Study of the prevalence of Multidrug-Resistant Pulmonary Tuberculosis (MDR-TB) in Western Rajasthan using line probe assay

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Objective: To determine the prevalence of MDR-TB and find out the incidence of drug resistance using molecular diagnostic method. Line probe assay (LPA) is based on the principle of multiplex PCR is used to detect MTB (Mycobacterium tuberculosis) complex as well as its sensitivity to rifampicin and isoniazid. Method: This is a hospital-based prospective observational study. The sputum of MDR-TB suspected patients was subjected to Ziehl-Neelsen staining and smear positive samples were analyzed by LPA. Decontamination and digestion of the samples was done using the NALC- NaOH method (as defined in RNTCP guidelines). DNA extraction was done from the decontaminated samples using Geno Lyse kit. After DNA extraction, detection of MTB complex and rifampicin and/or INH resistance was done with the help of line probe assay (LPA) using GenoType® MTBDRplus version 2.0. Results: Out of the 156 smear-positive sputum samples, 140 samples had LPA valid results. The most common age group of positive TB samples in this study was 30-40 years (26.42%). Twenty-five samples (17.85%) were found to be rifampicin resistant and 22 (15.71%) samples were found resistant to isoniazid. Sixteen patients (11.42%) were detected MDR. Nine patients (6.42%) were monoresistant to rifampicin and six patients (4.28%) were monoresistant to isoniazid. “Sputum positive retreatment cases” had the highest detection rate for MDR TB. Conclusion: Line probe assay is an economical and time saving method for the detection of MDR-TB and serves as a lifesaving tool for early diagnosis and treatment. This calls for a widespread national use of this assay. The detection of around 10% ZN-positive patients, who were not showing MTB complex in LPA may be a hidden iceberg for non-tubercular mycobacteria.

Keywords: Line probe assay, multidrug resistance tuberculosis, mycobacterium tuberculosis complex

Introduction

Tuberculosis (TB) remains a major health issue for developing countries, especially the Indian subcontinent. Approximately, 10 million people each year suffer from this disease and it is also one of the common causes of death in the last five years.¹ Tuberculosis most commonly affects the lungs; however, it may affect other organs also. Patients with infectious pulmonary tuberculosis can infect 10-15 persons in a year. The global incidence of TB is 39% of which India alone accounts for 24%.²,³ Multidrug-resistant tuberculosis (MDR-TB) caused by Mycobacterium tuberculosis resistant to both isoniazid and rifampicin with or without resistance to other drugs is among the most worrisome elements of the pandemic of antibiotic
resistance. The proportion is higher in patients who have previously received antitubercular treatment. While host genetic factors may probably contribute, incomplete and inadequate treatment is the most important factor leading to the development of MDR-TB. Misuse of antitubercular drugs, such as monotherapy or the addition of single drugs to failing regimens, results in the emergence of resistant mutants (acquired resistance). The transmission of such resistant strains to another person may result in infection and eventually disease (primary resistance). Outbreaks of highly fatal drug-resistant infection have been documented in several settings, especially those in which the prevalence of HIV infection is high. Recent reports describing totally drug-resistant tuberculosis require confirmation. The failure to detect drug resistance results in the prescription of inappropriate regimens, treatment failure, increased mortality, and further transmission of drug-resistant tuberculosis.

India is a high tuberculosis (TB) burdened country with an increasing prevalence of multidrug-resistant tuberculosis (MDR TB). Timely diagnosis and prompt treatment of infectious cases are the key elements in reducing the spread of TB. The conventional drug susceptibility testing (DST) considered as the “Gold standard” for the detection of drug-resistant TB is time consuming taking about 6–8 weeks. These systems have been supplemented with automated liquid culture systems in many diagnostic laboratories with decreased time to detection and greater sensitivity. However, the time for resistance testing is still about 7 to 10 days, beginning from the time a positive culture is obtained. The most rapid results could be achieved by molecular methods including commercial or in-house DNA hybridization or amplification methods which allow the detection of Mycobacterium tuberculosis as well as drug resistance in clinical samples within 1-2 days. Line probe assay (LPA) is based on the principle of multiplex PCR in combination with reversed hybridization that is used to detect the MTB complex as well as the drug sensitivity to rifampicin and isoniazid. MDR, which in fact is the consequence of spontaneous mutations in genes that encode either the target of the drug or enzymes involved in drug activation, needs to be studied in each of the cases referred for molecular diagnosis of TB. Moreover, a segment of MDR patients occupying a part in the overall burden of TB in a particular region needs to be documented to estimate the potential sources of MDR bacterial fauna (in vivo) capable of transmitting MDR tuberculosis even in a newly infected TB case. The present study has been undertaken to compute the regional burden of MDR and non-MDR tuberculosis and association of the biocological conditions of the region involving a particular disease burden of MDR-TB.

Materials and Methods

This is a hospital-based prospective observational study. It was carried out in the Department of Microbiology, TB (Tuberculosis) and Drug Susceptibility Test (DST) laboratory, at the tertiary care center in Western Rajasthan. A total of 220 sputum samples for the diagnosis of MDR-TB from suspect patients were received from January to June 2018. Among 220 cases, 155 cases were found suitable for study. Of these 155 cases, 14 cases were “any follow up smear positive,” two cases were “failure,” four cases were “Contact with MDR patient,” 126 cases were “retreatment, smear positive at diagnosis,” five cases were “smear negative at diagnosis, retreatment case,” four cases were “retreatment, smear positive at the fourth month.” All samples collected with aseptic measures and transported lab for further processing without delay. Samples having blood traces were excluded from the study.

Procedure

TB and Drug Susceptibility Test (DST) laboratory represents an accredited diagnostic laboratory for the molecular diagnosis of drug-resistant tuberculosis through LPA. Samples were received at the Laboratory within one day of the collection at various DTC’s/Tubercular units following the RNTCP protocol of transportation. Samples were then registered in the laboratory register and processed further. After collecting the specimen, the container was opened in a biosafety cabinet. Quality assurance of the samples was performed and a smear was made on a new clean labelled glass slide. After air-drying, Ziehl-Neelsen staining was performed. The samples tested smear positive were then subjected to LPA. Decontamination and digestion of samples were done using the NALC- NaOH method (as defined in RNTCP, guidelines). The process was carried out in the biosafety cabinet, from ESCO, class 2A2. DNA extraction was done from decontaminated samples by Geno Lyse kit (Hain Lifescience GmbH, Nehren, Germany). After the DNA extraction detection of MTB complex and rifampicin and/or ISONIAZID resistance were done with the help of Line Probe Assay (LPA) using GenoType® MTBDplus version 2.0 (Hain Lifescience GmbH, Nehren, Germany). The presence of a positive M. tuberculosis control (TUB) band in the sputum sample shows MTB complex. LPA was carried out only on the smear-positive samples. Approval from the institutional ethics committee was taken before initiation of the study. The study was initiated only after approval of Intuitional ethical committee dated 30/11/2017.

Observation

In our study, out of the 220 sputum samples processed, 156 samples were smear-positive. Of the 156 smear-positive sputum samples, 140 samples were found to be LPA valid results. The present study has been carried out from these 140 LPA valid smear-positive sputum samples. Most common age group of positive TB samples in this study was 30-40 years (26.42%), followed by 40-50 years (19.28%), 50-60 years (17.85%), 20-30 years (17.85%), 0-20 years (5.71%), 60-70 years (4.28%), >80 years (4.28%), and the least no. of samples were obtained from 70-80 years (3.58%). Males (77.85%) were more commonly affected by pulmonary tuberculosis than females (22.15%). The rural population (62.15%) were significantly infected than the urban population (37.85%; P value < 0.0001). [Table 1].
Among the 140 positive samples, 25 samples (17.85%) were rifampicin resistant and 115 samples (82.14%) were sensitive to rifampicin. Of the 25 samples, nine (37.50%) belonged to the age group of 30-40 years followed by 40-50 years (16.66%), 50-60 years (12.50%), 0-20 years (12.50%), 20-30 years (8.33%), 70-80 years (4.16%), and the least common age group is 60-70 years. Of the 25 rifampicin resistance cases, male were more commonly affected (83.33%) than females (16.66%). Out of 140 positive samples, 22 (15.71%) samples were resistant to isoniazid in which males (77.27%) were more resistant to than females (22.72%). Out of 140 positive samples, 109 (77.85%) were sensitive both to rifampicin and isoniazid. Sixteen patients (11.42%) were detected MDR, nine patients (6.42%) were mono-resistant to rifampicin, and six patients (4.28%) were mono-resistant to isoniazid. Among the positive samples, 13 male patients (11.92%) were detected with MDR and three female patients (9.67%) were detected MDR. There was no statically significant gender difference for MDR cases ($P$ value 0.75). [Table 1]. Residence wise distribution in MDR patients found that one patient (1.88%) out of 53 was from an urban background and 15 patients (17.24%) out of 87 belonged to the rural background. There was a significant difference for MDR-TB among the rural and urban populations. ($P$ value 0.01). Age-wise distribution of MDR detected patients shows that out of 98 patients who were age ≤50 years, 12 (12.44%) were detected MDR and among the 42 patients who were age >50 years, four patients (9.52%) were MDR. ($P$ value - 0.01). [Table 1].

MDR detection in suspected cases shows that a maximum of 113 (80.71%) were from sputum-positive retreatment cases, followed by 13 any follow-up sputum-positive cases (9.28%). Five were sputum-negative retreatment cases (3.57%) and two were from the failure cases (1.43%). [Table 2] [Figure 1]

**Discussion**

Line Probe Assay is used for the rapid detection of multidrug-resistant *M. tuberculosis* directly from smear-positive sputum samples. This assay has proved to be user-friendly and easy to perform. Line Probe Assay testing requires proper laboratory design, standard biosafety procedure, and quality control to avoid contamination. Increasing trends of TB and MDR-TB rates in high TB burden countries require the development and implementation of rapid diagnostic techniques and the ability to correctly detect MDR-TB in clinical specimens. Phenotypic DST is a time-consuming process because it requires culture, which may take 4-6 weeks or a longer time. The genotype MTBDRplus assay detects resistance based on

| Table 1: Demographic characteristic of cases and correlation with MDR detection |
|-------------------------------|---------------------------------|-----------------|-----------------|
| Total number of cases valid for LPA | 140 | Male | 109 |
| (Line Probe Assay) | | Female | 31 |
| Age wise distribution of cases | | Male (mean age in years) | 45.46±16.65 |
| | | Female (mean age in years) | 40.90±22.36 |
| Residence wise distribution cases | | Rural | 53 (37.85%) |
| | | Urban | 87 (62.15%) |
| LPA Results | | Sensitive | 109 (77.85%) |
| | | MDR + | 16 (11.42%) |
| | | Mono-resistant to ISONIAZID | 06 (4.28%) |
| | | Mono-resistant to RIFAMPICIN | 09 (6.42%) |
| Correlation of MDR Patients with gender distribution | | Male | 13 |
| | | Female | 03 |
| Correlation of Age with MDR cases | | Age ≤50 | 12 |
| | | Age >50 | 04 |
| Correlation of MDR Patients with Residence of cases | | Urban | 01 |
| | | Rural | 15 |

| Table 2: MDR detection in suspected cases. |
|-------------------------------|-----------|-----------------|-----------------|
| Type                          | MDR | Total Samples | Mono-Resistant to RIFAMPICIN | Mono-Resistant to ISONIAZID |
| Any follow up sputum positive | 02 | 13 | 01 | 00 |
| Failure                       | 00 | 02 | 00 | 00 |
| Contact with MDR patient      | 00 | 04 | 00 | 00 |
| Sputum positive at diagnosis, re-treatment case | 12 | 113 | 08 | 05 |
| Sputum negative at diagnosis, re-treatment case | 02 | 05 | 00 | 00 |
| Re-treatment case sputum positive at 4th month | 00 | 03 | 00 | 01 |
| Total                         | 16 | 140 | 09 | 06 |

![Figure 1: MDR detection in suspected cases]
the reverse hybridization method. Some previous studies have already demonstrated the feasibility of LPA as an effective tool in the early detection of MDR-TB.  

In the present study, a total of 140 LPA valid, smear-positive pulmonary tuberculosis MDR suspect patients of both genders and all age groups were assessed. The age of the patients ranged from 1 to 80 years, the mean age was 45 and 41 years for males and females, respectively. The age-group of 30-40 years was most frequently involved mainly because this is the group of people who are actively involved both in indoor and outdoor work. Children less than 10 years and the elderly greater than 80 years are less infected with TB&MDR because they are less mobile to outdoor activities. Similar findings were found by R Singhal et al., in which 16-35 years was the most frequent age group involved.  

As young adult males are an economically productive segment of society, high MDR-TB in this group has several socioeconomic implications. The maximum number of the MDR-TB suspect were from the rural area (62.15%), while 37.85% of cases from the urban area. Our study also showed the high prevalence of confirmed MDR-TB cases in the rural area (93%) than the urban area (7%). This type of distribution may be attributed to illiteracy, poor hygiene, overcrowding, and unawareness about the disease and improper treatment of tuberculosis in the rural area. Urban people are more literate and aware of treatment with better drug compliance. According to the current study, males were more susceptible to tuberculosis than females (Male: female ratio was 3.5:1). Among the TB-positive samples, MDR TB was also much more common in males than females. The exact cause is uncertain. This may be due to males travelling more frequently, having more social contacts, spending more time in outdoor activities, engaging in professions associated with a higher risk for TB like mining, other factors are smoking and alcohol. In the area of Western Rajasthan, there is “parda pratha” for females in which females are restricted to interact with unknown males; this may be a reason for those females are less commonly exposed to infected males and less chance of infection. Males (70.7%) were also found to be predominantly infected in a study conducted by R Singhal et al. The global male: female (M: F) ratio was 1.6:1 according to the Global tuberculosis report 2017.  

The prevalence of drug-resistant tuberculosis is increasing in India. Previously treated TB cases, who stop treatment before completion, residing in the area with a high prevalence of drug-resistant tuberculosis or close contact with an individual who is infected with MDR-TB are prone for developing drug-resistance tuberculosis. Drug resistance is usually acquired by spontaneous mutations as a result of the inappropriate use of antimicrobial agents to treat M. tuberculosis and the lack of patient compliance. In our study, the prevalence of MDR-TB was found to be 11.42% with mono-resistance to isoniazid and rifampicin 4.28% and 6.42%, respectively. The LPA valid smear-positive pulmonary samples sensitive to both drugs were 77.85%. Previous exposure to anti-tuberculosis agents is the most common cause of developing MDR-TB. Various Indian studies have reported MDR rates to be varying from 17.4% to 53% among re-treatment cases. The worldwide prevalence of MDR in re-treatment cases ranged from 9.4% to 36.5%, from 1994-2000 across the world. A similar study conducted by R Singhal et al. found 15.5% MDR-TB cases with mono-resistance to isoniazid and rifampicin 6.6% and 7.1%, respectively. Another study found 19.73% MDR-TB cases with mono-resistance to isoniazid and rifampicin 8.63% and 6.14%, respectively. Of all the MDR patients, most of the patients are found to be retreatment cases (87.5%).  

A major limitation of the molecular genetic detection of drug resistance remains that not all mutations conferring resistance to anti-TB drugs are known. This is especially true in detecting isoniazid resistance and explains the comparatively low sensitivity. LPA with GenoType MTBDRplus has revolutionized the MDR-TB diagnosis. Less staff is able to complete for a greater number of DSTs per day. The test provides additional information about the common mutations imparting resistance to rifampicin and isoniazid, which helps in understanding the epidemiology of the disease. More studies need to be instituted to assess the performance of the test in smear-negative patients at larger platforms. Currently, WHO recommended the use LPA for the initial drug resistance screening of sputum smear-positive samples.  

Line probe assay is available free of cost through the Revised National Tuberculosis Control Programme (RNTCP). This test still underutilized because of unawareness of primary care physician and use of this test in general and family physician practises significantly improve the diagnosis and treatment of multidrug resistance pulmonary tuberculosis. Use of this molecular test reduces the need of sputum culture which is a time-consuming method and not available easily in primary care setting.  

**Conclusion**  

Line Probe Assay is an economical and time-saving method for the detection of MDR-TB and serves as a lifesaving tool for early diagnosis and treatment. This calls for a widespread national use of this assay. Detection of around 10% sputum ZN staining positive patients, who were not showing MTB complex in LPA may be a hidden iceberg for non-tubercular mycobacteria.  

**Declaration of patient consent**  

The authors certify that they have obtained all appropriate patient consent forms. In the form, the patients have given their consent for their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.  

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Conflicts of interest

There are no conflicts of interest.

References

1. World Health Organization. WHO End TB Strategy: Global strategy and targets for tuberculosis prevention, care and control after 2015. Geneva: WHO; 2015. Available from: http://www.who.int/tb/post2015_strategy.
2. WHO Global Tuberculosis Report, 2014.
3. TB INDIA 2014 Revised National TB Control Programme ‘annual status report’.
4. Sharma SK, Mohan A. Multidrug-resistant tuberculosis. Indian J Med Res 2004;120:354‑76.
5. Zumla A, Raviglione M, Hafner R, von Reyn CF. Tuberculosis. N Engl J Med 2013;368:745‑55.
6. Frieden TR, Munsiff SS, Ahuja SD. Outcomes of multidrug‑resistant tuberculosis treatment in HIV‑positive patients in New York City, 1990‑1997. Int J Tuberc Lung Dis 2007;11:116.
7. Dheda K, Shean K, Zumla A, Badri M, Streicher EM, Page‑Shipp L, et al. Early treatment outcomes and HIV status of patients with extensively drug‑resistant tuberculosis in South Africa: A retrospective cohort study. Lancet 2010;375:1798‑807.
8. Udwadia ZF, Amale RA, Ajbani KK, Rodrigues C. Totally drug‑resistant tuberculosis in India. Clin Infect Dis 2012;54:579‑81.
9. Nathanson E, Nunn P, Uplekar M, Floyd K, Jaramillo E, Lönnroth K, et al. MDR tuberculosis-critical steps for prevention and control. N Engl J Med 2010;363:1050‑8.
10. Piersimoni C, Olivieri A, Benacchio L, Scarparo C. Current perspectives on drug susceptibility testing of Mycobacterium tuberculosis complex: The automated nonradiometric systems. J Clin Microbiol 2006;44:20‑8.
11. Watterson SA, Wilson SM, Yates MD, Drobniowski FA. Comparison of three molecular assays for rapid detection of rifampicin resistance in Mycobacterium tuberculosis. J Clin Microbiol 1998;36:1969‑73.
12. Hilleman D, Rusch‑Gerdes S, Richter E. Evaluation of the GenoType MTBDRplus assay for rifampin and isoniazid susceptibility testing of Mycobacterium tuberculosis strains and clinical specimens. J Clin Microbiol 2007;45:2635‑40.
13. Tripathi R, Sinha P, Kumari R, Chaubey P, Pandey A, Anupurba S. Detection of rifampicin resistance in tuberculosis by molecular methods: A report from Eastern Uttar Pradesh, India. Indian J Med Microbiol 2016;34:92‑4.
14. Central TB Division, Directorate General of Health Services, Ministry of Health and Family Welfare; 2005. Laboratory manual for sputum smear microscopy. Revised National Tuberculosis Control Programme (RNTCP).
15. Brossier F, Veziris N, Pernot CT, Jarlier V, Souqakoff W. Performance of the GenoType MTBDR line probe assay for detection of resistance to rifampicin and isoniazid in strains of Mycobacterium tuberculosis with low- and high-level resistance. J Clin Microbiol 2006;44:3659.
16. GenoType MTBDR Plus, VER 2.0, Instructions for Use, IFU‑304A‑02, Hain Lifesciences GmbH, Germany. 2012 Available from: https://www.ghdonline.org/uploads/MTBDRplusV2_0212_304A‑02‑02.pdf.
17. Raizada N, Sachdeva KS, Chauhan DS, Malhotra B, Reddy K, Dave PV, et al. A multi-site validation in India of the line probe assay for the rapid diagnosis of multi-drug resistant tuberculosis directly from sputum specimens. PLoS One 2014;9:e88626.
18. Singhal R, Myneedu VP, Arora J, Singh N, Bhalla M, Verma A, et al. Early detection of multi-drug resistance and common mutations in Mycobacterium tuberculosis isolates from Delhi using GenoType MTBDRplus assay. Indian J Med Microbiol 2015;33:S46‑52.
19. Global tuberculosis report 2017. Available from: http://www.who.int/tb/publications/global_report/MainText_13Nov2017.pdf.
20. Ramachandran R, Nalini S, Chandershekhar V, Dave PV, Sanghvi AS, Wares F, et al. Surveillance of drug resistant tuberculosis in the state of Gujrat, India. Int J Tuberc Lung Dis 2009;13:1154‑60.
21. Parmasivan CN, Rehman F, Wares F, Sundar Mohan N, Sundar S, Devi S, et al. First- and second-line drug resistance patterns among previously treated tuberculosis patients in India. Int J Tuberc Lung Dis 2010;14:243‑6.
22. Zignol M, van Gemert W, Falzon D, Sismanidis C, Glaziou P, Floyd K, et al. Surveillance of anti-tuberculosis drug resistance in the world: An updated analysis 2007–2010. Bull World Health Organ 2012;90:111‑9D.
23. Singhal R, Arora J, Sah GC, Bhalla M, Sarin R, Prasad Myneedu V. Frequency of multi-drug resistance and mutations in Mycobacterium tuberculosis isolates from Punjab state of India. J Epidemiol Glob Health 2017;7:175‑80.
24. Thakur C, Kumar V, Gupta AK. Detecting mutation pattern of drug-resistant Mycobacterium tuberculosis isolettes in Himachal Pradesh using GenoType® MTBDRplus assay. Indian J Med Microbiol 2015;33:547‑53.
25. Nguyen TNA, Anton-Le Berre V, Bañuls AL, Nguyen TVA. Molecular diagnosis of drug-resistant tuberculosis: A literature review. Front Microbiol 2019;10:794.