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Molecular Profiling of Drug Resistant Isolates of *Mycobacterium tuberculosis* in North India

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ABSTRACT

Multidrug-resistant tuberculosis (MDR-TB) is a major public health problem because treatment is complicated, cure rates are well below those for drug susceptible tuberculosis (TB), and patients may remain infectious for months or years despite receiving the best available therapy. To gain a better understanding of MDR-TB, we characterized isolates recovered from 69 patients with MDR-TB, by use of IS6110 restriction fragment-length polymorphism (RFLP) analysis; spacer oligonucleotide genotyping (*i.e.* spoligotyping). Clinical isolates from patients with tuberculosis have been considered to contain clonally expanded *Mycobacterium tuberculosis* (MTB) strain. Over the years, the identification method based on IS6110 insertion sequences has been established as the standard for typing strains of MTB. IS6110 RFLP fingerprinting is very convincing when it is applied to classify MTB isolates harboring a large number of IS6110 in their chromosomes. Therefore, in the present study we have characterized the isolates from the patients suffering from MDR TB, on the basis of conserved Variable Number Tandem Repeats (VNTR), Direct Repeats (DR) and Insertion Sequences (IS) IS6110 elements. The polymorphic data showed significant level of dissimilarities among all the MDR isolates of MTB. Comparative studies with the DR and VNTR data substantiate that polymorphism occur among MDR-TB cases as shown by the number of repeats present in different clinical isolates.

Keywords: Mycobacterium; Drug Resistance; IS6110; Polymorphism

1. Introduction

Although Tuberculosis (TB) is a preventable and treatable disease, it remains one of the leading infectious diseases worldwide. As a result of inadequate treatment, the proportion of patients with MDR-TB is constantly increasing, and the extensively drug resistant TB (XDR-TB) has become a new global threat. One important advance in the field of tuberculosis research has been the development of molecular techniques that allow the identification and tracking of individual strains of MTB. This new discipline, the molecular epidemiology of tuberculosis, began with the identification of IS6110, a novel mycobacterial insertion sequence which formed the basis of a reproducible genotyping technique for MTB [1].

The spread of MDR-TB, due to emergence of MTB isolates has increased worldwide and reached epidemic proportion in many countries [2-4]. MDR-TB, which is caused by MTB, isolates that are resistant to, at least, Rifampicin (RIF) and Isoniazid (INH), is a serious public health hazard [5,6]. Treating MDR-TB can be difficult because loss of use of the 2 most potent anti-TB drugs (*i.e.*, INH and RIF) means that only less MDR-TB can be cured by short-course chemotherapy [7-11], for other patients, bacillary growth is merely suppressed as long as treatment is continued [9,11]. Furthermore, 8% - 35% of patients with MDR-TB have persistently active disease that is refractory to multidrug therapy [12-16]. Consequently, in most studies, the cure rates for MDR-TB remain well below those for drug-susceptible TB, and mortality rates may be substantial, even among HIV-negative patients [12]. In addition, patients with MDR-TB those do not respond to treatment are a constant source of transmission of multidrug-resistant MTB [17-20]. In contrast to most bacteria, for MTB acquisition of drug resistance does not occur as a result of horizontal transfer of resistance-bearing genetic elements. Rather, acquisition of drug resistance by MTB results from mutations (caused by nucleotide substitutions, insertions, or deletions) in specific resistance-determining regions of the genetic targets (or their promoters) or activating enzymes of anti-TB chemotherapeutic agents [21]. Inadequate therapy or sub therapeutic drug level may provide a selective growth advantage and, thus, may favor the growth of a resistant phenotype that can ultimately predominate in

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persons in whom the disease was originally caused by drug susceptible isolates [5]. Moreover, in patients with MDR-TB, selection for additional mutations may be accomplished by adding a single drug to a failing regimen [20]. In the human lung, selection of drug-resistance mutations in MTB occurs predominantly within lung cavities for which high bacterial loads, active mycobacterial replication, and reduced exposure to host defense mechanisms have been reported [20-22]. Because MTB in sputum samples obtained from patients originates from lung cavities, molecular analysis of serially recovered sputum isolates allowed us to study aspects of the genetic evolution of drug resistance in the human host.

RIF and INH are two crucial bactericidal drugs helps in clearing nearly 80% MTB cells primarily in the cavities. Other drugs, Ethambutol (EMB) and Pyrazinamide (PYZ) are supporting drugs during the initial phase [23, 24]. Therefore, immediate identification of these resistant isolates is very important for adjustments in treatment [25-27]. RIF were introduced in1972 as an anti TB drug and has excellent sterilizing activity. It acts by binding to the *β*-subunit of RNA polymerase (*rpoB*) [28], the enzyme responsible for transcription and expression of mycobacterial genes, resulting in inhibition of the bacterial transcription activity and thereby killing the organism. Mutations in the 81-bp core region of *rpoB* were reported to be responsible for resistance in at least 95% of the isolates [27,29,30]. This region is located between codons 507 to 533 with the most common changes in codons Ser531.eu, His526Tyr and Asp16 Val [30,31].

The INH enters the bacterial cell as prodrug it is activated to a toxic substance in the cell by a catalase peroxides encoded by a *katG* gene [32] and subsequently affects intracellular targets such as mycolic acid biosynthesis, an important component of the cell wall, which eventually results in loss of cellular integrity and the bacterial death.

Genetic and biochemical studies have shown that resistance to EMB is mediated by mutations in the *embB* gene, which encodes arabinisyl transferase, an integral membrane protein that is inhibited by the drug. Various studies have identified five mutations in codon 306 ATG of the *embB* gene that alter its first or third base ATG to GTG CTG/ATA ATC or to ATT, resulting in three different amino acid substitutions (Met to Val, Leu or Ile) in the EMB resistant isolates. These five mutations are associated with 70% - 90% of all *embB* resistant isolates [11,33,34]. The early and rapid detection of multidrug resistance is essential for efficient treatment and control of MTB. The culture based methods for detection of MTB infection and drug susceptibility testing usually take more than a month, due to the slow growth of this bacterium. The use of molecular methods for the identification of mutations in the genes may offers means for rapid screening of the drug resistance among the MTB isolates and initiation of early treatment [27,28,30].

In the above context, we in the present study have typed the drug resistant isolates on the basis of DR and VNTR and compared those with the standard IS6110.

2. Materials and Methods

2.1. Collection of MTB Isolates

Three consecutive morning sputum samples from each patient were collected in properly labeled screw cap disposable plastic bottles after oral gurgling with normal water. Sputum samples were processed and stained for Acid Fast Bacilli (AFB). One sputum sample from each smear positive patient was processed, inoculated on Lowenstein Jensen (LJ) slants and incubated in automated culture system at 37˚C for six weeks (**Table 1**).

The preliminary identification of mycobacterium isolates depends on their growth on LJ slants. Specific identification is accomplished by the performance of Ziehl-Neelsen (Z-N) stain and battery of biochemical tests. The positive cultures include growth in LJ medium after decontamination of sputum samples and incubation at 37˚C for 4 - 6 weeks.

MTB isolates recovered from 69 HIV-negative, and smear positive cases of both sexes, age varied from 18 to 62 years with MDR-TB that was refractory to chemotherapy given for >12 months. All subjects were selected from Department of Pulmonary Medicine, CSM Medical University, Lucknow and residents from the peripheral region of Uttar Pradesh attending OPD of CSMMU, UP. Drug susceptibility was tested every 2 - 3 months. For all patients, treatment regimens were adjusted on the basis of the results of these evaluations, at month intervals. We performed a detailed microbiological analysis of MTB isolates recovered from these patients. History relevant to tuberculosis such as time and duration, AFB load, outcome of Patients was recorded in predesigned data sheet (**Table 2**).

2.2. Drug susceptibility Testing (DST)

The phenotypic resistance of all isolates was determined at baseline. Resistance to RIF and INH was in LJ medium that contained 2 µg/ml RIF or 0.1 µg /ml. INH.

Table 1. Results of culture of smear (AFB) positive sputum specimen (n = 69).

S. No.	Results of Culture	Number	Percentage (%)
1	Growth of Mycobacteria	**69**	**87.34**
2	Contamination	03	03.94
3	No growth of Mycobacteria	04	05.06
	Total	**76**	**100**

Table 2. Profile of selected tuberculosis (TB) patients.

S. No.	Age Range (in Years)	Total M/F[a] Ratio	Duration of Treatment (in Months) Mean	Sputum AFB Load[b]	Patients Outcome Status Alive/Deceased[c]
1	19 - 30	12 8/4	18 - 55 (36.5)	1.0 - 2.5	10/2
2	31 - 40	13 11/2	21 - 82 (51.5)	2.0 - 2.5	08/5
3	41 - 50	26 24/2	20 - 54 (37.0)	2.0 - 2.5	19/7
4	51 - 62	18 15/3	06 - 14 (10.0)	2.5 - 3.0	11/07

Note: [a]Male/Female: (M/F); [b]Sputum smears were recorded as having 1 - 4, 4 - 40, or 140 bacilli/high power fields, and they were given a score of 1, 2, or 3; [c]During the study.

Other anti tuberculosis agents were also determined on LJ medium that contained critical concentration of 7.5 µg/ml EMB, 10 µg/ ml Streptomycin(SM), 5 µg/ml, Kanamycin (KM), 2 µg/ml Ofloxacin (Ofx), 10 µg/ ml Ethionamide (ETO). Phenotypic susceptibility testing for PZA was not performed. All inoculated LJ drug and control media were incubated at 37˚C for 3 weeks. The media were examined at 48 h then weekly. The reading for drug susceptibility were taken at 3 weeks after that drug deterioration and the growth on control and drug containing media were recorded according to Kent and Kubica [35]. Drug resistance was expressed in proportion method, where a strain is considered to be drug resistant if the number of colonies that grow on a drug containing media is 1 % or more of colonies that grow on a drug free media. The control media must show good growth at least 50 to 150 colonies MTB H37Rv strain has been used as a control strain (**Tables 3** and **4**).

2.3. DNA Isolation

The mycobacteria were cultured in LJ medium for 3 weeks. The cells were harvested, and chromosomal DNA was extracted by an enzymatic lysis method [36,37]. The bacteria were pelleted by centrifugation and resuspended in a 10 mM Tris-HCl-1 mM EDTA buffer (pH 8.0) [36]. Cell walls were digested with Lysozyme (10 mg/ml), Proteinase K (10 mg/ml), and 10% SDS. DNA was extracted using 0.3 M cetyltrimethylammonium bromide (CTAB) and 5M NaCl, purified by Phenol chloroform extraction. DNA was precipitated by adding 1 volume of isopropanol to the aqueous supernatant. After 30 min incubation at –20˚C the mixture was centrifuged for 15 min. at 10,000 × g, the pellet was washed once with 70% ethanol, air-dried and finally suspended in Mili Q water [38].

2.4. PCR Amplification

The primers (**Table 5**) were used to amplify the flanking regions of the VNTR, DR and IS6110 insertion sequence [38]. PCR was performed using an automated gradient thermal cycler (Bio-Rad) and all reaction buffers contained 10 mM Tris/HC1 (pH 8.3), 50 mM KC1, 1 - 5 mM

Table 3. Drug resistance and susceptibility profile for 177 patients.

Name of drugs	No. of sensitive strains (%)	No. of resistant strains (%)
Isoniazid (INH)	110 (62.14)	67 (37.85)
Rifampicin (RIF)	101 (57.06)	76 (42.93)
Streptomycin (SM)	155 (87.57)	22 (12.42)
Ethambutol (EMB)	163 (92.09)	14 (07.90)
Pyrazinamide (PZA)	Not done	--
Ethionamide (ETO)	All (100)	Nil
Kanamycin (KM)	174 (98.0)	03 (1.69)
Capriomycin (CM)	173 (97.74)	04 (2.26)
Amikacin (AM)	All	Nil
Ofloxacin (Ofx)	All	Nil
Cycloserine (CS)	All	Nil
p-amino salicylic acid (PAS)	None	--
Sensitive to all drugs	None*	--
Resistance to any drugs	--	**All Total-177**

*MDR: Multi-drug resistant: Resistance to both Isoniazid and Rifampicin with or without Resistance to other drug.

Table 4. Multidrug resistance pattern of clinical isolates to anti tuberculosis drugs.

S. No.	No. of drugs	Name of drug	No. of resistant strains	Total (%) 69:177
1	**2 Drugs**	*RIF + INH	30 (43.47)	
		RIF + SM	21 (30.43)	
		RIF + EMB	05 (07.24)	**81.15:31.63**
2	**3 Drugs**	*RIF + INH + SM	07 (10.14)	
		*RIF + INH + SM	04 (05.79)	**15.93:06.21**
3	**4 Drugs**	*RIF + INH + EMB + SM	02 (02.89)	**02.89:01.12**

Rifampicin (RIF); Isoniazid (INH); Streptomycin (SM); Ethambutol (EMB).

Table 5. PCR primers used for gene amplification.

S. No.	Primer	Sequence
1	DR0272	F-5'AGCGATCCTGCTGGTGG3' R-3'TGCTGTTAGGGTCAAACG5'
2	DR0642	F-5'CCACTAGCAGATGGCCGTT3' R-3'GCTCCAAGCGTAGTGATCCT5'
3	DR2068	F-5'CACGACGTAGACGAATGC3' R-3'ATGACACGCTTTCTGCCC5'
4	DR3074	F-5'GTCACGATTGACACGCGGT3' R-3'CATGGCCTCCGTTGTACTC5'
5	DR3319	F-5'TGGTAGGTCTGGTTCCGC3' R-3'ATGTGCATCCTCAACGGG5'
6	DR3991	F-5'CCAACCTAGGCGTGTTCG3' R-3'GATGTTCACCCCGAATGG5'
7	DR4110	F-5'TTTAGACGATCGCACCGC3' R-3'AACGGAATCGTGGTCAGC5'
8	VNTR4052	F-5'GAGCCAAATCAGGTCCGG3' R-3'GAGGTATCAACGGGCTTGT5'
9	VNTR4120	F-5'GTTCACCGGAGCCAACC3' R-3'GAGGTGGTTTCGTGGTCG5'
10	VNTR4156	F-5'ACCGCAAGGCTGATGATCC3' R-3'GTGCATCTCGTCGACTTCC5'
11	VNTR4348	F-5'ACAAGGAGAGCGGTGTCG3' R-3'CATCCTGTAGATGGCGGC5'
12	IS6110	F-5'CCTGCGAGCGTAGGCGTCGG3' R-3'CTCGTCCAGCGCCGCTTCGG5'

$MgCl_2$, 0.2 mM of each dNTP (Fermentas, USA), 2 - 5 units Taq polymerase (Fermentas, USA), l µM of each primer, and 100 ng template DNA in a final volume of 100 µl. The amplification profile consisted of a denaturation step at 95˚C for five minutes, followed by 30 cycles with denaturation at 95˚C for one minute, primer annealing at 65˚C, 67˚C and 55˚C for one minute, and extension at 72˚C for one minute. The PCR products were electrophoresed through 1.5% - 2% agarose gels and stained with ethidium bromide. Visualization was done on a UV light illuminator (Chemidoc) the copy number of the amplified products was inferred from the difference between the molecular weights of the amplified products of the samples and those of the H37Rv strain. To estimate the length of the amplified products were used to compare with standard molecular weight markers (Fermentas, USA).

In the present study we have characterized 69 isolates from the patients suffering from MDR TB, on the basis of conserved VNTR, DR and IS6110 elements. The sets of DNA primers (VNTR = #4, DR = #7 and IS6110 = #1) were designed from the MTB genome and were used to amplify the genomic DNAs of isolates. Sequences of primers listed below were used for VNTR, DR, and IS elements. The position of each locus is reported earlier [39-41].

3. Results

3.1. Patients

A total of 177 sputum smear positive pulmonary tuberculosis patients were studied. Out of 177, 76 RIF resistant cases were selected. Among 76 cases 58 were male and remaining 18 were female (76.31% and 23.68%). All of them were in the age group of 19 - 62 years (**Table 2**). Of the 76 cases, 60 (78.94%) were in low income group and only 16 (21.05%) from middle-income group. Majority, of the patients came from urban area. Of these 76 smear positive cases, culture for Mycobacteria were positive in 69 (87.34%) cases, contamination in 3 (3.97%) and no growth of Mycobacteria in 4 (5.06%) cases (**Table 1**). Some of the patients were mono drug resistant initially but they converted into MDR cases. Study was carried out on 69 RIF resistant and other drugs resistant cases.

During the study period, sixty nine patients previously had TB; none of the patients had extra pulmonary TB and Diabetes mellitus. Most patients excreted large numbers of bacilli in sputum (median score, 2.0) (**Table 2**), some patients died during the study, most likely as a result of cachexia and/or chronic respiratory failure. Patients who died had more extensive disease compared with patients who survived. At the time that TB was originally diagnosed, all patients were treated with World Health Organization category I therapy (*i.e.*, treatment with INH, RIF, PZA and EMB for 2 months, followed by treatment with either INH and RIF or INH, RIF, and PZA for an additional 4 months) for varying lengths of time [36]. Once MDR-TB was diagnosed, the patients were switched to treatment regimens tailored to the phenotypic drug-susceptibility profile of their isolates. At entry to the study, therapy was again adjusted according to phenotypic drug susceptibility, treatment history, and the side effect profile.

3.2. Phenotypic and Genotypic Resistance Profile of *M. tuberculosis*

All 69 isolates displayed phenotypic resistance to RIF and taken together the isolates from all 69 patients were highly resistant to many of the most potent first and second line agents. Identification tests for *Mycobacterium* isolates were done in accordance with the standard procedures. **Tables 3** and **4** show the sensitivity and resistance pattern of 69 strains of MTB to 4 anti tuberculosis drugs. All strains were resistant to one or more drugs. Highest mono drug resistance (42.93%) was found in RIF either alone or in combination with other drugs [43.47%]. Our study identified 30 isolates were resistant to both INH and RIF; the other 39 isolates were resistant to all the three and four drugs tested (**Tables 3** and **4**).

Genotypic results are adding power to this approach that based on the detection of DNA polymorphism within the DR cluster and VNTR- PCR are gold standard techniques for strain typing (**Table 5**) and for the study of the global molecular epidemiology of MTB (**Tables 6** and **7**). These tools provide information like latent infection, Strain-specific patterns, and drug resistance in various isolates. The Polymorphic data showed significant level of dissimilarities among all the MDR isolates of MTB. Out of 69 patients, a number of VNTR's were detected, without showing any standard profile. The polymorphism of each tandem repeat locus was found to be different; they had moderate or high allelic diversity which are useful for the differentiation of MTB strains. Molecular genotyping based on VNTR-PCR analyses has several advantages over standard IS6110 RFLP and other typing methods. Five types of DR's were amplified with each other of the primer sets used. When compared the DR and VNTR data, we could only observe that polymorphism occur among clinical isolates of MDR-TB and there are number of fingerprints present (**Figures 1** and **2**, **Table 7**).

4. Discussion

In 1993, the National Tuberculosis Program (NTP) in India was strengthened in the form of Revised National Tuberculosis Control Program (RNTCP). Like HIV-AIDS, threat perception due to occurrence of multidrug resistance has assumed considerable gravity in constructing the epidemic situation analysis and appropriate intervention. In this study drug resistance of MTB to at least one drug were found in all selected cases. This situation is highly alarming. Resistances (37.85%) were found in INH which is the most popular drug, followed by RIF (42.9%) cases. Resistances to SM were found in 12.42% cases and to EMB 7.90% cases [42-44]. The efficiency of current tuberculosis control program in any

Table 6. Grouping of clinical isolates on the basis of IS6110.

Groups	No. of Patient Samples in Which IS6110 Positive	No. of Patients Samples in Which IS6110 Negative
A	1, 2, 9, 10, 17, 19, 23, 34	-
B	3, 4, 20, 21, 22	-
C	5, 11, 13, 14, 15, 16, 18, 24, 25, 26, 27, 28 to33, 35, 38 to 46, 48 to 63, 65 to 71	-
D	6	-
E	7	-
F	-	8, 36, 37, 47, 64

Table 7. Polymorphism in pulmonary isolates with various DRs and VNTRs.

S. No.	Primers Name (DR/VNTR)	Polymorphism Shows in Patients Samples
	Direct Repeats (DR) Band Size of Primers	
1	DR0272 305 kb	3 - 8, 18 - 22
2	DR0642 231 kb	1 - 6, All bands are of same size.
3	DR2068 336 kb	6 - 12, All bands are of same size.
4	DR3074 172 kb	6 - 12, All bands are of same size.
5	DR3319 574 kb	2 - 8, 2 - 5, 7 - 9.
6	DR3991 534 kb	2 - 9, All bands are of same size.
7	DR4110 531 kb	18 - 24
	Variable Number Tandem Repeats (VNTR)	
8	VNTR4052 879 kb	4 - 10
9	VNTR4120 447 kb	5 - 11
10	VNTR4156 704 kb	6 - 12
11	VNTR4348 516 kb	7 - 14

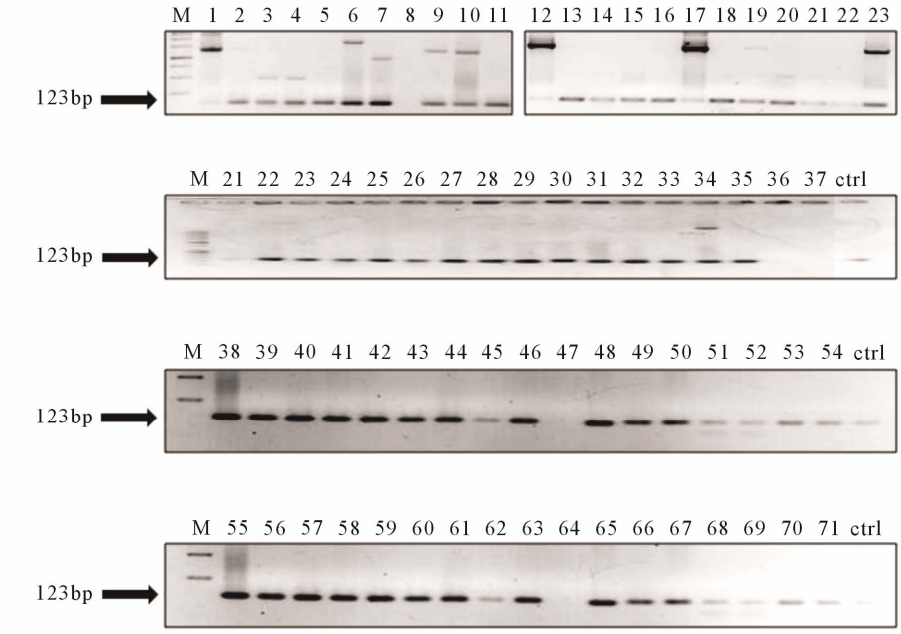

Figure 1. Primer IS6110, M-DNA ladder, lane 1-70-clinical isolates ctrl-control.

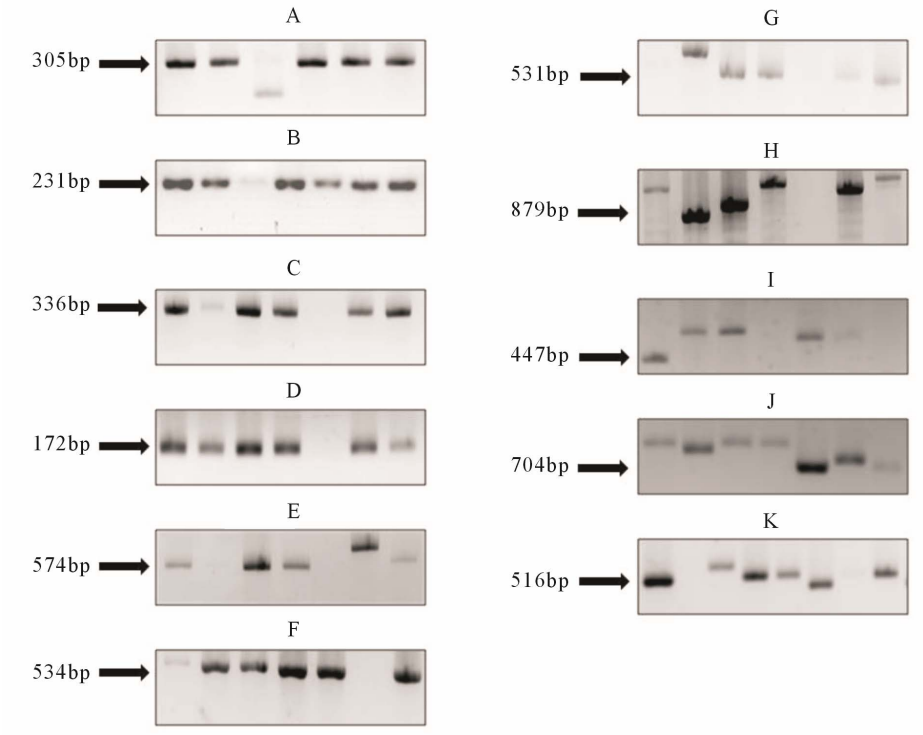

Figure 2. Polymorphism in clinical isolates with various primers; A-P1 [DR0272]; B-P2 [DR0642]; C-P3 [DR2068]; D-P4 [DR3074]; E-P5 [DR3319]; F-P6 [DR3991]; G-P7 [DR4110]; H-P8 [VNTR4052]; I-P9 [VNTR4120]; J-P10 [VNTR4156]; K-P11 [VNTR4348].

country is assayed by drug resistant pattern [45-47]. ICDDR'B, Dhaka reported resistance to any drug was 48.4%, resistance to INH, RMP, SM and EMB was 17.4%, 7.4%, 45.3% and 9.9% respectively [48]. Lina *et al.* [49] reported drug resistance to INH, RMP, SM, EMB and MDR-TB was 30.41%, 58.55%, 46.95%, 3.67% and 25.25% respectively. A similar study from Haryana, India shows MDR-TB of the same order (24%). In a recent review of the Indian situation [2] from the TRC, Chennai has concluded that the magnitude of the drug resistance

problem is principally due to acquired resistance (replaced in recent times by the term drug resistance among previously treated cases). In New Delhi, a similar extent of acquired drug resistance was reported. Institute of Thoracic medicine in Chennai had shown acquired resistance of about 63% among patients from District Tuberculosis Centers of Tamil Nadu. Resistance to INH and RIF (MDR TB) was of the order of 20.3%. It was considered 53% that initial drug resistance in India (freshly defined as, drug resistance among new cases) could be at a lower order than similarly placed countries globally, as distinct from the acquired drug resistance situation given above. There could be 5% - 10% resistance to INH, [20, 29,50,51]. This could be reflecting the primary drug resistance problem in the Indian context [2,52-54].

In this study 69 isolates resistant to two or more of the tested drug was identified. This is comparable to what has been reported in the neighboring countries, with resistance to INH and RIF being more common than resistance to EMB. The simultaneous resistance to INH and EMB that was detected in (3%) of the isolates is in agreement with previous reports [9,15,51], and the simultaneous resistance to RIF and EMB detected in (7.24%) of the isolates is consistent with a previous study [7-9]. Resistance to RIF is increasing because of widespread application that results in selection of resistant mutants, and is seen in cases noncompliant with TB treatment [51,52]. In this context, resistance to RIF can be assumed to be a surrogate marker for MDR-TB [11, 48]. Phenotypic susceptibility testing for PZA was not performed, because the results of this test can be difficult to reproduce and may not correlate well with drug susceptibility *in vivo* [12,13].

In conclusion, our results of MDR-TB underline the importance of strengthening classical case finding and treatment of smear-positive patients according to the ongoing DOTS program. The introduction of the rapid, specific and technically affordable molecular techniques can be used and interpreted in conjunction with conventional methods to detect more active cases of MDR-TB cases. The Polymerase Chain Reaction (PCR) appears to be a simple and accurate method that allows genotyping to be undertaken more quickly and in a less costly manner. It is applicable for direct detection in stained sputum smear preparations, which help in reducing the time needed for bacterial growth, and should facilitate the adequate choice of anti tuberculosis therapy [1,14,40,] that limits the extent and severity of MDR-TB transmission and infection.

INH and RIF's resistance in MTB complex (MTC) isolates are mainly based on mutations in a limited number of genes. However, mutation frequencies vary in different mycobacterial populations. In this work, we analyzed the distribution of resistance-associated mutations in MTB. The application of DNA fingerprinting can provide valuable insights into the pathogenesis of tuberculosis and may help in identifying strains of MTB with specific properties such as virulence and failure of drug response. Most of the epidemiological applications of RFLP analysis have used an insertion sequence known as IS6110 [39,52-55]. It was initially described by Thierry *et al.* [56] and has been shown to be distributed throughout the MTB complex.

Spoligotyping, in addition to IS6110 RFLP, can be useful in determining more distant relationships among isolates. In our current study, the relative instability of IS6110 RFLP was found in one of two MDR outbreak strains; however, not fewer than four of nine of the IS6110 RFLP patterns showed a minor and different alteration. Therefore, the transposition rate may be strongly related to the *M. tuberculosis* genotype represented. DNA fingerprinting of MTB has been shown to be a powerful epidemiologic tool because it exploits variability in both the no. and genomic position of insertion sequences and tandem repeats to generate strain specific patterns [2,54,56].

The integration of VNTR-typing with conventional approaches has the potential to be a powerful new technology, which provides a robust and high resolution tool for the molecular epidemiology of the MTB complex.

The direct repeat (DR) locus is the characteristic of the MTB complex. The DR locus consists of multiple tandem 36-bp repeats interspersed with variable spacers of about equal size. Polymorphism of the DR locus (absence or presence of single Direct variant repeat DVR), has been exploited widely for distinguishing among clinical isolates of the MTB by using spacer oligonucleotide typing., In the present study we have used all the three control group of genes and tried to demonstrate the differences among clinical isolates of MDR TB Isolates. In the present study, polymorphic data showed significant level of dissimilarities among all the MDR isolates of MTB. Out of 69 patients, a number of VNTRs were detected, without showing any standard profile. Similarly two types of DRs were amplified with each of the primer sets used (**Table 5**). When we compared the DR and VNTR data we could only claim that polymorphism occur among clinical isolates of MDR-TB and since there are number of fingerprints present [53-55].

Over the past decade, much has been learned of the drug targets and mechanisms of resistance to first-line and several second-lines anti tuberculosis agents (**Table 4**) [42,43,53,54]. As mentioned above, MTB generally acquires drug resistance via *de novo* nsSNP, small deletions, or insertions in specific chromosomal loci, unlike most other pathogenic bacteria, which often acquire drug resistance via horizontal transfer. This attribute of MTB drug resistance, coupled with fast and efficient DNA

sequencing methods, makes studying drug resistance highly amenable for molecular epidemiologic investigations [41,46,47,54,57]. Molecular epidemiologic studies on drug resistance have generally sought to examine the nature (e.g., genotype-specific mutations, association of specific mutations with phenotypic resistance) and extent (e.g., prevalence of specific mutations in a population) of drug resistance and patient risk factors (e.g., HIV) for acquiring resistance. The report by Bifani *et al.* [57,58] provides an example of a study of the nature and evolution of drug resistance during a clonal MDR-TB outbreak.

5. Conclusions

MTB is an obligate pathogen that does not naturally replicate outside of its host environment. As such, MTC members are believed to have coevolved with hominids for millions of years. Consequently, it is very possible that, unlike other opportunistic pathogens, viable tubercle bacilli encode the minimum ensemble of virulence genes required for successful infection, replication, and dissemination. Thus, the relative success of one clonal MTB family over another might rely on the relationship between levels of gene expression and environmental factors and the host.

Strain analysis, together with virulence studies, will help pinpointing isolates associated with higher morbidity and mortality, with the aim of directing efforts to limit the spread of those strains within the region.

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