Identification of ofloxacin-resistant *Mycobacterium tuberculosis* by PCR-RFLP and Sequencing

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Abstract: This study was planned to verify the resistance frequency of Ofloxacin (OFX) against *Mycobacterium tuberculosis* by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) technique and sequencing. Total 366 clinical samples of suspected TB patients were collected from various localities of central Punjab. All of them were found positive by ZN (Zeihl-Nelsen) staining method. Among them, 108 (29.5%) were found negative and 258 (70.5%) positive on PCR based study. The cases not responding to ATT were further characterized by proportion method and by PCR-RFLP to establish the drug resistance. Selected drug resistant case were further sequenced to confirm the results of amplified RFLP. The results showed that out of 118 drug resistant cases, 06 (5.08%), 03 (2.54%) were found resistant to OFX by drug susceptibility testing and PCR-RFLP respectively. The two strains were selected for sequencing procedure. The strain-79 showed point mutation at four points, at codon 70, 71, 76 and 78. The sequence of strain- 81 showed mutation at codon 95.PCR-RFLP is a useful molecular technique for the rapid detection of mutations and may be used to diagnose drug resistance but it should be confirmed by sequencing before starting 2nd and 3rd generation treatment because the restriction site is the cornerstone of PCR-RFLP and mutation may be occurring elsewhere.

Keywords: Tuberculosis, resistance, ofloxacin, PCR-RFLP, sequencing.

INTRODUCTION

Tuberculosis is a big malevolence tool for death and antibiotic drugs are life saving precious gift by the nature. Drug resistance became a fundamental dilemma of the globe from last several decades. MTB is a very important element of this resistant regime. In spite of the advancement in the medical research, it has been reported that about 70% of infections causing strains are resistant to at least one most commonly used antibiotic (Zell and Goldman, 2007). Multidrug-resistant tuberculosis (MDRTB) is defined as a disease caused by Mycobacterium resistant to first line anti TB drugs, including isoniazid and rifampin (Espinal *et al*., 2000). The MDRTB prevalent in a number of countries is an increasing threat to TB control, mainly in developing countries (Espinal *et al*., 2001). According to WHO (2015), 5% of TB cases were estimated to have had multidrug-resistant TB (MDR-TB) in 2014. It has been reported by CDC (2011) that in the United States, 63 cases of XDR TB have been reported between 1993 and 2011. Fluoroquinolones (FQs), ofloxacin (OFX), levofloxacin and moxifloxacin are broad-spectrum antibiotics widely used against the various bacterial infections. Most of them are also used as second line anti-tuberculosis drugs with resistance to first-line TB (Jassal and Bishai, 2009). In mycobacterium, FQs bind to DNA gyrase and inhibit DNA replication by inhibiting the movement of replication forks and transcription initiating complexes (Rathore *et al.,* 2011; Telenti *et al.,* 1997). Fluoroquinalones resistance is due to mutation in quinolone resistance determining region (QRDR) of *gyr A* and *gyr B* and severity of infection level depends upon the number of mutations (Kocagoz *et al*., 1996). Many researchers reported the different mutations at codons 84, 85, 86, 88, 90, 91, 94 and 95 as Tokue *et al*. (1995); Cheng *et al*. (2004); Shi *et al*. (2006); Cui *et al.* (2011); Zhao *et al*. (2012). There are various conventional and molecular methods have been used to detect resistant *M. tuberculosis* strains. The conventional methods are mostly based on culture assay (Abe *et al*., 2008; Mathuria *et al*., 2009). Most of the laboratories have adopted the agar proportion method, some of them used BECTEC (Becton Dickinson Diagnostic Systems, Sparks, MD) method with a liquid culture, drug susceptibility is determined by comparison of growth on a control medium (Ardito *et al*., 2001). But most of these assays are slow and time consuming and require 4-6 weeks for bacterial growth. The most rapid and advanced method has been recommended recently for the detection of drug resistance are molecular method, i.e., genotyping and sequencing (Huang *et al*., 2009). As twenty years back, many molecular methods have been developed for single nucleotide polymorphism genotyping by polymerase chain reaction, including allele specific PCR, hybridization (Iwasaki, 2002), oligonucleotide ligation (Pickering, 2002), primer extension (Meara *et al*., 2002), direct DNA sequencing. But the most convenient and less

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time consuming method is polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) which identifies location of resistant part on DNA within few hours (Abe *et al*., 2008; Mathuria *et al*., 2009). In this method amplified PCR product is digested with endonuclease restriction enzymes that cleaves the amplified DNA in different regions and produce different length and number of fragments in wild and mutant DNA (Rosdahl *et al*., 2007). Some other methods have also been used as DNA sequencing and single strand conformation polymorphism (SSCP) analysis, single gene is amplified and compared with known sequences available databases i.e., gene bank, but they require much more equipment in addition with thermal cycler and electrophoreses and still limited to research laboratories due to their extraordinary expenses. As Pakistan is among high TB burden countries with low socio-economic status, and standard protocol, procedures and techniques are not being practiced in the alignment of DOTS programme. Under these painful circumstances, the present study was planned to evaluate the ofloxacin resistance status among drug resistant cases by PCR-RFLP based technique along with supported by sequencing protocol analysis of selected strains.

MATERIALS AND METHODS

The patients having clinical symptoms with confirmed TB disease and showing positive radiograph of the chest, positive ZN (Zehil Nelson) staining were included in the study while, those with positive radio graph, negative ZN (Zehil Nelson) were excluded from the study. A total of 366 sputum samples from TB patients were collected. Out of which118 were suspected cases of drug resistance. These patients belonged to different hospitals of Faisalabad and Lahore. The experimental work was carried out in the Molecular Pathology research lab in the Department of Pathology, University of Agriculture Faisalabad, Pakistan. The study was formally approved by the synopsis scrutiny/ethical committee of the University of Agriculture, Faisalabad. The patients were informed about the study before samples and data collection.

Drug susceptibility testing by proportion method

Sputum samples were decontaminated by using Sodium hydroxide by the Modified Petroff method. Direct PCR was performed for the identification of bacteria from sputum samples by amplification of specific sequences to eliminate the samples with false positive ZN (Zehil Nelson) staining. Phenol Chloroform Method was used for the extraction of bacterial DNA from sputum samples (Sambrook *et al.,* 1989). Oligonucleotide primer set specific to 16S rRNA used for the identification of *Mycobacterium* genus and to MPB70 sequence identification of *M. tuberculosis* complex (Wilton and Cousins, 1992). The sputum samples collected from all the patients were initially cultured on LJ medium. The growth of a 1:100 dilution of the *M. tuberculosis* isolate on media without drug, with growth of the undiluted suspension on media containing drug were compared. If the undiluted suspension grows faster or more abundantly in the presence of the drug than does the 1:100 dilutions in the absence of the drug, the isolate is considered to contain a resistant population greater than 1% and is reported as resistant. The drug concentrations used in LJ medium 2.0µg/mL of Ofloxacin (Dinesh *et al*., 2012).

Drug susceptibility testing by PCR-RFLP

The oligonucleotide sets of primers were used for the amplification of *gyr A* genes (Zhao *et al.,* 2012). The PCR was carried out in a total volume of 25µl with 5ul of the template, 17µl of PCR-EZD-PCR master mix [Biobasic, Cat. No. BS294; 10mM KCl, 10mM $(NH_4)_2$ SO_4, 20mM TrisHCl, 0.1% Triton X-100, 0.1mg/ml BSA, 2mM $MgCl_2$, 200µM dNTPs), 1µl Taq DNA polymerase (Biobasic, Cat. No. B0089; 5u/µl) and 1µl each of the primers (Forward + Reverse). The tubes were placed into the thermal cycler (Qantarus) for the amplification of specific targets. The *Mycobacterium tuberculosis* control was run with these samples, which was already identified in the lab from previous study (Aslam, 2011). Amplified PCR products were electrophoresed using 1% agarose gel with 100 bp plus DNA ladder (Vivantis) at 90 V for 1.5 hours (Biorad) and visualized under Gel Doc Ez Imager (Biorad). The restriction fragment length polymorphism (RFLP) was performed on the PCR products for evaluating the drug resistance. For restriction analysis, 10 µL of PCR product, 1.0µL Taq1 (GeneMark) restriction enzymes and 2.0µL of the buffer supplied with the enzyme were used as described earlier (Zhao *et al*., 2012). The restricted products along with 100 bp ladder and negative control were run on 2% agarose gel at 90 volts for 2 hours and visualized. After restriction, the amplified fragment of *M. Tuberculosis* strains yielded two fragments were considered as wild type, while the amplified fragment of *M. Tuberculosis* strains yielded the single undigested fragment of 260 bp were considered as mutant (due to the loss of the restriction site, T↑CGA).

Sequencing and sample cleaning

The strains were randomly selected for sequence analysis among the drug resistant cases observed by proportion method and PCR-RFLP. Five µl PCR products were mixed with 1µl exonuclease and 1µl alkaline phosphatase. The products were then placed in thermal cycler with hot lid off. The cycling parameters were for 30 min at 37°C and 15 min at 80°C (Khan *et al*., 2013).

Sequencing of amplified PCR products were done by using ABI Genetic Analyzer 3730 in department of DNA Core Facility of the Centre of Applied Molecular Biology Lahore. Alignment of the DNA sequenced fragments of amplified product was carried out with the help of serial cloner software.

Table 1: Frequency PCR test and OFX resistance among total cases of drug resistance by Drug Susceptibility testing and by PCR- RFLP

Parameter		No of cases (N)	Frequency Percentage (%)	95% CI	
				Lower Limit	Upper Limit
PCR					
Negative		108	29.5	25.00	34.34
Positive		258	70.5	65.66	75.00
Ofloxacin resistance by Drug Susceptibility testing.					
1	Sensitive	112	94.92	89.73	97.91
2	Resistant	06	5.08	2.09	10.27
Mental-Haenszel Chi Square P< 0.0001; Odds ratio 154.86 = reciprocal 0.006457					
Ofloxacin resistance by PCR-RFLP					
1	Sensitive	115	97.5	93.2	99.4
2	Resistant	3	2.5	0.7	6.8
Mental-Haenszel Chi Square P< 0.0001; Odds ratio 1469 reciprocal 0.000681					

STATISTICAL ANALYSIS

The data obtained were analysed by applying the Chi-Square test and 95% confidence limits were worked out by using SAS software [SAS, 2004].

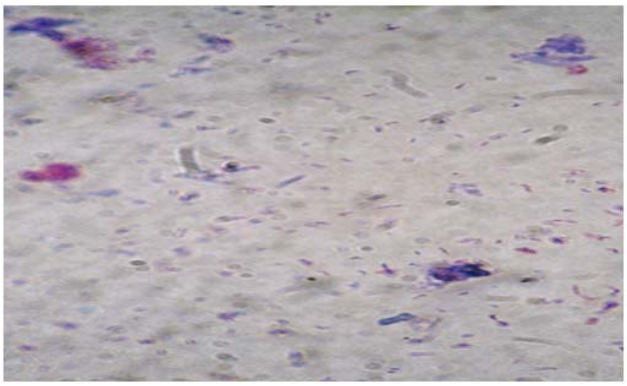
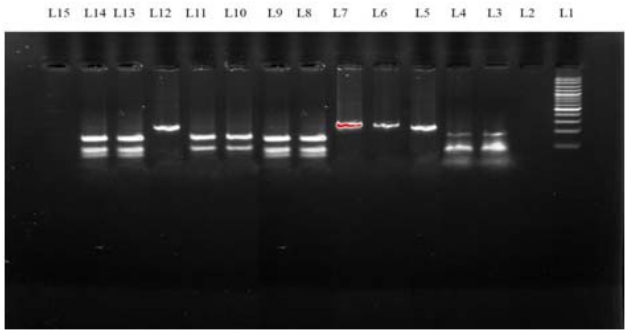


Fig. 1: ZN stained smear for Acid fast bacilli from the sputum sample showing pink colored rods of bacteria against blue back ground.

RESULTS

Out of 366, 118 suspected cases were found drug resistant. All of them were found positive by ZN (Zeihl-Nelsen) staining (fig. 1). ZN positive sputum samples were further studied with Polymerase Chain Reaction (PCR) by using specific set of primers for *Mycobacterium tuberculosis* complex. Among them, 108 were found negative (29.5%; CI 25.00 to 34.34) and 258 (70.5%; CI 65.66 to 75.00) were diagnosed positive on PCR based study (table 1). The suspected cases of drug resistance (118) which were not responding to ATT were further characterized by proportion method and by amplified restriction fragment length polymorphism (RFLP) to ascertain the drug resistance. Selected cases of these drug resistant organisms, as ascertain by amplified RFLP were further sequenced to confirm the results of amplified RFLP.

The results showed that out of 118 drug resistant cases ascertained by drug susceptibility testing, 06 (5.08%; CI 2.09 to 10.27) were resistant to OFX, while the out of 118 drug resistant cases ascertained by PCR-RFLP, 03 (2.54%; CI 0.65 to 6.76) were resistant to OFX (fig. 2) (table 1).



From right to left Lane 1, DNA Ladder; Lane 2, Negative control; Lane 5-7; un-digest PCR product, Lane 3,4, 8 to lane 11, digested *gyr A* gene PCR-RFLP product by *Taq1* restriction.

Fig. 2: PCR-RFLP results of *gyr A* gene for ofloxacin (OFX).

The two strains were selected to get further information about the mutation of gene sequence in these strains, which also showed different PCR-RFLP pattern (fig. 3 and 4). The sequences were aligned with the sequence of *gyr A* gene of *Mycobacterium tuberculosis* H37Rv. The alignment of *gyr A* sequence obtained by forward primer and reverse primer showed dis-similarities at many places. A consensus sequence was developed, which was again aligned with whole *gyr A* gene sequence of *Mycobacterium tuberculosisH37Rv* including coding and non-coding regions. The consensus sequence of strain- 79 was aligned from nucleotide 210 to 434 of *gyr A* gene, producing sequence of 224 nucleotide bases by removing errors. The consensus sequence showed point mutation at four points, at nucleotide 211 (codon 70) where transversion was noted with replacement of cytosine with

guanine, at nucleotide 213 (codon 71) where transition was noted with replacement of cytosine with thymine, at nucleotide 228 (codon 76) where transition was noted with the replacement of cytosine with thymine and at nucleotide 234 (codon 78) where transition was noted with the replacement of cytosine with thymine. The consensus sequence of strain- 81 was aligned from nucleotide 179 to 367 of *gyr A* gene, producing sequence of 188 nucleotide bases by removing errors. The alignment of consensus sequence showed mutation at one point, at nucleotide 284 (codon 95) showed transversion, where guanine is replaced by cytosine.

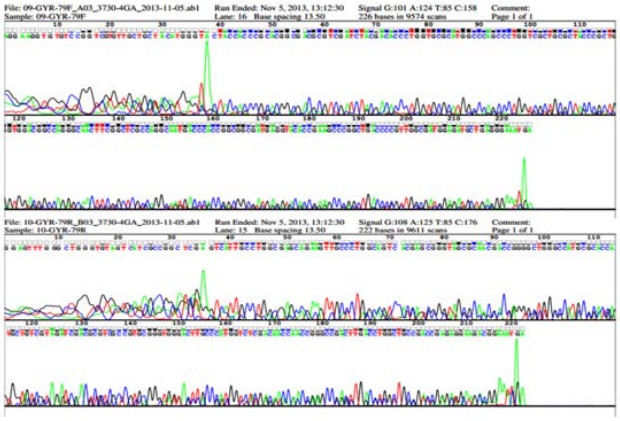


Fig. 3: Sequence analysis of *gyr A* gene of strain-79 with forward and reverse primer

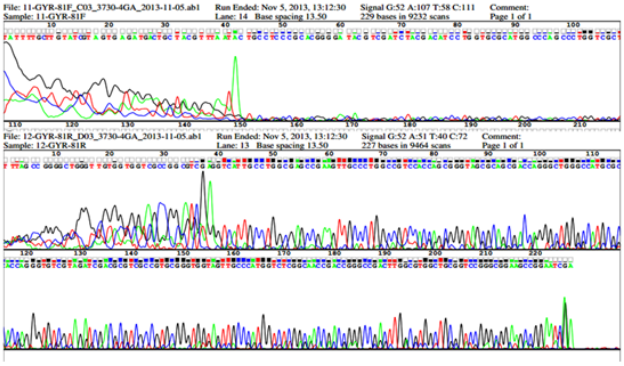


Fig. 4: Sequence analysis of *gyr A* gene of strain-81 with forward and reverse primer

DISCUSSION

A total of 118 suspected drug resistant cases, out of 366 were found drug resistant on the bases of proportion methods used for studying drug resistance with an overall drug resistance cases of 32%. The results of the present study revealed a lower percentage of drug resistance as compared with other reports as Khan *et al*. (2013) reported an overall resistance of 57% in Punjab province of Pakistan; Khoharo and Sheikh (2011) reported an overall resistance of 71.92%, while Akhtar *et al*. (2007) reported an overall prevalence of 60.5% in Sindh province of Pakistan, Assad and Alqahatani (2012) reported that 38.2% of patients were resistant to one or more than one drugs in Saudi Arabia, Sugavara *et al*. (2005) reported an overall resistance of 51.4% in China, Ndungu *et al*. (2012) reported an overall resistance of 30%, Purwar *et al*. (2011) reported an overall resistance of 47.3% in India, and Khan *et al.* (2013) reported an overall resistance of 63% in Punjab Pakistan. However, the resistance observed during present study was higher than the results reported by Nasiri *et al*. (2014) as they observed 16.3% resistance to MTB drugs in Iran, and Ayaz *et al*. (2012) observed overall resistance of 23.3% in Karachi.

The results of drug susceptibility testing revealed that out of 118 drug resistant isolates, 6 (5.08%) were resistant to ofloxacin (OFX), while according to results obtained by RFLP, 3 (2.54%) isolates were resistant to OFX and none of the OFX resistant were mono resistant. The results of present study revealed a much lower percentage of drug resistance to ultimate drug which can be used to treat MDR cases as compared with other reports as Chen *et al*. (2014) reported that out of 75 MDR isolates, 19 (25.3%) were resistant to OFX. Rao *et al*. (2010) reported that 7% isolates were MDR plus Quinolone-resistant. Purwar *et al*. (2011) reported that 10.42% of cases were resistant to OFX in India.

The alignment of consensus sequence of strain-79 and 81 showed mutation at different points as codon 70, codon 71, codon 76, codon 78 and codon 95 where transition was noted with the replacement of cytosine with thymine. Sreevatsan *et al*. (1997) reported that sequencing analysis of all flouroquinalone susceptible strains showed mutation at codon 95 and concluded that it was not associated with drug resistance. Zhao *et al*. (2012) reported different mutations, as at *gyrA*90, *gyr A* 91, *gyrA*94 and *gyrA*95 codon, while Tokue *et al*. (1995) reported different mutations at codon 84, 85, 86 and 88. Cui *et al.* (2011) reported mutations at codon 90, 91 and 94.

The results obtained by two methods, i.e., proportion and genotypic method had quite variation, where the genotypic method revealed less percentage of drug resistance. This can be related to multiple factors, including the mutation occurring in a non-coding region of drug resistant genes. The PCR-RFLP used to study the mutation in drug resistant genes mainly target the coding regions. Furthermore, the mutation may not yield increase or decrease in restriction site, which is the cornerstone of PCR-RFLP and mutation may be occurring elsewhere. Such a change will not be able to give a true picture of mutation by PCR-RFLP as a restriction enzyme will not be able to yield increase or decrease in number of bands than in drug susceptible cases. The results of sequencing support our statement as at many places mutation in amplified genes did not result in increase or decrease of restriction site(s), however in other cases it did. Therefore,

we suggest sequencing as a better tool than PCR-RFLP but yet further propose that whole gene may be sequenced for mutational changes to confirm drug resistance and such changes have to be correlated with results of proportion method. There is a dire need to have drug susceptibility testing before the start of the treatment as MDR and XDR strains are already circulating in the environment, which may be infecting people and blindly treating patients with routine ATT drugs may cause damage to liver and kidney and thus aggravate the condition of the patient. This may then complicate the situation where use of 3rd generation drugs may also not function normally under diseased liver stage, previously induced by ATT drugs and thus the patient is nearly taken to situation of no recovery. Therefore, we strongly suggest before the start of treatment every patient should undergo drug susceptibility testing by both proportion and genotypic methods and thus the patient identified who are suffering from MDR and XDR strain should be kept in separation under closed vigilance so that these strains should not spread in healthy population.

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