DRUG RESISTANCE PATTERN AND MOLECULAR CHARACTERIZATION OF *MYCOBACTERIUM TUBERCULOSIS* STRAINS IN PUNJAB, PAKISTAN

Hasnain Javed[1,2], Hafiza Jawairia Hashmi[1], Zarfishan Tahir[2] and Nazia Jamil[1]

[1]Department of Microbiology and Molecular Genetics, University of the Punjab, Lahore; [2]Provincial TB Reference Laboratory, Institute of Public Health, Lahore, Pakistan

Abstract. Tuberculosis (TB) is a cause of death from a single infectious agent *Mycobacterium tuberculosis* (MTB), leading to approximately 2.5 million deaths annually worldwide. Information regarding prevalence and pattern of drug resistance among TB patients in Pakistan remains inadequate due to the country's limited resources. This study compared conventional diagnostic techniques with a PCR-based assay targeting IS6110 sequence. In addition, MTB drug resistant profiles against four first-line drugs (ethambutol, isoniazid, rifampin, and streptomycin) from new and retreatment cases of TB. From 101 sputum samples microscopic examination of Ziehl-Neelsen-stained smears and culturing on Lowenstein Jensen medium resulted in 96% and 100% positives, compared to 98% by PCR. Prevalence of MDR-MTB was 41.5% and 58.5% among new (n = 51) and retreatment (n = 50) cases, but 10% of the former group were sensitive to all four first-line anti-TB drugs. Thus, MDR-MTB is highly prevalent among TB patients in Punjab Province, Pakistan (where the study was conducted) and, although PCR amplification of MTB-specific IS6110 sequence was rapid, it lacked the sensitivity of the culture assay.

Keywords: *Mycobacterium tuberculosis*, IS6110 sequence, isoniazid, multidrug resistance, rifampicin

INTRODUCTION

Tuberculosis (TB) is one of the most fatal infectious diseases, which has afflicted mankind for millennia. In 2014, approximately 1.5 million deaths and 9.6 million new TB cases were reported (Atif *et al*, 2016). The burden of disease is greatest in Africa and Asia (WHO, 2015). Pakistan is at the 5th position among the top 22 high burden TB countries and 4th amongst 27 multi-drug resistant (MDR) high burden TB countries in the world (Javed *et al*, 2016). TB is the main cause of mortality and morbidity in Pakistan (Turk *et al*, 2013). A probable prevalence of TB in Pakistan is 270/100,000 with a death rate of 26/100,000 and a case detection rate of 62/1,000 with 3.7% and 18% MDR TB in new and recurrent cases, respectively (WHO, 2015).

However, TB is curable if properly diagnosed and treated. Timely detection of its causative agent, *Mycobacterium tuberculosis* (MTB), is a vital factor in the control of this infection. By and large, TB is diag-

Correspondence: Nazia Jamil, Department of Microbiology and Molecular Genetics, Quaid-e-Azam Campus, University of the Punjab, Canal Bank Road, Lahore-54590, Pakistan.
Tel: +92 42 3595 3036
E-mail: nazia.mmg@pu.edu.pk

nosed through conventional methods, *viz*. microscopy examination of Ziehl-Neelsen-stained smears, chest radiography and culture technique. However, microscopy and culture-based assay are either time consuming or labor intensive (Lora *et al*, 2015). In order to overcome these difficulties, now-a-days molecular techniques for detecting MTB allow detection of the presence of MTB in any type of samples within few hours. These molecular techniques are based on PCR amplification of species- and strain-specific targets (Chaisson *et al*, 2014; Denkinger *et al*, 2014). In resource limited countries such as Pakistan, conventional microscopy and culture remain the standard diagnostic tool for TB as far as suspect cases are concerned (Denkinger *et al*, 2014).

MTB strain IS6110, identified in 1990, has a 1361-bp insertion sequence with two overlapping reading frames (orfA and orfB) and a 28-bp imperfect terminal inverted repeat. It is now considered as a contributor in MTB evolution due to its transposition ability by which it induces genome rearrangements or acts as a promoter to prompt expression of nearby genes (Lekhak *et al*, 2016). MTB IS6110 is an ideal molecular marker for PCR detection of MTB strains because of its multiple copy number and stability over long periods of MTB growth (Raveendran and Wattal, 2016).

Diagnosis of MTB and determination of its antibiogram are important measures not only for the patient but also for people in close contact, so that appropriate and timely anti-TB therapy can be initiated and thereby reduce the burden of disease in the community (Khan *et al*, 2016). Culture inoculation with subsequent phenotypic drug susceptibility testing to detect MTB drug-resistant strains is important for effective drug regimen and improvement of treatment outcome despite being much time consuming due to MTB long generation time of MTB (Geleta *et al*, 2015).

In the current study, we aim to characterize drug susceptibility pattern of MTB strains circulating in Pakistan and also to compare different diagnostic methods, *viz*. smear microscopy, culture, PCR and DNA sequence analysis, for rapid and robust identification and characterization of MTB strains.

MATERIALS AND METHODS

Sample collection methodology

From January to May 2015, 101 sputum samples from patients suspected of MDR-TB infection from different public sector hospitals of Punjab Province were sent to the Provincial TB Reference Laboratory, Institute of Public Health, Lahore, Pakistan. All samples were collected with informed consents of patients and their complete demographic information, contact history and clinical features were recorded.

Culturing and drug susceptibility testing

Samples were subsequently processed by a modified Petroff method for culture inoculation and Ziehl-Neelsen staining (Chandra *et al*, 2016). Drug susceptibility testing was performed using 1% indirect proportion method (Liu *et al*, 2016). In brief, ethambutol (ETH; 2 μg/ml), isoniazid (INH; 0.2 μg/ml), rifampin (RIF; 40 μg/ml), and streptomycin (STR; 10 μg/ml) were added to individual Lowenstein-Jensen (LJ) cultures. If growth on drug-containing medium was > 10% of the growth on the drug-free medium for STR and > 1% for ETM, INH and RIF, the strains were considered as drug-resistant. laboratory strain H37Rv was used as positive control.

PCR and DNA sequencing

MTB DNA from sediments of decontaminated sputum samples were extracted using CTAB/NaCl method (de Almeida, *et al,* 2013). All sediments were heated at 85°C for 30 minutes to kill viable bacilli. Presence of DNA was confirmed by 0.9% agarose gel-electrophoresis and ethidium bromide staining. PCR primer set was IS-F (5'CCTGCGAGCGTAGGCGTCGG3') and IS-R (5'CTCGTCCAGCGCCGCTTCGG 3') to amplify a 123 bp region of MTB insertion sequence IS6110 (Maurya *et al,* 2012). H37Rv strain was used as positive control and negative control contained sterile water. PCR mixture of 50 µl contained 25 µl of 2X Green PCR Master Mix (Thermo Fisher Scientific, East Grinstead, UK), 15 µl of nuclease-free water, 3 µl of ISF primer (10 nmol), 3 µl of ISR primer (10 nmol), and 4 µl of DNA template. Thermocycling was performed in C-1000 BIO-RAD instrument (Bio-Rad, Hercules, CA) as follows: 95°C for 5 minutes; 30 cycles of 95°C for 1 minute, 68°C for 1 minute, and 72°C for 1 minute; and a final step of 72°C for 10 minutes. PCR amplicons were analyzed by 1.5% agarose gel-electrophoresis as described above.

Gel-purified amplicons (GeneJet Gel Extraction Kit, Waltham, MA) were sequenced by 1st Base Asia Laboratories (Singapore). Sequences were aligned and compared using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and phylogenetic tree was constructed using MEGA version 6 (http://www.megasoftware.net/).

Statistical analysis

Data were compiled and analyzed using SPSS version 20.0 software (IBM, Armonk, NY). Correlation among variables such as gender, age, drug resistance and treatment history were analyzed using Fisher's exact and chi-square (χ^2) tests. A p-value ≤ 0.05 is considered statistically significant.

RESULTS

Demographic information

Mean age of the patients ($n = 101$) was 33 ± 14 years, with females ($n = 40$) 27 ± 13 years and males ($n = 61$) 38 ± 13 years. The majority of the patients were in their productive age group (14-34 years). Our study sample included suspected MDR TB subjects who were divided in two subsets: 51 (51%) were newly diagnosed TB cases and 50 (49%) were retreatment cases (Table 1). Newly diagnosed TB cases are defined as those who never received any anti-TB treatment, while retreatment cases were those who defaulted from the initial treatment, or failed to improve, or relapsed after initial treatment (Mustafa *et al,* 2016).

Microscopy and culture

Microscopy results revealed that 97 (96%) sputum samples were smear positive. The majority of MTB showed 2+ growth score (Table 2). All 101 sputum samples showed growth on LJ medium and formed colonies that were characteristics of MTB, namely, buff to yellow in color, with rough, raised, wrinkled and irregular margin. The majority of the samples were categorized as having 3+ growth (Table 2).

Drug susceptibility pattern

Based on the 1% indirect proportion method, 81% of the samples were characterized as MDR-MTB, with 41.5% and 58.5% among new and retreatment cases, respectively, with 20% and 23% among new and retreatment cases, respectively being resistant to all four first-line drugs (Table 3). However, 10% of new TB patients were responsive to all first-line drugs.

Table 1
Relatedness of demographic features with sputum multi-drug-resistant (MDR) *Mycobacterium tuberculosis*.

Feature	Number of cases (n =101)	Number of MDR cases (n = 82)	Chi-square test p-value[a]	Fisher's test p-value[a]
Age				
10-39 years	68	56	0.45	0.52
40-84 years	33	26		
Gender				
Male	61	49	0.80	0.50
Female	40	33		
Treatment status				
New cases	51	34	<0.0001[b]	<0.0001[b]
Retreatment	50	48		
Locality				
Lahore	63	45	0.38	0.36
Other cities	38	37		

[b]$p \leq 0.05$ is considered statistically significant. [b]Compared with total.

Table 2
Grading of *Mycobacterium tuberculosis* growth.

Growth grade	Number of samples analyzed by light microscopy	Number of samples analyzed on LJ medium
< 1+ (1-9 AFB/length; < 10 colonies)	9	1
1+ (10-99 AFB/length; 10-100 colonies)	22	17
2+ (1-10 AFB/HPF; > 100 colonies)	34	8
3+ (>100 AFB/1 Field; innumerable colonies or confluent growth)	33	75

AFB, acid-fast bacilli; HPF, high power field; LJ, Lowenstein Jensen medium.

PCR-based detection of MTB and sequence analysis of IS6110

From 101 MTB strains, whether MDR- or non-MDR-MTB strains, 123 bp fragments of IS6110 were successfully amplified in 99 (98%) samples (see Fig 1 for typical results). Amplicon sequences were refined and subjected to BLAST analysis. All 99 IS6110 123 bp sequences showed 95-100% similarity with MTB transposase gene (Accession number = PHA02517). A phylogenetic tree was constructed from the 123 bp sequences (Fig 2). This experiment was undertaken to determine if any heterogeneity exists among the sequences themselves and also with the reference strains (Table 4). Heterogeneity in amino acids was observed in some of the strains

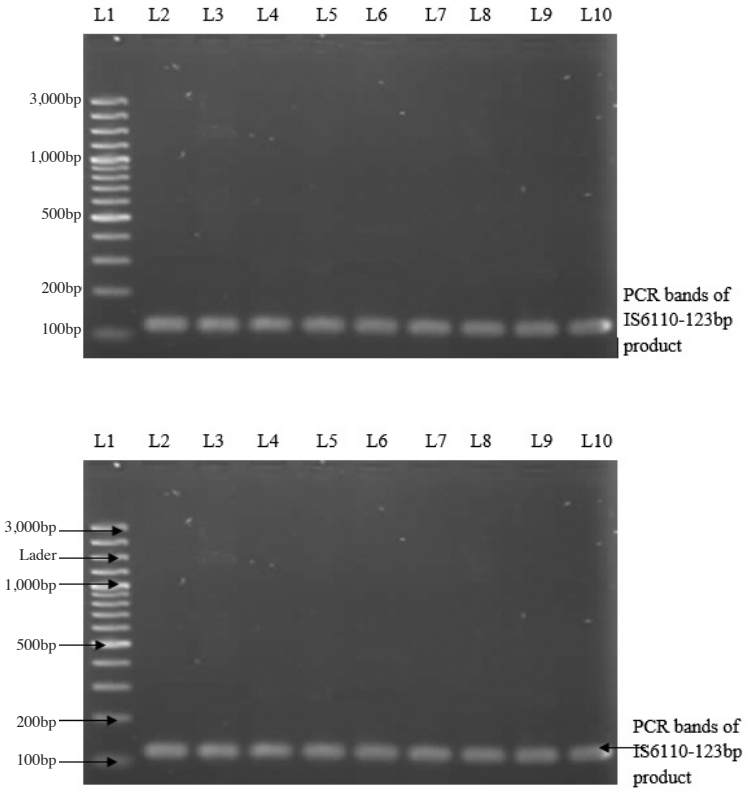

Fig 1–Gel electrophoresis of *Mycobacterium tuberculosis* (MTB) IS6110 amplicons. MTB DNA was amplified using IS6110 (transposase gene)-specific primers and analyzed by 1.5% agarose gel-electrophoresis. L1, DNA size markers; L2-L10, clinical MTB samples.

(Fig 3). These strains were chosen randomly.

DISCUSSION

The objective of the study was to compare the efficacy of conventional and molecular techniques in detecting MTB in sputum of new and retreatment patients, as well as comparing the antibiotic resistance profiles of MTB isolates between the two groups of patients. Demography data showed the prevalence of TB infection was not related to gender or locality (within Punjab Province, Pakistan), but, as expected, was more common among patients aged between 15-35 years. A recent study from Peshawar, Pakistan also reported that TB is more common in the age group 15-25 years (Iqbal *et al*, 2015), and an earlier study from Rawalpindi reported that TB is common in patients ≤ 35 years of age (Saqib *et al*, 2011).

For diagnosis of TB, sputum smear examination using Ziehl-Neelsen stain is the current technique as it is rapid, inexpensive and exceedingly specific but is less sensitive than culture, which requires a much longer time before results become known (Munir *et al*, 2015). Our results confirmed that culture method is more sensitive than Ziehl-Neelsen staining, as evidenced by the higher numbers of 3+ samples from culture than those from samples used for Ziehl-Neelsen staining. Other studies also reported the same results (Salam *et al*, 2015).

The existence of MDR-MTB was not related to age, gender or patients' locality in Punjab Province. It is worrying to note that that only 10% of new TB cases were treatable by any of the four front-line anti-TB drugs (ethambutol, isoniazid, rifampicin, and streptomycin) prescribed in Pakistan, emphasizing the necessity to determine the antibiotic resistance profile before commencing a drug regimen of treatment. Our studies confirmed the ma-

CCTGCGAGCGTAGGCGTCGGTGACAAAGGCCACATACGTAGGCGAXX*GCCAGG
TCGACACATAGGTGAGGTCTGCTACCCACAGCCGGTTAGGTGCTGGTGGTCCGA
AGCGGCGCTGGACGAGA

Fig 2–Sequence of most representative strain. X = C/T, X* = G/T (polymorphic bases).

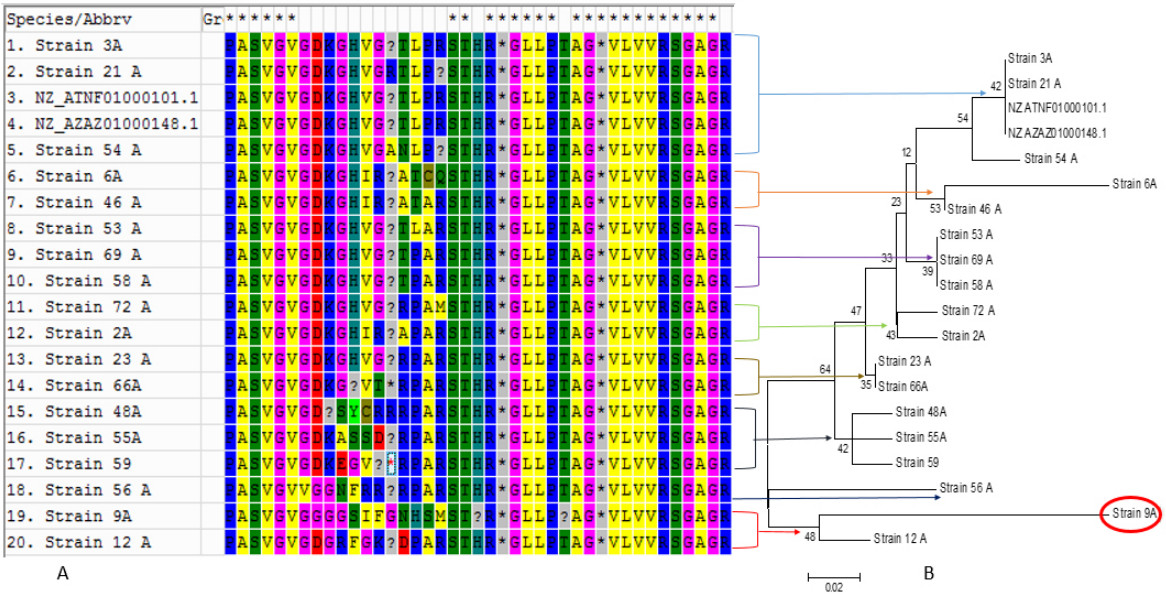

Fig 3–Comparison of genetic clusters on the basis of translated protein sequences. **A**- Aligned IS6110 sequences: different clusters are indicated with arrows of different colors. **B**- Phylogram of the *M. tuberculosis* sequences on the basis of IS6110. *The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.50949798 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method and are in the units of the number of amino acid substitutions differences per site. The analysis involved 20 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 32 positions in the final dataset. Two reference strains are from Central Asia (Accession Number = NZ_AZAZ01000148) and from Southeast Asia (Accession number = NZ_ATNF01000101). Scale bar represents the number of differences between sequences (*eg*, 0.002 means 0.2% difference between two sequences). Number at branch junction by convention refers to percent similarity. Evolutionary analyses were conducted using MEGA6 software.

jority (81%) of MTB strains were resistant to any combination of two first-line drugs (Meressa *et al*, 2015). It is not unexpected that overall drug resistance was higher in retreatment cases, with similar findings were from Pakistan and Bangladesh, India and other regions of Pakistan (Banu *et al*, 2012). In these cases infection with MDR-MTB depends, to some extent, on history of previous treatment.

Table 3
Resistance to first-line anti-TB drugs (FLDs) among TB patients, Punjab Province, Pakistan.

FLD	Number (%) of drug-resistant cases among newly diagnosed patients (n = 51)	Number (%) of drug-resistant retreatment patients (n = 50)
Inh	38 (74)	50 (100)
Rif	35 (69)	48 (96)
Stm	29 (57)	38 (76)
Emb	22 (43)	26 (52)
Inh, Rif	34 (67)	48 (96)
Inh, Stm	27 (53)	38 (76)
Inh, Emb	22 (43)	28 (56)
Rif, Stm	25 (49)	34 (68)
Rif, Emb	22 (43)	28 (56)
Stm, Emb	20 (39)	23 (46)
Inh, Rif, Emb	22 (43)	28 (56)
Inh, Rif, Stm	25 (49)	34 (68)
Inh, Stm, Emb	20 (39)	23 (46)
Rif, Stm, Emb	20 (39)	23 (46)
Inh, Rif, Emb, Stm	20 (39)	23 (46)

Emb, ethambutol; Inh, isoniazid; Rif, rifampicin; Stm, streptomycin.

Table 4
Heterogeneity in sequenced amplicons.

Strain	Deletion/insertion in sequenced amplicons
9A	40insG, 82delA, 61delT
21A	40insC, 54delG
54A	40insG,54delG
48A	40insG, 27delA
56A	40insG,42delG
66A	40insT, 32delA
59A	40insT, 38delG

Amplification of IS6110 of AFB-positive samples was successful in 99/101 samples. Two PCR reactions gave negative results due to insufficient quantity of samples.

In concludion. The study reveals that MDR-MTB is highly prevalent in all TB cases in Punjab Province, especially among retreatment patients, thus mandating that antibiogram profiles of bacilli cultures be determined. Although PCR-based identification of MTB, the use of IS6110 as target is less than 100% sensitive compared with conventional culture assay. More studies are required to develop molecular techniques comparable with

conventional well-tried methods of TB diagnosis.

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CONFLICT OF INTERESTS

The authors declare no conflict of interests.

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