The concordance between phenotypic and genotypic *M. tuberculosis* drug susceptibility tests results: observational study

Concordanța între rezultatele testelor de sensibilitate fenotipice și genetice pentru M. tuberculosis

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Abstract

Multi-drug resistant tuberculosis (MDR-TB) represents a major threat for TB control at the global level. Identification of mutations responsible for drug resistance by molecular methods can be used for rapid and specific detection of drug resistance.
The aim *of our study was to assess the concordance between phenotypic and genotypic tests results (GenoTypeMTBDRplus kit) for isoniazid and rifampicin resistance in M. tuberculosis isolated strains.*
Material and Methods. *The specific zone mutations in rpoB, katG and inhA gene for rifampicin and isoniazid were investigated with molecular methods in 198 recently isolated unique strains from patients diagnosed with pulmonary tuberculosis. These results were compared with the absolute concentration drug susceptibility test results.*
Results. *Sensitivity, specificity, predictive positive value, predictive negative value, efficiency of genotypic method, calculated by comparing with conventional method for INH and RMP were 93.85%, 100.00%, 100.00%, 63.33%, 94.44%, and 99.26%, 82.25%, 92.46%, 98.07% and 93.93%, respectively). Cohen coefficient showed Kappa values =0.746 (good strength of agreement) for INH, and Kappa value =0.853 (very good strength of agreement) for RMP.*
Conclusion. *The obtained results are consistent with those reported from other regions of the world. The use of rapid molecular assays reduces the time for drug resistance diagnostic to just a few days, and may help the control of the ongoing TB transmission.*
Keywords: *Multi-drug resistant tuberculosis (MDR TB), drug susceptibility tests, genetic testing, isoniazid, rifampicin*

Rezumat

Tuberculoza multidrug rezistentă (TB-MDR) reprezinta o amenințare majoră pentru controlul tuberculozei la nivel mondial. Identificarea prin metode de biologie moleculară a mutațiilor responsabile de rezistență la substanțele anti-tuberculoase poate fi folosită pentru detecția rapidă și specifică a rezistenței.
Scopul *studiului nostru a fost analiza concordanței între rezultatele testelor fenotipice și genetice (kitul GenoTypeMTBDRplus) pentru detectarea rezistenței la izoniazidă și rifampicină la tulpini de M. tuberculosis.*
Materiale și metodă. *Am analizat mutațiile din zonele specifice pentru rifampicină și izoniazidă din genele rpoB, katG și inhA la 198 tulpini unice, recent izolate de la pacienți diagnosticați cu tuberculoză pulmonară. Aceste rezultate le-am comparat cu cele obținute prin metoda concentrațiilor absolute.*
Rezultate. *Sensibilitatea, specificitatea, predicția valorii pozitive, predicția valorii negative, eficiența metodei genetice, calculate prin compararea cu metoda convențională pentru INH și RMP au fost 93,85%, 100,00%, 100,00%, 63,33%, 94,44%, respective 99,26%, 82,25%, 92,46%, 98,07%, 93,93%. Coeficientul de concordanță Cohen pentru INH este K= 0,746 (denotă putere bună a concordanței), iar pentru RMP, K= 0,853 (foarte bună putere a concordanței).*
Concluzii. *Rezultatele obținute de noi sunt similare cu cele obținute în alte regiuni din lume. Folosirea testelor moleculare rapide reduce timpul de diagnostic al rezistenței la substanțele anti-tuberculoase la doar câteva zile, și poate ajuta la controlul transmiterii tuberculozei.*
Cuvinte-cheie: *Tuberculoză multi-drog rezistentă (TB-MDR), Teste de sensibilitate, Teste genetice, Izoniazidă, Rifampicină*

Introduction

Tuberculosis (TB) is an important worldwide public health problem. Resistant tuberculosis and multi-drug resistant tuberculosis (MDR-TB) represents a major threat to the global TB control.[1,2] TB remains the leading cause of death among preventable and curable infectious diseases after human immunodeficiency virus (HIV)/AIDS[3,4]. It is estimated that in 2020, TB will remain among the 10 leading causes of global diseases[3,5].

M. tuberculosis drug resistance is due to the acquisition of mutations in chromosomally encoded genes by serial accumulation of mutations primarily due to inadequate therapy[6]. Early detection of MDR-TB is important for appropriate treatment and prevention of spreading resistant strains[7]. Mutations in the genome of *M. tuberculosis* that can confer resistance to anti-tuberculous drugs occur spontaneously, with an estimated frequency of 3.5×10^{-6} for isoniazid (INH) and 3.1×10^{-8} for rifampicin (RMP)[8]. Because the chromosomal loci responsible for resistance to various drugs are linked, the risk of double spontaneous mutations is extremely small (9×10^{-14} for both INH and RMP)[8]. Acquisition of drug resistance by *M. tuberculosis* (MTB) results from mutations (nucleotide substitutions, insertions, or deletions) in specific resistance determining

regions of the genetic targets[6]. The acquisition of drug resistance does not occur as a result of horizontal transfer of resistance-bearing genetic elements[6]. Inadequate therapy may favor the selection of a resistant bacterial population[6,8]. The mutations that predominate in MTB during in vitro selection of antibiotic-resistant strains may differ from those that develop in vivo and is important to perform genetic investigations in recently isolated strains[9].

The aim of our study was to assess the concordance between phenotypic and genetic drug susceptibility tests results for the detection of INH and RMP resistance in MTB isolated strains.

Material and Methods.

Patients and specimens

Pulmonary samples originating from 14 counties of Romania were collected and processed in 14 different county laboratories for microscopy in Ziehl Neelsen staining and for culture in Lowenstein Jensen medium between January 2010 and December 2011. MTB isolated strains were sent on solid medium, to Cluj Napoca National Reference Laboratory for drug resistance surveillance. We first performed the phenotypic susceptibility test (absolute concentration method). In the next step we performed the genetic test for all resistant strains to INH (44), RMP (1) or both of them (135), plus 18 randomly selected susceptible strains to a total of 198 strains from patients diagnosed with pulmonary tuberculosis.

This observational study used specimens and data collected in routine patient care and resistance surveillance, performed with informed consent at the diagnosis, and no ethic review was necessary for this research.

The indirect, absolute concentration method (ACM) in Lowenstein Jensen (LJ) medium was performed according to the national and international standard methods[10,11]. We defined MDR-TB as resistance to the most effective first line anti-tuberculous drugs, INH and RMP, according to the standard definition. As genetic assay, the GenoType MTB DR plus kit v.1.0 (Hain Life Science GmbH, Germany) based on the detection of a set number of mutations in a few genes associated with resistance to INH and RMP was used. This kit is unable to detect all isoniazid and all rifampicin resistance, and does not detect resistance to other first line or second line drugs.

The test includes three steps: DNA extraction from TB culture, a multiplex amplification with biotinylated primers, and reverse hybridization[12]. Mutational analysis was done by polymerase chain reaction amplification of extracted DNA. MTB-DR plus gave positive hybridization results with the mutation specific probes MUT and negative hybridization result with the corresponding wild type probes. The wild type probes comprise the most important resistance areas of the genes. When all wild type probes of a gene stain are positive, there is no detectable mutation within the examined regions. The absence of a signal for at least one of the wild type probes indicates a resistance to the respective antibiotic[12]. Mutations associated with INH and RMP resistance are located in several separated wild regions of the MTB genome. The banding pattern obtained with *rpoB* probes indicate RMP resistance, the *katG* and *inhA* banding pattern is linked with a high level, and a low level of INH resistance, respectively. Only the bands with intensities as strong as the Amplification Control zone are to be considered.

Statistical methods Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and efficiency of susceptibility test results, and the Cohen coefficient for agreement were calculated in order to establish the concordance between test results of phenotypic and genotypic assay.

Results

Recently isolated strains from 198 patients with pulmonary TB were enrolled in this study.

The results of genotype MTB DR plus and phenotypic drug susceptibility tests are presented in table 1.

Out of 44 INH resistant results in ACM, 39 were also INH resistant in genetic assay (29 INH resistant and 10 MDR TB). We found *katG* resistance pattern in 35 cases from 39 (35/39); 31 cases had wild type deletion and mutation (MUT1) at the *katG* level, and the other 4 cases had only deletion of wild type banding (DWT). *katG* mutations were associated with mutations in the *inhA* promoter region in 3 cases out of the 35, (all of them DWT1/MUT1 banding pattern). Four strains had only *inhA* banding pattern DWT1/MUT1, without mutations in the *katG* gene.

Seven cases had genotypic RMP resistance, with mutations located in *rpoB* region, with the next pattern: DWT8/MUT3 in 4 cases, and DWT4/DWT5, DWT7/DWT8, DWT8, in each other 3 cases. Out of the 7 cases, 6 were MDR and one RMP resistant in ACM test.

Out of 135 conventional MDR TB isolates, the genetic MDR TB pattern was identified in 129 strains, but in 6 strains only RMP genetic resistance was detected.

All 135 cases had *rpoB* banding modified pattern: one had wild type 2 deletion (DWT2, codons 510-513), three cases had wild type 2 and 3 deletions (DWT2/DWT3, codons 510-517), three cases had wild type 2, 3 and 4 deletions, (DWT2/DWT3/DWT4, codons 510-517, 513-519), 17 cases were with wild type 3, 4 deletions DWT3/DWT4 (codons 513-519), 23 cases with wild type 3, 4 deletions and mutation (DWT3/DWT4/MUT1), 1 case with wild type 3, 4 and 7 deletions DWT3/DWT4/DWT7 and 1 case with wild type 4, 5 deletions (DWT4/DWT5), 1 case had wild type 7 deletions and two mutations (DWT7/MUT2A/MUT2B, codon 526-529), 10 cases had wild type 7 deletions and one mutation (DWT7/MUT2A, codons 526-529), 2 cases had wild type 7 deletions and one mutation (DWT7/MUT2B, codons 526-529), 5 cases with wild type deletion DWT8 (codons 530-533). The most frequent mutations observed were DWT8/MUT3 at the codon 531 (63strains out of the 135).

From 135 isolates with MDR pattern (ACM), 127 had *katG* banding pattern mutation, and in 26 out of them were associated mutation in the *inhA* promoter. Two isolates with MDR had only *inhA* mutation without modification in *katG* gene.

We found 115/135 strains with *katG* banding mutations pattern DWT/MUT1, 5/135 isolates had type mutation

Table 1 Results of genotype MTB DR plus and conventional drug susceptibility testing in detecting rifampicin and isoniazid resistance

RMP	Conventional DST		
MTBDRplus	R	S	Total
R	135	11	146
S	1	51	52
Total	**136**	**62**	**198**
INH	Conventional DST		
MTBDRplus	R	S	Total
R	168	0	168
S	11	19	30
Total	**179**	**19**	**198**

R: resistant, **S:** susceptible

Table 2 Performance of genotype MTB DR plus in detecting rifampicin and isoniazid resistance

	RMP (%)	INH (%)
Sensitivity	99.26	93.85
Specificity	82.25	100.00
PPV	92.46	100.00
NPV	98.07	63.33
Efficiency	93.93	94.44

PPV: positive predictive value; NPV : negative predictive value;

DWT/MUT2, and 7/135 cases had only wild type deletion.

Besides the 18 susceptible isolates in the conventional test, another 5 additional strains showed no changes suggesting resistance in *rpoB*, *katG* and *inhA* genes.

Sensitivity, specificity, and positive predictive value (PPV), negative predictive value (NPV), and efficiency of susceptibility tests for INH and RMP are presented in table 2.

The Cohen coefficient for the agreement of the results for INH is Kappa =0.746 (95% confidence interval from 0.604 to 0.887). The strength of agreement is considered to be good. Number of observed agreement is 187 (94.44%).

The Cohen coefficient value of the results for RMP is Kappa =0.853 (95% confidence interval from 0.772 to 0.933). The strength of agreement is considered to be very good for RMP. Number of observed agreement is 186 (93.94%).

Discussion

Drug-resistant TB is increasing in many parts of the world, and high rates of drug-resistant and MDR-TB has been reported in several countries.

The World Health Organization (WHO) estimated the prevalence of MDR-TB between 2002-2006 years in 93 geographical settings ranging from 0% to 22% among newly diagnosed cases and from 0% to 60% among previously treated cases[4,13,14]. The WHO reported in 2013 an estimated 3.6% of new cases and 20.2% of treated cases with MDR TB[15]. In 2010, out of the 290,000 estimated MDR-TB pulmonary globally cases, only 16% were diagnosed and received an appropriate treatment[16].

The rapid and accurate detection of drug resistance (especially to first-line drugs) is imperative and essential for appropriate treatment to prevent the development of further resistance and the spread of resistant strains. More than 90% of RMP-resistant TB isolates are also resistant to INH, and screening for RMP can be a surrogate for checking MDR-TB[9,17,18]. In the last years the molecular methods exploring the genetic mechanism of drug resistance are commonly used as routine tests in laboratories from industrialized countries for quick and specific detection of *M.tuberculosis complex* and for rapid identification of drug resistance profiles[19,20,21]. That can reduce the propagation and the spread of drug resistant tuberculosis, and the early diagnosis can prevent aggressive treatment for resistant tuberculosis, with a marked reduction in the noncompliance[20,21].

RMP resistance of MTB is largely associated with point mutations in a region of *rpoB*. RMP inhibits the RNA polymerase at the level of the beta subunit encoded by the *rpoB* gene (point mutations predominantly located in the 511 to 533 region of the *rpoB* polypeptide). Resistance to INH is associated with mutation in *katG*, *inhA* promoter and other mutations in various genes, including *kasA*, *furA, iniA, iniB, iniC* and *oxyR- ahpC* intergenic region[18,22].

We reported the polymorphism of mutations (the frequency, location, and type of resistance), and we have to mention that 63 patients out of 146 with any RMP genetic detected resistance (43.15%) have the same type of mutation (DWT8/MUT3 in *rpoB* region), possibly due to the spread and transmission of resistant MTB.

In 11 cases we found in *katG* region deletion of wild type and in 5 cases DWT/MUT2. DWT/MUT1 profile was found in 146 cases. In promoter region *inhA*, the pattern with DWT2/MUT3A was in 5 cases, MUT1 in one case, and DWT1/MUT1 in 29 cases (14.6%).

Other studies reported the different range of mutation frequencies *katG*– 39.4% to 91.3%, *inhA* promoter – 4.3 % to 34.4 % and *oxyR – ahpC* 5.7% to 28.5 %[24,25]. In 5 phenotypic resistant strains we did not find any mutations, possible because the mutations were located in other genetic regions.

Similar observation (with no mutation presence of genotype test, although phenotypes resistant) have been reported by others and suggests that mutations located outside of the analyzed region can result in rifampicin resistance. The rates of genotyping indeterminate *M. tuberculosis* drug resistance ranged from 1.4% to 19.2% [21,24,25,26]. Compared with our results, similar distribution of genetic involvement in resistant strains were obtained in a Romanian assessment[27].

In this study MTB DRplus assay detected 93.85% of INH resistant strains and 99.26% of the RMP resistant strains, respectively. The results were comparable with previous

reports (100% detection of RMP resistance and 67% of INH resistance with the MTB DRplus Genotype test[17,21].

Rapid identification of *M. tuberculosis* and drug resistance profiles can reduce the spread of MDR tuberculosis. Each individual error in managing a MDR-TB case can be considered as a potential risk for generating new MDR-TB cases[13].

Surveillance of MDR is a major tool to document the situation at the regional and national level, and the trends in performance of TB care and control efforts[14]. Molecular methods are used to sustain the control of tuberculosis by early diagnosis of MDR. Because a current molecular technique based on DNA detects both live and dead bacteria, we can't use the molecular assays for treatment monitoring or for infection control[28].

In 2008, the World Health Organization recommended the use of molecular detection in high risk populations for MDR cases in a TB national program for preventing the development of resistance due to patient mismanagement[17].

The use of rapid molecular methods (GenoType MTB DR plus kit) does not replace conventional methods[29]. Romanian National TB program developed specific diagnostic settings, proposed diagnostic algorithm and diagnostic procedures for the access of all TB cases to modern, quality assured diagnostic tests.

Conclusion

The results of this study are consistent with those reported from other regions of the world. The use of rapid molecular assays reduces the time for drug resistance diagnostic to just a few days, and may help the control of the ongoing TB transmission. Identification of gene mutations can be applied at a national level for developing a rapid test in addition to the conventional methods, for the better control of tuberculosis.

Rapid detection of MDR-TB patients is necessary in order to refer these cases to special centers for adequate treatment, for prevention of developing further resistance, and for their isolation for a good TB infection control. ■

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