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Molecular analysis of multidrug resistant *Mycobacterium tuberculosis* isolates from Morocco

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Abstract - Tuberculosis remains a global threat to public health. Considerable efforts have been made to combat this disease. However, the emergence of *Mycobacterium tuberculosis* (Mtb) strains resistant to the major anti-tuberculosis drugs especially multidrug resistant (MDR) strains poses a deadly threat to control programs. The present study aims to identify the most common mutations within multidrug-resistant *M. tuberculosis* Moroccan isolates in order to use them as molecular markers for early and rapid detection of multidrug resistant strains. For that, all *M. tuberculosis* isolates received during 2002-2003 in the National Reference Laboratory of Tuberculosis in Morocco were subject to drug susceptibility tests for rifampicin and isoniazid and to a PCR probe method to detect specific mutation. Sequencing was performed for all genotypic rifampicin resistant isolates and also for four genotypic isoniazid resistant isolates randomly selected. Out of 187 *M. tuberculosis* positive cultures, 46 (24.6%) were phenotypically resistant to both rifampicin and isoniazid. Nucleotide mutations in rpoB531, rpoB526, rpoB516, katG315 and inh-15 codons associated with resistance to rifampin (RIF) and isoniazid (INH) were found respectively in 37/46 (80.4%) and 43/46 (93.5%) isolates. Genotypic multi drug resistance was then confirmed in 74% (34/46) isolates. The mutations at codon 315 of *katG* gene and at codons 531, 526 and 516 of *rpoB* gene are frequently found in MDR isolates which confirm their strong implication in the development of multidrug resistant tuberculosis. We concluded that these mutations are useful as molecular markers for detection of multidrug resistant isolates but are not yet sufficient to fully predict *M. tuberculosis* multidrug resistance.

Key Words*: Mycobacterium tuberculosis,* multidrug resistance, gene mutations, dot blot.

INTRODUCTION

Tuberculosis remains a global threat to public health. It has a significant impact on global morbidity. According to the World Health Organization (WHO), one third of the world population is infected by *Mycobacterium tuberculosis* (Mtb). About 9.2 million new cases of tuberculosis (TB) occur every year and around 1.7 million people die annually (WHO, 2007). If intense efforts are not made to control this disease, it could be responsible for 40 million deaths over the next 25 years (WHO, 2002, 2003). Further, the problem of TB is complicated by the emergence of multidrug-resistant tuberculosis (MDR-TB) as a consequence of (i) the widespread use and incautious administration of antibiotics, (ii) the poor quality of drugs, (iii) bad management of patients by public health and (iv) frequently, treatment default due to patient ignorance. This poses a real threat to the success of national TB control program and reduces the efficiency of standard treatment recommended by WHO (Ratsirahonana *et al.*, 2002). Rapid diagnosis and appropriate chemotherapy become the first priorities in controlling the TB growing epidemics. Unlikely, patients infected with drug resistant strains are less likely to be cured, because they may fail to respond to first line treatment. Patients are then subject to second line treatment which is longer, toxic and presents several side effects. Moreover, this treatment is more expensive than the treatment of patients infected with susceptible strains (Heyman *et al.*, 1999). Inadequate and/or interrupted therapy allows the selection of spontaneous mutations in favour of resistant organisms (Pfyffer, 2000) while sequential acquisition of these mutations in different genome loci results in the development of multidrug resistance (MDR).

In Morocco, with an estimated population of 37 million inhabitants, approximately 30000 new cases arise each year with an incidence of 85 per 100000 inhabitants in 2006. The age range of TB patients varies between 15 and 45 years and in general, 57% of the recorded cases are male (Unpublished data). The anti-TB National Program was restructured in 1990 and has adopted the Directly Observed Treatment Strategy (DOTS) recommended by

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WHO. Despite the standardised and supervised treatment for the new cases as well as for therapeutic failures and chronical cases of which the percentages are respectively 5% and of 1% compared to all treated cases, MDR-TB strains emerged. In Morocco, the rate of the MDR is 2.2% according to a study of the primary drug resistance carried out on Casablanca which is the greatest urban centre in Morocco (El Messaoudi *et al.*, 2000).

Isoniazid (INH) and rifampicin (RIF) are the most important components of the first-line anti-tuberculosis (anti-TB) drugs. Multiple genes responsible for conferring resistance to these major anti-TB drugs have been identified for Mtb. The majority (85-98%) of RIF resistant strains harbour mutations in the 81-bp Rifampin Resistance Determining Region (RRDR) of *rpoB* gene encoding DNA-dependent RNA polymerase β-subunit (Telenti *et al.*, 1993; Kapur *et al*., 1994; Ramaswammy and Musser, 1998). Resistance to INH is associated with a variety of mutations affecting one or more genes such as those encoding catalase peroxydase (*katG*), the eonyl-acyl carrier protein reductase involved in mycolic acid biosynthesis *(inhA)* and alkyl-hydroperoxide reductase *(ahpC)*. However, a predominant mutation in the katG locus, Ser315Thr, counts for up to 75% of INH resistant phenotypes, (Zhang *et al.*, 1992; Wilson and Collins, 1996; Rattan *et al.*, 1998).

The control of TB is associated with a difficulty in detecting Mtb in due time and determining its drug susceptibility by conventional microbiological assays. Determination of the drug susceptibility of Mtb usually takes 4 to 6 weeks, which leads to the failure to timely initiate efficient therapy. To minimise the transmission of drug resistant tuberculosis, rapid drug susceptibility testing are also required. Molecular genetic techniques yield results in a short time, which can solve the problem of initiating adequate antibacterial therapy in the shortest possible time after resistant TB is diagnosed (Ruiz *et al.*, 2004).

In this study, we aimed to evaluate the most common mutations associated to RIF and INH resistances within MDR-Mtb isolates from Morocco and then evaluate the usefulness of these mutations as molecular markers for early and rapid detection of MDR-Mtb strains.

MATERIALS AND METHODS

Specimens. The sample set included 187 consecutive strains of Mtb isolated during 2002-2003 at the Reference Laboratory of Tuberculosis in the National Institute of Hygiene (Morocco) and at different mycobacterial culture laboratories in different provinces of Morocco. These strains belong to TB patients with different therapeutic background. For each patient, only the first isolate was considered for this study. Each strain corresponds to one patient.

Bacteriological study. The sputa sent to Reference Laboratory were subject to direct examination according to Ziehl-Neelsen method and to culture according to Petroff method, which consists on the decontamination of sputa, then neutralisation and inoculation on Lowenstein (L/J) medium. The identification of the species consists on both a macroscopic analysis of colonies on L/J medium, and a microscopic analysis. In addition, complementary niacin, reductase nitrate, and catalase tests at room temperature and at 68 °C were done to confirm Mtb species (David *et al.,* 1987).

Drug susceptibility testing. Drug susceptibility tests were performed by the proportional method with L/J medium (David *et al.,* 1987). The critical drug concentrations were 0.2 µg/ml for INH and 40 µg/ml for RIF. The critical proportion of resistant bacillus necessary to define a resistant strain is 1% for the two tested drugs (David *et al.,* 1987).

DNA extraction. All the MDR strains were subject to molecular analysis by PCR probe method (Victor *et al.,* 1999). Crude DNA was obtained by scraping a few colonies of L/J slants using a sterile disposable loop, then added to a screw cap microfuge tube containing 400 µl of distilled water. The microfuge tube was then vortexed and placed in a heating block at 100 °C for 10 min to inactivate bacteria and release DNA. An aliquot of 5 µl of the resulting crude DNA was then used for PCR reactions.

PCR amplification. The *katG* and *rpoB* genes and *inhA* promoter were amplified by PCR using the corresponding primers (Table 1) (Victor *et al.,* 1999). Five micro-litres of genomic DNA were used as template for amplification in 100 µl reaction mixture consisting of magnesium chloride, 2.25 mM; dNTPs (dATP, dGTP, dCTP and dTTP), 200 µM each; forward primer and reverse primer, 0.2 µM each and 1 unit of Taq polymerase.

Reaction mixtures were first denatured at 94 °C for 7 min. Then, thirty-five cycles of PCR were performed with denaturation at 94 °C for 1 min, primer annealing for 1 min at Tm and DNA extension for 1 min at 72 °C. At the end of the last cycle, the mixtures were incubated at 72 °C for 7 min. For each batch of samples, a negative control in which DNA template was omitted from the amplification mixture, the reference strain H37Rv and molecularly well-characterised mutant and wild type clinical isolates were also amplified.

Efficient amplification was confirmed by electrophoresis on 1.5% agarose gel. Mutations leading to RIF and INH resistances were identified by PCR probe method.

Dot Blot hybridisation. Wild-type and mutant-specific oligonucleotides were designed in order to screen for the presence or the absence of specific mutations in *rpoB*, *katG* and *inhA* genes. Oligonucleotides sequences are given in Table 2 (Victor *et al.,* 1999). Ten micro-litres of each PCR product was added to 200 µl of 0.4 N NaOH and 25 mM EDTA, denatured at 95 °C for 10 min and applied under vacuum to a Hybond-N+ nylon filter (Amersham) in a dot-blot apparatus (Minifold I SRC 96-D).

TABLE 1 - Primers for PCR amplification

Gene	Primer	Fragment generated size	Sequence	Tm (°C)
rpoB	TR8	157 bp	TGCACGTCGCGGACCTCCA	58
	TR9		TCGCCGCGATCAAGGAGT	
katG	RTB 59	804 bp	GCTGGTGATCGCGTCCTTAC	66
	RTB 36		TCGGGGTCGTTGACCTCCCA	
InhA promotor	inhA P5	246 bp	CGCAGCCAGGGCCTCGCTG	60
	inhA P3		CTCCGGTAACCAGGACTGA	

TABLE 2 - Probes for hybridisation

Gene	Probe	Sequence	Tm (°C)
katG	KatG 315wt	GATCACCAGCGGCATCGAGG	66
	KatG315mu	GATCACCACCGGCATCGAGG	66
inhA	inhp-15wt	CGGCGAGACGATAGGTTGTC	64
rpoB	rpoB531wt	AGCGCCGACTGTCGGCGCTG	70
	rpoB531mu	AGCGCCGACTGTTGGCGCTG	68
	rpoB526mu	GGGTTGACCGACAAGCGC	60
	rpoB526wt	TTGACC CAC AAG CGC CGA	58
	rpoB516mu	TTCATGGTCCAGAACAACCG	60
	rpoB516wt	TTCATGGACCAGAACAACCCG	64

The amplicons of each gene were fixed on the filter by baking at 80 °C for 2 h. The specific probes for different genes were 5′ end-labelled by phosphorylation with [γ-^{32}P]-dATP (Amersham) and hybridised under stringent conditions to amplicons on the membrane as described previously (Victor *et al.,* 1999). The presence or the absence of specific mutations was confirmed by autoradiography. To reprobe, the membranes were stripped by incubation at 50 °C for 30 min in 200 ml 0.4 N NaOH with constant shaking. The NaOH was neutralised by a further 15 min of incubation at 42 °C in 200 ml of neutralising solution containing 0.2 M Tris-HCL (pH 7.5), 0.1% SDS, 0.1X SSC. The filters were stored in a sealed plastic bag in 5X SSPE buffer for further use and probing.

Sequence analysis. PCR products were purified using the Magnesil yellow solution (Promega) to eliminate the primers used for PCR reactions, then the sequence reaction for each purified product was done in the thermocycler. The sequencing reaction was performed in a final volume of 20 µl containing 20 pmol of one primer (TR8 or TR9 for *rpoB* gene and RTB59 or RTB36 for *katG* gene), 3 µl of Big Dye (version 1.1) and 2 µl of purified PCR product. Twenty-five cycles were performed: denaturation at 96 °C for 10 s, primer annealing at 55 °C for 10 s, and extension at 60 °C for 4 min. To eliminate the excess of labelled ddNTPs, sequencing reaction products were purified using the above mentioned Magnesil green solution.

Direct sequencing of amplified PCR products was performed on an ABI PRISM sequencing apparatus (ABPRISM 310 Genetic Analyser, Applied Biosystem) and data analysis was done by sequencing analysis software.

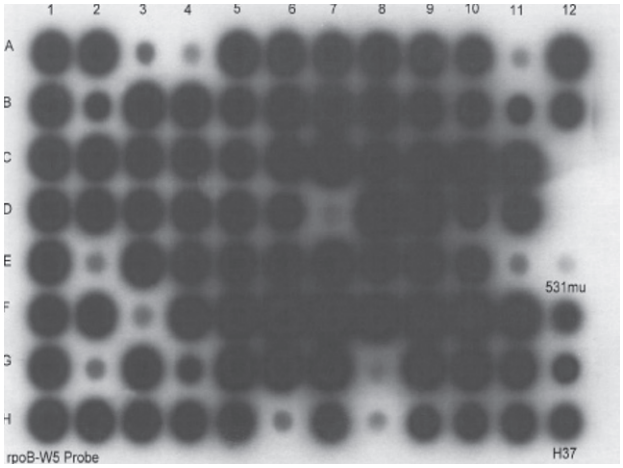

FIG. 1 - Filter obtained with wild type probe 531. PCR amplicons for rpoB gene were loaded by dot-blot in grid numbers A1-H12. Good discrimination after stringent hybridization with labelled allele specific probes was obtained between well characterised wild type (H12 in the filter) and mutant control (E12 in the filter). Ten samples had 531 mutation and are therefore resistant to RIF.

RESULTS AND DISCUSSION

All the 187 studied strains belong to the Mtb species. Of these 187 strains, 24.6% (46/187) were found resistant to RIF and to INH. The percentage of MDR strains is lower compared to other findings of similar studies carried out in Madrid (Spain) (75%) (Telenti *et al.,* 1997) and in Cape-Town (South Africa) (56%) (van Rie *et al.,* 2001). All the MDR strains were subject to molecular analysis by PCR based probe strategy.

Figure 1 shows a typical result obtained with rpoB531wt probe and allows the identification of Mtb isolates that are either resistant or susceptible to RIF.

In this study, we used a strategy that limits the number of targeted codons in specific genes associated with MDR resistance. Thus, we screened mutations in rpoB531; rpoB526 and rpoB516 codons of *rpoB* gene for RIF resistance, and in katG315 codon of *katG* gene and in inhp-15 codon of *inhA* gene promoter for INH resistance.

Of the 46 phenotypically MDR strains, 80.4% (37/46) were found to carry mutations in one of the three studied codons of the hot spot region of *rpoB* gene and are therefore genotypically RIF resistant (RIF^R). Likewise, a previous study has shown that 70 to 95% of phenotypically RIF^R isolates harbour mutations at codons 531, 526 and 516 (Cavusoglu *et al.*, 2002; Ramaswammy *et al.*, 2004).

Among the 37 genotypically RIF resistant isolates, the most common mutation occurred at codon 531. In fact, 58.7% (27/46) of the phenotypically MDR isolates did not hybridize with rpoB531 wild type probe. Furthermore, the codon 531 is well known to be a hot spot in *rpoB* gene for mutational changes in Mtb (Musser, 1995). The mutations at rpoB526 codon occurred in 10.9% (5/46) phenotypically MDR strains. Likewise, mutations at rpoB516 codon occurred in 10.9% (5/46) phenotypically MDR strains. Approximately, a similar result was found in Scotland (10%) (Fang *et al.*, 1999). However, in Cape town (South Africa),

only 5% of phenotypically MDR strains harbour this mutation (van Rie *et al.,* 2001). Furthermore, none of the MDR studied strains harboured simultaneously more than one of these point mutations.

Point mutations at 531, 526 and 516 positions of *rpoB* gene were confirmed by sequencing meaning that the dot blot analysis is accurate.

Among the phenotypically MDR isolates, 19.6% (9/46) did not carry any mutations at the three studied codons, this result was proven to be higher that the one found in China (10.4%) (Huang *et al.*, 2002) and in Cape-town (12%) (van Rie *et al.,* 2001). The sensitive genotype of phenotypically resistant strains might be due to many reasons (Brown *et al.,* 2000): (I) the existence of mutations in other codons of the hot spot region of *rpoB* gene, (II) the existence of additional mutations outside 81-bp core region of *rpoB* gene (Schilke *et al.,* 1999; Heep *et al.,* 2001), (III) RIF permeability (Tanaka *et al.*, 1996; Ouans *et al.*, 1997; Huang *et al.*, 2002), (IV) mutations in other genes, which ensure the RIF metabolism and (V) mutations in alternate subunits of RNA polymerase generating resistant phenotypes (Rattan *et al.*, 2002).

For INH resistance, the katG mutation was found in 89.13% (41/46) phenotypically MDR strains of which 33 are also resistant to RIF (classified as genotypically MDR-Mtb isolates), this result is almost the same as other findings: 95% in Cape Town (South Africa) (van Rie *et al.*, 2001) and 85.6% in Brazil (Cardoso *et al.*, 2004). However, 10.87% of phenotypically MDR strains (5/46) didn't have a mutation at the studied codon. These strains might have additional mutations in other codons of the amplified region of *katG* gene or outside this investigated region or in other genes involved in INH resistance. Furthermore, INH resistance could be due to unknown mechanisms (Lee *et al.*, 1999).

In order to test the accuracy of dot blot analysis, four MDR strains randomly selected were subject to sequence analysis which confirms the presence of mutation at codon 315 of *katG* gene.

Mutations *in inhA* promoter; associated to INH resistance, have been reported in several studies with higher percentages. Studies conducted in Madrid (Spain) (Telenti *et al.*, 1997) and in Brazil (Cardoso *et al.*, 2004) showed that respectively 46 and 26.8% of MDR cases harbour inhA substitution which confirms the implication of this putative promoter in INH resistance. In this work, we were interested by inhp-15 position of inhA putative promoter. Among the 46 phenotypically MDR strains, only 2 isolates (4.3%) were found to have mutations at inhp-15 codon of the putative promoter of *inhA* gene. Among them, one had mutation in *rpoB* gene and is classified as genotypically MDR Mtb strain. The result is higher compared to that found in Cape Town (2%) (van Rie *et al.,* 2001) but lower compared to the one found in a study carried out in china where 17% of MDR isolates had such mutation (Wu *et al.*, 2006).

Worldwide, molecular analysis of resistant Mtb was focused on the mutations at codons rpoB516, 526 and 531 as well as at codon katG315. As already reported, mutations in Ser531 and His526 codons of *rpoB* gene occured in more than 70% of the RIF-resistant isolates, these two mutations associated with rpoB516 and katG315 substitutions are known to be the most involved alterations within MDR Mtb strains (Ramaswammy and Musser, 1998; Huang *et al.*, 2002). Thus, the prevalence of mutations at codon 315 of *katG* gene and at codons 531, 526 and 516 of *rpoB* gene highlights their importance on the development of multidrug resistance within Mtb isolates. Furthermore, a previous outcome in South Africa has shown that 90% of all MDR-TB cases can be detected by screening for just 3 codons (rpoB531, rpoB526, and katG315) (Victor *et al.,* 1999; van Rie *et al.*, 2001).

The MDR strains identified by the proportional method were confirmed by dot blot analysis in 74% of the cases. Thus, the genotyping is a practical and fast way to have information on resistance profile of Mtb strains (Cockerill, 1999; van Rie *et al.*, 2001). It does not require living cells; moreover, the resistance can be detected directly from the pathological product but it requires exact knowledge of Mtb genes and genomic mutations associated with resistance (Nikolayevsky *et al.*, 2004). However, we should be aware of the presence of particular mutations because they are not systematically related to drug resistance (this is the case of KatG463 mutation) (van Doorn *et al.*, 2001). Background sequence information and prior knowledge of mutations associated with resistance is required. Indeed, genetic methods may predict resistant genotypes that are expressed at levels that may not be clinically relevant. In some cases, genotypic mutations may not lead to phenotypic expression, meaning that drug resistance cannot be manifested. Thus, the prescriptions of the appropriate treatment must not be done exclusively on the basis of the presence of such genetic mutations. Taken together, genetic mutations and conventional techniques must be used to have an effective diagnosis for a better treatment (Cockerill, 1999; Victor *et al.*, 2002). Also in some resistant strains, no mutation is detected meaning that resistance might be linked to other mechanisms of resistance not yet fully understood (Ramaswammy and Musser, 1998). Thus, the results of the antimycobacterial susceptibility tests must be taken into consideration since phenotypic tests still remain the "Gold standard" tool for the detection of drug resistance in spite of the slowness of results. Also, phenotypic results cannot always correlate with clinical resistance (Acar and Goldstein, 1998); the isolate might well contain a mixture of sensitive and resistant bacilli (Brown *et al.*, 2000).

The RIF and INH are the most potent first-line antituberculosis drugs used for standard treatment, and resistance to these two drugs known as MDR represents an important public health problem worldwide. Thus, knowledge of the susceptibility patterns of Mtb isolates is important for the effective clinical management of TB patients and for disease control. The treatment can then be adjusted earlier if drug resistance profile is determined. Thus, the transmission of MDR strains can be reduced in a country where MDR-TB burden is high (Carpel *et al.*, 1995; Perronne and Truchis, 1995; Nikolayevsky *et al.*, 2004). A better understanding of the molecular mechanisms involved in Mtb drug resistance may well contribute to develop new molecular techniques for conducting fast and accurate molecular diagnosis in order to prescribe an effective treatment for MDR-TB patients (Ozturk *et al.*, 2005). Although, these techniques require the expertise and an expensive equipment, which limit their broader application (Singh and Katoch, 2006)

The identification of the MDR strains should be quick in settings where MDR-TB is very common, and molecular techniques based on specific genetic markers might play a complementary role to culture based techniques which remain indispensable for definitive diagnosis in some cases and for determination of drug resistance.

Furthermore, the data of this study indicate that mutations at specific codons of *rpoB* and *katG* genes identified by PCR based probe assay are useful as molecular markers to detect MDR Mtb strains but are not yet fully sufficient to predict efficiently multidrug resistance.

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