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IS6110 AN INELUCTABLE MOLECULAR MARKER IN CLINICAL ISOLATES OF DRUG RESISTANT TUBERCULOSIS

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ABSTRACT

Background: *Mycobacterium tuberculosis (MTB)* seems to have found a liking for us human beings. The bacteria have been known to infect humans since the Vedic era and still continue to create havoc among medical and research community with no chance of resurgence in the near future. According to World Health Organization **(2016),** 0.48 million people are affected with MDR-TB globally, of which approximately 0.19 million have died. The Indian scenario is not so bright as well with 1/3rd of the MDR patients belonging to India and 2 TB patients dying every 5 minutes**. Materials and Method:** Drug susceptibility testing and genotyping of confirmed TB cases were done to identify for the polymorphism in IS6110.. The work was designed as an analytical cross-sectional study. **Result:** Our study has documented the importance/usefulness of *IS6110* in identifying DR-TB strains in clinical samples. Most isolates were resistant to single drug whereas few were to multidrug as well. **Conclusion:** The emergence of drug resistant strains (MDR/XDR) necessitated the need for better and faster diagnostic methods. Employing molecular techniques along with the conventional methods will allow us to find out new markers for diagnosis.

KEYWORDS: *Mycobacterium tuberculosis*, *IS6110*, biomarker**,** MDR/ XDR.

1. INTRODUCTION

Tuberculosis (TB), the disease caused by *Mycobacterium tuberculosis* (MTB), is one of the leading causes of mortality in developing and under-developed countries apart from HIV-AIDS. And now with the co-emergence of both of the diseases (TB and AIDS) the situation has taken a down side in the once TB free developed countries as well. Globally around 9.6 million people are affected with TB whereas in India as far as 40% of the individuals are under its influence. Various TB control efforts have been started, but the emergence of drug resistant (DR) strains have created an unwanted pressure on such programmes.[1,2,3] Treatment of DR strains understandably requires a rigorous treatment plan both in terms of time and drug. It is a phenomenon defined by the ability of a microbe to remain viable in the presence of a drug to which it was previously sensitive. Multi Drug Resistant (MDR) TB is the case where patient whose sputum is culture positive for MTB has *in vitro* resistance to Rifampicin (R) or Isoniazid (H) with or without anti-TB drugs based on drug susceptibility (DST) results from an Revised National Tuberculosis Control Program (RNTCP) certified culture and DST laboratory.[4] Extensively Drug Resistant (XDR) TB is an MDR-TB case whose MTB isolate is resistant to at least Rifampicin, Isoniazid, a fluoroquinolone (FQ) and a second line injectable anti-TB drug at a RNTCP certified laboratory.[5, 6]

Similar to other prokaryotes, MTB also contains insertion sequences *(IS)* in its genome.[7] Insertion sequences are different from typical transposons in two ways. Firstly *IS* are relatively smaller in size and secondly they simply encode for proteins responsible for transposition (jumping of genes). *IS6110*, a mobile MTB insertion sequence, has been identified as one of the very useful tool in diagnosing TB.[8] Studies in the recent past have given ample proof that it's not just a passive element; instead it actively contributes to phenotypic diversity among different strains of MTB and even alters the gene expression.[9,10]

IS6110 due to its high numerical and positional polymorphism has become a widely used marker in the epidemiological studies for the members of *Mycobacterium tuberculosis* complex (MTBC). Due to the genotypic variation created via its transposable activity this translates into a strain specific phenotypic variation.[11] The benefit of this could be enormous for setting up treatment plans. If a specific strain is identified accurately, the drug regimen could be adjusted accordingly. As per the current treatment regime followed all the suspected cases are primarily treated with first line drugs.[5,12] Subsequently after 2-3 months when it is confirmed that the strain causing disease is a resistant phenotype that the regimen is changed. Apart from this different individuals infected with bacteria (either sensitive or resistant) could very well contain different strains. With the emergence of *IS6110* as an epidemiological study marker many problems like drug resistance, side effects of TB drugs could be heavily reduced if not completely done away with.[13]

Keeping in mind the aim of this project analytical observational cross-sectional study was designed. The application of DNA fingerprinting/RFLP can provide valuable insights into the pathogenesis of TB and may help in identifying strains of MTB with specific properties, such as tissue tropism, virulence and failure of drug response.**[13, 14, 15]**

2. METHODOLOGY

The total number of samples collected for the study was 109.

2.1 SETTING

The study was conducted in Lucknow with the support of King George's Medical University (KGMU), Lucknow, UP and CSIR-Central Drug Research Institute, Lucknow for sample recruitments, data collection, molecular work and result analysis.

2.2 STUDY PLACE

Lucknow and its peripheral regions.

2.3 STUDY POPULATION

All newly diagnosed drug resistant patients attending KGMU OPD and admitted in the hospital from **July 2015 to November 2015** registered under RNTCP treatment. The recruited cases had their chest X-ray diagnosis, sputum microscopy done along with positive MTB culture and voluntary informed consent.

2.4 STUDY VARIABLES

- Two dependent variables: MTB strain genotype & drug resistance.
- Two independent variables: Demographic factors & symptoms of tuberculosis **(data not shown).**

3. SAMPLE AND DATA COLLECTION

Patient's willingness to participate in the study was confirmed by them signing the consent form.

3.1 INTERVIEW

Each TB patient was provided with an interview form. It was in easy to understand English language. If required the patients were made to understand the questions in Hindi. Anonymity and confidentiality of patients was maintained.

3.2 SPUTUM COLLECTION

The sputum was collected from all newly diagnosed and RNTCP registered Drug Resistant-Pulmonary Tuberculosis (DR-PTB) patients. The sample was collected in properly labeled screw cap disposable plastic bottles after oral gurgling with normal water at 8 to 24 hour intervals. Samples were collected for 3 consecutive mornings from each patient. Sputum samples were processed and stained for Acid Fast Bacilli (AFB).[16]

3.3 SPUTUM INDUCTION

Patients unable to cough up sputum on their own were given an aerosol of warm, sterile, hypertonic saline (3%-5%). It was labeled "induced" to ensure that the laboratory staff workers did not discard it,**[3,16,17]**

3.4 SPUTUM PROCESSING

Following the guidelines of IUALD (2000) [16] N-Acetyl-L-Cysteine-Sodium Hydroxide (NALC-NaOH) method /Petroff's method was employed and LJ medium was used to culture the bacteria.[3]

4. DIAGNOSTIC TESTS

4.1 CHEST X-RAY

4.2 SMEAR MICROSCOPY

Smear microscopy was done following RNTCP standard methods that are based on the guidelines from WHO.**[18]**

4.3 SPECIMEN CULTURE & MICROSCOPY

MTB from various clinical isolates of PTB were cultured on LJ slants at 37^0C until growth was observed (usually within 3-4 weeks)**.** It was a preliminary test and positive results confirmed the TB disease. The patients were given the treatment accordingly on monthly basis**[18]**

4.4 DRUG SUSCEPTIBILITY TESTING OF MTB ISOLATES

Susceptibility to the first line anti-tubercular drugs was tested for all the clinical isolates. Drugs used for the procedure were namely Rifampicin (R), Isoniazid (H), Ethambutol (E) and Streptomycin (S). The phenotypic resistance of all drugs was determined at baseline. Critical drug concentrations for drugs used were 0.2 µg/ml for H, 40 µg/ml for R, 2 µg/ml for E and 4 µg/ml for S**.** The inoculated LJ slants and control media were kept under standard conditions and checked every 48 hours followed by weekly observation. After 3 weeks the growth in control and drug containing media were recorded according to **Kent and Kubica (2000).[19]** The test was performed every 2-3 months and MTB H_{37}Rv strain was used as control.

Proportion method was carried out to indicate the results. Proportion method is a method where in a strain is considered to be drug resistant if the number of colonies that grow on drug containing media is 1 % or more of colonies that grow on a drug free medium. In control media 50-150 different colonies were obtained.

5. GENOTYPING OF CLINICAL ISOLATES

5.1 ISOLATION OF DNA

Isolation of genomic DNA from recruited clinical isolates was performed and standard protocol was followed for the purpose.[20]

5.2 PRIMER DESIGN

The sets of DNA primers of IS*6110* (*F5'CCTGCGAGCGTAGGCGTCGG-3'/R-3'CTCGTCCAGCGCCGCTTCGG5')* were designed and were used to amplify the genomic DNA of isolates. The position of each locus has been reported earlier.[21]

5.3 PCR AMPLIFICATION

The reagents used for the purpose were bought from GENETIX. Analysis and visualization of the PCR products was done in 1% agarose gel under the UV light transilluminator respectively. By comparing the difference between the molecular weights of the amplified products and that of control $H_{37}Rv$ strain, the copy number of amplified products was obtained. Length of amplified products was compared with the nucleic acid markers (Fermentas) [20].

6. RESULTS

A total of 109 sputum samples were collected from the DR-PTB cases attending the OPD in the Dept. of Respiratory Medicine, KGMU, Lucknow. Patients presented the symptoms prior to diagnosis, which were, sputum produced by cough(92.4%), fever(55.6%), weight-loss(50.2%), chest pain(49.8%), loss of appetite(38.8%), fatigue and weakness(30.2%), shortness of breath (22.5%), Haemoptysis (5.85%.).

All the samples included were positive for MTB and negative for HIV. Also none of the patients had Extra-pulmonary tuberculosis (EPTB) and Diabetes. Most of the clinical samples showed resistance to majority of the first line drugs. Some strains even showed resistance to more than one drug.[23]

Out of total 109 patients 17 (15.6%) were new cases (Group I). When comparing the patient's previous treatment history maximum number of cases (63.3%) was relapses due to inadequate treatment/incomplete treatment regimen (Group II) followed by the relapses in adequately treated cases (21.1%) (Group III).

Table 1: Distribution of cases according to history of previous treatment status

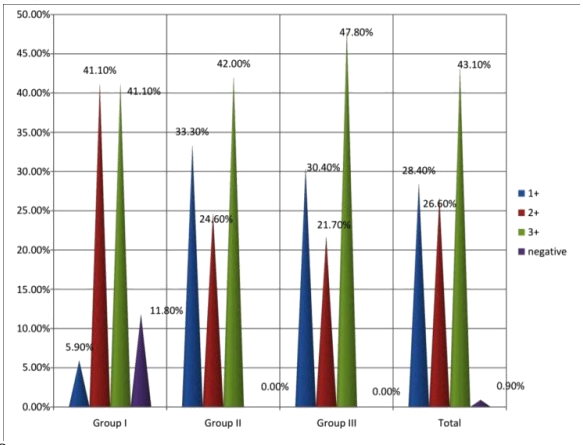
SN	Previous treatment history	Group	No. of cases	Percentage
1.	Not treated	I	17	15.6%
2.	Inadequately treated	II	69	63.3%
3.	Adequately treated	III	23	21.1%

Generally in case of recurrence, in completely/ adequately treated cases DR-MTB is known to cause the infection. There was significant difference seen in the age of the patients and previous treatment status; with younger people as expected responding better to the new treatment provided.

Analyzing the results of Group I, II and III for association between previous treatment status and sputum positivity, it was seen that all the cases were MDR positive with more cases falling under 2+ & 3+ [16]. All the groups showed complete resistance to rifampicin and extremely high resistance to isoniazid.

Table 2: Association between previous treatment status and sputum positivity

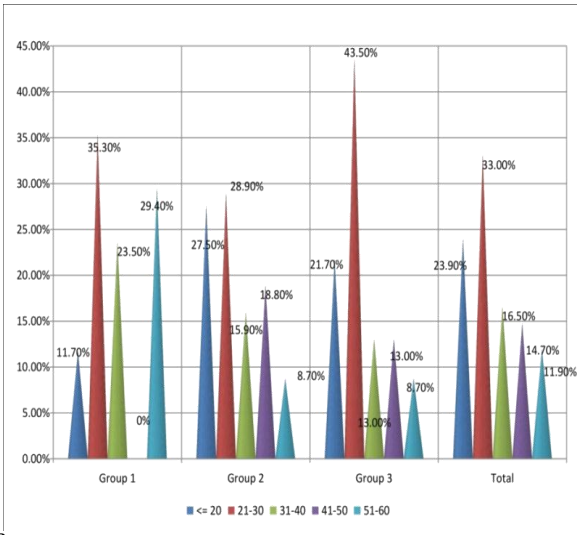
SN	Sputum Positivity	Group I (n=17)		Group II (n=69)		Group III (n=23)		Total (n=109)	
		No.	%	No.	%	No.	%	No.	%
1.	1+	1	5.9	23	33.3	7	30.4	31	28.4
2.	2+	7	41.1	17	24.6	5	21.7	29	26.6
3.	3+	7	41.1	29	42.0	11	47.8	47	43.1
4.	Negative	2	11.8	0	0.0	0	0.0	1	0.9



χ^2=11.05 (df= 6); p=0.087 (NS)

Table 3: Association between previous treatment status and age

SN	Age Group (Years)	Group I (n=17)		Group II (n=69)		Group III (n=23)		Total (n=109)	
		No.	%	No.	%	No.	%	No.	%
1.	≤20	2	11.7%	19	27.5%	5	21.7%	26	23.9%
2.	21-30	6	35.3%	20	28.9%	10	43.5%	36	33.0%
3.	31-40	4	23.5%	11	15.9%	3	13.0%	18	16.5%
4.	41-50	0	0%	13	18.8%	3	13.0%	16	14.7%
5.	51-60	5	29.4%	6	8.7%	2	8.7%	13	11.9%
Mean Age±SD (Range)		32.27±14.91		33.23±14.32		32.27±14.91		33.64±14.91	

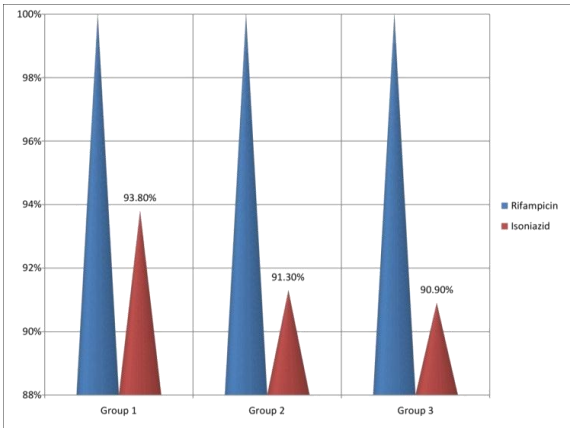


χ^2=14.861 (df= 10); p=0.137

As it could be clearly seen in the data collected for the experiment maximum percentage (63.3%) of cases were found in group II i.e., patients with inadequate treatment history. They were found to be 100% resistant to rifampicin and 91.3% resistant to isoniazid.

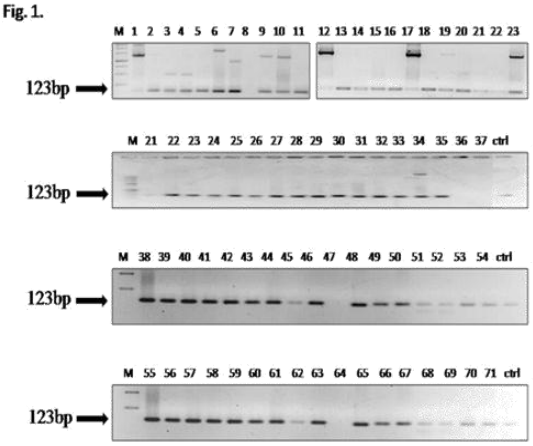
Table 4: Association between previous treatment status and specific ATT resistance

SN	Variable	Group I (n=17)		Group II (n=69)		Group III (n=23)		Statistical significance	
		No.	%	No.	%	No.	%	χ^2	"p"
1.	Rifampicin	17	100	69	100	22	100	-	-
2.	Isoniazid	15	93.8	63	91.3	20	90.9	0.117	0.943



Genotypic resistance profiling was specifically done for the multidrug resistant cases**.** Comparative analysis findings about *IS6110* have shown the presence of variation in this gene, which could very well be responsible for the specificity of different MTB strains to different anti-tubercular drugs.

Fig. 1.



Various clinical isolates of MDR possessing the IS6110 copy

These tools provide information like latent infection, strain- specific patterns, and drug resistance in various isolates**.** The polymorphic data showed significant levels of dissimilarities among all the MDR isolates of MTB. They had moderate or high allelic diversity which are useful for the differentiation of MTB strains [22, 24].

7. DISCUSSION

Discontinuation of treatment by patient leads to the exposure of MTB to sub-optimal dose of the drug there by permitting bacteria to make antigenic changes against that drug. Late detection of drug resistant strain by primary health care provider and over-use of anti tubercular treatment (ATT) in high –risk TB areas is also a major contributing factor to the emergence of DR.[26,27] Excessive use of drugs allow MTB to acclimatize to the rough environment created by drug inside host and over the given period of time as an evolutionary process the resistant strains are selected over the sensitive ones. This could be explained by the

"fall and rise phenomenon" [28, 29]. Initially when the drug is given to a patient, population comprising of sensitive strain declines but after a certain period of time the resistant strain population starts increasing thereby leading to the recurrence of disease.

Table 3 is clearly an indicative of MTB acquiring resistance to drugs and thereby converting into a DR strain.[29] Treating DR strains has its own disadvantages like the time period increases substantially thereby increasing the side-effects of drugs on the patients. Cost of treatment also goes-up owing to the long treatment duration and relatively expensive drugs. This becomes a hefty burden for patients coming from economically poor background. Hence a proper counseling of patients during the start of treatment must be considered, guiding them about completing the full course of treatment and drawbacks of not completing it.[5, 30]

Fixed dose combination (FDC) incorporating two or more anti-TB drugs into one tablet in fixed proportions have been used since late 1980s and is registered in more than 40 countries. Use of FDC, particularly in 3-4 drug combinations results in lower plasma levels of rifampicin with consequent treatment failures, relapses and/or emergence of rifampicin-resistant strains of MTB. Only FDCs for which bioavailability studies have been undertaken in human subjects should be used.[23]

Resistant strains are known to vary slightly among themselves in their genetic makeup. This variation could also be responsible for resistance shown to different class of drugs.[24,31] One way of determining it is by analyzing specific molecular markers found in *Mycobacterium tuberculosis* complex (MTBC) group, like *IS6110;* hsp65; *IS1081*; PGRS and MIRU. In our study we have studied *IS6110* gene, an *IS*, belonging to a larger family of *IS3.* Copies of *IS6110* can be found at 16 different positions in the genome of MTB $H_{37}Rv$ providing an important epidemiological tool.[10] In some strains as much as 25 copies are reported to be present in MTBC whereas in a few strains there are none.[32]

IS6110 is 1361 bp long and shows 28 bp imperfect IRs. It contains 2 overlapping *ORFs (orf A &orf B)* which code for the enzyme transposase. Despite the divergence seen in the *IS6110* sequences within the MTB strains, it is still a favorite target sequence for the diagnosis and epidemiological investigations to properly categorize mycobacteria.[33] Many functions have been attributed to *IS6110* which are activation of genes during infection, participation in evolution as an epidemiological marker and activation of downstream genes with an activity promoter.[10, 31, 34]

High degree of polymorphism seen in *IS6110* has made it highly useful in comparing different strains of MTB. Different isolates of MTB might have variation in *IS6110*, which could be a major contributing factor in patients responding differently to same drug. The patterns could be determined by the current molecular techniques employed for the epidemiological studies. Performing it could confirm the type of strain infecting patient and therefore the treatment regimen could be set accordingly. This could avoid the compulsory treatment for sensitive strain given to all the suspected TB patients; in the process stalling the further emergence of drug resistant cases.

In a hospital setting in a developing country like India; where the disease (TB) burden is very high, detecting and generating a profile via DNA amplification certainly boosts up the diagnostic efficiency at a relatively lower cost. Though it is an undecorated technique, it could be used in more than one ways to identify the mutations in the clinical samples efficiently. Polymerase chain reaction (PCR) appears to be a fast and accurate method that allows genotyping to be undertaken more quickly and in cost efficient manner.[32,34] Conventional diagnostic and identification methods though reliable are highly time consuming, and there is urgent need to use the molecular techniques which are fast to perform; because they give an accurate data. The technique has been employed in our study to generate the polymorphic profile of *IS6110* in MTB **(Fig. 1)**.

Hence it could be promulgated that *IS6110* is still a quite reliable marker for identifying different strains of DR-MTB and timely identification of resistant strains could lead to better treatment plans.[2,4, 32]

8. CONFLICT OF INTEREST

None declared

9. ACKNOWLEDGEMENT

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