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) 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.
The causative agent of TB, *Mycobacterium* TB (MTB) is an 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. 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. The disease takes no time spreading to other parts of the body viz. brain, lymph nodes, nervous system, bones, etc., and the condition is referred to as “extrapulmonary TB (EPTB).” Everyone infected with TB bacteria does not become sick and who are infected, but not sick, have latent TB infection.

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. When MTB becomes 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).

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. Direct repeat (DR) among previously treated patients is rising due to noncompliance to TB medications, lack of knowledge, poor management in health centers. 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. 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.

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.

### 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). 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

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 were addicted to 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.
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. The DR primers (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       |

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]. 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. 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.

A study carried out by Gupta et al., 2013 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. Our study was totally different from the study done by, 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 8]. A study carried out at Portugal 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%.

| Primer name | Primer sequence | Melting temperature |
|-------------|-----------------|---------------------|
| DR0272      | F-5’AGCGATCCTGCTGGTGG3’ | 50°C |
| DR0642      | F-5’CCACTAGCAGATGGCGGT3’ | 59.7°C |
| DR2068      | F-5’CACGACGTAGACGAATGC3’ | 63.4°C |
| DR3074      | F-5’GTCGCAATTTGACACCGGT3’ | 65.2°C |
| DR4110      | F-5’TCTGCCAACGAGCTTGCG3’ | 55°C |
| IS6110      | F-5’CCTGCGAGCGTAGGCGTGG3’ | 63.9°C |

**Table 8: Primers, their sequences, and melting temperatures**

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.[12]

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.

REFERENCES

1. Corbett EL, Watt CJ, Walker N, Maher D, Williams BG, Raviglione MC, et al. The growing burden of tuberculosis: Global trends and interactions with the HIV epidemic. Arch Intern Med 2003;163:1009-21.
2. TB India 2014 Revised National TB Control Programme Annual Status Report; 2014.
3. World Health Organization. Tuberculosis Control: WHO Report 2015. Geneva, Switzerland: WHO; 2015.
4. World Health Organization. Tuberculosis Control: WHO Report 2014. Geneva, Switzerland: WHO; 2014.
5. Tiemersma EW, van der Werf MJ, Borgdorff MW, Williams BG, Nagelkerke NJ. Natural history of tuberculosis: Duration and fatalitY of untreated pulmonary tuberculosis in HIV negative patients: A systematic review. PLoS One 2011;6:e17601.
6. Pérez-Martínez J, Ponce-De-León A, Bobadilla M, Villegas-Sepúlveda N, Pérez-García M, Sifuentes-Osorio, et al. A novel identification scheme for genus Mycobacterium. M. tuberculosis complex, and seven mycobacteria species of human clinical impact. Eur J Clin Microbiol Infect Dis 2008;27:451-9.
7. Sharma SK, Mohan A. Extrapulmonary tuberculosis. Indian J Med Res 2004;120:316-53.
8. Gideon HP, Flynn JL. Latent tuberculosis: What the host “sees”? Immunol Res 2011;50:202-12.
9. Fogel N. Tuberculosis: A disease without boundaries. Tuberculosis (Edinb) 2015;95:527-31.
10. Dye C, Scheele S, Pelan P, Pathania V, Raviglione M C. Consensus statement. Global burden of tuberculosis: Estimated incidence, prevalence, and mortality by country. WHO Global Surveillance and Monitoring Project, JAMA 1999;282:677-86.
11. Gaude GS, Hatthiholli J, Kumar P. Risk factors and drug-resistance patterns among pulmonary tuberculosis patients in Northern Karnataka region, India. Niger Med J 2014;55:327-32.
12. Prasad R, Gupta N, Singh M. Multidrug resistant tuberculosis: Trends and control. Indian J Chest Dis Allied Sci 2014;56:237-46.
13. Smittipat N, Palittapongampit P. Identification of possible loci of variable number of tandem repeats in Mycobacterium tuberculosis. Tuber Lung Dis 2000;80:69-74.
14. Menon S, Dharmshale S, Chande C, Gohil A, Lilani S, Mohammad S, et al. Drug resistance profiles of Mycobacterium tuberculosis isolates to first line anti-tuberculous drugs: A five years study. Lung India 2012;29:227-31.
15. van Soolingen D, Herrns PW, de Haas PE, Soll DR, van Embden JD. Occurrence and stability of insertion sequences in Mycobacterium tuberculosis complex strains: Evaluation of an insertion sequence-dependent DNA polymorphism as a tool in the epidemiology of tuberculosis. J Clin Microbiol 1991;29:2578-86.
16. Roring S, Brittain D, Bunschoten AE, Hughes MS, Skuce RA, van Embden JD, et al. Spacer oligotyping of Mycobacterium bovis isolates compared to typing by restriction fragment length polymorphism using PGRS, DR and IS6110 probes. Vet Microbiol 1998;61:111-20.
17. Huard RC, Fabre M, de Haas P, Lazzarini LC, van Soolingen D, Cousins D, et al. Novel genetic polymorphisms that further delineate the phylogeny of the Mycobacterium tuberculosis complex. J Bacteriol 2006;188:4271-87.
18. Warren RM, Streicher EM, Sampson SL, van der Spuy GD, Richardson M, Nguyen D, et al. Microevolution of the direct repeat region of Mycobacterium tuberculosis: Implications for interpretation of spoligotyping data. J Clin Microbiol 2002;40:4457-63.
19. Thierry D, Cave MD, Eisenach KD, Crawford JT, Bates JH, Gicquel B, et al. IS6110, an IS-like element of Mycobacterium tuberculosis complex. Nucleic Acids Res 1990;18:188.
20. Gupta H, Kant S, Jain A, Natu SM, Ablawalalia S. Initial drug resistance pattern among pulmonary tuberculosis patients. Indian J Tuberc 2013;60:154-61.
21. Gomes M, Correia A, Mendonça D, Duarte R. Risk factors for drug-resistant tuberculosis. J Tuberc Res 2014;2:111-8.
22. Kandi S, Prasad SV, Sagar Reddy PN, Reddy VC, Laxmi R, Kopu D, et al. Prevalence of multidrug resistance among retreatment pulmonary tuberculosis cases in a tertiary care hospital, Hyderabad, India. Lung India 2013;30:277-9.
23. Tripathi DK, Srivastava K, Kant S, Srivastava KK. Molecular profiling of drug resistant isolates of Mycobacterium tuberculosis in North India. Adv Microbiol 2012;2:317-26.