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Full Length Article

Identification of Single Nucleotide Polymorphism in *rpoB* Gene among *Mycobacterium tuberculosis* Isolates from Pakistan

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Abstract

The emergence of multidrug resistant strains of *Mycobacterium tuberculosis* is a threat to tuberculosis control programs. The most effective strategy to control the spread of these strains is to use an accurate and rapid drug susceptibility test. Resistance to rifampicin, one of the most potent first line anti-TB drugs, is found to be associated with mutations in "hot-spot" region of RNA polymerase gene (rpoB) are geographically distributed. *M. tuberculosis* isolates from various regions of Pakistan were collected and mutations were detected by reverse hybridization using in-house line probe assay. The *rpoB* gene from 45 isolates was cloned and sequenced to validate the results obtained by line probe assay. The most common mutations were found to be in codon 531 (56.45%) followed by codon 526 (9.68%), 516 (4.84%) and 518 (3.22%). Certain silent mutations and several novel mutations outside the "hot-spot" region were also found. © 2014 Friends Science Publishers

Keywords: Multiple drug resistance; Mutations; Rifampicin resistance; Line probe assay

Introduction

Tuberculosis, though curable, is still one of the leading causes of mortality all over the world. The severity of the problem is reflected by the fact that over one third of human population is infected with tuberculosis. The incidence of tuberculosis is particularly high in South East Asian countries, which harbor about 40% of global TB cases. In Pakistan, the magnitude of TB burden in terms of the number of cases per 100,000 populations is found to be 231 that rank Pakistan 5th amongst 22 high TB burden countries (WHO, 2012). The problem of TB is compounded by high rate of multiple drug resistance (resistance to at least rifampicin and isoniazid, the two most potent first line anti tuberculosis drugs). High rate of MDR is a continuous challenge to tuberculosis control program, not only in developing but also in developed countries. A laboratory based data has suggested that the incidence of MDR tuberculosis has increased from 14% to 47%, in Pakistan (Tanveer *et al*., 2008). Such an alarmingly high rate of MDR in communities poses continuous public health threat by hampering the efficacy of WHO recommended short course treatment regimen.

Rifampicin is a key component of WHO recommended short course chemotherapy. Patients, in whom resistance to rifampicin develops, show poor prognosis (Fischl *et al*., 1992). It has been observed that in more than 90% cases, rifampicin resistance is found to be coupled with isoniazid resistance (Drobniewski and Wilson, 1998). Thus rifampicin resistance can be used as a surrogate marker for the detection of MDR-TB (Hashmi *et al.,* 2013). Rifampicin binds with β subunit of DNA-dependent RNA polymerase and interferes with the transcription and elongation of RNA. Rifampicin resistance occurs by single step, high level mutations with a frequency of 10^{-9}. Genetic basis of the resistance to rifampicin lies in rifampicin resistance determining region (RRDR) of *rpoB* gene, where mutations occur in distinct 81 bp "hot-spot" region. This consists of 27 amino acids encompassing the codon 507 to 533 (Drobniewski and Wilson, 1998). Several groups of workers from different parts of the world have characterized the mutations associated with rifampicin resistance in RRDR of *rpoB* gene (Donnabella *et al*., 1994; Hirano *et al*., 1999; Valim *et al*., 2000; Mani *et al*., 2001).

Determining the mutation spectrum of *rpoB* gene among *M. tuberculosis* strains prevalent in Pakistan is important because it would not only help to identify new strains specific to this region of the world but will also be useful in designing an in-house molecular diagnostic assay for rapid screening of MDR strains. The study is of particular importance in the context, when commercially available line probe assay based diagnostic kits are being introduced in public sector for rapid MDR screening. Since type and frequency of mutations in RRDR of *rpoB* gene are known to bevaried geographically, hence, molecular tests based on mutations specific to this geographical setting will be more accurate to screen MDR cases (Jou *et al*., 2005;). So, the present study was aimed to screen mutations in *rpoB*

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gene by DNA sequencing and an in-house developed reverse hybridization line probe assay.

Materials and Methods

Collection of Specimens

A total of 141 samples, 97 cultures while 44 clinical (blood and sputum) specimens were included in this study. Culture isolates were obtained from different cities of Pakistan including Lahore (31 isolates), Peshawar (31 isolates), Karachi (11 isolates) and Faisalabad (10 isolates) and are given in Table 1. All the 44 clinical specimens were collected from Faisalabad and its periphery which included 38 blood and 6 sputum samples. Clinical specimens were confirmed as *M. tuberculosis* after amplification of IS*6110* element using specific primers.

Preparation of Genomic DNA

DNA from *M. tuberculosis* cultures on LJ slants was extracted by CTAB method (van Embden *et al*., 1993) while the extraction of DNA from clinical samples was performed using SDS/proteinase K method(Hill *et al*., 1972) Sputum samples were decontaminated by NaOH/N-acetyl-L-cystein solution before extraction of DNA. About 200 ng of DNA from culture isolates, while 5 µL of DNA from clinical specimens, was used as target for the amplification of hotspot region of *rpoB* gene by polymerase chain reactions.

Amplification of RRDR of *rpoB* Gene

Amplification of RRDR of *rpoB* gene of *M. tuberculosis* was achieved by nested PCR using LiPA OP1 (outer forward primer) 5´-GAGAATTCGGTCGGCGAGCTGATCC-3´, LiPA OP2 (outer reverse primer) 5´-CGAAGCTTGACCCGCGCGTACACC-3´; and LiPA IP1 (inner forward primer) 5´-GGTCGGCATGTCGCGGATGG-3´, LiPA IP2 (inner reverse primer) 5´-GCACGTCGCGGACCTCCAGC-3´. Inner primer LiPA IP2 was biotinylated at 3´ end (Innogenetics, Zwijndrecht, Belgium). The outer and inner pair of primers generated a fragment of 395 bp and 257 bp, respectively. The PCR was performed in a final volume of 25 µL containing 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each dNTPs (Fermentas Cat.# R0181) and 1U of Taq DNA polymerase (Fermentas Cat.# EP0402). Outer pair of primers was used in the first round of PCR cycle with 10 p*M* of each primer. The cycling parameters involved were denaturation at 95°C for 5 min followed by 30 cycles of 95°C for 60 sec, 58ºC for 30 sec and 72ºC for 30 sec. The second round of PCR was performed using inner set of primers with 20 p*M* of each primer and 1µL of PCR product from the first round of PCR cycle. The cycling parameters involved were; denaturation at 95°C for 5 min followed by 30 cycles of 95°C for 60 sec, 65ºC for 30 sec and 72ºC for 30 sec. The reaction was incubated at 72ºC for 7 min to allow final extension.

Table 1: Mutations in *rpoB* gene found by DNA sequencing and its comparison with in-house line probe assay

Isolate	Origin	Mutation detected by sequencing	Mutations detected by in-house line probe assay	Remarks
0607	Faisalabad	(531)TCG→TTG	(531)TCG→TTG	
0017	Karachi	(506)TTC→ATC	No mutation	Outside the hotspot
7	Karachi	(531)TCG→TTG	(531)TCG→TTG	
22	Karachi	(569)ATC→AAC	No mutation	Outside the hotspot
160	Lahore	(477)GAC→GAT	No mutation	Outside the hotspot
1188	Lahore	(494)AAC→AGC	No mutation	Outside the hotspot
145	Lahore	(520)CCG→CCA	No mutation	Outside the hotspot
640	Lahore	(531) TCG→TTG	(531)TCG→TTG	
1000	Lahore	(531) TCG→TTG (548)CGC→TGC*	(531)TCG→TTG	*Outside the hotspot
1249	Lahore	(538) TCT→TGT	No mutation	Outside the hotspot
1048	Lahore	(571)CTG→CCG** (531) TCG→TGG	(531) TCG→?	** Not included in LiPA
P95	Peshawar	(511) CTG→CGG (516)GAC→TAC**	(511)CTG—CGG (516)GAC→?	** Not included in LiPA
P81	Peshawar	(516) GAC→GTC	(516)GAC→GTC	
P79	Peshawar	(521) CTG→ CCG* (531) TCG→TTG (503)AAG→AAA**	(531)TCG→TTG	*Outside the hotspot **Not included in LiPA
P73	Peshawar	(526) CAC→TAC	(526)CAC→TAC	
P49	Peshawar	(531) TCG→TTG	(531)TCG→TTG	
P65	Peshawar	(531) TCG→TTG	(531)TCG→TTG	
P67	Peshawar	(531) TCG→TTG	(531)TCG→TTG	
P98	Peshawar	(531) TCG→TTG	(531)TCG→TTG	
P78	Peshawar	(531) TCG→TTG	(531)TCG→TTG	
P96	Peshawar	(531) TCG→TTG (554) CAC→CAT*	(531)TCG→TTG	*Outside the hotspot

Detection of Mutations in RRDR of*rpoB*Gene by Sequencing

For 45 culture isolates, 257 bp amplified PCR product was cloned in a T/A cloning vector from Fermentas (pTZ57R/T). The clones were commercially sequenced by M13 forward and reverse primers (Macrogen, Korea). Sequence information was stored, assembled, and analyzed using the Laser gene sequence analysis package (DNAStar Inc., Madison, WI, USA). The sequencing data was aligned and the mutations were analyzed in RRDR of *rpoB* gene.

Determination of Mutations in RRDR of *rpoB* Gene by In-house Reverse Hybridization Line Probe Assay

To screen the mutations associated with rifampicin resistance in RRDR of *rpoB* gene of *M. tuberculosis*, an in-house reverse hybridization line probe assay was used. Briefly, 10µL of each PCR product was heat denatured after diluting in 140 µL of hybridization solution (5XSSPE/0.5%SDS) and applied to nylon filters at right angle to the oligonucleotides using Miniblotter45. Hybridization was carried out at 45ºC for 30 min. To remove unbound PCR products, filters were washed briefly with 2XSSPE/0.1%SDS at room temperature and then with 2XSSPE/0.1%SDS twice at 55ºC for 10 min. Detection of hybridization signals was carried out using Biotin Chromogenic Detection kit (Fermentas Cat.# K0662).

Results

Sequencing analysis of *rpoB* gene

Among randomly selected 45 sequenced isolates, 24 (52.17%) showed no mutation, while 21 (46.67%) isolates were found to have 26 point mutations of 14 different types (Table 1). Among these isolates, 17 had single mutation, 3 had double mutations and only one had triple mutations. Codon 531 was found to be highly mutated as 11 isolates possessed mutation 531 (TCG→TTG) while one isolate exhibited 531 (TCG→TGG). Other mutations in RRDR region of *rpoB* gene of *M. tuberculosis* found in the present study by sequencing include *rpoB* 511 (CTG→CCG), *rpoB* 516 (GAC→GTC), *rpoB* 516 (GAC→TAC), *rpoB* 521 (CTG→CCG) and *rpoB* 526 (CAC→TAC). Mutations in codon *rpoB* 477 (GAC→GAT), *rpoB* 503 (AAG→AAA) and *rpoB* 520 (CCG→CCA) were silent mutations. Novel mutations found in the present study included *rpoB* 494 (AAC→AGC), *rpoB* 503 (AAG→AAA), *rpoB* 505 (TTC→TCC), *rpoB* 506 (GAC→ATC), *rpoB* 538 (TCT→TGT), *rpoB* 548 (CGC→TGC), *rpoB* 554 (CAC→CAT), *rpoB* 569 (ATC→AAC) and *rpoB* 571 (CTG→CCG). Two isolates had double mutations, of which one had mutation in codon 531 (TCG→TTG) along with a novel mutation 548 (CGC→TGC), while second had a novel mutation in codon 554 (CAC→CAT) that coexisted with 531 (TCG→TTG) mutation. Another isolate had mutation in codon 511 (CTG→CCG) and 516 (GAC→TAC). One isolate that contained triple mutations had mutations in codon 531 (TCG→TTG) and 521 (CTG→CCG). This particular isolate also had a novel mutation 503 (AAG→AAA) that resides outside the RRDR of *rpoB* gene (Table 2).

The Detection of Common Mutations by In-House Line Probe Assay

Results of in-house line probe assay were in agreement with those obtained by DNA sequencing (Table 2).

Table 2: Mutation profile in *rpoB* gene in *Mycobacterium tuberculosis* isolates. Shown are the nucleotide mutations with frequency (%) and the resulting change in amino acids

Codon	Nucleotide mutation	Amino acid change	Type of mutation	Mutation frequency (%)
477	GAG→GAT	Glu→Asp	Substitution	1.61
494	AAC→AGC	Asn→Ser	Substitution	1.61
503	AAG→AAA	Lys→Lys	Silent	1.61
505	TTC→TCC	Phe→Ser	Substitution	1.61
506	TTC→ATC	Phe→Ile	Substitution	1.61
511	CTG→CCG	Leu→Pro	Substitution	8.06
511	CTG→CGG	Leu→Arg	Substitution	
515	ATG→?	Met→unknown	unknown	1.61
516	GAC→TAC	Asp→Tyr	Substitution	
516	GAC→GTC	Asp→Val	Substitution	4.84
516	GAC CAG	Asp Gln	Deletion	
518	AAC	Asn	Deletion	3.22
520	CCG→CCA	Pro→Pro	Silent	1.61
521	CTG→CCG	Leu→Pro	Substitution	1.61
526	CAC→TGC	His→Cys	Substitution	
526	CAC→CGC	His→Arg	Substitution	9.68
526	CAC→TAC	His→Tyr	Substitution	
526	CAC→GAC	His→Asp	Substitution	
526	CAC→?	His→unknown	Unknown	
531	TCG→TTG	Ser→Leu	Substitution	56.45
531	TCG→TGG	Ser→Trp	Substitution	
538	CTG→GTG	Leu→Val	Substitution	1.61
548	CGC→TGC	Arg→Cys	Substitution	1.61
554	CAC→CAT	His→His	Silent	1.61

*could not be sequenced

Hence, the sensitivity and specificity of in-house line probe assay was found to be 100% in comparison to sequencing.

Mutations In the present study the mutations were mostly located at codon at codon 531,526,516 and 511of *rpoB* gene. At codon 531, two different substitution mutations were observed while 6 different substitution mutations were observed in codon 526. On the other hand, codon 511 showed 5 different substitution mutations and codon 516 showed 2 substitution and one deletion mutations.

Use of Line Probe Assay for Screening Mutations in Clinical Specimens

Forty-four clinical samples were screened for the mutations in RRDR of *rpoB* gene. Out of 44 specimens, only two isolates showed the presence of mutation in RRDR of *rpoB* gene which were found to be in codon 511 (CTG→CCG) and codon 531 (TCG→TTG). Specimen that had mutation in codon 531 also gave positive signal with wild type probe showing the presence of mixed infection.

Discussion

About 50% (24 out of 45) of sequenced isolates had wild type *rpoB* gene sequence, while 21 samples showed 14 different point mutations in *rpoB* gene in 26 isolates.

Mutations in codon 503, 520 and 554 were silent mutations as they did not change the encoded amino acid. An interesting finding in this study was the presence of multiple mutations in some resistant isolates, where mutation in codon 531 was found to be coexisting with some novel mutations. Mutations at codon 511 and 516 also coexisted in one isolate. Presence of multiple mutations in the rifampicin resistant isolates is not uncommon and is also reported previously (Moghazeh *et al*., 1996; Mani *et al*., 2001; Huitric *et al*., 2006). Certain novel mutations in codons *rpoB* 477, *rpoB* 494, *rpoB* 505, *rpoB* 506, *rpoB* 521, *rpoB* 538 and *rpoB* 548 were also found in the present study. A mutation that resides in codon outside the RRDR of *rpoB* gene is not a new phenomenon. Several other studies (Mani *et al*., 2001; Cavusoglu *et al*., 2002) reported the novel mutations outside the RRDR of *rpoB* gene.

It is remarkable that all the mutations that reside in RRDR of *rpoB* gene were correctly identified by the in-house assay resulting in 100% concordance between the in-house assay results and the results of DNA sequence analysis of *rpoB* gene. The isolate having double mutation (in codon 511 and 516) was also detected by in-house assay, adding to the sensitivity of this assay.

The overall mutation spectrum was quite varied with the most frequently mutated codon found to be 531 followed by 526, 511 and 516 with frequencies of 56.45%, 9.68%, 8.06 % and 4.84, respectively. Contribution of the mutations that reside inside the RRDR was 87.09%. Dominance of the substitution mutation at codon 531 followed by 526 and 516 was also observed in other studies (Ramaswamy and Musser, 1998; Lingala *et al*., 2010). The increased prevalence of mutations at codon 531 is because of the molecular and physiological processes that result in the development of the resistance, without decrease in fitness of these strains (Arnold *et al*., 2005; Jenkins *et al*., 2009).

A low mutation frequency (4.84%) at codon 516 observed in the present investigation is contrasting to the results of Hungarian study (Bartfai *et al*., 2001) and the study conducted on Asian isolates (Hirano *et al*., 1999) where mutations at codon 516 were found in 37.9% and 13.3% isolates, respectively. These comparative studies indicate that the frequency of mutations in codon 531, 526, 16 and 511 is subjected to strong geographical variations.

The frequency of mutations in RRDR of *rpoB* gene in clinical samples was 4.54%, which is similar to the incidence of drug resistance reported from Pakistan (WHO, 2012). One patient was found to be infected with two strains of *M. tuberculosis* but it is difficult to determine the relative abundance of the subpopulations in the sample as PCR cannot discriminate between the templates from the wild and the mutant organism. This is one of the major drawbacks of the molecular assay for the diagnosis of MDR-TB, while dealing with the clinical isolates which not only affects the sensitivity of the molecular assay particularly in a situation where percentage of the resistant population is low but also is an obstacle for the successful therapy. Therefore, molecular tests are preferred to be used in conjunction with the conventional tests.

The degree of rifampicin resistance in *M. tuberculosis* isolates strongly correlates with the site and type of mutation in *rpoB* gene (Zaczek *et al*., 2009). Lower resistance is attributed by the mutations at codon 522, 518, 516 (Williams *et al*., 1998) and 511 (Moghazeh *et al*., 1996; Bodmer *et al*., 1995) while mutations at codon 531 (Huang *et al*., 2002) and 526 are usually known to cause high level of resistance (Hwang *et al*., 2003). This shows that the position of mutation in the allele at molecular level is critically affecting the complex drug target interaction that leads to the varied levels of drug resistance. Pakistan is sharing its geographical boundaries with the countries like India, Afghanistan and Iran where tuberculosis is highly endemic. Prevalence of *M. tuberculosis* isolates in Pakistan, harboring mutations associated with high level of drug resistance, is critical as similar mutation profiles are observed in these countries. Lingala *et al*. (2010) showed that the majority of strains circulating in this particular geographical setting are highly resistant to rifampicin. One of the possible explanations of such similar trends is historical connections between these countries and this probably represents prevalent strains of this region.

Our data serves as the baseline information regarding the geographical and temporal trends in the prevalence of specific mutations as well as for geographical differences in mutation frequencies for the development of new diagnostic tool. This data may also enable to feature effective and better policies in this particular geographical setting for the control of tuberculosis. However, level of resistance by different novel mutations identified in the present study demands further reseach work respective fields.

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