Journal of Infection and Public Health 12 (2019) 496–501

Contents lists available at ScienceDirect

Journal of Infection and Public Health

journal homepage: http://www.elsevier.com/locate/jiph

Mutational analysis of *gyrB* at amino acids: G481A & D505A in multidrug resistant (MDR) tuberculosis patients

Nasir Mahmood[a,b,*], Shahzada N. Abbas[c], Neelam Faraz[c], Saman Shahid[d]

[a] Department of Biochemistry & Department of Human Genetics and Molecular Biology, University of Health Sciences, Lahore, Pakistan
[b] Department of Cell and Systems Biology, University of Toronto, Canada
[c] Department of Biology, Lahore Garrison University, Lahore, Pakistan
[d] Department of Sciences and Humanities, National University of Computer & Emerging Sciences (NUCES), Foundation for Advancement of Science and Technology (FAST), Lahore, Pakistan

ARTICLE INFO

Article history:
Received 30 April 2018
Received in revised form 3 January 2019
Accepted 15 January 2019

Keywords:
Multi-drug resistance (MDR) TB
Semi-multiplex allele specific (MAS) PCR
gyrB
Gly481Ala
Asp505Ala

ABSTRACT

Background: The MDR (multidrug resistance) tuberculosis is a serious public health concern. Fluoroquinolones are in use to treat tuberculosis, but M. tuberculosis strains have now become resistant due to several mutations in different genes. We evaluated mutations in *gyrB* gene at amino acid positions G481A and D505A of *M. tuberculosis* by semi-multiplex allele specific (MAS) PCR.
Methods: The information on gender, age, type of tuberculosis (TB), positive/negative for MDR-TB and HIV infection was gathered. The genomic DNA isolation from sputum culture samples (n = 53) was carried out by non-column based method. The *gyrB* mutations were investigated by using self-designed primers in semi MAS-PCR, at mentioned amino acid positions.
Results: There were 38% male patients and 62% were female patients. Most of MDR-TB patients (58.5%) were in the age between 16–30 years. There were 90.5% cases of pulmonary TB and 9.4% cases of extra pulmonary TB. Only 1.8% patients were co-infected with HIV. The 24 samples had mutation in *gyrB* gene out of 53 (45.28%), on both of positions of amino acids Gly481Ala and Asp505Ala. All samples had mutations at Gly481Ala, whereas, 24 samples (45.28%) had mutations at Asp505Ala.
Conclusion: Mutations at amino acids positions 481 and 505 were involved in MDR-TB, which could further develop into an extensively-drug resistance (XDR) TB. Therefore, there is a need to explore all mutations in *gyrB* gene in MDR-TB, because it can result in a Fluoroquinolones resistance.

Introduction

The multi-drug resistant tuberculosis (MDR-TB) and extensively drug resistant tuberculosis (XDR TB) are emerging as a most catastrophic ailments around the world [1–4]. The multi-drug resistant (MDR) TB is defined as a resistance against first-line anti-tuberculosis drugs, i.e., resistance to at least Isoniazid (INH) and Rifampin (RIP) [5,6]. In Pakistan, 2.0–3.2% of new cases of MDR-TB are reported each year, with 35% of post-diagnosed cases are already existing [7]. The MDR-TB occurs due to under or over dosage of anti-tuberculosis drugs [5,6,8]. Primarily, the treatment of Fluoroquinolones is important as they reduce the infection [9,10]. In current research, the tuberculosis patients (n = 53) with a history of multidrug resistance (MDR) were included to evaluate mutations in *gyrB* gene at amino acid positions G481A and D505A of *M. tuberculosis* (Mycobacterium tuberculosis) by semi-multiplex allele specific (MAS) polymerase chain reaction (MAS-PCR). There is no report in literature about G481A and D505A mutation spectrum in local MDR-TB patients from Pakistan. Moreover, the method used in this study is very simple which involved semi-multiplex allele specific PCR strategy in which we used self-designed primers and the reported mutations were detected by using this simple method first time. Cigarette smoking and low socioeconomic status are considered significant risk factors for TB [11]. The immunocompromised patients and those undergoing chemotherapy are at a higher risk [1,2,4]. The prevalence of extensively drug-resistant (XDR) TB is elevating, because the use of second-line TB drugs is also increasing. Any previous treatment with second-line drugs

* Corresponding author at: Department of Cell and Systems Biology, University of Toronto, Canada.
E-mail address: nasir.sbs1@outlook.com (N. Mahmood)

https://doi.org/10.1016/j.jiph.2019.01.056

is also a major risk factor for this resistance. The World Health Organization [12] WHO, has mentioned that worldwide, 450,000 people had developed MDR-TB with 170,000 deaths. In 2012, the MDR-TB cases were reported 29% from South-East Asia, 27% from Africa, 19% from Western Pacific regions, 26% from India and 12% from China. The high prevalence of MDR-TB cases were also found in Central Asia and Eastern Europe. In the WHO report [12], Pakistan has been included in the list of high-burden countries for tuberculosis [12]. In Pakistan, approximately 51% cases of tuberculosis were recorded from Punjab, 23% from Sindh, 15% from Khyber Pakhtunkhwa and 3.5% from Azad Kashmir and Baluchistan [13].

The genetic variations can induce drug resistance and hence these variations transfer from one generation to another [14]. The drug resistance of *M. tuberculosis* can be due to an insertion or deletion of single base and may also due to base substitutions [15]. A group of mutations may also responsible for this resistance in MDR-TB strains [16]. The *M. tuberculosis* also shows resistance against enzymes of drug inactivation or efflux pump mechanisms [17]. In pleiotropism, many phenotypes are affected by a single gene [18] and mutations caused by pleiotropism that lead MDR production are not reported in *M. tuberculosis*. A mutation in chromosomes by sequential accumulation generates a resistance to many drugs. Sometimes, if a strain gets resistant directly by one drug, it becomes resistant to one or more drugs without a direct exposure (i.e., cross resistance). There is a probability that a strain can be resistant to two drugs on different resistance mechanisms. The rate of mutation for Rifampin (RIF) is approximately one in one hundred and eight organisms [19]. The *M. tuberculosis* has an efflux mechanism which is an important factor in developing a resistance against antibiotics, such as Amino-glycoside, Fluoroquinolones and Tetracyclines [20]. It was mentioned by Stephan et al. [21], that certain species of mycobacterium lack *mspA* gene, which encode major porins mspA Msp, and lacking this gene can increase MIC (minimum inhibitory concentration) of Vancomycin, Erythromycin, Ampicillin and Cephaloridine by 2–10 folds. The deletion of the porins MspA and MspC can increase the resistance against β-lactam antibiotics and hydrophilic Fluoroquinolones such as Norfloxacin [22]. Song et al. [23] has mentioned that mycobacterial outer membrane proteins (*RB1698* and *RB1973)* are involved in natural resistance in mycobacterium [23]. The acquired drug resistance in *M. tuberculosis* is also due to spontaneous mutations in chromosomal gene [24].

The Rifampin (RIF) drug targets the β-subunit of RNA polymerase in *M. tuberculosis* and inhibits the elongation of mRNA [24]. It was mentioned by Telenti et al. [19], that Rifampin (RIF) resistant strain develops due to the mutation in *rpoB* gene that encodes the β-subunit of RNA polymerase and then conformational changes occur in RNA polymerase. Due to this, Rifampin (RIF) shows lower binding affinity with the RNA polymerase [19,24]. The extensive drug resistance (XDR TB) strains can be resistant to Fluoroquinolones and to the other injectable second-line of drugs such as Amikacin (AMK), Capreomycin (CAP) and Kanamycin (KAN) [25]. The Fluoroquinolones mainly affect the topoisomerase-II or DNA gyrase [26]. The DNA gyrase is tetramer and contains two α-subunits and two β-subunits. The α-subunits are encoded by *gyrA* gene and β-subunit is encoded by *gyrB* gene [27]. Resistance against Quinolones is due to mutations occurrence in *gyrA* and *gyrB* genes [28]. Quinolones bind with *gyrA* and *gyrB* genes to stop DNA relaxing. Bacteria become resistant by substituting the amino acid in the Quinolones' binding region and this region is called Quinolones resistance-determining region (QRDR) of *gyrA* and *gyrB* genes [29,30].

Materials and methods

Background information and sample collection

The information on gender, age, positive/negative for MDR-TB, type of tuberculosis (pulmonary/extra-pulmonary) and HIV infection (positive/negative) was gathered. In this research work, fifty three (n = 53) MDR-TB sputum culture samples were collected from patients by informed consents. The clinical history of tuberculosis resistance against first-line anti-tuberculosis drugs was taken. The samples were collected from Gulab Devi Hospital, Lahore, in collaboration with University of Health Sciences (UHS), Lahore. After sample collection, each sample tube was labeled with a random number. Each sputum culture sample was mixed with 0.5 ml autoclaved sterile water to make suspension in an autoclaved Eppendorf tube. The Eppendorf tubes were stored at −70 °C for molecular analyses. During sampling and processing of samples in molecular work, proper biosafety conditions were followed.

Genomic DNA isolations

The genomic DNA isolation from sputum culture samples was carried out by non-column based method from the genomic DNA isolation kit (Gene All, Inc. Korea) with some alterations. The suspension of sputum culture sample was centrifuged at 12000 rpm for 3 min. The supernatant was discarded, 300 μl RL buffer was added in bacterial cell pellet and mixed well until cell-pellet completely homogenized. The 300 μl AL buffer was added into the pellet and mixed well with vortex machine until it completely homogenized. The tubes were incubated at 37 °C for an hour in case of inhomogeneous mixing of buffer with the pellet. A 3 μl of Proteinase K was added into microfuge tube and incubated it at 37 °C overnight. The Proteinase K was used to degrade all types of proteins from the sample. The sample containing microfuge tube was cooled at room temperature for 5 min. The 100 μl of PP buffer was added in tubes and mixed well by using a vortex machine to precipitate protein. The PP buffer was a protein precipitating buffer. These samples were then incubated in a freezer at −10 °C for 5 min. These samples were then centrifuged at 10,000 rpm for two minutes and removed the supernatant carefully. This supernatant was then transferred into a new autoclaved and labeled microfuge tube. The 300 μl chilled absolute ethanol was then added into the supernatant. The solution was gently mixed and incubated in a freezer for an hour. This solution was centrifuged at 12,000 rpm for 8 min after incubation. The supernatants were immediately discarded and the DNA pellet was dried at 50 °C for half an hour. After drying, the 50–100 μl injection water was added into the DNA pellet and mixed well for 4–5 min. Finally, the DNA sample was stored at −10 °C.

Quantitative & qualitative analyses of genomic DNA

The UV (ultraviolet) spectrophotometer was used to quantify the isolated genomic DNA. The dilution of isolated DNA was prepared and the optical density (OD) was checked at the ratio of 260 mm and 280 mm. The DNA concentration was determined by using following equation: [μg/ml of nucleic acid = $OD_{260} \times 50 \times$ dilution factor]. In 260/280 ratio with the value above 1.5, the DNA quality was considered good enough to proceed further for subsequent molecular analysis. The genomic DNA analysis on 0.8% agarose gel was also performed for samples. The intact DNA band showed a non-degraded DNA.

```
aagagcgccaccgacatcggtggattgcccggcaagctggccgattgccgttccacggat
 K  S  A  T  D  I  G  G  L  P  G  K  L  A  D  C  R  S  T  D
ccgcgcaagtccgaactgtatgtcgtagaaggtgactcggccggcggttctgcaaaaagc
 P  R  K  S  E  L  Y  V  V  E  G  D  S  A  G  G  S  A  K  S
ggtcgcgattcgatgttccaggcgatacttccgctgcgcggcaagatcatcaatgtggag
 G  R  D  S  M  F  Q  A  I  L  P  L  R  G  K  I  I  N  V  E
aaagcgcgcatcgaccgggtgctaaagaacaccgaagttcaggcgatcatcacggcgctg
 K  A  R  I  D  R  V  L  K  N  T  E  V  Q  A  I  I  T  A  L
ggcaccgggatccacgacgagttcgatatcggcaagctgcgctaccacaagatcgtgctg
 G  T  G  I  H  D  E  F  D  I  G  K  L  R  Y  H  K  I  V  L
atggccgacgccgatgttgacggccaacatatttccacgctgttgttgacgttgttgttc
 M  A  D  A  D  V  D  G  Q  H  I  S  T  L  LL T  L  L  F
Cggttcatgcggccgctcatcgagaacgggcatgtgtttttggcacaaccgccgctgtac
 R  F  M  R  P  L  I  E  N  G  H  V  F  L  A  Q  P  P  L  Y
Aaactcaagtggcagcgcagtgacccggaattcgcatactccgaccgcgagcgcgacggt
 K  L  K  W  Q  R  S  D  P  E  F  A  Y  S  D  R  E  R  D  G
Ctgctggaggcggggctgaaggccgggaagaagatcaacaaggaagacggcattcagcgg
 L  L  E  A  G  L  K  A  G  K  K  I  N  K  E  D  G  I  Q  R
tacaagggtctaggtgaaatggacgctaaggagttgtgggagaccaccatggatccctcg
 Y  K  G  L  G  E  M  D  A  K  E  L  W  E  T  T  M  D  P  S
gttcgtgtgttgcgtcaagtgacgctggacgacgccgccgccgccgacgagttgttctcc
 V  R  V  L  R  Q  V  T  L  D  D  A  AAA D  E  L  F  S
atcctgatgggcgaggacgtcgacgcgcggcgcagctttatcacccgcaacgccaaggat
 I  L  M  G  E  D  V  D  A  R  R  S  F  I  T  R  N  A  K  D
Gttcggttcctggatgtctaa.
 V  R  F  L  D  V  -
```

Fig. 1. Physical mapping of primers on the *gyrB* gene (NCBI accession number L27512). Key: Yellow: Forward primer (gyrBG481A, 5′CTGAATTCGCAAAAAGCGG′3); Grey: Forward primer (gyrBD505A, 5′GAGAAAGCGCGCATCGA′3); Blue: Common reverse primer (gyrBR, 5′TGCGAATTCCGGGTCAC′3).

Primer designing & translation of gyrB *gene*

The designing of primers was performed by using the *gyrB* gene sequence (NCBI accession number L27512) and an oligonucleotide properties calculator. The BLAST analysis of designed primers was performed for the specificity. The *gyrB* nucleotide sequence along with its protein and its physical position of designed primers are described in Fig. 1. At amino acid position 481, the Gly is converted into the Ala, while at amino acid position 505, Asp is converted into the Ala, in case of mutation. Therefore, two forward primers and one reverse primer were designed. The gyrBG481A forward primer 3′ end contained GG nucleotide, because Gly is encoded by GGT,GGC,GGA,GGG codons and Ala encoded by GCT,GCC,GCA,GCG codons. The Gly is a normal amino acid and Ala is a mutated amino acid. The gyrBD505A forward primer 3′ end contained GA nucleotide, because Asp is encoded by GAC and GAT codons and Ala encoded by GCT, GCC, GCA, GCG codons. The Asp is a normal amino acid and Ala is a mutated amino acid. A common reverse primer gyrBR was designed as described in Fig. 1. The primers used in current research were self-designed and confirmed by physical mapping on *gyrB* gene and BLAST analysis therefore, these were mutations specific primers. Secondly, these were also checked on healthy individuals DNA samples and no any amplification was observed in all healthy individuals under optimized semi multiplex PCR conditions. The designed primers were used in semi multiplex allele specific (MAS) PCR, the product sizes were determined on 1.2% agarose gel. In this research, the primers for each mutation type were designed and were physically mapped on *gyrB* gene and further validated by NCBI BLAST analysis. Moreover, experimentally, the DNA isolated from five healthy individuals were used as control and primers did not produce any amplification in those samples. This showed that molecular assay was validated and was accurate. Further, PCR was performed under aseptic conditions and water was also used as negative control in the PCR reactions instead of DNA in each set of PCR reactions performed for MDR-TB patients. In the current research, two types of negative controls were used; one was pure water instead of *Mycobacterium tuberculosis* DNA and second negative control was DNA of healthy individuals. In both types of negative controls, no amplification was observed.

The semi-multiplex allele specific (MAS) polymerase chain reaction (PCR)

The semi multiplex allele specific PCR was carried out to detect mutations (G481A, D505A) in the *gyrB* gene of *M. tuberculosis*. The PCR was carried out for 40 cycles. The melting temperature was 55 °C for 1 min. In PCR, the initial denaturation was conducted at 94 °C for 4 min, denaturation at 93 °C for 30 s, annealing at 55 °C for 1 min, polymerization at 72 °C for 45 s and final extension was carried out at 72 °C for 7 min. After PCR amplification, the fragments of 347 bp and 278 bp were amplified by forward primer gyrBG481A and gyrBD505A respectively, whereas the reverse primer (gyrBR) was common. The presence of 347 bp fragment showed no mutation, whereas the absence of 347 bp fragment showed G481A mutation in samples. Similarly, the presence of 278 bp fragment showed no mutation, whereas the absence of 278 bp fragment showed D505A mutation in samples. The semi multiplex allele specific PCR master mixture (45.5 µl) for each sample was prepared by mixing PCR buffer (1X), $MgCl_2$ (1.5 mM), dNTPs (0.2 mM) and dH_2O (32.5 µl) in a PCR reaction tube. Further, the 2.5 µl of purified DNA, 0.5 µl (2.5 units) of Taq DNA polymerase and 0.5 µl (50 pmols) of each of three primers were added into PCR tube to make a total PCR mixture volume equal to 50 µl. After that, all reaction tubes were transferred in PCR machine with predefined PCR conditions for 40 cycles. After PCR, the mixture was loaded into the wells of 1.2% agarose gel. The DNA ladder was used to determine the required PCR fragments sizes for mutation(s) detection. After gel electrophoresis, the gel-documentation system was used for gel imaging.

Results

Clinical information for tuberculosis patients

Among 53 patients included in this study, 20 (38%) were male and 33 (62%) were females. Most of the patients (31; 58.5%) were infected with MDR-TB and were found in the age group of 16–30 years. This is the highest ratio of infected patients in this study. According to the cultured sputum samples, the 48 (90.5%) patients

Table 1
Presence and absence of mutations G481A and D505A based on amplified PCR fragments of *gyrB* gene from sputum cultured samples of MDR-TB patients products.

S. No.	Sample Number	347 band (Gly481Ala)	278 band (Asp505Ala)
1	TB-1	–	+
2	TB-2	–	–
3	TB-3	–	–
4	TB-4	–	–
5	TB-5	–	–
6	TB-6	–	–
7	TB-7	–	–
8	TB-8	–	–
9	TB-9	–	–
10	TB-10	–	–
11	TB-11	–	–
12	TB-12	–	+
13	TB-13	–	+
14	TB-14	–	+
15	TB-15	–	+
16	TB-16	–	+
17	TB-17	–	+
18	TB-18	–	+
19	TB-19	–	+
20	TB-20	–	–
21	TB-21	–	–
22	TB-22	–	+
23	TB-23	–	+
24	TB-24	–	+
25	TB-25	–	–
26	TB-26	–	+
27	TB-27	–	+
28	TB-28	–	–
29	TB-29	–	+
30	TB-30	–	+
31	TB-31	–	+
32	TB-32	–	+
33	TB-33	–	+
34	TB-34	–	+
35	TB-35	–	–
36	TB-36	–	–
37	TB-37	–	+
38	TB-38	–	+
39	TB-39	–	+
40	TB-40	–	+
41	TB-41	–	–
42	TB-42	–	–
43	TB-43	–	+
44	TB-44	–	–
45	TB-45	–	+
46	TB-46	–	+
47	TB-47	–	–
48	TB-48	–	–
49	TB-49	–	+
50	TB-50	–	+
51	TB-51	–	–
52	TB-52	–	–
53	TB-53	–	–

Key: + means amplification present and no mutation present at that position. – means amplification absent and mutation present at that position.

were suffering from pulmonary TB and only 5 (9.4%) patients were suffering from extra-pulmonary TB. The patient history showed that only 1 (1.8%) patient was co-infected with HIV.

Quantification of DNA by UV spectrophotometer

The highest quantity of DNA was 67 ng/μL and the minimum quantity was 20 ng/μL.

Mutational analysis: the gyrB gene

With PCR (polymerase chain reaction) setup, the amplified PCR product was run on the 1.5% gel. Table 1 shows the presence and absence of bands on both amino acid positions i.e., Gly481Ala and

Table 2
The Prevalence of G481A and D505A Mutations in *gyrB* Gene.

Targeted Positions	No. of Occurrence[a]	Percentages Prevalence (%)
Gly481Ala (G481A)	53	100
Asp505Ala (D505A)	24	45.28
Gly481Ala (G481A) Asp505Ala (D505A)	24	45.28

[a] Total Sample Size, n = 53.

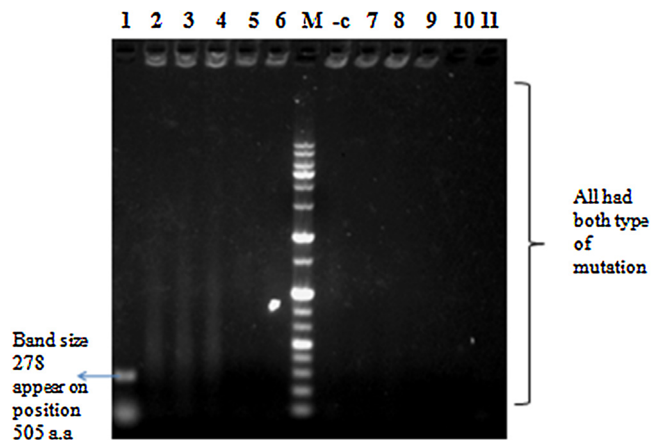

Fig. 2. Semi-Multiplex PCR analysis of *gyrB* gene of *Mycobacterium tuberculosis* from MDR-TB patients on 1.8% agarose gel electrophoresis. Lane 1: Band appears on the position of amino acid 505 which means normal amino acid was not replaced by mutated amino acid (Asp505Ala). Mutation present on the position of 481. Lane 2,3,4,5,6: both type of mutation present (Gly481Ala,Asp505Ala). Lane M: DNA marker. Lane-c: negative control only had water to check the contamination. Lane 7,8,9,10,11: also had both type of mutation.

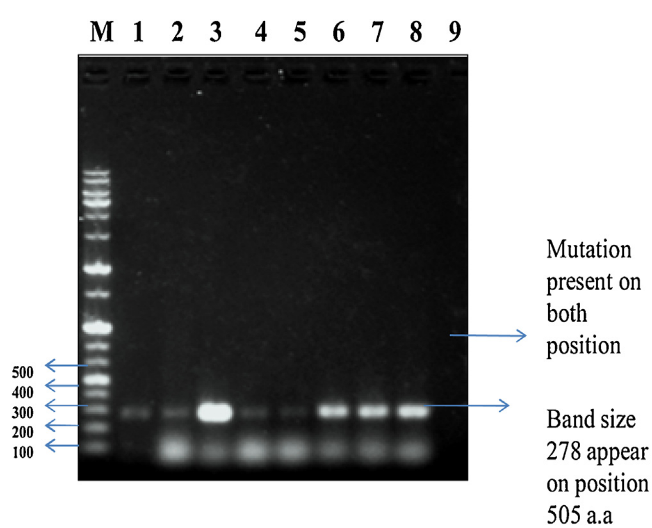

Fig. 3. Semi-Multiplex PCR analysis of *gyrB* gene of *Mycobacterium tuberculosis* from MDR-TB patients on 1.8% agarose gel electrophoresis. Lane M: DNA marker or a ladder sequence. Lane 1,2,3,4,5,6,7,8: showing that mutation present on the position of 481 while on the position of 505 bands were appear which means normal amino acid not replaced by mutated amino acid (Asp505Ala).

Asp505Ala. The 24 samples had mutation in *gyrB* gene out of 53 (45.28%) at both positions of amino acid: Gly481Ala and Asp505Ala (Table 2). All samples were having mutation (100%) at amino acid position Gly481Ala. The 24 samples (45.28%) had mutation at position Asp505Ala (Table 2). The images of gel electrophoresis are represented by Figs. 2–5.

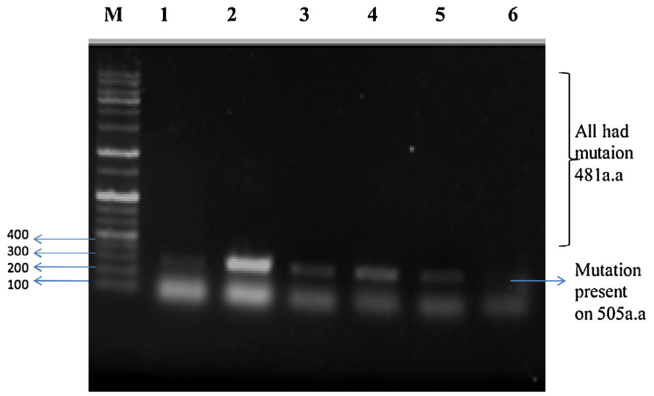

Fig. 4. 1.5% agarose gel showing PCR products. Lane M: DNA marker. Lane 1,2,3,4,5: had one type of mutation. Lane 6: both type of mutation present (Gly481Ala, Asp505Ala).

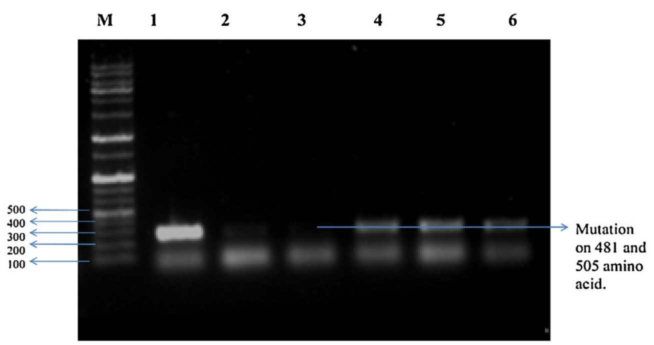

Fig. 5. The 1.5% agarose gel showing PCR products. Lane M: DNA marker. Lane 2,3: mutation are absent. Lane 3: mutation present on the position of 481 and 505 amino acid. Lane 4,5,6: mutations are absent on the position of 481 because normal amino acid is not replaced by mutated amino acid (Gly481Ala).

Discussion

The *M. tuberculosis* has become resistant toward many drugs and treatment is getting difficult now. The MDR-TB is a very serious public health concern. The Fluoroquinolones are used to treat tuberculosis, but *M. tuberculosis* strains have also become resistant to the Fluoroquinolones due to many gene(s) mutations [31]. The *gyrB* gene protein amino acid positions 481 and 505 were not studied earlier in MDR-TB patients of Pakistan. Moreover, the methodologically used was cost effective i.e., semi multiplex allele specific PCR by using self-designed primers. We selected these positions, because, the amino acid position 481 is very close to QRD region of *gyrB* gene protein of *Mycobacterium tuberculosis* while, 505 is within QRD region. The main objective of this study was to determine the Fluoroquinolones resistance in MDR tuberculosis patients based on mutational analysis of *gyrB* gene. The multidrug resistance (MDR) tuberculosis patients were evaluated for the presence of mutations in *gyrB* gene at amino acid positions G481A and D505A of *M. tuberculosis* by semi-multiplex allele specific polymerase chain reaction (MAS-PCR). This research work targeted two new amino acid positions 505 and 481. Pakistan stands at fifth position of top TB burden countries of the world [32]. Further, this research work was carried out with semi multiplex allele specific PCR, which was cost-effective. In our study, 90% patients were having pulmonary TB whereas, 9.4% were having extra-pulmonary. We found that MDR tuberculosis was more reported in young individuals of age between 16–30 years. In the current study, the ratio of female patients was higher (62%) as compared to male patients (38%). It was mentioned by the WHO report [12], that although more TB related deaths prevail with men, but TB is considered among the top three causes of death in women as well. It was estimated that 410,000 TB deaths had occurred in women in the year 2012 [12]. Moreover, half of HIV-positive people who died from TB were women [12].

The current research's results have shown that 24 samples (45.28%) had mutations in *gyrB* gene out of 53 at both positions of amino acids i.e., Gly481Ala and Asp505Ala. All samples (100%) had mutations at amino acid position Gly481Ala. The 24 samples (45.28%) had mutation at the position Asp505Ala. The mutations in *gyrB* gene at positions 481 and 505 can cause resistance and can convert MDR-TB into XDR TB. The gyrase enzyme contains two genes *gyrA* and *gyrB* and it was found that *M. tuberculosis* had a gyrase enzyme that carries both genes *gyrA* and *gyrB* [26]. Pitaksajjakul et al. [33] used 35 isolates of MDR TB; among them there were 22 samples having mutations at *gyrA* (codons 88, 90, 91, 94) and one sample had mutation at *gyrB* (codon 493). It is known that the *M. tuberculosis* shows drug resistance against Quinolones. This resistance can be due to mutation in both of these genes. The A90V, D94G and D94H mutations at *gyrA* and N510D mutation in *gyrB* were responsible for resistance against Ofloxacin as reported by Aubry et al. [26]. The mutations occur in different genes and develop Isoniazid (INH) resistance. Zhang et al. [34] reported mutations in following: *katG, ahpC, inhA, ndh* and *kasA*. The *M. tuberculosis* becomes resistant toward following second-line drugs TB drugs: Viomycin, Capreomycin Amikacin and Kanamycin [35] due to the mutation in *rrs* gene (A1401G) as reported by Jugheli et al. [36]. The *M. tuberculosis* strains become resistant toward Capreomycin and Viomycin due to mutation in the *tlyA* gene as well [37]. Soudani et al. [9] discovered mutations in *gyrA* gene of gyrase in all samples, whereas, no mutation was detected in *gyrB* gene in any sample. Yin & Yu [31] found that all *M. tuberculosis* strains were having mutations in QRD region of *gyrA* gene at codon positions 90, 91 & 94 and only one strain had mutation in QRD region of *gyrB* gene at codon position 511. It is now believed that several mutations in different genes can prompt Fluoroquinolones resistance in *M. tuberculosis* strains [31]. Maruri et al. [10] reviewed different articles and mentioned that Fluoroquinolones resistance is due to mutations in *gyrA* and *gyrB* genes. In 42 researches, 780 out of 1220 *M. tuberculosis* samples were found with mutations in *gyrA* gene in QRDR and 17 samples had mutations in *gyrB* gene. Chernyaeva et al. [38] reported mutations in *gyrA* and *gyrB* genes of *M. tuberculosis* strains with both high level (54.3%) and low level (76.9%) resistances. Chernyaeva et al. [38] found mutations in QRD region of *gyrA* gene and few mutations in *gyrB* gene. Singh et al. [39] also reported that *M. tuberculosis* becomes resistant toward Fluoroquinolones due to mutations in *gyrB* and *gyrA* genes. According to the research conducted by An et al. [40], Fluoroquinolone resistant isolates were identified and QRDR of the *gyrA* and *gyrB* genes were used to identify the Beijing genotype of *M. tuberculosis*. They found 82.6% of isolates were having mutations in *gyrAB*. They further discovered nine other mutations in *gyrB* gene.

Conclusion

The mutations at amino acid positions G481A and D505A of *gyrB* gene of *Mycobacterium tuberculosis* are involved in MDR-TB and can be further developed into XDR-TB. Therefore, there is a need to explore all the mutations in *gyrB* gene as well as in other genes involved in MDR-TB, because they may result in the development of Fluoroquinolones resistance. We recommend that the mutation frequency at amino acid position 481 of *gyrB* gene protein may be further validated in MDR-TB samples of different geographical regions.

Funding

No funding sources.

Competing interests

None declared.

Ethical approval

Informed consents were obtained from all. Patients conformed to institutional ethical standards.

Acknowledgments

Authors are thankful to University of Health Sciences, Lahore and School of Physical Sciences, University of the Punjab, Lahore for molecular work assistance. Authors are also thankful to Gulab Devi Hospital, Lahore for help in clinical aspects of research.

References

[1] Raviglione MC, Uplekar MW. WHO's new stop TB strategy. Lancet 2006;367:952–5.
[2] Migliori G, De Iaco G, Besozzi G, Centis R, Cirillo D. First tuberculosis cases in Italy resistant to all tested drugs. Euro surveill 2007;12:E070517.
[3] Velayati AA, Masjedi MR, Farnia P, Tabarsi P, Ghanavi J, ZiaZarifi AH, et al. Emergence of new forms of totally drug-resistant tuberculosis bacilli: super extensively drug-resistant tuberculosis or totally drug-resistant strains in Iran. Chest J 2009;136:420–5.
[4] Udwadia ZF, Amale RA, Ajbani KK, Rodrigues C. Totally drug-resistant tuberculosis in India. Clin Infect Dis 2012;54:579–81.
[5] Kethireddy S. Tuberculosis-a review of clinical features, differential diagnosis and treatments available. Int J Pharm Technol 2010;2.
[6] Kethireddy S, Light RB, Mirzanejad Y, Maki D, Arabi Y, Lapinsky S, et al. Mycobacterium tuberculosis septic shock. Chest J 2013;144:474–82.
[7] Javaid A, Hasan R, Zafar A, Ghafoor A, Pathan A, Rab A, et al. Prevalence of primary multidrug resistance to anti-tuberculosis drugs in Pakistan. Int J Tuberc Lung Dis 2008;12:326–31.
[8] Lienhardt C, Glaziou P, Uplekar M, Lönnroth K, Getahun H, Raviglione M. Global tuberculosis control: lessons learnt and future prospects. Nat Rev Microbiol 2012;10:407–16.
[9] Soudani A, Hadjfredj S, Zribi M, Messaoud T, Masmoudi A, Majed B, et al. First report of molecular characterization of fluoroquinolone-resistant Mycobacterium tuberculosis isolates from a Tunisian hospital. Clin Microbiol Infect 2010;16:1454–7.
[10] Maruri F, Sterling TR, Kaiga AW, Blackman A, van der Heijden YF, Mayer C, et al. A systematic review of gyrase mutations associated with fluoroquinolone-resistant Mycobacterium tuberculosis and a proposed gyrase numbering system. J Antimicrob Chemother 2012;67:819–31.
[11] Hershkovitz I, Donoghue HD, Minnikin DE, Besra GS, Lee OY, Gernaey AM, et al. Detection and molecular characterization of 9000-year-old Mycobacterium tuberculosis from a Neolithic settlement in the Eastern Mediterranean. PloS One 2008;3:e3426.
[12] Organization WH. Global tuberculosis report 2013. World Health Organization; 2013.
[13] Hasan R, Jabeen K, Mehraj V, Zafar F, Malik F, Hassan Q, et al. Trends in Mycobacterium tuberculosis resistance, Pakistan , 1990-2007. Int J Infect Dis 2009;13, se377–e82.
[14] Wiuff C, Zappala R, Regoes R, Garner K, Baquero F, Levin B. Phenotypic tolerance: antibiotic enrichment of noninherited resistance in bacterial populations. Antimicrob Agents Chemother 2005;49:1483–94.
[15] Sandgren A, Strong M, Muthukrishnan P, Weiner BK, Church GM, Murray MB. Tuberculosis drug resistance mutation database. PLoS Med 2009;6:e1000002.
[16] Safi H, Sayers B, Hazbón MH, Alland D. Transfer of embB codon 306 mutations into clinical Mycobacterium tuberculosis strains alters susceptibility to ethambutol, isoniazid, and rifampin. Antimicrob Agents Chemother 2008;52:2027–34.
[17] Zaunbrecher MA, Sikes RD, Metchock B, Shinnick TM, Posey JE. Overexpression of the chromosomally encoded aminoglycoside acetyltransferase eis confers kanamycin resistance in Mycobacterium tuberculosis. Proc Natl Acad Sci 2009;106:20004–9.
[18] King R, Stansfield W, Mulligan PA. Dictionary of genetics. 7th ed. Oxford, UK: Oxford University Press; 2006.
[19] Telenti A, Imboden P, Marchesi F, Schmidheini T, Bodmer T. Direct, automated detection of rifampin-resistant Mycobacterium tuberculosis by polymerase chain reaction and single-strand conformation polymorphism analysis. Antimicrob Agents Chemother 1993;37:2054–8.
[20] De Rossi E, Aínsa JA, Riccardi G. Role of mycobacterial efflux transporters in drug resistance: an unresolved question. FEMS Microbiol Rev 2006;30:36–52.
[21] Stephan J, Mailaender C, Etienne G, Daffé M, Niederweis M. Multidrug resistance of a porin deletion mutant of Mycobacterium smegmatis. Antimicrob Agents Chemother 2004;48:4163–70.
[22] Danilchanka O, Pavlenok M, Niederweis M. Role of porins for uptake of antibiotics by Mycobacterium smegmatis. Antimicrob Agents Chemother 2008;52:3127–34.
[23] Song H, Sandie R, Wang Y, Andrade-Navarro MA, Niederweis M. Identification of outer membrane proteins of Mycobacterium tuberculosis. Tuberculosis 2008;88:526–44.
[24] Blanchard JS. Molecular mechanisms of drug resistance in Mycobacterium tuberculosis. Annu Rev Biochem 1996;65:215–39.
[25] Liu CH, Li L, Chen Z, Wang Q, Hu YL, Zhu B, et al. Characteristics and treatment outcomes of patients with MDR and XDR tuberculosis in a TB referral hospital in Beijing: a 13-year experience. PloS One 2011;6:e19399.
[26] Aubry A, Veziris N, Cambau E, Truffot-Pernot C, Jarlier V, Fisher LM. Novel gyrase mutations in quinolone-resistant and-hypersusceptible clinical isolates of Mycobacterium tuberculosis: functional analysis of mutant enzymes. Antimicrob Agents Chemother 2006;50:104–12.
[27] Wang JC. Cellular roles of DNA topoisomerases: a molecular perspective. Nat Rev Mol Cell Biol 2002;3:430–40.
[28] Takiff HE, Salazar L, Guerrero C, Philipp W, Huang WM, Kreiswirth B, et al. Cloning and nucleotide sequence of Mycobacterium tuberculosis gyrA and gyrB genes and detection of quinolone resistance mutations. Antimicrob Agents Chemother 1994;38:773–80.
[29] Cheng AF, Yew WW, Chan EW, Chin ML, Hui MM, Chan RC. Multiplex PCR amplimer conformation analysis for rapid detection of gyrA mutations in fluoroquinolone-resistant Mycobacterium tuberculosis clinical isolates. Antimicrob Agents Chemother 2004;48:596–601.
[30] Sun Z, Zhang J, Zhang X, Wang S, Zhang Y, Li C. Comparison of gyrA gene mutations between laboratory-selected ofloxacin-resistant Mycobacterium tuberculosis strains and clinical isolates. Int J Antimicrob Agents 2008;31:115–21.
[31] Yin X, Yu Z. Mutation characterization of gyrA and gyrB genes in levofloxacin-resistant Mycobacterium tuberculosis clinical isolates from Guangdong Province in China. J Infect 2010;61:150–4.
[32] WHO, Eastern Mediterranean. Pakistan — tuberculosis; 2018. Available at: http://www.emro.who.int/pak/programmes/stop-tuberculosis.html.
[33] Pitaksajjakul P, Wongwit W, Punprasit W, Eampokalap B, Peacock S, Ramasoota P. Mutations in the gyrA and gyrB genes of fluoroquinolone-resistant Mycobacterium tuberculosis from TB patients in Thailand. Southeast Asian J Trop Med Public Health 2005;36:228–37.
[34] Zhang Y, Heym B, Allen B, Young D, Cole S. The catalase-peroxidase gene and isoniazid resistance of Mycobacterium tuberculosis. Nature 1992;358:591.
[35] Tsukamura M, Mizuno S. Cross-resistance relationships among the aminoglucoside antibiotics in Mycobacterium tuberculosis. Microbiology 1975;88:269–74.
[36] Jugheli L, Bzekalava N, de Rijk P, Fissette K, Portaels F, Rigouts L. High level of cross-resistance between kanamycin, amikacin, and capreomycin among Mycobacterium tuberculosis isolates from Georgia and a close relation with mutations in the rrs gene. Antimicrob Agents Chemother 2009;53:5064–8.
[37] Johansen SK, Maus CE, Plikaytis BB, Douthwaite S. Capreomycin binds across the ribosomal subunit interface using tlyA-encoded 2′-O-methylations in 16S and 23S rRNAs. Mol Cell 2006;23:173–82.
[38] Chernyaeva E, Fedorova E, Zhemkova G, Korneev Y, Kozlov A. Characterization of multiple and extensively drug resistant Mycobacterium tuberculosis isolates with different ofloxacin-resistance levels. Tuberculosis 2013;93:291–5.
[39] Singh P, Jain A, Dixit P, Prakash S, Jaiswal I, Venkatesh V, et al. A novel gyrB gene mutation in fluoroquinolone resistant clinical isolates of Mycobacterium tuberculosis. BMC Infect Dis 2014;14:O14.
[40] An DD, Duyen NTH, Lan NTNL, Hoa DV, Ha DTM, Kiet VS, et al. Beiing genotype of Mycobacterium tuberculosis is significantly associated with high-level Fluotoquinolone resistance in Vietnam. Antimicrob Agents Chemother 2009;11:4835–9.