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Variations in the occurrence of specific *rpoB* mutations in rifampicin-resistant *Mycobacterium tuberculosis* isolates from patients of different ethnic groups in Kuwait

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***Background & objectives*: Frequency of resistance-conferring mutations vary among isoniazid- and ethambutol-resistant *Mycobacterium tuberculosis* isolates obtained from patients of various ethnic groups. This study was aimed to determine the occurrence of specific *rpoB* mutations in rifampicin-resistant *M. tuberculosis* isolates from tuberculosis patients of various ethnic groups in Kuwait.**

***Methods*: Rifampicin-resistant *M. tuberculosis* isolates (n=119) from South Asian (n=55), Southeast Asian (n=23), Middle Eastern (n=39) and other (n=2) patients and 107 rifampicin-susceptible isolates were tested. Mutations in *rpoB* were detected by DNA sequencing. Polymorphisms at *katG463* and *gyrA95* were detected by PCR-RFLP for genetic group assignment.**

***Results*: None of rifampicin-susceptible but 116 of 119 rifampicin-resistant isolates showed *rpoB* mutation(s). Mutations among isolates from South Asian patients were distributed at *rpoB516* (20%), *rpoB526* (24%) and *rpoB531* (27%) while 78 and 51 per cent of isolates from Southeast Asian and Middle Eastern patients, respectively, contained a mutated *rpoB531*. All isolates with *rpoB* N-terminal and cluster II mutations were obtained from Middle Eastern and South Asian patients. Most isolates from South Asian (84%) and Southeast Asian (70%) patients belonged to genetic group I while nearly all remaining isolates belonged to genetic group II. Isolates from Middle Eastern patients were distributed among genetic group I (46%), genetic group II (33%) and genetic group III (21%).**

***Interpretation & conclusions*: The occurrence of specific *rpoB* mutations varied considerably in rifampicin-resistant *M. tuberculosis* isolates obtained from patients of different ethnic groups within the same country. The present data have important implications for designing region-specific rapid methods for detecting majority of rifampicin-resistant strains.**

Key words Ethnic differences - *Mycobacterium tuberculosis* - rifampicin resistance - *rpoB* mutations

The global burden of tuberculosis (TB) is being sustained by the expanding human immunodeficiency virus (HIV) infection and its association with active TB disease and increasing resistance of *Mycobacterium tuberculosis* to the most-effective (first-line) anti-TB drugs[1,2]. Incomplete or improper treatment of TB patients leads to selection of strains with resistance-conferring mutations in genes encoding drug targets[3]. Sequential accumulation of mutations in target genes generate multidrug-resistant (MDR, resistant at least to

rifampicin and isoniazid) *M. tuberculosis* (MDR-TB) strains[3,4]. Rifampicin (RMP) is an important anti-TB drug in the current therapy regimens. Monoresistance to RMP is rare except in TB patients co-infected with HIV or with other underlying conditions[3-6]. Resistance of *M. tuberculosis* to RMP is also a surrogate marker for MDR-TB since about 90 per cent RMP-resistant strains are also resistant to isoniazid[3,7]. While proper treatment of drug-susceptible TB has a cure rate >95 per cent, proper management of MDR-TB is difficult, particularly in resource-poor settings, due to delays in diagnosis and chemotherapy with less effective but more expensive and toxic second-line drugs for an extended period that makes adherence to therapy more difficult[3].

Rapid drug susceptibility testing (DST) of *M. tuberculosis* isolates ensures effective treatment of TB patients and limits further transmission of infection and emergence of additional drug resistance, MDR-TB and extensively drug-resistant (XDR)-TB[3,4]. The DST on solid medium takes about 3 wk while broth-based radiometric, semi-automated BACTEC 460 TB and nonradioactive, fully-automated BACTEC MGIT 960 TB systems report results within 4-12 days[8]. Other low cost rapid methods have also been developed for resource-poor settings but still require 10-14 days to report the results[8]. Molecular methods have the potential to report DST results within 1-2 days[3].

The RMP binds to β-subunit of RNA polymerase (encoded by *rpoB*) and inhibits RNA transcription and protein synthesis in *M. tuberculosis*[3,7]. Mutations within 81-bp hot-spot region of *rpoB* gene (mainly involving codons 516, 526 and 531) are primary mechanism conferring RMP resistance in 90-95 per cent RMP-resistant *M. tuberculosis* strains[7,9]. Resistance in 5-10 per cent RMP-resistant isolates is due to mutations in N-terminal or other (such as cluster II) *rpoB* gene regions[3,10]. Frequency of specific mutations in hot-spot region codons 516, 526 and 531 was found to vary among *M. tuberculosis* isolates collected from different geographical locations[3,11-13]. However, it has not been ascertained whether these variations are due to differences in the genetic background of *M. tuberculosis* isolates or due to differences in ethnic origin of the infected TB patients or both. The frequency of mutations at *katG* codon 315 (*katG315*) conferring resistance to isoniazid and *embB306* conferring resistance to ethambutol have also been shown to vary among *M. tuberculosis* isolates obtained from different geographical locations[3,13-18]. We have previously reported that the occurrence of *katG315* mutations in isoniazid-resistant isolates vary considerably among patients of different ethnic groups at the same geographical location[19]. However, similar studies have not been carried out with RMP-resistant *M. tuberculosis* isolates from patients of different ethnic background within the same country.

This study was undertaken to determine the occurrence of specific *rpoB* mutations in rifampicin-resistant *M. tuberculosis* isolates from TB patients of various ethnic groups in Kuwait.

Material & Methods

Clinical M. tuberculosis isolates and drug susceptibility testing: A total of 7268 *M. tuberculosis* isolates from TB patients were obtained during the study period (1998 to 2008) at National Tuberculosis Reference Laboratory in Kuwait. The isolation and identification of mycobacterial isolates was done by using the mycobacterial growth indicator tube (MGIT) 960 TB system (Becton Dickinson, Sparks, MD, USA) as described previously[20]. The identity of *M. tuberculosis* was further confirmed by a multiplex PCR assay[21]. The phenotypic DST was performed for all the 7268 clinical *M. tuberculosis* isolates using BACTEC 460 TB system by recording bacterial growth in the presence of RMP (2 µg/ml), isoniazid (0.1 µg/ml), ethambutol (2.5 µg/ml) or streptomycin (2 µg/ml) as described previously[20]. A total of 226 *M. tuberculosis* isolates were analyzed in this study. These included all RMP-resistant *M. tuberculosis* isolates (n=119) that were isolated during the study period. The RMP-resistant *M. tuberculosis* strains were recovered from TB patients of South Asian (n=55), Southeast Asian (n=23), Middle Eastern (n=39) and other ethnic (n=2) origin. A total of 107 randomly selected drug-susceptible *M. tuberculosis* isolates were also included to ensure that RMP resistance-conferring mutations in the *rpoB* gene are not found in pansusceptible *M. tuberculosis* strains. Most RMP-resistant isolates included in this study have been analyzed previously for *rpoB* mutations by the line probe assays and/or DNA sequencing[13,22], however, the frequency of *rpoB* mutations in the context of ethnic origin of the TB patient and genetic background of *M. tuberculosis* was not determined. The *M. tuberculosis* reference strain $H_{37}Rv$ was used as a control in DST, DNA sequencing of three *rpoB* gene regions and genetic group analysis of clinical *M. tuberculosis* isolates.

DNA extraction for molecular assays: One ml of MGIT 960 culture of reference or clinical *M. tuberculosis*

isolate was heated with 40 mg Chelex-100 (Sigma-Aldrich, St. Louis, MO, USA) at 95°C for 20 min and then centrifuged at 12,000 x g for 15 min. For a PCR, 2 µl of supernatant was used as a source of DNA.

Sequencing of hot-spot, N-terminal and cluster II regions of rpoB gene: The *rpoB* gene fragment containing codons 462 to 591 including hot-spot region codons 507 to 533 from *M. tuberculosis* isolates was amplified by touchdown PCR with primers RPOHSF (5'-GACGACATCGACCACTTCGGCAAC-3') and RPOHSR (5'-GAACGGGTTGACCCGCGCGTACA-3') and reaction and thermal cycling conditions, as described previously[23,24]. The 426 bp amplicons were purified by using QIAQuick PCR product purification kit (QIAGEN, Hilden, Germany). Both strands of purified amplicons were sequenced by using DTCS CEQ2000 DNA sequencing kit (Beckman-Coulter, Fullerton, CA, USA) as described previously[23] except that HSRFS (5'-AAACCAGATCCGGGTCGGCATGT or HSRRS (5'-GCGTACACCGACAGCGAGCCGA-3') was used as sequencing primer. For all RMP-resistant *M. tuberculosis* isolates with wild-type sequence of the hot-spot region of *rpoB* gene and 10 selected pansusceptible *M. tuberculosis* isolates, N-terminal and cluster II regions were also sequenced. The N-terminal *rpoB* gene region was amplified by touchdown PCR by using primers RPONF (5'-CGACGAGTGCAAAGACAAGGACA-3') and RPONR (5'-GACGGTGTCGCGCTTGTCGAC-3') and reaction and thermal cycling conditions as described previously[23,24]. The PCR generated 310 bp amplicons were purified and both strands were sequenced by using internal primers (RPONFS, 5'-TTCGTCACCGCCGAGTTCATCAA-3' or RPONRS, 5'-CTTGACGCTGTGCAGCGTCTTGT-3')[23,24]. The cluster II region of *rpoB* gene was also PCR amplified by using primers RPOIIF (5'-TCATGGACCAGAACAACCCGCTGT-3') and RPOIIR (5'-ACATCACTGTGATGCACGACAACG-3') with reaction and thermal cycling conditions as described previously[22,23]. The 679 bp amplicons were purified and sequenced with internal primers RPOIIFS (5'-CGCGACGTGCACCCGTCGCACT-3') and RPOIIRS (5'-ACATCACTGTGATGCACGACAACG-3') as describe above for the hot-spot region of *rpoB* gene. The nucleotide and deduced amino acid sequences were compared with corresponding sequences from susceptible strain *M. tuberculosis* $H_{37}Rv$ using BLAST.

Genetic group analysis of clinical M. tuberculosis isolates: Clinical *M. tuberculosis* isolates belong to one of three principal genetic groups (genetic group I, II and III) based on polymorphisms at *katG463* (R463 or L463) and *gyrA95* (S95 or T95)[25]. Thus, genetic group of each clinical *M. tuberculosis* isolate was determined by detecting polymorphisms at *katG463* and *gyrA95*. The presence of R463/L463 at *katG463* was detected by PCR amplification of *katG463* DNA region with KATG463F (5'-CCCGAGGAATTGGCCGACGAGTTC-3') and KATG463R (5'-GGTGCGAATGACCTTGCGCAGATC-3') primers followed by purification of 360 bp amplicons with QIAQuick PCR product purification kit and digestion with restriction enzyme *Nci* I, to generate RFLP patterns, as described previously[26]. The presence of S95/T95 at *gyrA95* was also determined by PCR amplification of *gyrA95* DNA region with primers GYRA95F (5'-CGCAGCTACATCGACTATGCGATG-3') and GYRA95R (5'-GGGCTTCGGTGTACCTCATCGCC-3') followed by purification of 322 bp amplicons with QIAQuick PCR product purification kit and restriction digestion with *Ale* I, to generate RFLP patterns, as described previously[26]. Based on these polymorphisms, the isolates were then assigned to one of the three genetic groups (L463 + T95, genetic group I; R463 + T95, genetic group II and R463 + S95, genetic group III).

Fingerprinting of RMP-resistant M. tuberculosis isolates: Molecular fingerprinting of RMP-resistant *M. tuberculosis* isolates carrying identical *rpoB* mutation was carried out by double-repetitive-element (DRE)-PCR, and those yielding unique DNA banding patterns were classified as genotypically distinct isolates[13].

Statistical analysis: Differences in proportions were compared using Fisher's two-tailed Exact test. The 95% confidence interval (CI) was also calculated by large-sample method. All statistical analyses were performed by using WinPepi software ver. 3.8 (PEPI-for Windows, *www.brixtonhealth.com*).

Results

Phenotypic DST is performed on all cultured *M. tuberculosis* isolates as part of routine patient care in Kuwait. Based on the results of phenotypic DST by BACTEC 460 TB system, 119 of 7268 *M. tuberculosis* isolates were resistant to RMP with or without additional resistance to other first-line drugs. A total of 107 *M. tuberculosis* isolates susceptible to all first-line drugs (pansusceptible strains) were also tested to ensure that resistance-conferring mutations in the *rpoB* gene are not present in RMP-susceptible strains. All 226 clinical isolates were identified as *M. tuberculosis* based on

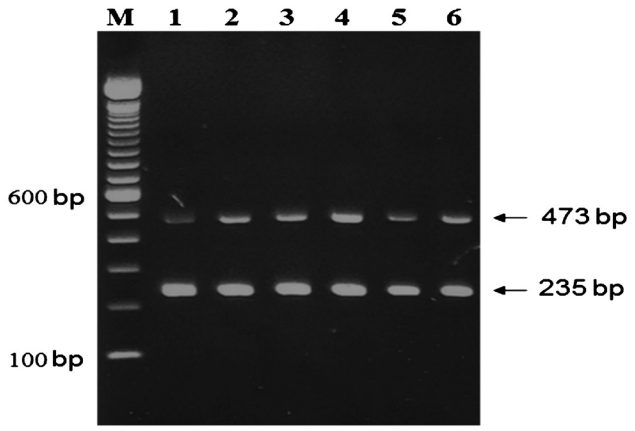

Fig. Representative agarose gel of multiplex PCR products from six selected multidrug-resistant isolates (lanes 1-6) showing *M. tuberculosis*-specific amplification of 473 bp and 235 bp fragments (marked by arrows) of *oxyR* and *rpoB* genes, respectively. Lane M is 100 bp DNA ladder and the position of migration of 100 and 600 bp fragments are marked.

specific amplification of two DNA fragments of 473 bp and 235 bp (Fig.)[21]. Only six of 119 RMP-resistant *M. tuberculosis* isolates were resistant to RMP alone (monorifampicin-resistant) while the remaining 113 isolates were additionally resistant at least to isoniazid. Thirty-nine (33%) and 40 of 119 (34%) RMP-resistant *M. tuberculosis* isolates were resistant to three and all four first-line drugs tested, respectively (Table I).

Of the 119 RMP-resistant *M. tuberculosis* isolates, 96 (81%) were recovered from pulmonary specimens while the remaining 23 (19%) isolates were cultured from specimens collected from extra-pulmonary sites. Majority (76 of 119, 64%) of RMP-resistant *M. tuberculosis* isolates were cultured from male patients. All RMP-resistant *M. tuberculosis* isolates were obtained from adult (range 18-65 yr) TB patients. Only 10 were isolated from Kuwaiti nationals while the remaining isolates were cultured from expatriate workers or their family members. The date of arrival in Kuwait or history of previous treatment with anti-TB drugs was not available for expatriate patients. Amplification and interpretable sequencing results were obtained for all isolates. No mutation was detected by DNA sequencing in any of the pansusceptible *M. tuberculosis* isolate in the three (hot-spot, N-terminal and cluster II) regions of *rpoB* gene (Table I). Most (116 of 119, 97%) RMP-resistant *M. tuberculosis* isolates contained at least one RMP resistance-conferring mutation while three isolates contained wild-type sequences in all the three regions of the *rpoB* gene (Table I).

Table I. Phenotypic susceptibility testing by BACTEC 460 TB system and detection of rifampicin resistance-associated mutations in *rpoB* gene by DNA sequencing among 226 *M. tuberculosis* isolates

Resistance pattern of *M. tuberculosis* isolate	No. of isolates tested*	No. (%) of isolates with *rpoB* mutation(s)
None	107	0 (0)
RMP	6	6 (100)
RMP, INH	34	33 (97)
RMP, INH, EMB	27	27 (100)
RMP, INH, SM	12	11 (92)
RMP, INH, EMB, SM	40	39 (98)

RMP, rifampicin; INH, isoniazid; EMB, ethambutol; SM, streptomycin; *A total of 226 *M. tuberculosis* isolates were tested by both phenotypic and molecular susceptibility testing methods and the numbers shown are specific for a particular pattern only

Three major ethnic groups were identified among TB patients yielding RMP-resistant *M. tuberculosis* isolates and included patients of South Asian (n=55), Southeast Asian (n=23) and Middle Eastern (n=39) origin (Table II). The country of origin of South Asian patients included India (n=47), Bangladesh (n=6) and Nepal (n=2). Southeast Asian patients were from Philippines (n=19) and Indonesia (n=4). The countries of origin of Middle Eastern patients were Egypt (n=17), Kuwait (n=10), Syria (n=4), Pakistan (n=4), Iran (n=2), and Iraq (n=2). The remaining two patients were from Ethiopia and Nigeria. Overall, 106 of 119 (89%) RMP-resistant *M. tuberculosis* isolates contained mutation(s) in hot-spot region of *rpoB* gene. Among these 106 isolates, only five contained two mutations in the hot-spot region (Table II). Of the remaining 13 isolates, seven contained a mutation in the N-terminal region (V176F) and three in the cluster II region (I572F) whilst three other isolates contained wild-type sequences in all the three regions of the *rpoB* gene. Interestingly, all the three isolates with I572F mutation in cluster II region and the three isolates with no mutation in *rpoB* gene were cultured from patients of South Asian origin. The seven isolates with V176F mutation in the N-terminal region were recovered from patients of Middle Eastern origin only (Table II). Among isolates recovered from South Asian patients, mutations at *rpoB516* (11 of 55, 20%), *rpoB526* (13 of 55, 24%) and *rpoB531* (15 of 55, 27%) were nearly evenly distributed and several other codons were mutated in the remaining isolates. On the contrary, most of RMP-resistant isolates cultured from

Table II. Distribution of specific *rpoB* mutations in rifampicin-resistant *M. tuberculosis* isolates from TB patients belonging to the three major ethnic groups in Kuwait

Ethnic group of TB patient	No. of *M. tuberculosis* isolates tested	No. of strains with mutation at *rpoB* hot-spot or N-terminal or cluster II region codon									No *rpoB* mutation
		Q513	Ins. 514	D516	S522	H526	S531	L533	2 codons	V176	I572	
South Asian	55	3	2	11*	0	13	15	1	4[a]	0	3	3
Southeast Asian	23	2	0	0	0	3	18*	0	0	0	0	0
Middle Eastern	39	2	3	0	2	4	20*	0	1[b]	7*	0	0
Others	2	0	0	0	0	1	1	0	0	0	0	0

[a]Two mutations were identified as M516E + S522L in one isolate, as D516G + H526Q in another isolate and as M515I + D516Y in two strains; [b]The two mutations were identified as M515I + D516Y; *The frequency of occurrence of these mutations was statistically significant in the indicated ethnic groups compared with that in South Asians (P<0.05 and <0.001)

Southeast Asian (18 of 23, 78%) and Middle Eastern (20 of 39, 51%) patients contained a mutation at *rpoB531* (Table II). Although no statistically significant difference was noted among isolates containing a mutation at *rpoB526* isolated from patients of the three ethnic groups, mutations at *rpoB516* were detected only among isolates from patients of South Asian origin (Table II). Further, frequency of mutations at *rpoB531* was also different among isolates cultured from South Asian patients compared to patients of Southeast Asian (P<0.001) and Middle Eastern (P<0.05) origin. The second most common mutation among the isolates from Middle Eastern patients was in the N-terminal region which was not detected among patients of South Asian and Southeast Asian origin (P<0.001) (Table II). The DRE-PCR data showed that majority of RMP-resistant *M. tuberculosis* isolates obtained from different patients but carrying identical *rpoB* mutation were genotypically distinct (data not shown).

The genetic group analysis based on polymorphisms at *katG463* and *gyrA95* showed that majority of RMP-resistant *M. tuberculosis* isolates from South Asian (46, 84%) and Southeast Asian (16, 70%) patients belonged to genetic group I while nearly all remaining isolates from patients of these two ethnic groups belonged to genetic group II (Table III). However, the isolates from Middle Eastern patients were nearly equally distributed among genetic group I (46%) and genetic group II (33%). The frequency of genetic group I isolates among patients of Middle Eastern origin was significantly (P<0.001) different compared to patients of South Asian origin. Nearly all (8 of 9) genetic group III isolates were recovered from Middle Eastern patients compared to only 1 of 55 (P<0.01) and none (P<0.05) from patients of South Asian and Southeast Asian origin (Table III). The frequency of mutations at *rpoB516*, *rpoB526* and *rpoB531* varied considerably among *M. tuberculosis* isolates belonging to different genetic groups. Nearly all (18 of 21, 90%) isolates with a mutation at *rpoB526* belonged to genetic group I. Eight of 11 (73%) isolates with a mutation at *rpoB516* belonged to genetic group I while the remaining three belonged to genetic group II. On the contrary, only 33 of 54 (61%) isolates with a mutation at *rpoB531* belonged to genetic group I while the remaining belonged to genetic groups II or III.

Table III. Distribution of rifampicin-resistant *M. tuberculosis* isolates belonging to principal genetic group (GP) I, GP II and GP III among TB patients from the three major ethnic groups in Kuwait

Ethnic group of TB patient	No. of *M. tuberculosis* isolates tested	No. (%) of isolates belonging to[a]		
		GP I	GP II	GP III
South Asian	55	46 (84)	8 (15)	1 (2)
Southeast Asian	23	16 (70)	7 (30)	0 (0)
Middle Eastern	39	18* (46)	13* (33)	8* (21)
Others	2	1 (50)	1 (50)	0

[a]Genetic group (GP) I, KatG L463 + GyrA T95; genetic group (GP) II, KatG R463 + GyrA T95; genetic group (GP) III, KatG R463 + GyrA S95; *The frequency of occurrence of these genetic groups was statistically significant in the indicated ethnic group (P<0.001, P=0.003 for GP I, GP II and GP III respectively), compared to South Asian patients

Discussion

This study was focused to determine the frequency of specific *rpoB* mutations in RMP-resistant *M. tuberculosis* isolates from TB patients of various ethnic groups in Kuwait, a low TB incidence country in the Arabian Gulf region of the Middle East[20]. Three major ethnic groups (Middle Eastern, South Asian and Southeast Asian) are represented among TB patients in Kuwait. The data were also correlated with the genetic group of *M. tuberculosis* strains that are circulating among different populations. Consistent with low incidence of TB and RMP-resistant TB in Kuwait, only 4 per cent RMP-resistant *M. tuberculosis* isolates

contained mutations in two codons of *rpoB* gene. Since nearly all drug-resistant strains in low TB incidence countries contain single point mutations while >10 per cent drug-resistant strains in high TB incidence countries contain multiple mutations in target genes[24,26-28], the data suggest limited previous exposure of TB patients to anti-TB drugs. Based on fingerprinting studies, majority of RMP-resistant isolates from different TB patients were genotypically distinct. The data rule out active transmission of infection in majority of TB patients infected with RMP-resistant *M. tuberculosis* strain in Kuwait. This is contrary to high TB incidence countries where recent acquisition of infection with drug-resistant strains is more common[2,3].

Although only 8 per cent RMP-resistant isolates contained a mutation in N-terminal or cluster II region (outside the hot-spot region) of *rpoB* gene, their distribution in the three ethnic groups was interesting. Our data suggest the inclusion of probes that interrogate the N-terminal codon V176 for molecular detection of RMP resistance among *M. tuberculosis* isolates collected from Middle Eastern patients and cluster II region probes for isolates from South Asian patients for greater sensitivity.

Previous studies carried out on RMP-resistant *M. tuberculosis* isolates from TB patients at various geographic locations have shown that the frequency of specific *rpoB* mutations vary considerably[9,11,13,29]. Initially, these variations were attributed to the geographical differences in RMP-resistant *M. tuberculosis* strains circulating in different settings and their clonal propagation[11-13,29]. For instance, the high (90%) frequency of *rpoB531* mutation in MDR-TB strains from Samara region in Russian Federation was attributed to the high frequency of Beijing genotype strains in that setting[29]. Similarly, molecular epidemiological studies carried out on isoniazid-resistant *M. tuberculosis* have also shown that the frequency of *katG315* mutations varies from 50 to 95 per cent at different geographical locations[14-16,19]. The high (90-95%) frequency of *katG315* mutations among *M. tuberculosis* strains in Russia was also attributed to the high frequency of Beijing genotype strains[14,16,19]. However, the frequency of *rpoB531* and *katG315* mutations in RMP-resistant and INH-resistant strains, respectively, are much lower in *M. tuberculosis* strains isolated in China and Taiwan, even though the frequency of Beijing genotype strains in these settings are also high[9,27,28]. Multiple studies carried out at different time periods in the same country/geographical setting have yielded variable frequency of specific *rpoB* mutations[11,13,24,27,28]. Since majority of active disease cases in low TB incidence countries occur as a result of reactivation of previously acquired infection[30], the present data suggest that the evolution of a mutation at *rpoB516*, *rpoB526* and *rpoB531* is also influenced by genetic background of *M. tuberculosis*. Taken together these observations suggest that the selection of specific *rpoB* mutation during evolution of RMP resistance in *M. tuberculosis* is influenced by both, the genetic background of the infecting *M. tuberculosis* strain and by the ethnic origin of the TB patient.

The major drawback of the present study was lack of information about the date of arrival of expatriate subjects in Kuwait, their travel history, and history of previous exposure to anti-TB drugs. Another limitation was the small number of TB patients representing various nationalities and ethnic groups that were studied which may not be representative of the entire ethnic group.

In conclusion, our findings showed that the occurrence of specific *rpoB* mutations varied considerably in RMP-resistant *M. tuberculosis* isolates obtained from patients of different ethnic groups within the same country. The data have important implications for designing region-specific and ethnic group-specific rapid methods for detecting majority of RMP-resistant strains.

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