probe and rpsL43 mutant probe to screen mutations respectively in *katG* and *rpsL* genes are given in figures 1 and 2.

The amplicons of *KatG* gene were first hybridised with katG315 wild type probe because the katG315 site is known as the most frequently mutated codon. No hybridisation signal was obtained with 51 of the 66 INH^R strains suggesting that all these strains (77.3%) have mutations either in the KatG315 codon or in their neighboring codons covered by the corresponding probe. Then, the *katG* amplicons were hybridised with katG315 ACC mutated probe to identify the exact substitution. The substitution AGC to ACC (Ser/Thr) was confirmed in 35/51 (68.6%) isolates. A total of 16 isolates did not hybridise either with katG315wt or with katG315ACC.

All INH^R *M. tuberculosis* isolates were also tested for mutations in codon inhp-15 of the putative promoter of *inhA* gene, which is also known to be frequently altered. Only two (3%) isolates had mutation in inhp-15 position. All the INH susceptible strains did not harbor mutations in these two studied codons.

SM resistance genotyping was also analyzed. A total of 12.1% (7/58) of phenotypically SM^R isolates harbored mutations at codon 43 of the *rpsL* gene; 1.7% (1/58) had mutations at codon 88 of the *rpsL* gene; and 3.4% (2/58) harbored mutation at codon 512 of the *rrs* gene. All the isolates harboring these mutations are MDR or at least are polyresistant. Finally, the results of EMB resistance genotyping showed the presence of mutations at codon 306 of the *embB* gene in 42.3% (11/26) of *M. tuberculosis* isolates phenotypically EMB^R (Table 3).

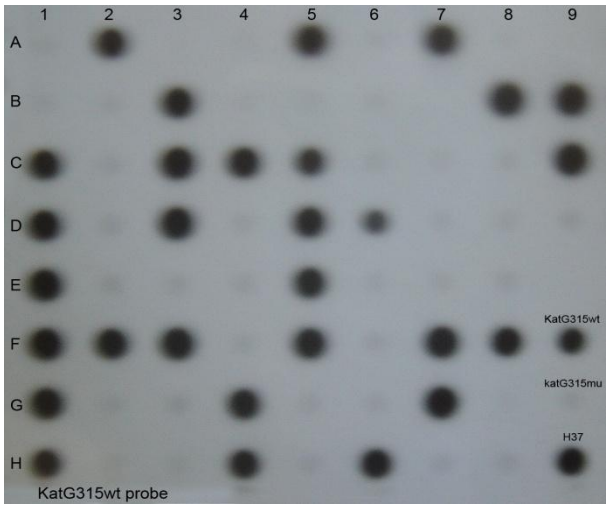
Discussion

The main problem currently associated with TB is the rise in frequency of *M. tuberculosis* strains resistant to many drugs [24] and the slowness to get phenotypic DST results. Thus, alternative methods are needed to improve the speed of diagnosis of DR-TB, especially MDR-TB [25]. The PCR probe-based assay developed by Victor et *al*. [20] is one of the methods widely used for the identification of mutations linked to *M. tuberculosis* drug resistance [26]. In this study, we have limited the number of the studied codons to katG315 and inhp-15 for INH resistance, rrs512, rpsL43 and rpsL88 for SM resistance, and embB306 for EMB resistance, since they are known to be the most frequently altered. The different frequencies of distribution of the mutations in the studied genes might be due to the local frequency of the relevant mutations [20]. The result of this study demonstrates the high prevalence of the katG315 mutation within *M. tuberculosis* isolates from Morocco and highlights its importance in the development of INH resistance [8]. The katG315 codon can therefore be selected as molecular target for the rapid detection of the majority of INH^R *M. tuberculosis* isolates. In addition, the *inhA* promoter gene is known to harbour mutations, especially in *inhp-15* site. However, this mutation is responsible only for 3% of the INH^R cases in this study. Approximately 20% of the INH^R isolates did not have these two mutations on *katG* and *inhA* genes, which indicates that mutations conferring INH resistance might also occur in additional codons of the amplified region of the *katG* gene or outside the investigated regions [8], or in other genes also involved in INH resistance (*kasA*, *ahpC* and *ndh.*). Also, other mechanisms of INH resistance except genomic mutations might occur [8].

Table 1: Primers for PCR amplification.

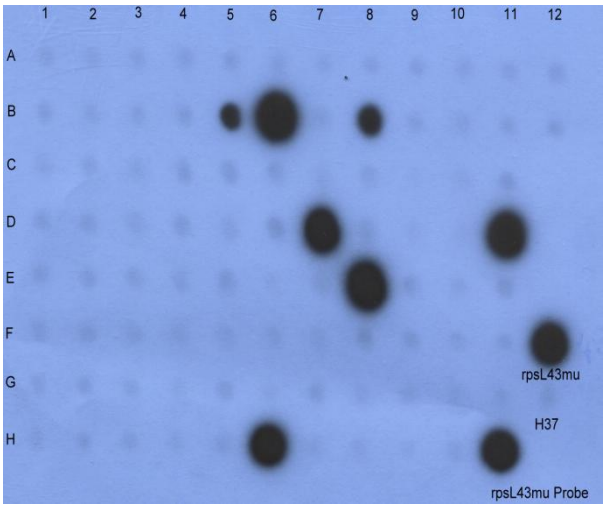
Gene	Primer	Primer sequence	Tm	Size of PCR product	Ref
katG	RTB 59	GCTGGTGATCGCGTCCTTAC	66°C	804bp	[22]
	RTB 36	TCGGGGTCGTTGACCTCCCA			
inhA promotor	inhA P5	CGCAGCCAGGGCCTCGCTG	60°C	246bp	[23]
	inhA P3	CTCCGGTAACCAGGACTGA			
rpsL	STR 52	GTCAAGACCGCGGCTCTGAA	60°C	272 bp	[21]
	STR 34	TTCTTGACACCCTGCGTATC			
rrs	STR 53	TCACCATCGACGAAGCTCCG	64°C	570 bp	[21]
	STR 31	CTAGACGCGTCCTGTGCATG			
embB	emb 151	CGGCATGCGCCGGCTGATTC	65°C	260 bp	[20]
	emb 131	TCCACAGACTGGCGTCGCTG			

Figure 1: filter obtained with wild type probe katG315



PCR amplicons for the *katG* gene were loaded by Dot-Blot in grid numbers A1-H9. Good discrimination after stringent hybridisation with labelled allele specific probes was obtained between the well-chacacterized wild type (F9 in the filter) and the mutant control (G9 in the filter). Forty clinical samples did not hybridise with the wild type probe and are therefore resistant to INH.

Figure 2: filter obtained with mutant probe rpsL43



PCR amplicons for the *rpsL* gene were loaded by Dot-Blot in grid numbers A1-H12. Good discrimination after stringent hybridisation with labelled allele specific probes was obtained between the well-chacacterized wild type (G12 in the filter) and the mutant control (F12 in the filter). Eight clinical samples showed mutations and are therefore resistant to SM.

Table 2. Probes used for hybridisation [23].

Gene	Probe	Probe sequence	Tm
katG	katG315wt	5'- GATCACCAGCGGCATCGAGG- 3'	66°C
	KatG315mu	5'- G ATCACCACCGGCATCGAGG- 3'	66°C
inhA	inhp-15wt	5'- CGGCGAGACGATAGGTTGTC- 3'	64°C
rpsL	rpsL43mu	5'- ACCACTCCGAGGAAGCCGAA-3'	64°C
	rpsL88 wt	5'- CGGGTGAAGGACCTGCC-3'	60°C
rrs	rrs512/3 wt	5'- ACGTGCCAGCAGCCGCG-3'	60°C
embB	Emb 306wt	5'- CCTGGGCATGGCCCGAGTCG-3'	70°C

Table 3: Percentage of drug-resistant isolates found by genotypic and phenotypic tests

Drug	No (%) of resistant to the corresponding drug found by			
	Phenotypic test	Genotypic test		
		No of resistant isolates (%)	Mutated codon	No of mutations (%)
INH	66	53 (80.3%)	katG315	51 (77.3%)
			inhp-15	2 (3%)
SM	58	10 (17.2%)	rrs512	2 (3.4)
			rpsL43	7 (12.1%)
			rpsL88	1 (1.7%)
EMB	26	11 (42.3%)	embB306	11 (42.3%)

Mutations in *rpsL* and *rrs* genes have not been found in SM monoresistant isolates showing that genetic resistance to SM is often associated with the resistance to other drugs, especially RIF and INH. Thus, resistance to SM might be a surrogate marker for MDR-TB.

For EMB resistance, the PCR probe-based assay enables screening of mutations in the emb306 codon in 42.3% of phenotypically EMB^R isolates. Interestingly, EMB resistance seems to be closely linked to multidrug resistance since 100% of the isolates genotypically EMB^R were resistant to both INH and RIF [27]. However, the frequency of emb306 mutation compared to other findings is low [27 - 29]. Furthermore, several studies have shown that mutations might occur in other codons, such as mutations in Phe330Val and Thr630Ile of the *emb* gene, although they are less frequent [15].

Considerable progress has been made in recent years toward understanding the molecular basis of antimicrobial resistance in Mycobacteria [30]. However, the genetic basis of M. *tuberculosis* drug resistance is not yet fully understood, since not all TB drug resistance cases are associated with a specific mutation. Thus, the presence of such mutations is clinically relevant, whereas the lack of the mutation must be interpreted with caution. Furthermore, not all mutations conferring resistance to anti-TB drugs are known. Thus, molecular techniques will play only a complementary role to classical and culture based techniques, which will remain indispensable for definitive diagnosis in some cases and determination of drug resistance.

Genotypic tests are now well known to be rapid, especially for slow-growing organisms. The PCR probe-based assay, in particular, has proved in several settings to be reproducible, technically undemanding, and takes only two working days to provide results from the start of amplification to the final autoradiography step [23,29]. However, it has limitations, for example: (1) PCR may not be able to amplify the gene targets from samples that have a few organisms [25]; and (2) Background sequence information and prior knowledge of mutations associated with resistance is required [26].

For instance, a "Reverse Line Blot" assay was developed to detect the mutations in the *rpoB*, *inhA*, *ahpC*, *rpsL*, *rrs* and *embB* genes (RLB) [31]. Additional work is necessary to find other mutations in these genes and also in other genes involved in INH resistance (especially in the *katG* gene) and then to develop a specific membrane (RLB) which combines different targets in a single assay for rapid prediction of antituberculosis drug resistance. This method would be the most convenient tool used routinely in TB diagnostic laboratories.

In conclusion, rapid drug resistance genotyping can be used as an adjunct to the traditional culture-

based methods in reference laboratories. This procedure would facilitate the adjustment of treatment regimens in time to reduce the chances of developing further drug resistance and of transmitting resistant strains, especially MDR strains.

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