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Short Communication

Characterization of *rpoB* Mutations by Line Probe Assay in Rifampicin-Resistant *Mycobacterium tuberculosis* Clinical Isolates from the Aegean Region in Turkey

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SUMMARY: The nature and frequency of mutations in the *rpoB* gene of rifampicin (RIF)-resistant *Mycobacterium tuberculosis* clinical isolates vary considerably according to the geographical location, and very little information is available regarding specific mutational patterns in our country. The main objective of this study was to determine the frequency of mutations in the hypervariable region of the *rpoB* gene in RIF-resistant *M. tuberculosis* isolates recovered from tuberculosis patients in our region by using the INNO-LiPA Rif. TB kit and to evaluate the performance of the kit for the detection of RIF-resistance. Mutations associated with RIF resistance were studied by line probe assay (LiPA) in 65 RIF-resistant and 56 RIF-susceptible *M. tuberculosis* strains isolated from different patients in the Aegean region of Turkey. The LiPA identified all susceptible strains (100%) as RIF-sensitive and 63 of 65 (96.9%) phenotypically documented RIF-resistant *M. tuberculosis* isolates as RIF-resistant, with specific detection of mutation in 44 (67.7%) isolates, whilst 2 strains were identified as RIF-susceptible. The R5-pattern (Ser-531-Leu mutation) was the most frequently observed (35 of 65, 53.8%), followed by the ΔS2-pattern (7.7%) and ΔS4-pattern (7.7%).

More than 90% of rifampicin (RIF)-resistant *Mycobacterium tuberculosis* isolates are also resistant to isoniazid, making RIF resistance a useful surrogate marker for multidrug-resistant tuberculosis (MDR-TB) (1). More than approximately 95% of RIF-resistant *M. tuberculosis* strains have mutations in an 81-bp hot-spot region (codons 507-533) of the *rpoB* gene that encodes the RNA polymerase β subunit (2). This region is therefore an ideal target for molecular tests for RIF-resistance (3,4). The frequency and nature of mutations in the *rpoB* gene in RIF-resistant *M. tuberculosis* strains vary considerably according to the geographical location or the ethnic populations (5). There is limited information regarding the prevalence of specific mutations within the *rpoB* gene in RIF-resistant *M. tuberculosis* strains from Turkey. The commercially available INNO-LiPA Rif. TB kit (Innogenetics, N.V., Gent, Belgium) is a line probe assay (LiPA) able to detect genetic mutations in the *rpoB* gene region related to RIF-resistance (6,7). The aims of this study were (i) to detect and to characterize the specific base changes within the hypervariable region of the *rpoB* gene in RIF-resistant *M. tuberculosis* isolates recovered from TB patients in Aegean region, Turkey by using the INNO-LiPA Rif. TB kit and (ii) to evaluate the performance of the kit for the detection of RIF-resistance.

Of the 121 clinical isolates of *M. tuberculosis* selected from our strain collection included in this study, 65 were RIF-resistant and 56 were susceptible. All isolates had been collected from different patients from the Aegean region of Turkey, particularly from Manisa city. Isolation and identification of the isolates was carried out and the RIF susceptibility was determined using the BACTEC 460 system (Becton Dickinson, Sparks, Md., USA) by following the standard procedures and as recommended by the manufacturer (8); all samples were stored at −70°C. All strains were freshly subcultured on Lowenstein Jensen (LJ) medium before use. *M. tuberculosis* strain H37RV was used as a control. The line probe assay kit (INNO-LiPA Rif. TB) was used according to the manufacturer's instructions. The RIF resistance-determining region of the *rpoB* gene was amplified with specific biotin-labeled primers by using 1 U of *Taq* DNA polymerase per reaction mixture in a thermocycler (Gene Amp PCR System 9700; Applied Biosystems, Foster City, Calif., USA). The PCR product was then denatured and hybridized to a strip with 10 specific oligonucleotide probes by using an automated system (AUTO-LiPA; Innogenetics) according to the manufacturer's instructions. *M. tuberculosis* was detected in the samples by use of the *M. tuberculosis* complex-specific probe. The reactivities of an amplified fragment with the S-type probes for the wild type (probes S1 through S5) were used to detect the mutations that lead to RIF resistance in *M. tuberculosis*. Furthermore, four probes (R-type probes) were specifically designed to hybridize to the sequences of the four most frequently observed mutations: R2 (Asp-516-Val), R4a (His-526-Tyr), R4b (His-526-Asp), and R5 (Ser-531-Leu). In conclusion, when all the wild-type S probes gave a positive signal and all the R probes reacted negatively, the *M. tuberculosis* isolate was considered susceptible to RIF. When at least one negative signal was obtained with the wild-type S probes, the isolate was considered RIF-resistant (INNO-LiPA Rif. TB S patterns). When the resistance to RIF was due to one of the four most frequently observed mutations described above, a positive reaction was obtained with one of the four R probes and was always accompanied by a negative reaction with the corresponding S probe (INNO-LiPA

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Table 1. Distribution of LiPA patterns and resistance mutations among the RIF-resistant isolates of *M. tuberculosis*

LiPA pattern	No. of isolates (%) $n = 65$	Mutation
R5	35 (53.8)	Ser-531-Leu
ΔS2	5 (7.7)	
ΔS4	5 (7.7)	
R4a	3 (4.6)	His-526-Try
R4b	3 (4.6)	His-526-Asp
ΔS1	3 (4.6)	
ΔS5	3 (4.6)	
R2	2 (3.1)	Asp-516-Val
ΔS3	1 (1.5)	
R4a + ΔS5	1 (1.5)	His-526-Try
ΔS1 + ΔS2	1 (1.5)	
ΔS2 + ΔS3	1 (1.5)	
Wild type	2 (3.1)	

Rif. TB R patterns).

MDR-TB is a rapidly increasing health problem. According to a recent World Health Organization report (9), the median prevalence of MDR-TB and resistance to RIF among combined cases are 1.7 and 2.2%, respectively. The rate of MDR-TB and resistance to RIF among combined cases in Manisa city were found to be 7.3 and 8.7%, respectively (10), while in another study from the Aegean region of Turkey (11) they were found to be 6.8 and 8.2%. RIF is the key element in the treatment of TB, and the development of resistance to RIF is generally considered to be a marker for MDR-TB. As such, there is a need for rapid and more accurate detection of resistance to RIF both for optimal patient treatment and control of the disease. Since conventional drug-susceptibility tests are usually time-consuming, tremendous efforts have been put into the development and use of genotypic methods. For a genotypic method to be clinically useful, it needs to be accurate, reproducible, and capable of detecting the range of mutations seen in isolates from the patient population to which it is applied.

In the present study, all of the 56 phenotypically RIF-susceptible strains yielded hybridization patterns with all 5 wild-type S probes and none of the resistant R probes, indicating that no RIF resistance-associated mutations were present by the INNO-LiPA Rif. TB kit. Of the 65 phenotypically RIF-resistant isolates, 63 were determined as RIF-resistant by LiPA and 2 were found to be wild-type. In compartion with the BACTEC 460TB system, the sensitivity and specificity regarding the ability of LiPA to detect RIF resistance were 96.9 and 100%, respectively. Similarly, the majority of previous studies showed a sensitivity of 90% or greater, and a specificity of almost 100% (6,12-17). In the present study, the overall accuracy of LiPA for the detection of susceptibility and resistance, when compared to phenotypic RIF susceptibility testing, was 98.3% (119 of 121 isolates). The LiPA Rif. TB kit failed to diagnose resistance in only two strains (1.7%). It has previously been reported that this test is unable to determine resistance because in less than 4% of RIF-resistant strains the mutation is located outside the 81-bp region of the *rpoB* gene covered by the assay (5,18). On the other hand, 100% specificity has been found in most studies, indicating that silent mutations are rare and that there is a high correlation between phenotypic resistance and mutations within the 81-bp region of the *rpoB* gene (19).

The LiPA assay can identify only the four most common mutations. Therefore, this method might be unreliable in geographic areas where less common or silent mutations are more frequent (12). The rates of identification of particular mutations by LiPA vary among different countries (Table 2). The interpretation of the LiPA results may be more difficult in geographic areas where less common or novel mutations of the *rpoB* gene occur more frequently (18). Therefore, by the help of further studies on mutations in different geographic regions, this assay could be improved by including more specific mutation probes in the kit. In the present study, four distinct nucleotide substitutions (Ser-531-Leu, His-526-Tyr, His-526-Asp, and Asp-516-Val) accounting for resistance in 44 (67.8%) of 65 RIF-resistant isolates were identified by the kit R probes specific for these mutations (INNO-LiPA patterns R5, R4a, R4b, and R2, respectively), while a genetic alteration within the 81-bp core region of *rpoB* gene was detected in 19 (29.2%) of the 65 isolates, but a specific mutation was not identified by LiPA (INNO-LiPA patterns ΔS1-5) (Table 1). Although the rate of identified specific mutations in the present study (67.7%) was higher than that reported by Cavusoglu et al. (20) in the Aegean region, the difference did not reach the level of significance. The rates in

Table 2. Rates of identifying of particular mutations by LiPA, and the frequency of mutations in the *rpoB* gene in RIF-resistant *M. tuberculosis* strains from the different geographic regions

Geographic region (Reference no.)	No. of RIF-resistant isolates	No. correctly identified by LiPA		% Frequency of mutations detected by LiPA			
		No. isolates undetected particular mutation (ΔS1-ΔS5)	No. isolates detected particular mutation (%) (R2, R4a, R4b, R5)	Asp-516-Val (R2)	His-526-Tyr (R4a)	His-526-Asp (R4b)	Ser-531-Leu (R5)
Belgium (6)	91	64	27 (29.7)	8.8	5.5	5.5	9.9
United States (13)	51	5	41 (80.4)	3.9	39.2	3.9	33.3
Greece (14)	17	3	13 (76.5)	5.9		17.6	52.9
Asia (15)	90	13	70 (77.8)	13.3	8.9	4.4	51.1
Hungary (18)	29	3	23 (79.3)	41.4		6.9	31.0
Turkey (20)	41	17	23 (56.1)	2.4	4.9	2.4	46.3
United States (12)	64	15	46 (71.9)	4.7	9.4	4.7	53.1
Brazil (16)	113	30	80 (70.8)	8.0	8.0	10.6	48.6
Sweden (19)	27	6	21 (77.8)	7.4	3.7	7.4	59.3
Kuwait (21)	32	5	19 (59.4)	6.3	6.3	9.4	37.5
Latvia (17)	33	2	29 (93.5)	24.2		3.0	60.6
This study	65	19	44 (67.7)	3.1	6.2	4.6	53.8

most studies from many regions of the world vary between 60 and 80%, while Rossau et al. (6) have reported 29.7% in Belgium (Table 2).

The types and the frequency of the mutations of RIF-resistant strains at the region of the *rpoB* gene differ according to geographic region. Results reported from many areas of the world and Turkey (20) show that the most frequent type of mutation is the Ser-531-Leu mutation (Table 2). In agreement with these results, we found that 35 of the 65 (53.8%) RIF-resistant strains from Manisa and surrounding areas have the Ser-531-Leu mutation (LiPA pattern R5). The second most dominant LiPA pattern was ΔS2 (7.7%) and ΔS4 (7.7%). A double mutation was detected in three strains (Table 1). In contrast, Bartfai et al. (18) have found that the most common type of mutation in Hungarian isolates is Asp-516-Val (41.4%) by LiPA, while Cooksey et al. (13) have found the His-526-Tyr mutation to be more frequent in isolates from the United States (39.2%). Similar to another study from Turkey (20), we observed that three mutations occurring less frequently in Turkish strains (His-526-Asp, His-526-Tyr, and Asp-516-Val) have different relative frequencies in strains from different countries (Table 2).

In conclusion, it has been shown that the most frequent mutation causing RIF resistance is the Ser-531-Leu mutation in the Aegean region of Turkey. Furthermore, this study showed that the rate of concordance of the results of the LiPA with the results of the in-vitro susceptibility test is high (98.3%). LiPA may therefore be useful for the rapid diagnosis of RIF-resistant TB and for epidemiological analyses of the types of mutation in the region of *rpoB* gene in our country.

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