Molecular Detection of Fluoroquinolone Resistance in Multidrug Resistant *Mycobacterium tuberculosis*Using*gyrA*

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Abstract—Tuberculosis is one of the leading causes of death worldwide. WHO 2015 report indicates that Pakistan ranks fourth among multidrug resistant tuberculosis (MDR-TB) cases reportedglobally.Fluoroquinolones (FQ) are the primary broad spectrum antibiotics used for the treatment of MDR-TB and recent studies indicate a rapid rise in FQ resistance. This study focuses on the molecular detection of fluoroquinolone resistance in multi-drug resistant tuberculosis patients and the analysis of mutations occurrence in *gyrA*, at codons 90, 91 and 94.A total of 60 sputum cultured samples were analyzedthrough allele specific polymerase chain reaction (ASPCR) with self-designed primers. Among the 60 isolates examined, 36 (60%) were positive for *gyrA* mutations at codons 90, 91 and 94 while 24 (40%) were negative. The highest occurrence of mutations was found at codon 94 (37%), followed by mutations at codon 90 (20%) and at codon 91 (3%). Results indicate an increasing trend of FQ resistance in Pakistan. This study indicates molecular detection of TBto be faster and consistent and holds promise as a diagnostic tool for rapid screening of FQ resistance among MDR-TB patients to ensure its control and appropriate treatment.

Index terms— Multidrug resistant *Mycobacterium tuberculosis*, Fluoroquinolone resistance, *gyrA* gene, allele specific polymerase chain reaction, ASPCR.

1 INTRODUCTION

Tuberculosis (TB) has become the leading cause of death worldwide along with human immune deficiency virus, reportedly causing 1.5 million deaths in 2014. Global survey indicates Pakistan to be among the 6th highest burden countries and 4th highest with reported MDR-TB cases [1].Pakistan declared tuberculosis a national emergency in 2001 and continues to face TB as a major public health challenge, despite the implementation of numerous programmes and strategies to control it. In 2014, a very high 316,577 cases were notified with an MDR-TB prevalence of 78% and XDR-TB of 3.6% among the reported Drug resistant TB cases [2].

The increased resistance among TB isolates has been alarming and studies have shown spontaneous mutations in genes closely or directly interacting with anti-TB drugs to be the primary cause, enhanced by less than the required standard of health care among low income developing countries [3], [4], [5], [6], [7], leading to MDR-TB (resistant to two first line anti-TB drugs), Pre-XDR-TB (MDR-TB with resistance to one second line anti-TB drug) and XDR-TB (MDR-TB with further resistance to fluoroquinolones and one aminoglycoside) [1], [8].

Fluoroquinolones (FQ) are the most important second line anti-TB drug used to treat MDR-TB patients but increased use has led to high levels of resistance [10], leading to decreased treatment options as MDR-TB patients, who already exhibit a low cure rate and a comparatively high mortality, develop Pre-XDR-TB [11], [12].Mutations in*gyrA* are the leading cause of fluoroquinolone resistance occurring mostly within QRDR, codons 88 to 94, successfully explaining 60% to 95% of FQ resistance among isolates and associated with a high XDR potential [13], [14], [15], [16], [17], [18], [19] , [20]. Molecular studies have indicated codons 90, 91 and 94 of GyrA protein actively interacting with fluoroquinolones and therefore exhibiting the most frequent mutations to induce hydrophobic zones decreasing affinity for FQ drugs [4], [21].

This study focuses on mutation detection and analysis among codons 90, 91 and 94 of *gyrA* to detect fluoroquinolone resistance among MDR-TB isolates using Allele Specific Polymerase Chain Reaction (ASPCR). Speed and accuracy, are paramount in TB burden areas for greater control hence molecular tests being rapid and highly sensitive, are a potential diagnostic tool of great interest for large scale screening among high TB burden areas, disease diagnosis and control [22], [23]. ASPCR is fast, provides consistent results and shows great potential as a rapid diagnostic tool.

2 METHODOLOGY

2.1 Samples

60 cultured samples of confirmed MDR-TB were obtained from Gulab Devi Hopital, Lahore, Pakistan, in collaboration with University of Health Sciences, Lahore, Pakistan. Genomic DNA was extracted using TIANamp Genomic DNA isolation kit and analyzed by 1% agarose gel electrophoresis.

2.2 Primer Designing

Gyrase gene *gyrA* was translated and codons physically mapped to design specific primers. Codon 90 was amplified with primer GCG (5′- CCC GCACGG CGA CGC -3′), codon 91 with primer TCG (5′- CGC ACG GCG ACG CGT -3′), codon 94 with primer GAC (5′- CGA CGC

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GTC GAT CTA CGA -3′) and a common reverse primer (5′- ATG ACC GCG GCC AGG G -3′) were deigned.
Each PCR tube carrying 50 µl of reaction mixture contained 46.5 µl of Master mix, 2.5 µl of Genomic DNA, 0.5 µl of Forward Primer and Reverse Primer each. The thermal cycling parameters were pre-denaturation at 94˚C for 4 minutes, 40 cycles at 93 ˚C for 30 seconds, 50 ˚C for 1 minute and 72 ˚C for 45 seconds, followed by final extension at 72 ˚C for 7 minutes.

2.3 Mutational Analysis through Agarose Gel Electrophoresis

1% agarose gel electrophoresis of PCR products was done to analyze the PCR products. Successful amplification of wild type sequences produced fragments of 406 bp for codon 90, 404 bp for codon 91 and 397 bp for codon 94.

3 RESULTS

3.1 Patient profiles

In this study 60 cultured sputum samples of MDR-TB patients were examined, patient profiles indicated 18 (30%) were females and 42 (70%) were males. Their distribution in age groups was; 1 (1.6%) case of MDR-TB occurred in less than 15 years of age, 29 (48.3%) cases occurred in between 15 to 29 years, 15 (25%) of the cases occurred in ages between 30 to 44 years, 10 (16.6%) were within 45 to 60 years, and 55 (8.3%) were more than 60 years of age. The highest occurrence observed within the age group 15-29 years.

3.2 Mutational analysis

After the ASPCR of the 60 MDR-TB samples, the isolates positive for mutations in *gyrA* were 36 (60%) showing bands at target codons, while 48 (40%) were negative for this mutation. Among the samples studied, about 12 (20%) were positive for codon 90 mutation, 2 (3%) at codon 91 and 22 (37%) at codon 94. one showed a double mutation at codon 94 and codon 90.

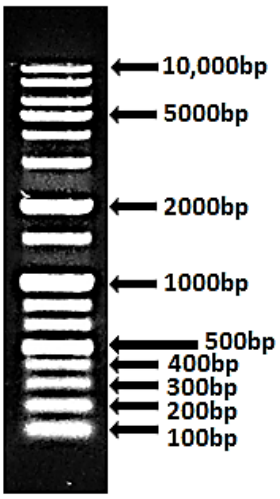

Figure 1. DNA ladder used in the current study, on 1% agarose gel electrophoresis. 1kb(+) DNA ladder, Enzynomics (Inc.).

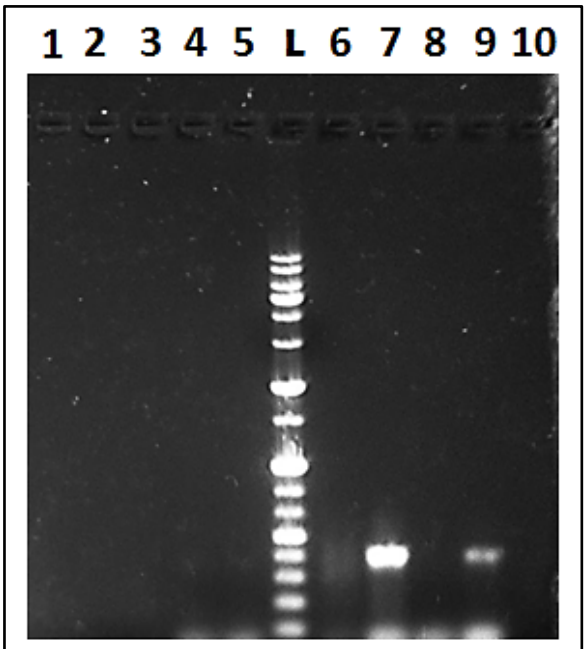

Figure 2. Representative photograph showing PCR analysis of mutations at codon position 90 in *gyrA* gene in MDR-TB patients on 1% agarose gel electrophoresis. Lane 1-5, 6, 10 show mutation positive samples; Lane 7 and 9 show mutation negative samples by creating bands at 406 bp; Lane 8 was the negative control; L: 1Kb DNA ladder.

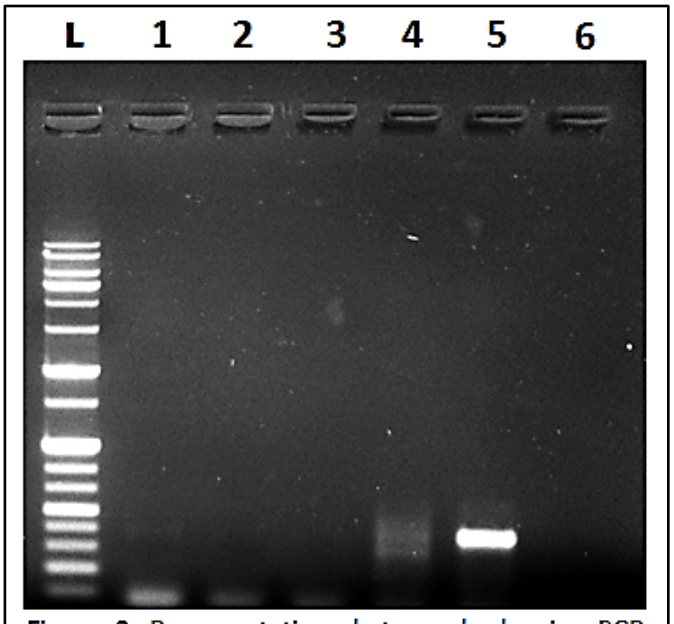

Figure 3. Representative photograph showing PCR analysis of mutations at codon position 94 in *gyrA* of MDR-TB patients on 1% agarose gel electrophoresis. Lane 5 showed mutation negative sample by creating band at 397 bp. Lanes 1, 2, 4 and 6 showed mutation positive samples; Lane 2 was the negative control; L: 1Kb DNA ladder.

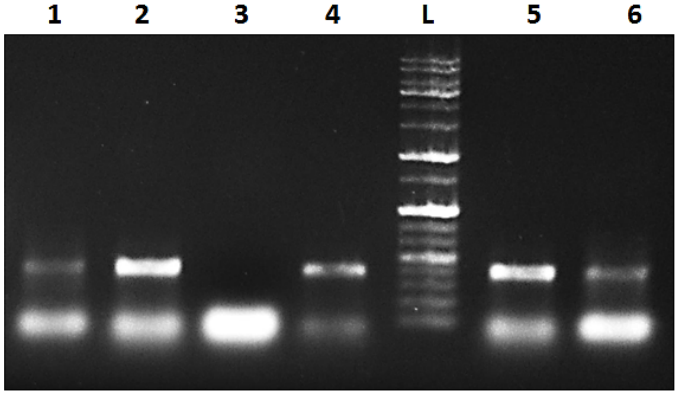

Figure 4. Representative photograph showing PCR analysis of mutations at codon position 91 in *gyrA* of MDR-TB patients on 1% agarose gel electrophoresis. Lanes 1, 2, 4, 5 and 6 show mutation negative samples by producing bands at 406 bp; Lane 3 shows the negative control; L: 1Kb DNA ladder.

4 DISCUSSION

This study aimed at the detection of *gyrA* mutations among sixty (n= 60) MDR-TB isolates to analyze the presence of fluoroquinolone resistance among the samples and study the mutational type and frequency. The patients under consideration had a history of resistance against first line anti tuberculosis drugs and were undergoing treatment with second line anti TB drugs.

The samples analyzed in this study indicated a greater occurrence of MDR-TB in males 70% as compared to Females 30%, and the maximum MDR-TB cases to lie between the age group 15- 29 years followed by 30 to 44 years. Regional studies within Pakistan support these findings. Although some studies have shown a higher prevalence of tuberculosis among women from 15–24 years, but after 40 years the male occurrence becomes significantly higher. Recent studies indicate a greater occurrence of confirmed and reported TB cases to be higher among men as compared to women, and treatment outcomes to have a greater failure rate among men as well. Sample analysis from this study also indicates a higher male TB incidence of 72% in ages greater than 40. A greater unreported TB prevalence is highly suspected among women. Reasons to this disparity have been attributed to factors such as; delay in seeking health care, seeking out low quality health care leading to unreported cases, taking care of family members sick with TB and therefore being more prone to its transmission, nutritional deficiencies and lack of awareness [24], [25], [26], [27], [28].

Previous studies in Pakistan have reported the rise in FQ resistance among the MDR-TB isolates over the years, 54%, in 2010, 57% in 2011, 59% in 2012 and an average FQ occurrence over 2010 to 2014 was about 56% [11]. The results of this study indicate the occurrence of fluoroquinolone resistance to be 60% among the MDR-TB isolates,following the increasing trend and justifying the recognition by WHO as one of the most TB burdened countries, even though the actual occurrence of TB is still unaccounted for as only half the cases are reported and recorded in international studies. The lack of facilities, public awareness and literacy causes TB cases from rural areas to go unreported. The actual TB occurrence is suspected to be double the cases recognized by statistical studies [29].

The largest frequency of mutations lied within the codon 94 being 64%, followed by codon 90 mutations at 33%, the second most frequent among the *gyrA* mutations recorded globally and codon 91 has the least mutation occurrence of 6%, consistent with multiple global*gyrA* mutational studies [14], [23], [30]. Among all the isolates, one showed a double mutation at codon 94 and codon 90, the most frequently recorded double mutation in *gyrA* [13], [14], [20], [31].

Pakistan being a developing country continues to face TB as a major public health challenge, still reporting a very high TB and drug resistant TB incidence, an MDR-TB occurrence of 32% among the total TB being treated, 3.5% among newly reported TB cases and 78% among the drug resistant TB cases [1], [32]. The occurrence of FQ resistance continues to increase and Considerable data is available reporting delayed diagnosis of FQ resistance through culture based tests and the resultant poor outcomes such as treatment failure or death of patients [11]. Improper treatment regimens are a major factor contributing to increased resistance among tuberculosis isolates. Molecular methods are being stressed upon and utilized by global studies as a rapid tool for the detection of drug resistance, being more sensitive, accurate and rapid as compared to culturing methods [22], [33]. In this study, ASPCR has shown to be fast, consistent and holds promise as a rapid molecular diagnostic tool requiring small sample volumes for better treatment regimens and tuberculosis control.

5 CONCLUSION

This study aimed at the detection of *gyrA* mutations among sixty (n= 60) MDR-TB isolates for mutational analysis and molecular detection of fluoroquinolone resistance to calculate the Pre-XDR-TB potential among patients and its impact upon the total TB burden faced by Pakistan.

About 60% of the isolates showed resistance associated mutations, 64% at codon 94, making it the most frequent mutation detected, while the mutational occurrence at codon 90 was 33% and 6% at codon 91. One isolate showed a double mutation at codon 94 and codon 90. The occurrence of MDR tuberculosis was found to be 41 (70%) in males and 18 (30%) in females. The distribution in age groups was; 1 (1.6%) case of MDR-TB in less than 15 years of age, 29 (48.3%) cases lied between 15 to 29 years, forming the highest occurrence rate among all age groups, 15 (25%) of the cases occurred in ages between 30 to 44 years, 10 (16.6%) were within 45 to 60 years, and 55 (8.3%) were more than 60 years of age.

Results of this study indicates a rise of MDR-TB occurrence in Pakistan, suggesting the need for a rapid gene based detection system here as well, since improvement in diagnostic facilities and their approach

to the general public is essential for the much required TB control in Pakistan and a fast diagnosis ensures immediate and appropriate treatment, forming one of the most essential steps for TB control. Results of this research support the superior aspects of molecular diagnosis tools, being rapid, consistent and highly sensitive, and aims to aid in the improvement of molecular diagnostic tools while adding to the scarcely available data of tuberculosis resistance in Pakistan.

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