RPOB GENE MUTATIONS FOR RIFAMPICIN RESISTANCE IN STRAINS OF *MYCOBACTERIUM TUBERCULOSIS* FROM A BUCHAREST CLINIC

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ABSTRACT

Introduction: The incidence of multidrug-resistant tuberculosis (MDR-TB) is increasing worldwide through selection of antibiotic-resistant mutants. This may occur either spontaneously or as a result of inappropriate therapies. Early and quick detection of genetic mutations occurring in strains of *Mycobacterium tuberculosis* (*M. tuberculosis*) can help the clinician to select the appropriate regimen.

Objectives: The objective is to show which gene mutations occur most frequently in drug resistant *M. tuberculosis* strains and to investigate the efficiency of two commercial systems in comparison with reference techniques.

Materials: The study was conducted on strains of *M. tuberculosis* by using the INNO-LiPA Rif.TB system and GenoType MTBDRplus test, as well as the standard antibiogram on Lowenstein Jensen (LJ) growth medium containing both rifampicin (RIF) and isonicotinhydrazide (INH).

Results: Fifty strains were evaluated using the above-mentioned techniques. Using the INNO LiPA system, 21 strains (42%) were wild-type and therefore RIF-sensitive, one strain showed no amplification band suggesting a different species, and 29 strains (56%) had various mutations of the rpoB gene, indicating resistance to RIF. GenoType MTBDRplus offered similar results to the INNO-LiPA Rif.TB assay, with only minor differences – three strains for which drug susceptibility results were discordant. Final results were obtained from phenotypic drug susceptibility testing.

Conclusions: Mycobacterial strain testing using both the INNO-LiPA and the GenoType MTBDRplus system offered quick and accurate results. The GenoType MTBDRplus system highlights not only RIF resistance but also INH resistance, thereby more accurately assigning a certain strain to the MDR group.

Keywords: INNO-LiPA Rif.TB, antimicrobial therapy, genetic variations.

REZUMAT

Introducere: Incidența tuberculozei multidrog rezistente (MDR-TB) este în creștere la nivel global, prin selecția de mutanți rezistenți. Aceștia pot apărea fie spontan, fie ca rezultat al terapiilor nepotrivite. Detecția precoce și rapidă a mutațiilor genetice ce apar în *Mycobacterium tuberculosis* (*M. tuberculosis*) poate fi de ajutor medicilor clinicieni în selectarea tratamentul corect.

Obiective: Acest studiu cataloghează cele mai frecvente mutații întâlnite și să investigheze eficiența a două sisteme comerciale în comparație cu tehnicile de referință.

Materiale: Studiul a fost efectuat pe tulpini de *M. tuberculosis*, utilizând sistemul INNO-LiPA Rif.TB și cu GenoType MTBDRplus, cât și antibiograma standard pe mediul de cultură Lowenstein Jensen (LJ) conținând rifampicină (RIF) și isonicotinhidrazidă (INH).

Rezultate: Cincizeci de tulpini au fost evaluate folosind tehnicile sus-menționate. Utilizând sistemul INNO-LiPA, 21 de tulpini (42%) au fost sensibile la RIF, neprezentând mutații. O tulpină nu a putut fi amplificată cu sonde specifice complexului *M. tuberculosis*, sugerând că este vorba de o mycobacterie atipică. Douăzeci și nouă de tulpini (51%) au prezentat diverse alte mutații ale genei rpoB, indicând rezistența la RIF. GenoType MTBDRplus a oferit rezultate similare cu INNO-LiPA Rif.TB, cu mici diferențe – în cazul a trei tulpini, rezultatele pentru rezistență au fost discordante. Rezultatele definitive au fost tranșate prin metoda fenotipică de testare a susceptibilității la antibiotice.

Concluzii: Testarea tulpinilor mycobacteriene utilizând atât INNO-LiPA, cât și GenoType MTBDRplus oferă rezultate rapide și precise. Sistemul GenoType MTBDRplus permite depistarea rezistenței atât la RIF, cât și la INH, facilitând încadrarea în categoria MDR a tulpinilor.

Cuvinte-cheie: INNO-LiPA Rif.TB, terapie antimicrobiană, variații genetice.

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INTRODUCTION

Tuberculosis (TB) is one of the most pressing public health problems worldwide, having a huge impact on society. Through efforts in the last two decades, the incidence of TB has slightly declined. In 2013, about nine million people were diagnosed with TB and 1.5 million people died [1]. Despite the overall decline in the incidence of TB, a new challenge presents strains that are resistant to anti-TB drugs. Multidrug-resistant tuberculosis (MDR-TB) is defined as a *M. tuberculosis* strain resistant at least to rifampicin (RIF) and isoniazid (INH). Multidrug-resistant strains of *M. tuberculosis* have been reported in many parts of the world. In 2013, 3.5% of all new cases and 20.5% of previously treated TB cases worldwide were estimated as MDR-TB. The incidence of MDR-TB is alarming in the WHO European Region, especially in Eastern European countries: Estonia, Latvia, Lithuania, Azerbaijan, Moldova, Armenia, Kazakhstan, Russia and Romania [2].

Early detection of *M. tuberculosis* strains by rapid laboratory tests and identifying the various mutations associated with antituberculosis drug resistance is among the 5 WHO prerogatives addressing the MDR-TB epidemic [1].

Currently, the best studied mutation is the one associated with *M. tuberculosis* resistance to RIF. In over 96% of RIF resistant cases, mutations appeared in an 81 bp segment of the *rpo*B gene, which encodes the β subunit of the RNA polymerase [3, 4, 5]. *M. tuberculosis* resistance to RIF occurs spontaneously with an estimated frequency of 10^{-8} [6]. Most RIF-resistant strains present a single mutation, with occurrences of more than one mutation being relatively rare [4, 7]. Mutations affecting codons 531 and 526 cause both a high phenotypic resistance, as well as cross-resistance with other rifamycins. Conversely, mutations 511, 516, 518 and 522 are associated with low resistance to RIF and rifapentine [6, 7].

In less than 5% of RIF-resistant *M. tuberculosis* strains, no mutation was detected in the *rpo*B resistance region [8].

In over 90% of cases, RIF resistance is associated with INH resistance, and this is definitory for MDR strains [5, 9, 10]. For this reason, the detection of RIF-resistance has been suggested as a possible surrogate molecular marker for MDR-TB [11]. Confirmation of RIF resistance may represent a screening criterion when identifying MDR strains.

This study aimed to analyse *rpo*B gene mutations from a total of 50 *M. tuberculosis* strains, isolated from patients admitted to Marius Nasta Pneumophtisiology Institute (MNPI), Bucharest.

MATERIALS AND METHODS

We analyzed a total of 50 strains of *M. tuberculosis* isolated on Lowenstein Jensen (LJ) medium in the first six months of 2011. Strains originated from patients hospitalized at the MNPI for pulmonary tuberculosis. Microscopy was positive for acid-fast bacilli (AFB) in all collected samples (48 sputum and two bronchial aspirates).

RIF susceptibility testing was done using the INNO-LiPA Rif.TB test, whereas the GenoType MTBDRplus test was used for both RIF and INH. At the same time, susceptibility testing of each strain was performed by standard absolute concentration method using LJ medium with added RIF (40 g/ml) and INH (0.2 g/ml) [12]. A reference H_{37}Rv *M. tuberculosis* strain was used as positive control.

Mycobacterium DNA extraction was done using standard protocol. Initially, bacterial colonies were harvested from LJ solid medium using an inoculation loop (2-3 streaks) and placed in 1 ml of sterile distilled water in a 1.5 ml Eppendorf tube. The tube was heated for 30 minutes at 95 °C using a Thermomixer in order to inactivate the mycobacteria, then placed for 12 minutes in an ultrasonic bath, and centrifuged for 10 minutes at 13,000 rpm. The supernatant containing the DNA of interest was transferred to a clean Eppendorf tube. The extracted DNA from all 50 strains grown on LJ medium was identified and susceptibility to RIF determined, using the INNO-LiPA Rif.TB test. At the same time, a standard antibiogram was performed for each of these strains on LJ medium supplemented with antibiotics (RIF and INH).

The INNO-LiPA Rif.TB test was performed by manufacturer recommended protocol. DNA regions associated with RIF resistance were

amplified using specific primers and 1 unit of AmpliTaq DNA polymerase per reaction mixture. Amplification was done using a thermocycler unit by the following protocol: denaturation for 3 minutes at 94 °C, and 35 cycles of 45 seconds at 94 °C, 1 minute at 64 °C and 45 seconds at 72 °C, followed by a final cycle of 10 minutes at 72 °C. The final amplification product was then hybridized at 62 °C in a shaking water bath (80 rpm) for 30 minutes.

The INNO-LiPA Rif.TB assay involves reverse hybridisation [13]. DNA extracted from the sample of interest is hybridized with specific oligonucleotide probes, deposited as parallel lines on a strip of nitrocellulose. Following the addition of streptavidin alkaline phosphatase and subsequent incubation with a chromogenic substrate, results can be viewed and interpreted.

Using a target amplicon generated by the INNO-LiPA Rif.TB system, the test detects the presence of *M. tuberculosis* and at the same time identifies point mutations, insertions and deletions in the *rpo*B gene (codon 509-534), through a set of five overlapping S-probes. These probes only hybridize to the wild-type sequence. When a mutation is present, the amplicon and the corresponding S-probe only partially overlap and cannot hybridize. Therefore, the absence of hybridization in a given sample was indicative of the presence of a mutation, suggesting the resistant genotype. In addition, the presence of four specific mutations was confirmed by four corresponding R-probes (R2=D516V, R4a=H526Y, R4b=H526D, R5=S531L). Results were interpreted by comparing them to a control strip.

Results were then validated using water as negative control and the reference H37Rv (ATCC 27294) *M. tuberculosis* strain as positive control.

RESULTS

Evaluation of the 50 mycobacterial strains using the INNO-LiPA Rif.TB system (Table 1) revealed that 21 strains (42%) were wild-type and therefore RIF-sensitive, one strain showed no amplification band suggesting a different species, and 28 strains (56%) had various mutations of the *rpo*B gene, indicating resistance to RIF.

The interpretation of mutations present on the LiPA band and their assignment to a resistance pattern according to the instructions provided by the manufacturer are shown in Table 2.

The most frequent mutations in RIF-resistant strains were H526Y (seven strains, 24.13%) and mutations of the S4 region of the *rpo*B gene (seven strains, 24.13%), followed by the H531L mutation (five strains, 17.24%), H526D (four strains, 13.79%) and mutations of the S1 region (two strains, 6.89%).

Interestingly, previous studies indicated that the most common *rpo*B gene mutations are S531L, H526Y and H526D [5, 14, 15, 16].

As already stated, the INNO-LiPA Rif. TB test was conducted in parallel with the standard drug sensitivity tests (using RIF and INH), as well as the GenoType MTBDRplus test, on all 50 strains.

The results of the GenoType MTBDRplus assay for the 50 strains are shown in Table 3.

There was only slight disagreement between the INNO-LiPA Rif.TB and the GenoType MTBDRplus test results. Specifically, for strain 14 INNO-LiPA Rif.TB testing showed resistance to RIF, while the GenoType MTBDRplus analysis indicated it as sensitive to the same drug. This inconsistency was addressed by using the standard antibiogram on LJ medium supplemented with antibiotics, which indicated that this strain is resistant to both RIF and INH. We have also found an inconsistency between GenoType and the phenotyping method at strain 20 to which the GenoType showed resistance to INH and the phenotypic method showed sensibility. But we suppose that in some cases the GenoType assay is more sensitive than the absolute concentration method. The resistance detected in strain 20 by GenoType assay is low, due to the MUT3B in the *inh*A gene and this mutation has not yet manifested phenotypically. Another inconsistency was present in strain 23, which was resistant (Δ S1 pattern) through INNO-LiPA Rif.TB, while no *rpo*B mutation was detected by GenoType MTBDRplus. However, the GenoType system detected a mutation in the *inh*A gene. The standard antibiogram confirmed an MDR strain, with phenotypic resistance to both isoniazid and rifampicin.

Table 1 - INNO-LiPA Rif.TB testing of the 50 bacterial strains

No	*Marker line*	Conj.control	MTC	S1	S2	S3	S4	S5	R2	R4a	R4b	R5
1	+	+	+	+	+	+	+	+	-	-	-	-
2	+	+	+	+	+	+	+	+	-	-	-	-
3	+	+	+	+	+	+	+	+	-	-	-	-
4	+	+	+	+	+	+	+	+	-	-	-	-
5	+	+	+	+	+	+	+	+	-	-	-	-
6	+	+	+	+	+	+	+	+	-	-	-	-
7	+	+	+	+	+	+	-	+	-	+	-	-
8	+	+	+	+	+	+	+	+	-	-	-	-
9	+	+	+	+	+	+	-	+	-	-	-	-
10	+	+	+	+	+	-	+	+	-	-	-	-
11	+	+	+	+	+	+	-	+	-	-	-	-
12	+	+	+	+	+	+	+	+	-	-	-	-
13	+	+	+	+	+	+	-	+	-	-	-	-
14	+	+	+	+	+	+	+	-	-	-	-	+
15	+	+	+	+	-	+	+	+	-	-	-	-
16	+	+	+	+	+	+	-	+	-	+	-	-
17	+	+	+	+	+	+	+	+	-	-	-	-
18	+	+	+	+	+	+	+	+	-	-	-	-
19	+	+	+	+	+	+	+	+	-	-	-	-
20	+	+	+	+	+	+	+	-	-	-	-	+
21	+	+	+	+	+	+	-	+	-	-	+	-
22	+	+	+	+	+	+	+	+	-	-	-	-
23	+	+	+	-	+	+	+	+	-	-	-	-
24	+	+	+	-	+	+	-	+	-	-	-	-
25	+	+	+	+	+	+	-	+	-	-	-	-
26	+	+	+	+	+	+	+	-	-	-	-	+
27	+	+	+	+	+	+	-	+	-	+	-	-
28	+	+	+	+	+	+	-	+	-	+	-	-
29	+	+	+	+	+	+	+	+	-	-	-	-
30	+	+	+	+	-	+	+	+	+	-	-	-
31	+	+	+	+	+	+	+	+	-	-	-	-
32	+	+	+	+	+	+	-	+	-	-	+	-
33	+	+	+	+	+	+	-	+	-	-	-	-
34	+	+	+	+	+	+	-	+	-	-	-	-
35	+	+	+	+	-	+	+	+	+	-	-	-
36	-	-	-	-	-	-	-	-	-	-	-	-
37	+	+	+	+	+	+	-	+	-	+	-	-
38	+	+	+	+	+	+	+	-	-	-	-	+
39	+	+	+	+	+	+	-	+	-	-	+	-
40	+	+	+	+	+	+	-	+	-	+	-	-
41	+	+	+	+	+	+	-	+	-	+	-	-
42	+	+	+	+	+	+	+	-	-	-	-	+
43	+	+	+	+	+	+	+	+	-	-	-	-
44	+	+	+	+	+	+	+	+	-	-	-	-
45	+	+	+	+	+	+	+	+	-	-	-	-
46	+	+	+	+	+	+	+	+	-	-	-	-
47	+	+	+	+	+	+	+	+	-	-	-	-
48	+	+	+	+	+	+	+	+	-	-	-	-
49	+	+	+	+	+	+	-	+	-	-	+	-
50	+	+	+	+	+	+	+	+	-	-	-	-

Importantly, the analysis of mycobacterial strains using the GenoType MTBDRplus system also showed resistance to INH and thereby allowed the classification of the various strains as MDR, whereas RIF resistance alone only indicated a high probability that the strains of interest were MDR.

A comparison between RIF-resistant strains detected using INNO-LiPA Rif.TB assay, antibiograms on LJ medium and the

Table 2 - Interpretation of mutations occurring

No. of strains	Mutations	Resistance pattern
1	Mutations in S1 region	Δ S1
1	Mutations in S2 region	Δ S2
1	Mutations in S3 region	Δ S3
7	Mutations in S4 region	Δ S4
0	Mutations in S5 region	Δ S5
2	D516V	R2
7	H526Y	R4a
4	H526D	R4b
5	S531L	R5
Total = 28		

GenoType MTBDRplus assay is shown in Table 4. When using the GenoType MTBDRplus test 26 RIF-resistant strains were identified, 25 of them also showed resistance to INH, strongly indicating these as MDR strains.

DISCUSSION AND CONCLUSION

There is a growing number of MDR-TB strains worldwide and Romania makes no exception. Early and accurate detection of multi-drug resistant cases is a necessity. Consequently, the use of the RIF resistance as a surrogate marker is becoming a very important diagnostic tool. The present study has identified the RIF resistance of 50 bacterial strains using INNO-LiPA Rif.TB and compared the results with those obtained using the GenoType MTBDRplus assay as well as the standard antibiogram on LJ medium supplemented with RIF and INH.

Of the 50 strains analyzed, 28 showed resistance to RIF, 21 were susceptible and one strain showed no amplification band, suggesting that it does not belong to the *M. tuberculosis* family.

The most frequent mutations found by the present study are mutations H526Y (24.13%), H531L (17.24%) and H526D (13.79%). Genetic variations were also found in a 81 bp region of the *rpo*B gene, specifically S1 (24.13%) and S4 (6.89%); however, no specific mutations could be identified.

Mutation types and their frequency vary by geographical area. [9,10] Previous studies have shown H531L as the most frequent mutation worldwide, followed by H526Y and H526D.

GenoType MTBDRplus offered similar results to the INNO-LiPA Rif.TB assay. One strain detected by the INNO-LiPA Rif.TB test as resistant was found to be sensitive with the GenoType MTBDRplus test; however, this strain was found to be resistant to RIF and INH when using a standard antibiogram on LJ medium. Another strain found to be resistant by INNO-LiPA Rif.TB showed no mutation in the *rpo*B gene through GenoType MTBDRplus system, but showed a mutation in the *inh*A gene. The standard antibiogram confirmed an MDR strain. This shows that INNO-LiPA Rif. TB can consistently detect RIF resistance and as such, may indicate the presence of MDR strains.

By contrast, mycobacterial strain testing using the GenoType MTBDRplus system not only highlights RIF resistance but also INH resistance, thereby more accurately assigning a certain strain to the MDR group. Of the 50 strains tested using the GenoType MTBDRplus system, 26 strains showed resistance to RIF, of which 24 (92.3%) were unambiguously classified as MDR due to also showing resistance to INH.

In conclusion, the present study showed that the most frequent mutations causing resistance to RIF in Romania are H526Y and mutations of the S4 region of the *rpo*B gene. At the same time, it revealed a high degree of agreement between the INNO-LiPA Rif. TB, GenoType MTBDRplus assays and the standard antibiogram on LJ medium, supplemented with RIF and INH.

Table 3 - GenoType MTBDRplus testing of the 50 bacterial strains

Nr	*rpoB*	WT 1	WT 2	WT 3	WT 4	WT 5	WT 6	WT 7	WT 8	MUT 1	MUT 2A	MUT 2B	MUT 3	*katG*	WT	MUT 1'	MUT 2'	*inhA*	WT 1''	WT 2''	MUT 1''	MUT 2''	MUT 3''	MUT 4''
1		■	■	■	■	■	■	■	■							■			■		■			
2		■	■	■	■	■	■	■	■							■			■			■		
3		■	■	■	■	■	■	■	■							■			■		■			
4		■	■	■	■	■	■	■	■							■			■				■	
5		■	■	■	■	■	■	■	■							■			■					■
6		■	■	■	■	■	■	■	■							■			■					■
7		■	■	■	■	■	■		■		■				■	■			■					
8		■	■	■	■	■	■	■	■							■			■					■
9		■	■	■	■	■	■	■	■						■				■	■				■
10		■	■	■	■	■	■	■					■			■								■
11		■	■		■	■	■	■	■				■			■								■
12		■	■	■	■	■	■	■	■						■				■	■				
13		■	■		■	■	■	■	■				■				■			■				■
14		■	■	■	■	■	■	■					■				■		■					■
15		■	■	■	■	■	■	■					■		■				■	■				
16		■	■		■	■	■	■	■				■		■				■					■
17		■	■	■	■	■	■	■	■						■									
18		■	■	■	■	■	■	■	■						■				■	■				
19		■	■	■	■	■	■	■	■							■			■		■			
20		■	■		■	■	■	■					■		■				■	■				■
21		■	■		■	■	■	■	■				■			■			■		■			
22		■	■	■	■	■	■	■	■						■				■					■
23		■	■	■	■	■	■	■	■						■				■			■		
24		■	■		■	■	■	■	■				■		■				■					■
25		■	■		■	■	■	■	■				■		■				■		■			
26		■	■	■	■	■	■	■					■		■				■					■
27		■	■	■		■	■	■	■		■						■		■			■		
28		■	■	■	■	■	■	■				■				■			■	■				■
29		■	■	■	■	■	■	■	■						■				■	■				
30		■			■	■	■	■	■		■					■			■		■			
31		■	■	■	■	■	■	■	■						■				■		■			
32		■	■			■	■	■	■		■					■			■		■			
33		■	■		■	■	■	■	■		■					■			■				■	
34		■			■	■	■	■	■		■	■				■			■		■			
35		■			■	■	■	■	■		■					■			■		■			
36																								
37		■	■	■	■	■	■	■			■					■				■				■
38		■	■	■	■	■	■						■		■				■		■			■
39		■	■	■	■	■	■	■			■					■			■		■			
40		■	■	■	■	■	■	■					■			■				■			■	
41		■	■		■	■	■		■	■						■			■		■			
42		■	■	■	■	■	■	■					■			■			■		■			
43		■	■	■	■	■	■	■	■						■				■	■				
44		■	■	■	■	■	■	■	■						■				■	■				
45		■	■	■	■	■	■	■	■						■				■	■				
46		■	■	■	■	■	■	■	■						■				■	■				
47		■	■	■	■	■	■	■	■						■				■	■				
48		■	■	■	■	■	■	■	■						■				■		■			
49		■	■		■	■	■		■	■						■			■		■			
50		■	■	■	■	■	■	■	■						■				■	■				

Table 4 - A comparison between RIF-resistant strains detected using INNO-LiPA Rif.TB assay, antibiograms on LJ medium and the GenoType MTBDRplus assay

	RESISTANCES				
	INNO LiPA Rif.TB	GenoType MTBDRplus		Line I ABG	
Strain number	RIF	RIF	HIN	RIF	HIN
7	R	R	R	R	R
9	R	R	R	R	R
10	R	R	R	R	R
11	R	R	R	R	R
13	R	R	R	R	R
14	R	S	S	R	R
15	R	R	S	R	S
16	R	R	R	R	R
20	R	R	R	R	S
21	R	R	R	R	R
23	R	S	R	R	R
24	R	R	R	R	R
25	R	R	R	R	R
26	R	R	R	R	R
27	R	R	R	R	R
28	R	R	R	R	R
30	R	R	R	R	R
32	R	R	R	R	R
33	R	R	R	R	R
34	R	R	R	R	R
35	R	R	R	R	R
37	R	R	R	R	R
38	R	R	R	R	R
39	R	R	R	R	R
40	R	R	R	R	R
41	R	R	R	R	R
42	R	R	R	R	R
49	R	R	R	R	R

Conflict of interests: None to declare.

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