Original Article

Assess drug resistance pattern and genetic profile of Mycobacterium tuberculosis clinical isolates by molecular typing methods using direct repeats and *IS6110* in pulmonary tuberculosis cases

Deepika Kalo, Surya Kant[1], Kanchan Srivastava[1], Ajay K Sharma

Department of Zoology, University of Lucknow, [1]Department of Respiratory Medicine, King George's Medical University, Lucknow, Uttar Pradesh, India

ABSTRACT

Background: Tuberculosis (TB), a highly contagious disease that sees no gender, age, or race is mainly a disease of lungs. According to World Health Organization, a TB patient can be completely cured with 6–9 months of anti-TB treatment under directly observed treatment short course. **Objectives:** The aim of this study was to check the mono, multi- and triple-drug resistance to first line drugs (FLDs) among TB patients and to access their genetic profile using DR 3074, DR 0270, DR 0642, DR 2068, and DR 4110 using molecular techniques. **Material and Methods:** To gain a better understanding of drug resistant TB, we characterized 121 clinical isolates recovered from 159 drug resistant pulmonary tuberculosis patients by *IS6110* genotyping. MTB isolates recovered from HIV- negative, and smear positive cases of both genders, age varied from 18 to 70 years with drug resistant-TB that was refractory to chemotherapy given for > 12 months. Of a total of 159 sputum smear positive patients sum number of male and female patients was 121 (76.10%) and 38 (23.89%), respectively. Among these patients, number of literate and illiterate patients were 123 (77.3%) and 36 (22.6%). 25 (15.7%) patients had farming as their occupation, 80 (50.3%) had nonagricultural occupation and 54 (33.9%) women were housewives. **Results:** Mono drug resistant, multi-drug resistant, and totally drug resistant (TDR) cases of TB were calculated as 113.83%, 125.1%, and 67.9%. Isoniazid showed the highest percentage of resistance among the patients. **Conclusion:** Any noncompliance to TB medications, lack of knowledge, and poor management in health centers, etc., results in the emergence of deadly direct repeat forms of TB, which are further complicated and complex to treat.

KEY WORDS: Drug resistance, isoniazid, molecular typing, *Mycobacterium tuberculosis*

Address for correspondence: Dr. Surya Kant, Department of Respiratory Medicine, King George's Medical University, Lucknow - 226 003, Uttar Pradesh, India. E-mail: skantpulmed@gmail.com

INTRODUCTION

Tuberculosis (TB), one of the oldest and contagious infectious diseases is a major cause infection related morbidity and mortality. Worldwide, the disease TB ranks at 1st number as the infectious disease after human immunodeficiency virus (HIV)[1] and India accounts for the second most populous country in the world with one-fourth of the global incident TB cases annually.[2] India accounted for 27% of global TB notifications in 2014, followed by China (14%).[3] According to World Health Organization (WHO) reports; worldwide, TB has engulfed about 9 million people out of which 1.1 million were HIV

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infected.[4] The most commonly observed age group among TB patients is between 15 and 59 years.[5]

The causative agent of TB, *Mycobacterium* TB (MTB) is acid fast bacilli, rod-shaped nonmotile; obligate intracellular pathogen whose length and width are 2–4 and 0.2–0.5 μm, respectively. The bacterium may have killed more persons than any other microbial pathogen and is having the capacity to cause both symptomatic as well as asymptomatic infection.[6] The transmission takes place via inhalation of aerosol droplets by a healthy person expelled by the infected host. According to studies, around 70% of patients with sputum smear-positive cases of pulmonary tuberculosis PTB, died within 10 years.[5] The disease takes no time spreading to other parts of the body viz. brain, lymph nodes, nervous system, bones, etc., and the condition are referred as "extrapulmonary TB (EPTB)."[7] Everyone infected with TB bacteria does not become sick and who are infected, but not sick, have latent TB infection.[8]

At times the excreting bacilli become resistant to one or more anti-tubercular drugs, the case is then referred to as drug-resistant TB (DR-TB). DR-TB can take place in several forms: Mono-resistance, poly resistance, multi-drug resistant TB (MDR-TB), extensively drug resistant TB, and totally drug resistant TB (TDR-TB). It can take place either in primary or secondary form.[9] When MTB become resistant to any one first line anti-TB drug (FLD) the case is called Confirmed mono-resistance. The FLDs are Isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA), ethambutol (EMB), and streptomycin (SM).[10]

India accounts for more than 50% of global MDR-TB cases. The rising trend of DRTB can be clearly observed in India. It contributes to more than 50% of global MDR-TB cases.[11] Direct repeat (DR) among previously treated patients is rising due to noncompliance to TB medications, lack of knowledge, poor management in health centers.[12] According to WHO report, out of six other countries, India (2.0 million–2.3 million) was reported to be at the first position of high incidence of TB.[4] According to WHO report statistics (2013), the estimated prevalence and incidence for TB in India was recorded as 2.6 million and 2.1 million respectively out of the global incidence of TB recorded as 9 million.[4]

The first-line drugs are considered as a boon in the treatment of TB patients and any mismanagement in consumption of these drugs results in a serious health hazard. To study the epidemiology of MTB, based on its DNA polymorphism various molecular techniques have been established. These include DR, variable number of tandem repeat typing, insertion sequence 6110 fingerprinting (*IS6110*), spoligotyping, and much more.[13]

Study design

The aim of this study was to check the mono, multi- and triple-drug resistance to FLDs among TB patients and to access their genetic profile using DR 3074, DR 0270, DR 0642, DR 2068, and DR 4110 using molecular techniques.

MATERIALS AND METHODS

Study population

After a verbal consent from one hundred fifty-nine (159) PTB patients showing ZN stained smear positive who had visited the hospital for diagnosis and treatment were included in the study. The study was carried out at King George's Medical University, Lucknow, Uttar Pradesh, India, and sampling was done from April 2014 to October 2014.

Patients collected their sputum samples in a labeled sterile disposable container as directed and were also advised to collect it every morning after rinsing their mouths with plain water for 3 subsequent days. Strict exclusion and inclusion criteria were taken into concern. Subjects who satisfied the criteria were included in the study whereas; those not fulfilling the criteria were excluded from the study. New pulmonary TB patients of both the sexes, HIV^-, aged between 18 and 70 years who agreed to participate were included and patients who had EPTB, HIV^+ and did not agree to involve their selves were excluded from the study.

For identification of *Mycobacterium* isolates, sample smear positivity test was done. Isolation and identification was done by the conventional methods and thus subjected to drug susceptibility testing (DST) against the FLDs by the proportion method against INH (0.2 μg/ml), EMB (2 μg/ml), SM (4 μg/ml), and RIF (40 μg/ml).[14] Incubation of samples was done at 37°C for 6 weeks which therefore produced a visible growth on the LJ slants and helped in the identification of the *Mycobacterium* isolates. DST using proportion method was performed to check the mono, multi, and triple DR among TB patients.

RESULTS

Patients

A total of two hundred patients were studied. Sputum sample of all the patients was collected aseptically and scientifically. Of 200 patients, 159 cases were found to be sputum smear positive. This was analyzed by ZN staining method. Pink colored, rod-shaped TB bacilli were observed under microscope. All the subjects were interviewed based upon for information on characteristics of education, occupation, and residence. Among these patients, number of literate patients were 123 (77.3%) and 36 (22.6%) as illiterate. 25 (15.7%) patients had farming as their occupation, and 80 (50.3%) had nonagricultural occupation. Furthermore, 54 (33.9%) women were housewives. 122 patients (76%) were reported from urban areas and 37 (23.2%) from rural areas. Most of the patients were males [Table 1].

In TB patients' addiction to certain addictives was a common factor. Patients' addictions to several drugs were also observed. The addictives were bidi (B), cigarette (C), tobacco (T), alcohol (A), and ganja (G). Some of the patients were addicted to mono addictives like B, T, and C and some patients were addicted to more than one addictives. A number of patients

who were addicted to B, T, and C were 67 (42.13%), 9 (5.66%), and 19 (11.94%), respectively. On the other hand, patients addicted to C-B-G; C-A; and B-T were 3 (1.88%), 21 (13.20%), and 14 (8.80%) respectively. Furthermore, number of patients who were addicted to B-A and B-C were 19 (11.94%) and 7 (4.40%) respectively [Table 2].

Mono drug-resistant cases for INH, SM, EMB, and RIF were recorded as 62, 45, 47, and 27 [Table 3].

Whereas, MDR cases for INH + RIF, INH + SM, INH + EMB, and RIF + SM was recorded as 49, 53, 52, and 45, respectively [Table 4] and TDR cases for INH + RIF + SM, INH + RIF + EMB, INH + EMB + SM were recorded as 40, 36, and 31, respectively [Table 5].

Number of patients who were resistant and sensitive to INH was 62 (38.9%) and 97 (61%) respectively. Furthermore, patients' resistance and sensitivity toward SM was 45 (28.3%) and 114 (71.6%) respectively. Resistivity and sensitivity toward EMB was found to be 47 (29.5%) and 112 (70.4%) respectively. Similarly, number of patients resistant and sensitive to RIF was 27 (16.9%) and 132 (83.01%) respectively. Total number of patients calculated to be resistant and sensitive was 30 (18.8%) and 74 (46.5%) respectively [Table 6].

Among all the MTB clinical isolates a noteworthy level of distinction was observed. DR primers were amplified with the primer sets used. A total of 159 cases were studied out of which the polymorphism with various DRs was seen in 47 patients [Table 7].

Polymorphism among MTB isolates was significantly observed by the amplification of primers.[15-17] The primers DR (3074, 0272, 2068, and 0642) and *IS6110* were designed from MTB genome in such a way that it can help in studying the MTB epidemiology, detecting DNA polymorphisms, and strain typing. The melting temperature and primer sequence of the same is given as

Table 1: Characteristics of patients on the basis of education and occupation

Characteristics	Number of patients (*n*=159)	Percentage
Education		
Literate	123	77.3
Illiterate	36	22.6
Occupation		
Farming	25	15.7
Nonagricultural	80	50.3
Housewives	54	33.9
Residence		
Urban	122	76
Rural	37	23.2
Sex		
Male	121	76
Female	38	23.8

Table 2: Distribution of patients according to their drug habits

Addictives	Number of patients addicted (*n*=159)	Percentage
Bidi	67	42.13
Tobacco	9	5.66
Cigarette	19	11.94
Cigarette, bidi, ganja	3	1.88
Cigarette, alcohol	21	13.20
Bidi, tobacco	14	8.80
Bidi, alcohol	19	11.94
Bidi, cigarette	7	4.40

Table 3: Number of patients infected with mono direct repeat pattern

Pattern of DR-TB	Drugs	Number of resistant strains	Percentage
Mono DR-TB	Isoniazid	62	38.9
	Streptomycin	45	28.3
	Ethambutol	47	29.5
	Rifampicin	27	16.9

DR-TB: Drug resistant tuberculosis

Table 4: Number of patients infected with multi-drug resistant tuberculosis pattern

Pattern of drug resistant tuberculosis	Drugs	Number of resistant strains	Percentage
Multi drug resistant-tuberculosis	INH + RIF	49	30.81
	INH + SM	53	33.33
	INH + EMB	52	32.70
	RIF + SM	45	28.30

INH: Isoniazid, RIF: Rifampicin, SM: Streptomycin, EMB: Ethambutol

Table 5: Number of patients infected with triple drug resistance pattern

Pattern of DRTB	Drugs	Number of resistant strains	Percentage
Triple DRTB	INH + RIF + SM	40	25.1
	INH + RIF + EMB	36	22.6
	EMB + RIF + SM	31	19.4

DRTB: Drug resistant tuberculosis, INH: Isoniazid, RIF: Rifampicin, SM: Streptomycin, EMB: Ethambutol

Table 6: Number of patients resistant and sensitive to first-line antituberculosis drugs

Drugs	Resistance (%)	Sensitive (%)
Isoniazid	62 (38.9)	97 (61)
Streptomycin	45 (28.3)	114 (71.6)
Ethambutol	47 (29.5)	112 (70.4)
Rifampicin	27 (16.9)	132 (83.01)
Resistant to all drugs	30 (18.8)	-
Sensitive to all drugs	-	74 (46.5)

Table 7: Patients with direct repeats

Primer name (direct repeats)	Band size of primers (kb)	Number of clinical isolates
DR3074	172	10
DR0272	305	10
DR0642	231	11
DR2068	336	8
DR4110	531	8

DR: Direct repeat

below [Table 8].[13] The polymorphism among the isolates was checked by running the PCR products on agarose gel. Ladder (fermentas) of 1 Kb was run through the gel [Figures 1-3]. The representative gel pictures depict the different band sizes which can be observed below:

DISCUSSION

Consumption of anti-TB drugs in a prescribed and regular manner helps combating TB. Discontinuation of drugs as advised increases the risk of DR-TB, treatment failure and relapse. Hence, this study was undertaken to study three types of drug resistance patterns, i.e., mono DRTB, MDR-TB, and TDR-TB to FLDs in newly diagnosed cases of PTB. PCR is a rapid and accurate technique for genotyping. It reduces the time of patient's ailment and prevents the transmission of infection to others.[18] Insertion Sequence-*IS6110* initially described by Thierry *et al*. is distributed throughout the MTB complex and has been in great use in the epidemiological applications of restriction fragment length polymorphism analysis.[19]

A study carried out by Gupta *et al*., 2013,[20] showed the DR pattern to INH, rifampin, SM and EMB as 18.3%, 4.7%, 10.1%, and 10.7%, respectively which did not coincide with our study.[20] Our study was totally different from the study done by,[21] the percentage of patients resistant to INH, RIF, EMB, and SM was 1.4%, 0.2%, 0%, and 7.3%, respectively. MDR cases for INH + RIF, INH + SM, INH + EMB, and RIF + SM was recorded as 49, 53, 52, and 45, respectively [Table 4]. A study carried out at Portugal[21] showed resistance to INH + RIF and INH + SM as 1.1% and 3.3% respectively which did not match with our study. A similar type of study carried out at Belgaum showed the highest resistance to RIF (80.4%), while resistance to INH, PZA, EMB, and SM were 60%, 58.7%, 52.1%, and 63%,

Table 8: Primers, their sequences, and melting temperatures

Primer name	Primer sequence		Melting temperature
	Forward sequence	Reverse sequence	
DR0272	F-5'AGCGATCCTGCTGGTGG3'	R-3'TGCTGTTAGGGTCAAACG5'	50°C
DR0642	F-5'CCACTAGCAGATGGCCGTT3'	R-3'GCTCCAAGCGTAGTGATCCT5'	59.7°C
DR2068	F-5'CACGACGTAGACGAATGC3'	R-3'ATGACACGCTTTCTGCCC5'	63.4°C
DR3074	F-5'GTCACGATTGACACGCGGT3'	R-3'CATGGCCTCCGTTGTACTC5'	65.2°C
DR4110	F-5'TTTAGACGATCGCACCGC3'	R-3'AACGGAATCGTGGTCAGC5'	55°C
IS6110	F-5'CCTGCGAGCGTAGGCGTCGG3'	R-3'CTCGTCCAGCGCCGCTTCGG5'	63.9°C

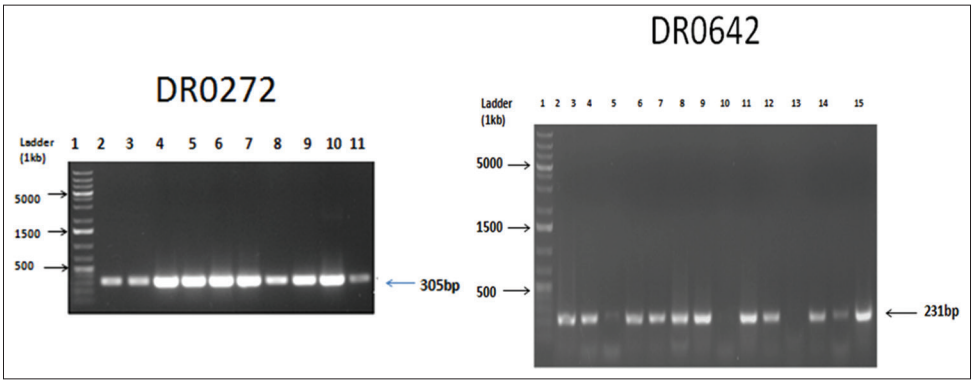

Figure 1: Representative gel pictures of clinical isolates: Primer: DR0272; Lane 1: Ladder (1 Kb); Lane 11: Control; Lane 2–10: Clinical isolates; Primer: DR0642; Lane 1: Ladder (1 Kb); Lane 2: Control; Lane 3–15: Clinical isolates

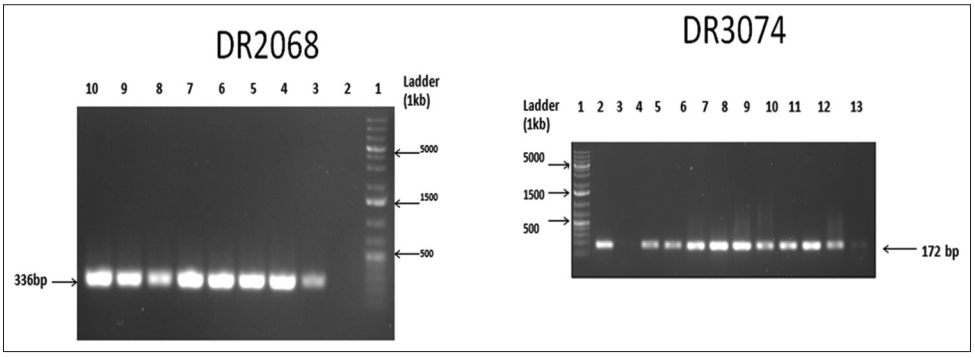

Figure 2: Representative gel picture of clinical isolates; Primer: DR2068; Lane 1: Ladder (1 Kb); Lane 6: Control; Lane 2–5; 7–10: Clinical isolates; Primer: DR3074; Lane 1: Ladder (1 Kb); Lane 2: Control; Lane 3–13: Clinical isolates

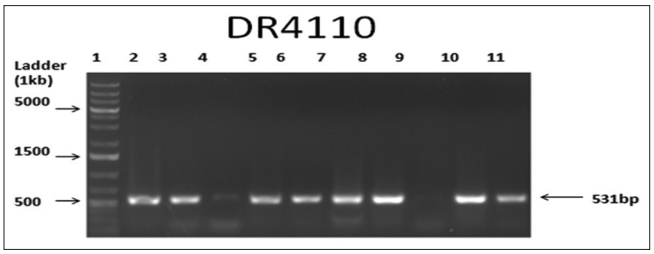

Figure 3: Representative gel picture of clinical isolates. Primer: DR4110; Lane 1: Ladder (1 Kb); Lane 2: Control; Lane 3–11: Clinical isolates

respectively. Resistance to one drug, three drugs, four drugs were (17.9%), (17.9%), and (8.7%), respectively. MDR isolates were obtained in 24 patients (52.2%).[11] According to a study carried out in Karnataka, 24 (52.2%) isolates showed MDR strains while 8 (17.9%) and 4 (8.7%) isolates confirmed mono and poly resistance, respectively.[11] Similarly, in another study being carried out at Hyderabad 28% of the cases were confirmed with MDR-TB whereas polydrug resistance was reported in 42% of the cases.[22]

A study was carried out at Lucknow, India,[23] in which a total of 69 patients were studied, and five types of DR's were amplified out of the total number of patients. In a study carried out at Thailand,[13] polymorphism with various DRs was observed in 39 out of 91 patients. Males were found to be more prone to TB disease. Literate patients were in majority and non-agricultural occupation was seen in most of the patients. The study shows the pattern of drug resistance to FLDs among new pulmonary cases.

CONCLUSION

DR-TB is a major public health problem because treatment is complicated, cure rates are well below those for drug susceptible TB, and patients may remain infectious for months or years despite receiving the best available therapy. The data showed significant level of dissimilarities among all the DR isolates of MTB and number of repeats of *IS6110* were present in different clinical isolates. Over the years, the identification method based on *IS6110* has been established as the standard for typing strains of MTB. *IS6110* genotyping is very convincing when it is applied to classify MTB isolates harboring a large number of *IS6110* in their chromosomes.

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Conflicts of interest
There are no conflicts of interest.

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