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Original paper

***Genotypic detection of multidrug resistant Mycobacterium tuberculosis* strains**

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Abstract

Multidrug-resistant *Mycobacterium tuberculosis* strains can now be rapidly detected using genetic methods. Most mutations that induce resistance to rifampicin and isoniazid appear in the core region of rpoB gene and, respectively, in codon 315 of katG gene and in the promoter region mabA-inhA:-15. Our objective was the assessment of cost and test performance of multiplex allele-specific polymerase chain reaction (MAS-PCR) for the detection of mutations that induce rifampicin and isoniazid resistance in *M. tuberculosis*. We have analyzed 83 non-duplicate *M. tuberculosis* isolates using conventional phenotypic drug susceptibility testing and a single-step MAS-PCR assay for simultaneous detection of mutations in codons 531, 526 and 516 of rpoB gene, as well as in codon 315 of katG and in the promoter region mabA-inhA:-15. We have calculated the cost of genotypic testing/strain. MAS-PCR technique detected rifampicin resistance with 88.88% sensitivity and 100% specificity in comparison to phenotypic testing, while isoniazid resistance was detected with 95.06% sensitivity and 100% specificity. The cost of genotypic testing/strain was approximately US$ 1.62. MAS-PCR technique demonstrated good sensitivity and specificity for detection of mutations that induce rifampicin and isoniazid resistance, and therefore can be used as a rapid and inexpensive method for diagnosis of multidrug resistant tuberculosis.

Keywords Resistance, rifampicin, isoniazid, MAS-PCR, cost.

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Introduction

Tuberculosis (TB) is a major worldwide public health issue and the emergence of drug-resistant strains poses a great concern for the control of this disease. Multidrug-resistant tuberculosis (MDR-TB) is defined by resistance to at least rifampicin (RIF) and isoniazid (INH). These strains are spreading worldwide and can acquire additional resistance to fluoroquinolones and one of the three injectable drugs: capreomycin, kanamycin or amikacin becoming extensively drug-resistant (XDR-TB). Difficulties in MDR-TB detection contribute to the dissemination of these strains, since conventional drug susceptibility testing is time-consuming, costly and poses technical challenges. New molecular biology techniques allow for rapid diagnosis and could represent a solution to those problems, improving the management of MDR-TB cases.

According to World Health Organization Global TB report 2015, MDR-TB accounted for 3.3% of new TB cases and 20% of previously treated cases worldwide. In 2014 there were 300,000 estimated MDR-TB cases among notified pulmonary cases but only 41% of them were detected (123,000) (WORLD HEALTH ORGANIZATION [1]). In Romania, the TB incidence and prevalence rates were estimated at 81 per 100,000 populations (71-91%000) and 99 per 100,000 populations (41-184%000), respectively. These were the highest values for TB incidence and prevalence rates in the European Union/European Economic Area countries in 2014 (ECDC/WHO REGIONAL OFFICE FOR EUROPE [2]). In our country, MDR-TB represented 2.8% of notified new TB cases and 11% of notified previously treated TB cases. The number of estimated MDR-TB cases among notified pulmonary TB cases was 650 and 578 of them (89%) were confirmed (WORLD HEALTH ORGANIZATION [1]). In this situation, the revision of diagnosis algorithm in order to improve the cost-benefit ratio and to support patients' treatment, especially for MDR-TB cases, becomes a crucial objective.

Our objective was to evaluate the opportunity of using molecular techniques for rapid detection of MDR-TB strains in routine diagnosis. To reach this objective, we have analyzed the performance parameters of MAS-PCR technique (sensitivity, specificity, accuracy) for detection of RIF and INH resistance and identification of MDR strains, in comparison to conventional phenotypic drug susceptibility testing. We have also calculated the cost of genetic testing/strain.

Materials and Methods

Selected strains

The study included a collection of 83 non-duplicate *Mycobacterium tuberculosis* strains from the Bacteriology Laboratory of the Clinical Hospital of Pulmonology Iasi, isolated from patients with pulmonary TB between January 2008 and December 2012.

Drug susceptibility testing (DST)

We used conventional phenotypic testing (proportion method) on Löwenstein-Jensen medium for antibiotic susceptibility to first line antituberculous drugs (G. CANETTI & al [3]; G. CANETTI & al [4]; S.J. KIM [5]). Reference strain *M. tuberculosis* H37Rv ATCC 27294 was used as control.

DNA extraction procedure

DNA extraction was done using a method previously described (C. CAVASOGLU & al [6]) and modified: a 2 McFarland culture suspension of *M. tuberculosis* was inactivated by boiling for 20 min, followed by sonication for 15 min and centrifugation for 5 min at 14000 rpm. DNA yield in the supernatant was determined by spectrophotometry using NanoPhotometer® P 300 (*Implen GmbH*, Germany).

MAS-PCR

For the molecular detection of RIF and INH resistance we have applied a single-step multiplex allele-specific PCR, which allowed the simultaneous detection of mutations in codons 531, 526 and 516 of *rpo*B gene and in codon 315 of *katG* gene, as well as in promoter region *mabA-inhA*:-15. We used the Corbett Palm-Cycler (*Qiagen*, SUA) and primers synthesized by Eurogentec (*Eurogentec* S.A., Liège, Belgium) (Table 1).

Table 1. Description of primers for MAS-PCR

Genes	Primer	Sequence (5'-3')	Size (bp)	References
*rpo*B	*RIR*	TGA CCC GCG CGT ACA C		I. MOKROUSOV & al [7]
	R531B	ACA AGC GCC GAC TGT C	167	
	R526B	GTC GGG GTT GAC CCA	181	
	R516B	GCT GAG CCA ATT CAT GGA	214	
katG	katGOF	GCA GAT GGG GCT GAT CTA CG		I. MOKROUSOV & al [8]
	katG5R	ATA CGA CCT CGA TGC CGC	292	
mabA-inhA:-15	InhAPF2	CAC CCC GAC AAC CTA TCG		Z. ALLEGUI & al [9]
	InhAP15	GCG CGG TCA GTT CCA CA	270	

Each PCR reaction had a final volume of 50 µl and included: template DNA (4 µl), *RIR* (500 pmol), *R531B* (300 pmol), *R526B* and *R516B* (200 pmol each), *katGOF*, *katG5R*, InhAPF2, InhAP15 (10 pmol each), 1× *ImmoMix*™ Red Master Mix (*Bioline* GmbH, Germany) (25 µL), *nuclease-free* water (5 µl). PCR amplification was carried out according to the protocol described by Allegui *et al* (Z. ALLEGUI & al [9]) and modified: initial denaturation 3 min at 96ºC, followed by 23 cycles as follows: 50 s at 95ºC, 40 s at 63ºC and 1 min at 72ºC, with a final extension of 7 min at 72ºC. Reference strain *M. tuberculosis* H37Rv ATCC 27294 represented the control for amplification. Each reaction was performed in triplicate. Analysis of amplification products was done by electrophoresis in 2.5% agarose gel. The absence of amplification signified mutation in analyzed codon.

The cost of genotypic testing / strain

We have calculated the cost of testing/strain depending on the acquisition price of reagents for MAS-PCR (primers, master mix, nuclease-free water) and electrophoresis (agarose, buffer solution, ethidium bromide). All prices included VAT 20%. The cost did not include the expenses for infrastructure, equipment, consumables and staff (training and salaries).

Statistical analysis

Statistical analysis was performed using Epi Info™. A p-value of less than 0.05 was considered significant.

Results and Discussion

In our study, the phenotypic testing of resistance to first line antituberculous drugs showed that, of the total 83 strains, 80 were MDR (96.38%), one strain had monoresistance to RIF (1.2%), and one to INH (1.2%), while one strain was sensitive to first line antituberculous drugs (1.2%). The rates of resistance to first line antituberculous drugs are summarized in Figure 1.

MAS-PCR technique detected RIF resistance in 72 strains, with a sensitivity of 88.88% (81-95%, a 95% confidence interval, p=0.0002). The specificity of MAS-PCR in detecting RIF resistance was 100%, the positive predictive value was 100%, and the test accuracy was 89.15% (Table 2).

The mutations responsible for INH resistance were detected in 77 strains, MAS-PCR technique showing a sensitivity of 95.06% (90-99%, a 95% confidence interval, p=0.0000003), specificity of 100%, positive predictive value of 100%, and test accuracy of 95.18% (Table 3).

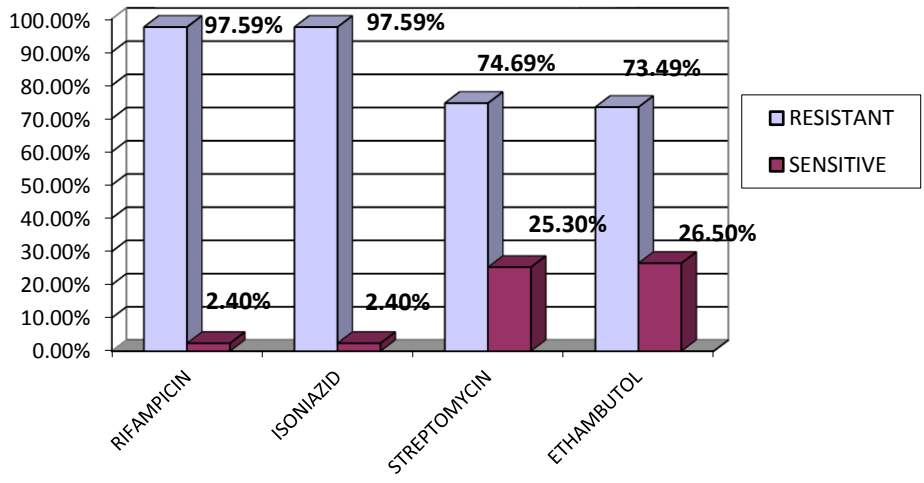

Figure 1. The results for DST on Löwenstein-Jensen medium for first line antituberculous drugs.

Table 2. MAS-PCR results for detection of RIF resistance, compared with DST results

Test		DST (No. of strains)		Total (No. of strains)
		Resistant	Sensitive	
MAS-PCR (No. of strains)	Resistant	72	0	72
	Sensitive	9	2	11
Total (No. of strains)		81	2	83

Table 3. MAS-PCR results for detection of INH resistance, compared with DST results

Test		DST (No. of strains)		Total (No. of strains)
		Resistant	Sensitive	
MAS-PCR (No. of strains)	Resistant	77	0	77
	Sensitive	4	2	6
Total (No. of strains)		81	2	83

Of the 80 MDR-TB strains, MAS-PCR technique identified 71 strains, with a sensitivity of 88.75% (80-95%, a 95% confidence interval, p=0.00001). Positive predictive value was 100%, and test accuracy was 89.15% (Table 4).

Table 4. MAS-PCR results in detection of MDR-TB compared with DST results

Test		DST (No. of strains)		Total (No. of strains)
		Resistant	Sensitive	
MAS-PCR (No. of strains)	Resistant	71	0	71
	Sensitive	9	3	12
Total (No. of strains)		80	3	83

Our results were comparable with those reported by studies that have been conducted in different countries and geographical regions.

In a study by Gupta *et al.* (India), the sensitivity of MAS-PCR technique was 93% for detection of RIF resistance and 83% for detection of INH resistance, with 100% specificity and positive predictive values in detection of each type of resistance. The accuracy of the test was 98% for RIF resistance detection and 94% for INH resistance detection, when compared to DST (A. GUPTA & al [10]). Another study, conducted in India by Vadwai *et al*, which compared MAS-PCR performances with DST, reported 94.9% and 89.2% sensitivity of MAS-PCR technique for detection of RIF resistance and INH resistance, respectively. The specificity was 86.9% for detection of RIF resistance and 94% for INH resistance. In the same study, the positive predictive value was 94% for RIF resistance detection and 97.9% for INH resistance detection (V. VADWAI & al [11]).

In a study by Mohammed *et al* (Iraq), the sensitivity of MAS-PCR in detecting RIF resistance was 94.5%, while detection of resistance among MDR strains was 90.9%. The INH resistance was detected in 78.2% of MDR strains. The sensitivity of MAS-PCR in detecting MDR-TB strains was 72.7% (S.H. MOHAMMED & al [12]), a value lower than in our study (88.75%).

In Central America, Chia *et al* showed that MAS-PCR detected RIF resistance and INH resistance with 98.4% and 82.8% sensitivity, respectively, while the specificity was 100% *versus* DST (B.-S. CHIA & al [13]). These findings were supported by a study conducted in China by Wang and collaborators that showed that MAS-PCR technique had a sensitivity of 97.9% in detecting RIF resistance and 83.3% for INH resistance, with specificities of 100% (X. WANG & al [14]).

In our study the most frequent mutation associated with RIF resistance appeared in codon 531 of *rpoB* gene (69.13%). The frequencies of mutations in codons 526 and 516 were much lower (11.11% and 8.64%, respectively). No mutations were detected in 9 cases (11.11%). No strain was found to present multiple mutations associated with RIF resistance. INH resistance caused by mutations in codon 315 of *katG* gene had the highest frequency (87.64%). Mutations in promoter region *mabA-inhA* had a frequency of 25.91%. Fifteen strains (18.51%) had simultaneous mutations in *katG*315 and *mabA-inhA-15*. The testing showed no mutations in 4 strains.

The same pattern of mutation frequencies was observed in other studies.

In India, several studies have reported frequencies of mutations in codons 531, 526 and 516 of *rpoB* gene between 54.4-67.31%, 15.38-45.29% and 3.85-22.2%, respectively, while the frequency of multiple mutations was between 4.4-19.66% (A. GUPTA & al [10]; A. GUPTA & al [15]; R. THIRUMURUGAN & al [16]). In the same studies, the frequency of mutations in codon 315 of *katG* gene was between 82.95-92.31% (A. GUPTA & al [10]; A. GUPTA & al [15]).

Using MAS-PCR assay, Wang and collaborators detected mutations in codons 531, 526 and 516 of *rpoB* gene with frequencies of 79.1%, 14.9% and 5.9%, respectively. Frequencies of mutations in codon 315 *katG* and *mabA-inhA-15* were 68.9% and 19.8%, respectively (X. WANG & al [14]). Very similar results were obtained

by Chia *et al.* who found the following frequencies: 79.1% for 531 *rpoB* mutations, 13.4% for 526 *rpoB* mutations, 6.0% for 516 *rpoB* mutations, 70.1% for 315 *katG* and 19.4% for *mabA-inhA-15* (B.-S. CHIA & al [13]).

Other studies conducted in Eastern European countries reported frequencies of mutations that induced resistance to INH and RIF similar to our findings (Figure 2).

These results demonstrate that using MAS-PCR technique for highlighting five key mutations that induce resistance to INH and RIF provides good sensitivity in detection of MDR-TB strains.

We have evaluated the cost of MAS-PCR assay at about US$ 1.62/strain (Table 5). In a study conducted by Tho *et al.*, the cost of MAS-PCR for detection of RIF resistance was about US$ 1.60 per sample (excluding salary and equipment costs) (D.Q. THO & al [20]). In another study, Imperiale and collaborators estimated a cost of MAS-PCR of US$ 14 including staff, overheads and medical supplies (B.R. IMPERIALE & al [21]).

Our technique had a lower cost than the commercial molecular kits for MDR-TB diagnosis whose costs were estimated between US$ 50 (D.Q. THO & al [20]) and US$ 94 per strip (B.R. IMPERIALE & al [21]) and also lower than the automated culture methods whose costs were approximately US$ 10 (S. SOLOMON & al [22]) to US$ 31 (B.R. IMPERIALE & al [21]). In comparison to microscopic observation drug susceptibility assay (US$ 2) (S. SOLOMON & al [22]) and DST on Löwenstein-Jensen medium (US$ 16) (B.R. IMPERIALE & al [21]), MAS-PCR was less costly.

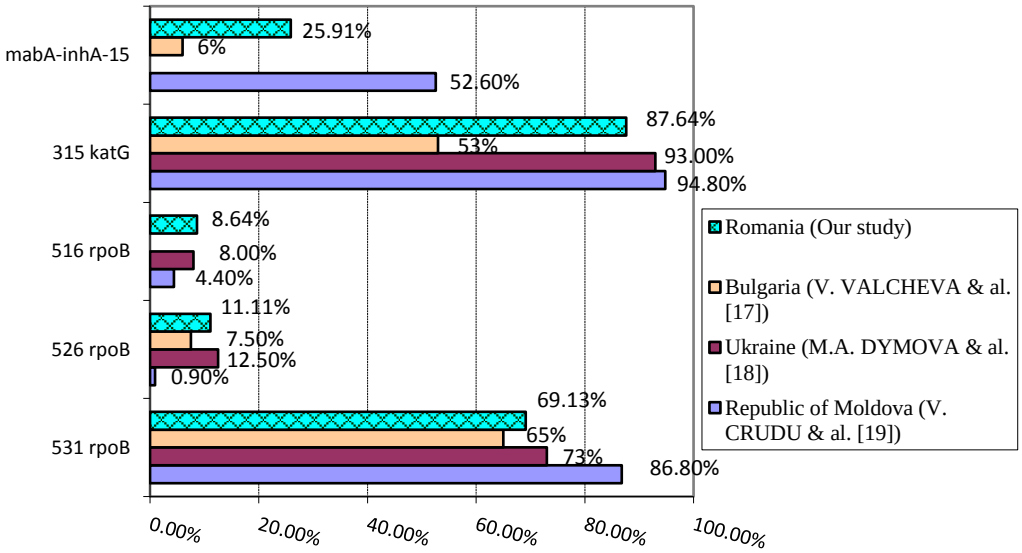

Figure 2. The frequencies of mutations that induce resistance to INH and RIF in Eastern European countries.

Table 5. The cost of MAS-PCR assay

Steps	Cost / strain* (US$)
MAS-PCR	1.37
Electrophoresis	0.25
TOTAL	1.62

*including VAT 20%

Using MAS-PCR assay in MDR-TB detection allowed us to obtain results in 4 hours (amplification and electrophoresis), compared with automated culture methods or microscopic observation drug susceptibility assay which required 7-15 days each (B.R. IMPERIALE & al [21]; L. SOLARI & al [23]). Also, MAS-PCR saved approximately 28-41 days in MDR-TB diagnosis compared with DST on Löwenstein-Jensen medium, the gold standard assay (B.R. IMPERIALE & al [21]).

Conclusion

MAS-PCR proved to be a low-cost and time-saving technique, with good sensitivity and specificity for detection of mutations that induce resistance to RIF and INH that can be used in the management of MDR-TB cases.

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