

Prevalence of multidrug resistant *Mycobacterium tuberculosis* in Lucknow, Uttar Pradesh

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Background & objectives: Multi-drug resistant (MDR) *Mycobacterium tuberculosis* isolates may be transmitted within communities due to dense population and poor hygienic conditions. For proper management and control of MDR-TB, understanding drug susceptibility pattern of *M. tuberculosis* isolates and their transmission pattern in every health care setting are essential. In the present study, we attempted to describe the current prevalence of MDR-TB in Lucknow district, Uttar Pradesh, and our observations on transmission of MDR isolates among populations in and around this area.

Methods: Patients diagnosed as that of pulmonary tuberculosis (PTB) were enrolled from primary level (PLH), secondary level (SLH) and tertiary level (TLH) healthcare centres from Lucknow district. Detailed history of intake of antitubercular drug in the past was taken to decipher initial/ acquired drug resistance. Sputum samples were cultured on Lowenstein- Jensen media to isolate mycobacteria. Drug susceptibility patterns of isolated *M. tuberculosis* isolates were recorded using 1 per cent proportion method. Transmission of MDR isolates in community was accessed by random amplified polymorphic DNA (RAPD). Isolates showing same band pattern on RAPD were retyped using different primers targeted to the inverted repeat sequence of IS6110 copies in *M. tuberculosis* genome.

Results: A total of 686 *M. tuberculosis* isolates were obtained from 1162 patients, of which 318 were from untreated subjects and 368 were from patients who were treated for tuberculosis in the past. Prevalence of MDR was 19.8 per cent, initial and acquired being 13.2 and 25.5 per cent respectively. Prevalence of resistance to any drug, MDR and individual drug resistance to isoniazid, streptomycin, ethambutol and rifampicin was significantly higher in patients who were treated in the past. Drug resistance was significantly higher at tertiary level health care compared to primary level health care. Genotypically similar clusters were seen at all levels of health care. It was not always possible to establish geographic connections within clusters.

Interpretation & conclusion: High prevalence of both initial and acquired MDR was noted in *M. tuberculosis* isolates collected from pulmonary tuberculosis patients. Presence of small clusters of MDR isolates at all health care levels suggests transmission within the studied community.

Key words Multi drug resistance - *Mycobacterium tuberculosis* - RAPD - transmission

Infection with *Mycobacterium tuberculosis* that is resistant to isoniazid and rifampicin, is defined as multi-drug resistant tuberculosis (MDR-TB) and often

culminates in incurable disease¹. MDR-TB isolates constitute 1 to 3 per cent of global isolates and worldwide distribution is characterized by localized

“hot zones”^{2,3}. The rate of MDR-TB mortality is estimated to range from 40 to 60 per cent, which is similar to the mortality of patients with untreated tuberculosis (TB) cases⁴ and has been identified as a significant problem in every region under World Health Organization (WHO) surveillance⁵.

Molecular typing of *M. tuberculosis* can facilitate investigation into the transmission of TB⁶. Data on drug resistant TB as well as transmission of MDR-TB from most of developing world are limited. These isolates may be transmitted within communities due to dense population and poor hygienic conditions in resource-poor countries and countries with high incidence of TB like India⁷. In the present study, we planned to investigate the prevalence of drug resistant tuberculosis at three different levels of health care systems, and collected information on transmission of MDR-TB within Lucknow district of northern India.

Material & Methods

Settings, patients and samples: A total of 1162 clinically suspected pulmonary tuberculosis patients, who gave written consent to participate in the study, were enrolled consecutively from three different levels of tuberculosis (TB) clinics, *i.e.*, Primary Health Centre, Mohanlalganj, Lucknow (primary level of health care, PLH), District Tuberculosis Centre, Lucknow (secondary level of health care, SLH) and Department of Pulmonary Medicine, Chhatrapati Sahuji Maharaj Medical University, Lucknow (tertiary level of health care, TLH) over a period of two years (November 2000 to October 2002). The age of patients varied from 12-60 yr and included both male and female populations in urban and rural settlements. Institutional ethical clearance was taken for the study. Patients with extrapulmonary tuberculosis, immunocompromised patients and those who did not consent, were excluded. Detailed clinical history to decipher the intake of antitubercular therapy in past was taken. Three sputum samples (one on spot and two early mornings) were collected in sterile containers from each patient (minimum 5 ml of sputum) for sputum microscopy and mycobacterial culture. Sputum samples were transported to TB laboratory, Postgraduate Department of Microbiology, C.S.M. Medical University, Lucknow, as soon as possible, preferably within 24 h. If delay was expected, samples were transported at 4°C. All the samples were processed at TB laboratory, Postgraduate Department of Microbiology, C.S.M. Medical University, Lucknow. Initial resistance was defined as *in vitro*

resistance in isolates from patients who did not give a history of antituberculosis treatment in the past, while acquired resistance is defined as *in vitro* resistance in isolates from patients previously treated with any antituberculosis medication, for more than four weeks.

Sputum microscopy: A smear from yellow purulent portion of the sputum using a wire loop was made on clean slide. Smear was air dried for 15-30 min, and fixed by passing the slide over the flame for 3-5 times for 3-4 seconds each time. The smear was stained by Ziehl-Neelsen method and examined under Nikon ECLIPSE E-400 light microscope (Nikon, Japan) under 100 X-oil immersion objectives. Known positive and negative slides were prepared with every batch of the specimen. A minimum of 100 fields of smear were observed, before the smear was reported negative. Grading of acid fast bacilli (AFB) positivity was done as per Revised National Tuberculosis Control Programme (RNTCP) Protocol⁸.

Isolation and identification of mycobacteria: One of the three sputum samples from each patient showing maximum numbers of AFB, was decontaminated by Petroff's method⁹, and inoculated on to two Lowenstein-Jensen tubes. The culture bottles were incubated at 37°C and read weekly for eight weeks. No growth after eight week of incubation was treated as negative. Growth of *M. tuberculosis* was typed by colony appearance, niacin production, catalase activity at 68°C and pH 7 and susceptibility to p-nitrobenzoic acid¹⁰.

Drug susceptibility testing for *M. tuberculosis*: Drug susceptibility testing (DST) was performed by conventional 1 per cent proportion method against streptomycin (4 µg/ml), isoniazid (0.2 µg/ml), rifampicin (40 µg/ml) and ethambutol (2 µg/ml)¹¹. All the drugs and chemicals were procured from Sigma, USA. External quality control (EQA) for DST was provided by the Tuberculosis Research Centre (TRC, ICMR), Chennai (India).

Genotyping: Genomic DNA was extracted from mycobacterial cultures by the method described earlier. Briefly, a loopful colony of *M. tuberculosis* was mixed with 200 µl of Tris-EDTA buffer and placed in boiling water bath at 100°C for 10 min. It was followed by adding 200 µl lysis buffer (pH 8.0) and 10 µl of proteinase K (10 mg/ml) and incubated at 56°C in water bath for 2 h. The sample was then vortexed and boiled at 100°C for 10 min to inactivate proteinase K, after that DNA was extracted by phenol: chloroform (24:1) followed by chloroform only.

DNA fingerprinting was done by random amplified polymorphic DNA (RAPD) method described by Linton *et al*¹³. Briefly, a single primer (5' ACG CTC AAC GCC AGA GAC CA 3') [Genei Bangalore, India] that was targeted to the inverted repeat sequences of the IS 986 was used to amplify the DNA between closely spaced copies of this element. PCR was performed in a total volume of 20 µl and the reaction mixture contained 0.5U TAQ polymerase, reaction buffer [10 mM-Tris HCl (pH9.00), 1.5 mM MgCl₂, 50 mM KCl, 0.1% [v/v] triton X-100, 0.01% (wt/vol) gelatin], 200 µM of each deoxynucleoside triphosphate and 100 pmol primer with 2 µl of template DNA. A total of 40 cycles of PCR was performed by using a thermal cycler (Techne, USA) consisting of a denaturation step for 20 sec at 94°C, an annealing step for 1 min at 36°C, and an extension step for 1 min at 72°C. After the final cycle, there was a final step of 7 min at 72°C. The PCR products generated were visualized by ethidium bromide staining after electrophoresis in gel containing 2 per cent agarose.

Isolates showing same band pattern on RAPD were retyped by different primer (5'GAGTCTCCG GACTCACCG- 3') [Genei, Bangalore, India] targeted to the inverted repeat sequence of IS6110 copies in *M. tuberculosis* genome by methods described by Yates *et al*¹⁴. The reaction mixture was same as in RAPD. A total of 40 cycles of PCR was performed by this method using a thermal cycler (Techne, USA) consisting of a denaturation step for 120 sec at 95°C, 1 cycle of 95°C for 20 sec, 45°C for 360 sec, and 72°C for 120 sec, 30 cycles of 95°C for 20 sec, 62°C for 30 sec. After the final cycle, there was a final step of 10 min at 72°C. The PCR products generated were visualized by ethidium bromide staining after electrophoresis in gel containing 0.8 per cent agarose.

Gels were photographed and patterns of bands generated by RAPD were compared visually and the amplification products were recorded as binary data, presence (1) and absence (0). Pair-wise genetic similarities among isolates were calculated from these data using Jaccard's coefficient¹⁵. The genetic relationships among isolates were established by cluster and ordination analyses performed on the matrix of genetic similarities. Cluster analysis was performed by mean of the unweighted paired group method with arithmetic average (UPGMA) using multivariate statistical package software (MVSP version 3.1, USA, www.kovacom.com/mvsp/down/2.html).

Statistical analysis: Statistical analysis was done by STATA software (version 9.2, Texas, USA). Prevalence

were reported and supported by 95 per cent confidence intervals. Comparison of proportion was done by chi square test.

Results

Of the 1162 enrolled subjects, 1014 (87.3%) were AFB positive on smear microscopy (even if one of three sputum samples was positive, patient was labeled as AFB positive). Total 724 (62.3%) cultures were mycobacteria positive, of which 686 (59.0%) were *M. tuberculosis* and 38 (3.2%) were MOTT (mycobacteria other than tuberculosis). MOTT were not further characterized/ tested.

Of the 686 *M. tuberculosis* isolates, 136 were MDR (19.8%), initial and acquired resistance being (42/318) 13.2 per cent and (94/368) 25.5 per cent respectively (Table I). Prevalence of MDR at tertiary level health care (85/304 - 27.9%) and secondary level health care was significantly higher (37/188 - 19.6%) than at primary level health care (14/195 - 7.2%) (Table II). Antimycobacterial drug resistance was significantly higher in treated patients compared to new cases (Table I). RAPD profiles of the MDR isolates from each centre were found to be constant, discriminatory and reproducible as seen by band patterns elicited by both primers and analyzed by dendrogram. Two of the MDR isolates from PLH with same drug susceptibility pattern were genotypically similar by both of the primers used for the fingerprinting (Fig. 1A, 2). The patients were residents of the same geographic locality. Two clusters of isolates were seen in MDR isolates both from secondary and tertiary care hospitals (Fig.1B & 1C). These isolates had same drug susceptibility pattern however their geographical connections could not be established. Fig. 3 shows the five clusters of two strains each and one each of 3, 4 and 5 isolate. Except in one cluster of isolates from PHC no other geographical connection could be established.

Discussion

In the present study a high prevalence of MDR-TB, both initial and acquired was shown. Ample literature from India and other developing Asian countries regarding prevalence rate of MDR has been reported showing a wide variation from study to study. The overall per centages of resistance to different antituberculosis drugs obtained from different surveys done by WHO¹⁶ using standardized guidelines showed that the levels of primary resistance to isoniazid as single agent ranged from 0 - 16.9 per cent and for streptomycin 0.1 - 23.5 per cent. Initial resistance to

Table I. Culture positivity and drug resistance pattern of *M.tuberculosis* isolates from treated and new cases of pulmonary tuberculosis

| | Total no. (%) | New cases no. (%) | Treated cases no. (%) |
|---------------------------|---------------|-------------------|-----------------------|
| Enrolled cases | 1164 | 556 | 608 |
| Smear positive | 1014 (87.3) | 478 (85.9) | 536 (88.1) |
| Culture positive | 724 (62.3) | 321 (57.7) | 403 (66.5) |
| MOTT | 38 (3.2) | 3 (0.53) | 35 (5.7) # |
| 95% CI, (<i>P</i> value) | | 0.1,1.1 | 3.9,7.6 (<0.0001) |
| * <i>M. tuberculosis</i> | 686 (59.0) | 318 (57.1) | 368 (60.7) |
| 95% CI, (<i>P</i> value) | | 53.1,61.3 | 56.8,64.6 (0.2213) |
| Any drug resistant | 262 (38.1) | 95 (29.8) | 167 (45.3) # |
| 95% CI, (<i>P</i> value) | | 24.8,34.9 | 40.3,50.5, (<0.0001) |
| MDR | 136 (19.8) | 42 (13.2) | 94 (25.5) # |
| 95% CI, (<i>P</i> value) | | 9.5,16.9 | 21.1,30.0 (0.0001) |
| Resistant to STR | 190 (27.6) | 64 (20.1) | 126 (34.2) # |
| 95% CI, (<i>P</i> value) | | 15.7,24.5 | 29.4,39.1 (<0.0001) |
| Resistant to INH | 204 (29.7) | 64 (20.1) | 140 (38.0) # |
| 95% CI, (<i>P</i> value) | | 15.7,24.5 | 33.1,43.0 (<0.0001) |
| Resistant to RIF | 142 (20.6) | 40 (12.5) | 102 (27.7) # |
| 95% CI, (<i>P</i> value) | | 8.9,16.2 | 23.1,32.3 (<0.0001) |
| Resistant to EMB | 122 (17.8) | 39 (12.2) | 83 (22.5) # |
| 95% CI, (<i>P</i> value) | | 8.6,15.9 | 18.3,26.8 # |

*Denominator for calculating MDR and resistant to antituberculosis drugs; # significant compared to new cases by 95 % CI ($P < 0.005$); MOTT, mycobacteria other than tuberculosis; STR, streptomycin; INH, isoniazid; RIF, rifampicin; EMB, ethambutol; MDR, Multidrug resistant; 95% CI = 95% Confidence interval, treated cases = Pulmonary tuberculosis patient having treated for antituberculosis drugs in past for at least 4 wk.

Table II. Centre-wise drug resistant pattern of *M. tuberculosis* isolates

| | PLH | SLH | TLH | Total |
|--------------------------------|------------|-------------|--------------|-------------|
| Enrolled cases n | 348 | 328 | 486 | 1162 |
| Smear positive n (%) | 312 (89.6) | 284 (86.5) | 418 (86.0) | 1014 (87.2) |
| Culture positive n (%) | 204 (58.6) | 200 (60.9) | 320 (65.8) | 724 (62.3) |
| MOTT n (%) | 10 (2.8) | 12 (3.6) | 16 (3.2) | 38 (3.2) |
| 95 % CI | 1.4, 5.2 | 1.9, 6.4 | 1.9, 5.3 | |
| * <i>M. tuberculosis</i> n (%) | 194 (55.7) | 188 (57.3) | 304 (62.5) | 686 (59.0) |
| 95 % CI | 50.4, 61.0 | 51.8, 62.7 | 58.0, 66.9 | |
| Any drug resistant n (%) | 47 (24.2) | 76 (40.4) | 139 (45.7) # | 262(38.1) |
| 95 % CI | 18.4, 30.9 | 33.4, 47.9 | 40.1, 51.6 | |
| MDR n (%) | 14 (7.2) | 37 (19.6) # | 85 (27.9) # | 136 (19.8) |
| 95 % CI | 4.0, 11.9 | 14.3, 26.1 | 22.9, 33.4 | |
| Resistant to STR n (%) | 39 (20.1) | 51 (27.1) | 100 (32.8) # | 190 (27.6) |
| 95 % CI | 14.8, 26.5 | 20.9, 34.1 | 27.6, 38.5 | |
| Resistant to INH n (%) | 31 (15.9) | 59 (31.3) | 114 (37.5) # | |
| 95 % CI | 11.2, 21.9 | 24.9, 38.6 | 32.0, 43.3 | 204 (29.7) |
| Resistant to RIF n (%) | 22 (11.3) | 35 (18.6) | 85 (27.9) | |
| 95 % CI | 7.3, 16.7 | 13.4, 24.9 | 16.6, 24.6 | 142 (20.6) |
| Resistant to EMB n (%) | 14 (7.2) | 31 (16.4) | 77 (25.3) # | |
| 95 % CI | 4.0, 11.9 | 11.5, 22.6 | 20.6, 30.7 | 122 (17.8) |

PLH, Primary level health care centre; SLH, Secondary level health care centre; TLH, Tertiary level health care centre; *Denominator for calculating MDR and resistant to antituberculosis drugs; # TLH prevalence is significantly higher compared to PLH by 95 %CI ($P < 0.005$); MOTT, mycobacteria other than tuberculosis; STR, streptomycin; INH, isoniazid; RIF, rifampicin; EMB, ethambutol; MDR, multidrug resistant

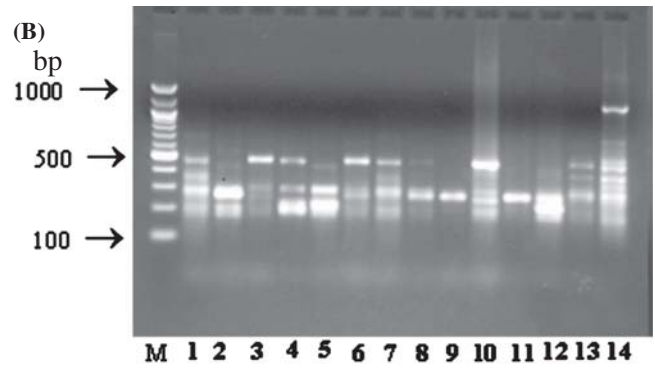
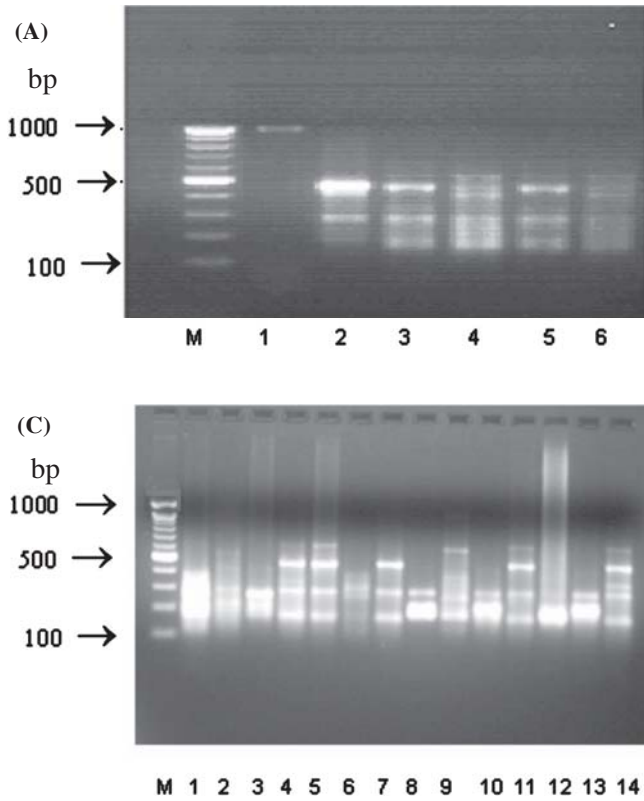


Fig. 1. RAPD profile of *M. tuberculosis* isolates with primer targeted to IS 986. (A) Isolates from primary level health care system (lane no 1-6, from centre, cluster I: lane nos 3 & 5, M= 100 bp marker). (B) Isolates from secondary level health care systems (lane no 1-14, from centre, cluster-I: lane no 3, 6 & 7, cluster-II lane 9 & 11, M= 100 bp marker). (C) Isolates from tertiary level health care systems (lane no 1-14, from centre, cluster-I lane; no 4, 5 & 11, cluster II: lane no 8, 10 & 13, M = 100 bp marker).

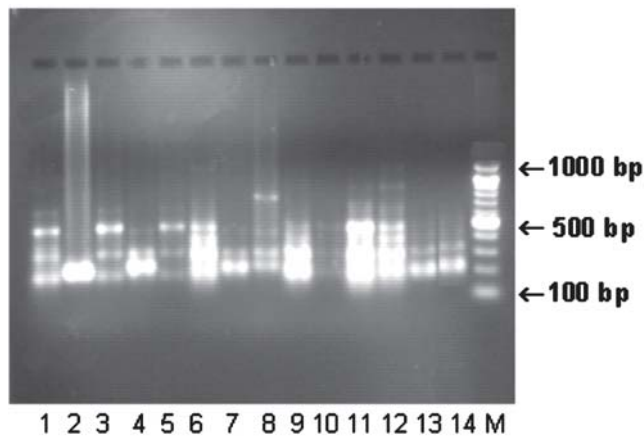


Fig. 2. Genotypic profile of *M. tuberculosis* strains with primer targeted to IS 6110. M = 100 bp marker; lane no. 1, 3 & 5 isolates from tertiary level health care system identical as in RAPD (lane no. 4, 5 & 11 of Fig.1-C); lane no. 11 and 12 from secondary level health care system identical as in RAPD (9 & 11 of Fig.1-B); lane no. 13 & 14 from primary level health care system identical as in RAPD (3& 5 of Fig. 1-A); lane no. 2, 4, 6 from secondary level health care system identical as in RAPD (3,6 & 7 of Fig. 1-B); lane no 7, 8, 9 isolates from tertiary level health care system identical as in RAPD (lane no. 8, 10 & 13 of Fig. 1-C) showing different genetic pattern found clusters by RAPD with IS 986 primer in all centers.

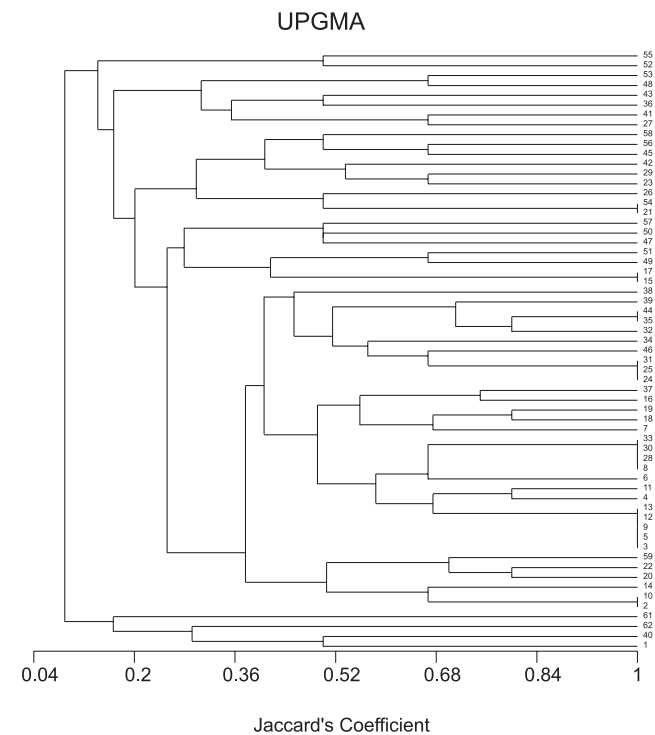


Fig. 3. Dendrogram generated by the UPGMA method from the RAPD data of isolates showing the relationships among the clustering isolates.

rifampicin as single agent was unusual with a rate ranging from 0 - 3 per cent. The rate of initial resistance to ethambutol was low ranging from 0 - 4.2 per cent globally. High rates of primary resistance to isoniazid have been reported from Kenya, India, and Haiti while it is reported to be low in southeastern England, Melbourne and Argentina. High rates of resistance to streptomycin were reported in Zaire, Pakistan and Brazil while low levels of resistance were reported from China, Ethiopia, Bosnia and Herzegovina. There are fewer surveys of acquired drug resistance and the rates are usually higher than those of primary resistance. The rate of acquired resistance to isoniazid ranged from 4 - 53.7 per cent, to streptomycin from 0 - 19.4 per cent, to rifampicin from 0 - 14.5 per cent and to ethambutol from 0 - 13.7 per cent. Initial drug resistance reported from India is 18 - 20 per cent for isoniazid, 4.8-14 per cent for streptomycin. Prevalence of initial MDR-TB reported from India varied between 0-5 per cent^{17,18}. Data from India on acquired resistance are limited and concluded that any resistance to isoniazid was in between 47.7¹⁹ to 87.1 per cent²⁰. For rifampicin, it was 28.3 - 80.6 per cent and for MDR it was between 9.6 to 80.6 per cent²¹. It is evident that prevalence of drug resistant tuberculosis varied considerably throughout the world and particularly in India. The reasons for this variation in different studies were the criteria of selection of patients studied, the extent of misuse of drugs, the quality of questionnaire used for eliciting history of previous treatment, inadequate laboratory support and reporting systems. Moreover, previously published data from India on drug resistant tuberculosis and MDR-TB had been from referral centres and institutions¹⁷, and did not reflect the overall status of drug resistance problem in India. We have shown that cases at TLH have a referral bias hence significantly higher prevalence of drug resistance and MDR-TB was seen at TLH compared to PLH and SLH. Department of Pulmonary Medicine at Chhatrapati Sahu Ji Maharaj Medical University, Lucknow (TLH) is a referral center for TB treatment from all parts of the UP state and some neighbouring States and neighbouring countries like Nepal also. Significant increased isolation of MOTT isolates in treated cases and also prevalence of these isolates in new cases as well, highlighted their role in presenting the disease. These can become a challenge for TB control programme in this area in future.

Molecular typing provides an important epidemiological tool to investigate the transmission of tuberculosis and provides novel information about

the spread of tubercle bacilli in mini epidemics and outbreaks, to analyze the transmission dynamics of tuberculosis and to distinguish exogenous re-infection from endogenous reactivation⁶. Molecular epidemiologic studies on drug resistance are 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 patients risk factors (*e.g.*, HIV) for acquiring resistance. Some studies have queried the contribution of initial versus acquired drug resistance in specific populations^{22,23}, while others have aimed to describe the evolutionary dynamics of drug resistance during clonal expansion or dissemination between and within patients^{24,25}. Bifani *et al*²⁵ described the genotypic drug resistance profile of strain W and its variants during an outbreak in the early 1990s in New York city. Since then, at least 11 MDR W variants with subtle variations in IS6110 RFLP profiles have been recovered from New York patients. DNA sequence analyses of drug resistance targets confirmed these variants as descendants of the original outbreak strain (*i.e.*, mutations identical to those of strain W); however, in some variants additional resistance to fluoroquinolone and capreomycin was noted^{25,26}.

In India, several studies on molecular epidemiology of *M. tuberculosis* have been done showing diverse polymorphism in the host genome. A study²⁷ from south India suggested that the majority of TB cases in that part were due to re-activation of the isolates. Demonstration of clusters in MDR-TB strains in our study signifies transmission of MDR-TB within the studied community. However, the limited data reported in the present study are not sufficient to conclude anything about transmission of MDR-TB. More studies and rigorous testing of these isolates with other molecular epidemiological tools will be needed.

In conclusion, our findings showed a high prevalence of drug resistant *M. tuberculosis* isolates, especially MDR-TB, in the study area. Transmission of MDR-TB within community is an emergent situation which has to be tackled. There is an urgent need to further study the risk factors for development of transmission and MDR-TB in these settings.

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