

Risk factors for *Mycobacterium tuberculosis* resistance and detection of resistance mutations to Rifampin and Isoniazid by Real-Time PCR

Detectarea mutațiilor de rezistență ale *Mycobacterium tuberculosis* la Rifampin și Isoniazid prin Real Time PCR

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Abstract

Objectives The objectives of our study were 1) to define risk factors for antimicrobial-resistant *Mycobacterium tuberculosis* (MTB) 2) to evaluate the use of real-time PCR-based technique for the prediction of phenotypic resistance of MTB. **Methods** We performed a prospective study in which we considered all 120 patients with smear-positive tuberculosis who were admitted to two clinical wards of the Marius Nasta Institute of Pneumology within a period of one year. Only data from 103 patients who underwent bacteriological investigations, including sensitivity tests, and who had filled out a questionnaire was analyzed. Fourteen patients with culture-confirmed tuberculosis resistant to any first line drug were compared to patients with fully susceptible disease. Sixty-seven MTB strains (26 drug-resistant and 41 drug-susceptible) were tested using a method recommended for the Light Cycler platform. For rifampin resistance, two regions of *rpoB* gene were targeted, while for identification of isoniazid resistance, we searched for mutations in *katG* and *inhA* genes. The susceptibility testing was performed using the absolute concentration method. **Results** We noted that while MTB resistance tends to be lower in new cases (3.7%), in patients with retreatment this resistance is much higher (44%), especially multidrug resistance (MDR), which accounts for 36% of cases of retreatment vs. 1.2% of new cases. Drug resistance was significantly associated only with previous antituberculous treatment (OR 19.56, CI 95% (3.96-94.41, $p < 0.005$). The sensitivity and specificity of the rapid detection of mutations for isoniazid were 96% (95% CI: 88-100), respectively 95% (95% CI: 89-100), while for the rifampin resistance, sensitivity and specificity were 92% (95% CI: 81-100%), and 74% (95% CI: 61-87%) respectively. In our isolates the main resistance mechanism to isoniazid was related to changes in the *katG* gene that encodes catalase. Using this method, the best accuracy for genotyping compared to phenotypic resistance testing was obtained in what concerned detecting isoniazid resist-

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ance mutations. We found that for rifampin resistance the correlation between the predicted and observed phenotype was less than satisfactory. **Conclusion** MTB resistance was significantly associated only with previous anti-tuberculous treatment. Real-time PCR assays represent a valuable diagnostic tool but they are not yet completely satisfactory for the detection of drug-resistant MTB.

Keywords: *Mycobacterium tuberculosis*, risk factors, resistance, Real-Time PCR

Rezumat

Obiective Scopul acestui studiu a fost acela de a evalua tehnica real-time PCR pentru determinarea rezistenței la izoniazidă și rifampicină a tulpinilor de *Mycobacterium tuberculosis*. **Metode** Un număr de 67 de tulpini de *Mycobacterium tuberculosis* (26 rezistente și 41 sensibile la tuberculostatice) au fost testate folosind o metodă descrisă anterior pentru platforma Light Cycler. Testarea fenotipică a rezistenței s-a realizat prin metoda concentrațiilor absolute. În vederea identificării rezistenței la rifampicină au fost căutate mutații de rezistență în gena *rpoB*, iar pentru izoniazidă, în genele *inhA* și *katG*. **Rezultate** Sensibilitatea și specificitatea detectării rapide a mutațiilor de rezistență pentru izoniazidă au fost 96% (95% CI: 88-100), respectiv 95% (95% CI: 89-100). Pentru detectarea rezistenței la rifampicină, sensibilitatea și specificitatea au fost 92% (95% CI: 81-100%) și respectiv 74% (95% CI: 61-87%). Principalul mecanism de conferire a rezistenței la izoniazidă întâlnit la tulpinile studiate a fost modificarea în gena *katG*, codificatoare a catalazei micobacteriene. În privința rezistenței la rifampicină, concordanța dintre testarea fenotipică și cea genotipică nu a fost mulțumitoare. **Concluzii** Folosind această metodă, acuratețea cea mai bună între testarea fenotipică și cea genotipică a rezistenței a fost obținută pentru izoniazidă. Deși real-time PCR este o tehnică de diagnostic valoroasă, în cazul detectării rezistenței la antituberculoase a *Mycobacterium tuberculosis*, ea nu este pe deplin satisfăcătoare.

Cuvinte cheie: *Mycobacterium tuberculosis*, factori de risc, rezistența,

Introduction

Although antituberculous therapy has been available for the past 50 years, tuberculosis is still a major worldwide public health problem, with the World Health Organization (WHO) estimating that approximately one third of the planet's population is infected with *Mycobacterium tuberculosis* (MTB). Romania is one of the European countries with the highest incidence of both new and relapse cases of tuberculosis (TB) with more than 25,000 total cases recorded every year (1,2).

The emergence of multi-drug resistant (MDR) tuberculosis - defined as the resistance to at least isoniazid (INH) and rifampin (RMP) - has been a major public health concern since the early 1990's. Although it remains unclear whether MDR strains are less transmissible than sensitive strains,^[3] infections with drug-resistant tuberculosis represent a major public

health issue because patients can remain infectious for longer periods of time. Therefore, public health consequences of drug-resistant TB might be more serious than those of drug-susceptible disease, as MDR renders TB more difficult and costly to treat.

According to a national survey performed in Romania between 2003 and 2004, 3.6% of the strains isolated from newly diagnosed patients and 8.6% from relapse cases were resistant to one anti-tuberculosis drug (INH) (2). The study also showed that MDR tuberculosis represented 2.9% of the strains isolated from newly diagnosed patients and 11% of those isolated from relapse cases. Taking into account that in Romania more than 25,000 TB cases (new cases and relapses) are reported each year, we can estimate that more than 1,100 patients are infected with MDR-TB strains.

Drug-resistant strains emerge by selective multiplication of resistant mutants within the lesions, as a result of inappropriate and/or inad-

equate treatment. High rates of MDR-TB have been observed not only among previously treated TB patients, but also among new cases due to the transmission of MDR-TB in the community. The main risk factors associated with acquired drug resistance are cavitory disease, poor compliance with medication, inappropriate prescription of drugs, HIV infection and immigration from areas with high prevalence in MTB resistance.

Rapid identification of resistance is essential in reducing the time between diagnosis and appropriate therapy and thus in limiting the transmission of drug-resistant strains. Classical phenotypic methods of determining resistance require a long time (up to 10 weeks after referral of a sample to the laboratory) which can be significantly reduced by nucleic acid amplification assays. Given this major advantage there has been a lot of effort invested in the past few years in designing new performance protocols for genotyping MTB strains. The real-time PCR came to be the main approach because of its unique features: high sensitivity, specificity, and speed that require no post-PCR sample manipulation. Specific primers and probes that can be used to identify gene mutations associated with drug resistance in MTB have been developed using results from fundamental research such as the complete MTB genome sequencing.

RMP binds to the bacterial RNA polymerase and interferes with the RNA synthesis by binding to the bacterial RNA polymerase; resistance to RMP is conferred by mutations resulting in at least eight amino acids substitutions in the *rpoB* subunit of the RNA polymerase. Mutations in a limited region of *rpoB* have been found in >95% of RMP-resistant clinical isolates of MTB and results in high-level resistance (MIC >32 µg/ml) to RMP and cross-resistance to all rifamycins.^[4] INH acts by inhibiting an oxygen-sensitive pathway in the mycolic acid biosynthesis of the cell wall. At least four genes have been described to be involved in resistance to isoniazid: the *katG* gene, which encodes a catalase; the *inhA* gene, whose

product is a target for INH and the *oxyR* gene and the neighboring *aphC* gene as well as their intergenic region (5). Several real-time PCR-based methods targeting these specific genomic regions have been described (6-13).

The purpose of the present study was to define risk factors for MTB resistant to antimicrobials and to evaluate the use LightCycler instrument in detecting these mutations associated with resistant MTB strains isolated from Romanian patients.

Materials and Methods

Strains and resistance testing

For the first objective we performed a prospective study in which we included 120 smear-positive TB patients admitted to two clinical wards of the Marius Nasta Institute of Pneumology within a period of one year. We analyzed data from 103 patients who underwent bacteriological investigations including sensitivity tests and who had filled out a questionnaire. The data collected in the questionnaire were:

- Employment status
- History of exposure data:
- Living conditions
- History of imprisonment, homelessness, employment in TB wards
- History of hospitalization in pneumology or other medical wards
- Other TB cases of family members or friends
- History of TB chemoprophylaxis
- History of previous TB diagnosis & treatments
- Drug & alcohol-abuse
- HIV status
- Other immune-compromising conditions
- Type of case by previous exposure to treatment according to the WHO definition

We defined the type of case according to previous exposure to treatment as: new case, relapse, retreatment after failure or default and chronic case, as follows:

- **New case:** new TB patient, never treated with anti tuberculous drugs or treated for less than 1 month

- **Relapse:** TB in a patient who received a complete treatment in the past
- **Retreatment after failure:** TB patient who continues to be positive or turned to be positive again at sputum examination after the 4th month of treatment.
- **Retreatment after default:** restart of TB treatment in a TB case that interrupted treatment for more than 2 consecutive months and turned to positive sputum results.
- **Chronic case:** failure of the first retreatment

Forty-one susceptible and 26 resistant clinical isolates of MTB (23 resistant to both INH and RMP, 1 mono-RMP resistant and 2 resistant to INH only) from 62 different patients were studied. The susceptibility testing was performed by using the absolute concentration method (Meissner) (14). This method is based on the comparison between the growth of mycobacteria on drug-free medium and that from drug-containing media (antituberculosis drugs incorporated in the medium at different concentrations), after 21 days from inoculation with a standardized inoculum. Two critical concentrations were used for every tested drug: INH 0.2 and 1 µg/ml for INH, 20 and 40 µg/ml for RMP. According to this method, resistance to a drug is defined by the growth of more than 20 colonies on drug-containing media (INH 1 µg/ml, RMP 40 µg/ml).

Extraction of mycobacterial DNA

The MTB DNA was extracted using the thermal lysis procedure in the presence of Chelex 100 (Amersham Pharmacia Biotech, Uppsala Sweden). One loopful of bacteria scraped from Löwenstein-Jensen solid medium was suspended in 100 µl of sterile water; 10% suspension of Chelex was added volume to volume and the mixture was incubated for 45 minutes at 45°C and 5 minutes at 100°C. The samples were centrifuged at 12000xg for 5 minutes and the supernatant was harvested for subsequent use in the following steps of the experiment.

Real-time PCR

We adapted a previously described protocol for genotyping the MTB drug resistance.^[6] The method published by Torres et al. was designed as a single tube method able to detect RMP and INH resistance mutations; one set of primers and two fluorescently labeled hybridization probes were used for each targeted region. One set of primers and two sets of probes (rpoB1 and rpoB2) that targeted the *rpoB* gene have been used for detection of RMP resistance and one set of primers and probes for each *katG* and *inhA* gene in order to test INH resistance. All primers and probes were synthesized by TIB MOLBIOL (DNA Synthesis Service; TIB MOLBIOL GmbH, Berlin, Germany). Real-time PCR was followed by melting curve analysis, both performed on the Light-Cycler instrument (Roche Diagnostics, Mannheim, Germany). We used the same PCR conditions (components concentration, cycling and melting programs) as previously described, but we added 10 more cycles of amplification to the recommended 35 (7). We included into each experimental run one negative control (the DNA template was replaced with PCR-grade water) and one positive control (the DNA template was isolated from *MTB* H37Rv, strain susceptible to the INH and RMP).

Statistical analysis

In order to determine the cut-off of T_m changes to predict mutations associated with resistance, we generated ROC curves. A ROC curve is a graph of sensitivity (or true positive rate) on the Y-axis as a function of 1-specificity (the false-positive rate) on the X-axis. The graph is used to assess the global value of a test whose result is a continuous variable (the greater the area under the curve, the greater accuracy of the test), and to select different cut-off points for which to calculate sensitivity and specificity. For a test with a large area (>0,850), one can have good sensitivities and specificities, while for a test with lesser accuracy (0.600-0.800), one can choose a cut-off point in order to increase

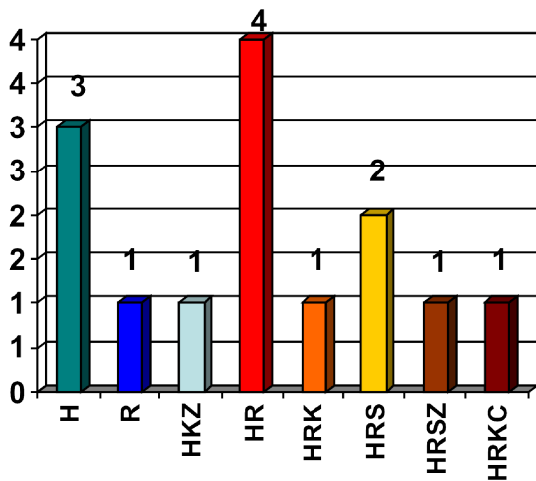


Figure 1A. Susceptibility testing for the whole group
(H - isoniazid, R - rifampicin, K - kanamycin, Z - pyrazinamide, S - streptomycin, C - cyprofloxacin)

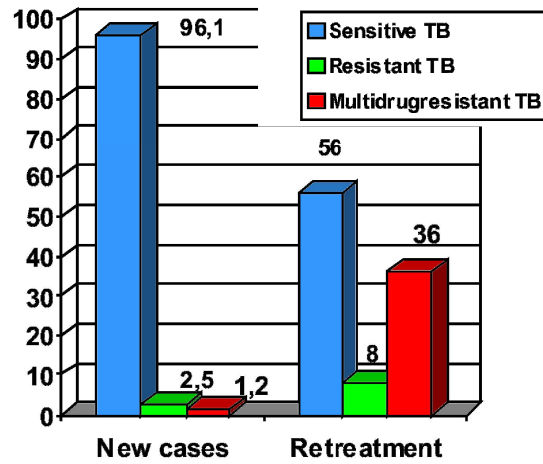


Figure 1B. *M. tuberculosis* susceptibility according to the previous antituberculous treatment

sensitivity or specificity, but with the cost of a low specificity, respectively sensitivity.

The area under the ROC curves was then determined, and the cut-off points were identified to maximize test sensitivity (and thus to decrease the false negative rate). To further enhance sensitivity, we also assessed in parallel the tests detecting the presence of either *rpoB1* or *rpoB2* for RMP resistance and the presence of either *katG* or *inhA* gene for INH resistance. SPSS 10.0 software (SPSS, Inc., Chicago, IL, USA) was used for the database construction and ROC curves, and CAT maker 1.1 (Centre for Evidence-Based Medicine, Oxford, GB, 2004) to calculate the attributes of the diagnostic tests studied.

Results

103 out of 120 questionnaires were eligible for analysis. Most patients were new cases (78, representing 75.7%), but we noted 12 cases (11.6%) of relapses, 8 cases (7.8%) of chronic TB and 5 cases (4.8%) of retreatment either for default (3 cases) or failure (2 cases). The majority of patients were less than 50 years old (similar to the national incidence) and 65% of them were unemployed/retired, or with poor socio-economical status.

The results for the susceptibility testing for the whole group (Figure 1A) and according to previous exposure to antituberculous treatment (Figure 1B) were as follows: 89 strains (86.4%) fully susceptible and 14 strains (13.6%) resistant. The resistant strains were either monoresistant (4 representing 3.9%), or multidrug-resistant (9 representing 9.5%). We noted that while MTB resistance tends to be low in new cases (3.7%), in retreatment cases this resistance is much higher (44%).

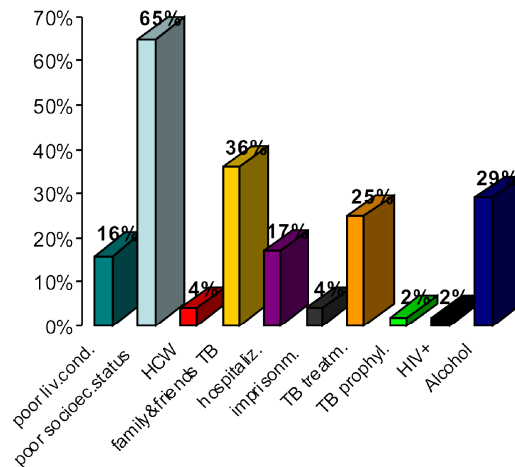


Figure 2. Potential risk factors for drug resistance in our study group

Table 1. Risk factors for resistance

Risk factors	Resistance strains	Susceptible strains	OR (CI)
History of previous TB diagnosis & treatments	11/14	15/89	18,09 (3,96-94,41)
Alcohol-abuse	6/14	24/89	2,03 (0,55-7,38)
Other TB cases in family	4/14	23/89	1,15 (0,27-4,55)
History of TB chemoprophylaxis	2/14	0/89	0,44 (0,09-1,89)
Unemployment	3/14	34/89	0,44 (0,09-1,89)

When studying the association between different factors (*Figure 2*) and drug resistance, we found that this was significantly correlated only with previous TB treatment (OR 19.56, CI 95%, (3.96-94.41) $p < 0.005$) (*Table 1*)

During the real-time PCR experiments, the amplification of the DNA template was monitored by continuously measuring the fluorescence level. The Ct for samples and for the positive control ranged between 20 and 35 cycles. The T_m s for the probes annealed to the PCR product were generated by running the melting analysis program (ramping from 50 to 85°C with 0.1°C per second) and calculated using the LightCycler software. While running different sets of samples along with the positive control, we observed that the melting temperature (T_m) for the MTB H37Rv was variable, ranging between

70.08 and 71.26°C. Therefore, for each experimental run we analyzed the changes in T_m for the PCR products derived from our collection of resistant and susceptible MTB clinical isolates as compared with the T_m of the H37Rv tested in the same run rather than using directly the observed T_m s.

We also noticed that the T_m values for the H37Rv strain as well as for other wild-type (wt) field strains tested were lower than expected (70.08°C for H37 as compared with 72.8°C in the original communication). For the resistant strains, in one case (sample 2312), we obtained a lower T_m value than the expected T_m ; the δT_m was however consistent with AGC > ACC mutation in position 315. In *Figure 3* we represented the melting profiles for the positive control (H37Rv) and other 7 samples when we analyzed the *katG* PCR

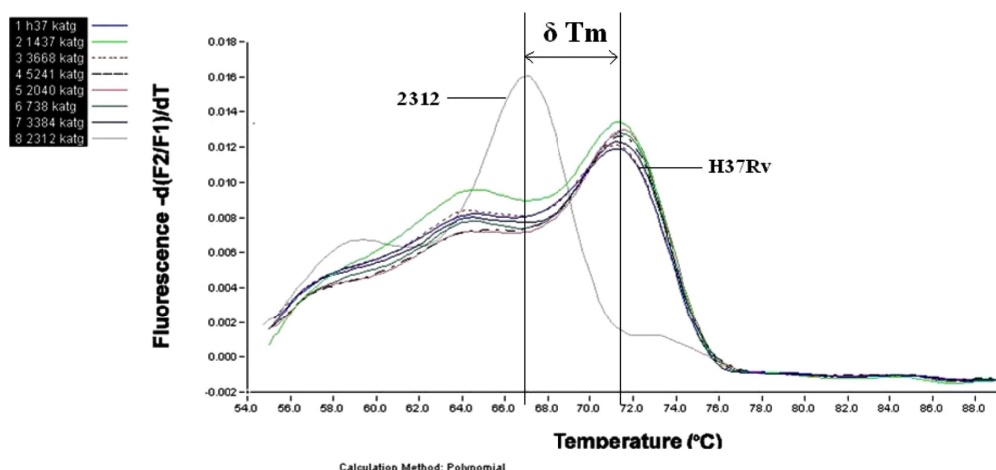


Figure 3. Melting profiles for H37Rv and other 7 MTB strains tested

products. It can be seen that the sample 2312 has a melting profile different than H37Rv, in agreement with the phenotypic results which scored this sample as resistant to INH. The other isolates had the same melting profile with the positive control and were also found to be susceptible to INH by the phenotypic analysis.

As compared with the T_m of the susceptible strain, the T_m changes for the products derived from our collection of resistant and susceptible MTB clinical isolates varied largely: 0.00-2.29 (*rpoB1*), 0.00-4.63 (*rpoB2*), 0.01-3.32 (*inhA*) and 0.01-5.86 (*katG*).

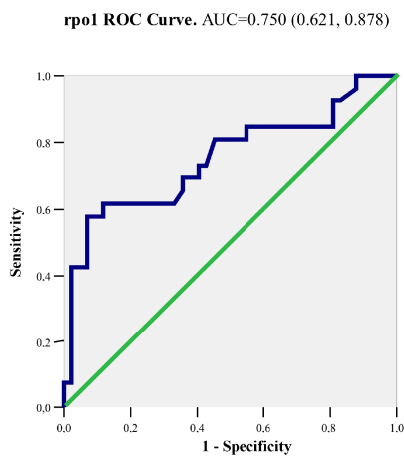


Figure 4A. Analysis of T_m changes in order to define the cut-off predictable for mutations associated with rifampin resistance

In order to determine the cut-off of T_m changes for mutations associated with resistance we used the ROC curves. Concerning the RMP resistance, the area under the ROC curves with the 95% confidence intervals for *rpoB1* and *rpoB2* (Figure 4A) were 0.750 (0.621, 0.878) and 0.711 (0.584, 0.839) respectively, while for the INH resistance, the areas for *katG* and *inhA* (Figure 4B) were 0.935 (0.862, 1.008) and 0.666 (0.530, 0.802) respectively. The cut-off points of T_m changes predictable for mutations associated with resistance for *rpoB1*, *rpoB2*, *inhA* and *katG* were respectively 0.90, 0.95, 1.30 and 1.10.

For INH resistance, the genotyping results correctly matched classical resistance testing in 24 (96%) of 25 isolates. There were 2 isolates reported as genotypically resistant and phenotypically susceptible (Table 2). We found that 20 strains had mutations in the *katG* gene, while only one had mutations in the *inhA* gene; 4 strains had mutations in both genes. We can conclude that the main isoniazid resistance mechanism identified in the MTB Romanian isolates is related to changes in the gene that encodes catalase.

For RMP our results only partly matched those generated by conventional testing. There were 11 isolates reported as genotypically resistant while their phenotype was susceptible; however, only 2 isolates among 24

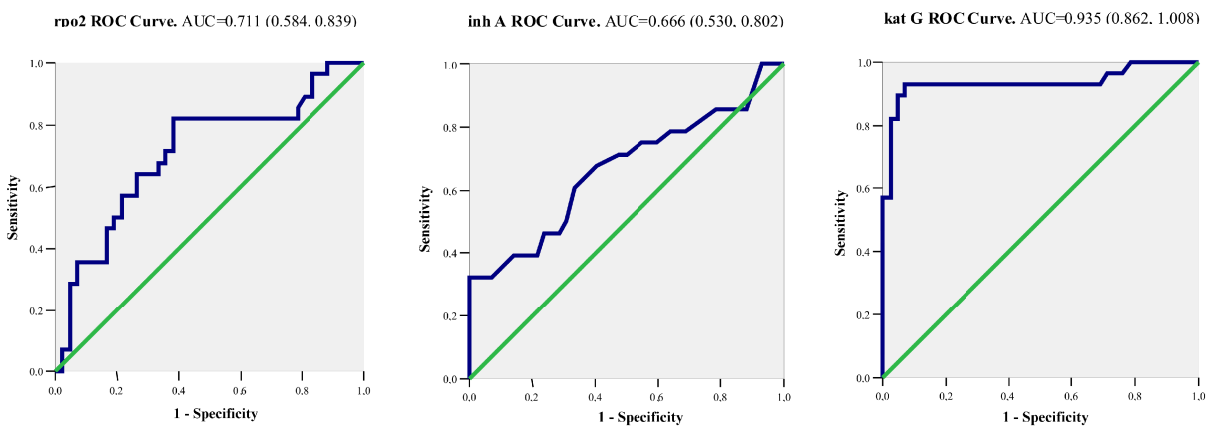


Figure 4B. Analysis of T_m changes in order to define the cut-off predictable for mutations associated with isoniazid resistance

Table 2 Rapid detection of resistance associated mutations for INH (presence of either *inhA* or *katG*) as compared to the INH resistance phenotype

		Isoniazid phenotype		Total
		<i>Resistant</i>	<i>Susceptible</i>	
Isoniazid genotype	<i>Resistant</i>	24	2	26
	<i>Susceptible</i>	1	40	41
Total		25	42	67

Table 3 Rapid detection of resistance associated mutations for RMP (presence of either *rpoB1* or *rpoB2*) as compared to the RMP resistance phenotype

		Rifampin phenotype		Total
		<i>Resistant</i>	<i>Susceptible</i>	
Rifampin genotype	<i>Resistant</i>	22	11	33
	<i>Susceptible</i>	2	32	34
Total		24	43	67

phenotypically RMP-resistant strains generated a susceptible hybridization pattern (Table 3).

The sensitivity and specificity of the rapid detection of mutations for isoniazid (presence of either *inhA* or *katG*) were 96% (95% CI: 88-100), respectively 95% (95% CI: 89-100), with a positive likelihood ratio (LR+) of 20 and a negative likelihood ratio (lr-) of 0.04. For RMP (presence of *rpoB1* or *rpoB2*), sensitivity and specificity were 92% (95% CI: 81-100%), and 74% (95% CI: 61-87%), respectively, with a LR+ of 3.58, and a lr- of 0.10.

Discussions

MTB can develop resistance to an antimicrobial agent spontaneously or under the selective pressure of antibiotics, but spontaneous development of resistance to both IHN and RMP in a properly treated patient is virtually impossible. Therefore, MDR arises under the selective pressure of inadequate therapy.

Between 1990 and 1992 the Centers for Disease Control and Prevention reported several outbreaks of MDR-TB. In Europe such outbreaks have also been reported. In these outbreaks the

AIDS epidemics has been found to be associated with MDR. We did not find an association between MDR-TB and HIV infection, but the number of HIV infected patients in our study group was very limited. The major risk factor is considered to be a previous and inadequately administered therapy and our results support this finding. Alcohol abuse and a poor socio-economic status are risk factors rather for TB than for resistant TB. Other factors associated with MTB resistance were the deterioration of the public health infrastructure and inadequate training of health care workers in the epidemiology of TB (14).

None of the methods used to determine the susceptibility of MTB to antituberculous drugs is perfect (15).

One of the objectives of our study was to evaluate to which extent differences in sequence between circulating strains might hamper the use of real-time PCR based techniques for the prediction of phenotypic resistance of MTB strains. We used a technique recommended for the Light Cycler platform to analyze 67 sequences from 26 drug-resistant and 41 drug-susceptible MTB strains.

Our results suggest that this platform can be used but there are some limitations. One

of them is related to reproducibility. While testing for the resistance to INH and RMP with real time PCR, we found that the T_m for sensitive as well as for resistant strains varied proportionally from one experiment to another when compared to the control strains due to factors that could not be identified. However, the differences between the T_m s of resistant and sensitive strains were consistently observed and could be reliably associated with predicted resistance.

Although the genotypic assays are very useful for rapid detection of drug resistance, there are some limitations. Firstly, not all MTB-DR isolates have mutations in the so-called *hot spots* of the genes associated with resistance. For instance, about 20-30% of the INH-resistant strains do not have mutations in *katG*, *inhA*, *kasA* or *aphC* genes (4). For that reason, it is very difficult to design a test that could identify all the possible mutations that confer resistance to anti-MTB drugs. This was the case with the MTB isolates from Romanian patients. Here, only two of the main genes involved in conferring resistance to INH were targeted by PCR. We found that for the Romanian strains, *katG* was adequate to detect INH resistance. In the analysis of data from other studies, geographical differences in the frequencies of specific mutations are also apparent: the *katG* gene was mutated at codon 315 in 64% of INH resistant strains from South Africa and Central and Western Africa but in only 26% of Singaporean isolates (16,17). Furthermore, even the commercial tests for genotyping MTB-DR have been reported to present some limitations: a recent study has evaluated the results of the two commercially available line probe assays and it showed that while the accuracy for RMP resistance was very good, the sensitivity for INH was variable (17).

We found that for RMP resistance, the concordance between the predicted and observed phenotype was less than satisfactory. This is not entirely unexpected, because a single mutation, although implicated in resistance, might not be enough to generate a resistant phenotype. Two explanations can account for these observations. The most important is

the presence of mutations within the *rpoB* locus that are not associated with resistance but nevertheless influence the annealing properties of the probes; this is most likely the reason for which a significant number of strains were classified as resistant to RMP by genetic analysis and sensitive by phenotypic testing.

On the other hand, it should be kept in mind that isolates that are susceptible according to molecular assays targeted on specific mutations may contain other unknown mechanisms of resistance, and these mechanisms will be missed by these techniques.

A much smaller number of strains were reported sensitive by the hybridization analysis and resistant by the phenotypic analysis. The explanation for this is that a small albeit significant number of strains have determinants of resistance outside the area targeted by the assays we used. A similar phenomenon has been reported by others (18-20). Another possibility is that changes have occurred in genes whose products participate in antibiotic permeation or metabolism (18).

In addition, the results of the absolute concentration method used in the phenotypic test are less reliable compared to the proportion method (the most preferred choice); errors in the susceptibility testing may be related to: cultures older than 21-30 days, incorrect size of inoculum, incorrect dilution, errors in incorporation of antibiotics in culture media (14). This technique should be further evaluated since the circulating strains in different geographical regions might behave differently when genotypically tested.

This real-time PCR assay could be useful when investigation of drug resistant TB is mandatory: in cases with a history of one or more previous treatment(s) with several failing or discontinued regimens or in the situation of exposure to a known source of drug resistant TB. Although real-time PCR assays may be a valuable diagnostic tool, they are not yet completely satisfactory for MTB drug-resistance detection. Phenotype-based assays will continue to have a place in the clinical mycobacteriology laboratory.

Conclusion

Based on our experience, real-time PCR assay could be used in clinical practice but only with caution, in cases with risk factors for resistance. The results can be used for guiding the initiation of therapy especially in patients with prior inadequate antituberculous treatment. Nevertheless, the treatment should be adjusted accordingly as soon as phenotypic testing results are available. The best accuracy for genotyping compared to phenotypic resistance testing was obtained for detecting INH resistance mutations targeting the *katG* gene.

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