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Molecular mechanisms of drug-resistance in MDR-strains of M. tuberculosis

Seema P.[1*], Meera M.[2], Bodhankar M.[3]

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[1*] Patil Seema, Department of Medical Microbiology, Bharati Vidyapeeth deemed University- Rajiv Gandhi Institute of Information technology and Biotechnology, Pune, Maharashtra, India.

[2] Modak Meera, Department of Medical Microbiology, Bharati Vidyapeeth deemed University, Pune, Maharashtra, India.

[3] MG Bodhankar, Department of Microbiology, Bharati Vidyapeeth deemed University- Yashwantrao Mohite college of Science, Pune, Maharashtra, India.

Aim: The Present study was conducted to detect pattern and mechanisms of drug resistance in isolates of Mycobacterium tuberculosis in a tertiary care hospital in Western Maharashtra. **Material and Methods:** Total 1186 sputum samples from clinically suspected Tuberculosis patients were screened for presence of M. tuberculosis, acid-fast bacilli (AFB) by Ziehl-Neelsen staining technique. Out of that, 130 were smear positive showing presence of AFB. Out of these, 123 were successfully cultivated on Lowenstein-Jensen's media showing typical colonies of M.tuberculosis, which were confirmed by standard biochemical tests. The drug susceptibility testing (DST) of all these isolates for first line anti-tubercular drugs was performed by phenotypic –gold standard "proportion method" and only 10 isolates were resistant to rifampicin and isoniazid i.e. multi-drug resistant (MDR) strains. The molecular mechanisms of resistance to these drugs was identified by detecting mutations in rpoB gene (for Rifampicin resistance) and inhA gene (high level INH resistance) and katG gene (low level INH resistance) by using MTBDRplus assay. **Results:** Out of 10 phenotypically identified MDR-TB strains, in 8 strains, Rifampicin (RMP) resistance was associated with missing of rpoB WT8 gene while missing of rpoB WT7, 8 and rpoB WT4, 5 was observed in two different strains. High level Isoniazid (INH) resistance was observed in 5 strains showing presence of katG MUT1 band while low level Isoniazid (INH) resistance was observed in 5 strains; showing presence of inhA MUT3A band in 2 strains and inhA MUT 3B band in 3 strains. In present study MDR-TB, rate was 8.13%. **Conclusion:** Identification of mutations in genes of M. tuberculosis by MTBDRplus is a rapid method to detect MDR-TB strains of M. tuberculosis.

Keywords: AFB, MDR-TB, ZN staining, L.J. medium, Proportion method, MTBDRplus assay

Corresponding Author

Patil Seema, , Department of Medical Microbiology, Bharati vidyapeeth deemed university- Rajiv Gandhi Institute of Information technology and Biotechnology, Pune, Maharashtra, India.
Email: simaapatil@gmail.com

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Introduction

Tuberculosis is an ancient disease caused by M. tuberculosis. Robert Koch discovered this pathogen in 1843 but still the disease in not in control due to development of new drug resistant strains of M. tuberculosis. First drug-resistant strain of Mycobacterium tuberculosis showing resistance to streptomycin was observed immediately after its discovery in 1944. Wide clinical use of streptomycin promptly followed, eventually in combination with other drugs like isoniazid, Pyrazinamide, ethambutol and rifampicin to attack resistant bacilli. Mismanagement of TB paves the way to drug resistant tuberculosis like Multi Drug Resistant TB (MDR-TB, 1990), Extensively Drug Resistant TB (XDR-TB, 2006) and Extremely Drug Resistant TB (XXDR-TB)/ Totally Drug-Resistant TB (TDR-TB, 2007).

Multi Drug Resistant Tuberculosis (MDR-TB) is defined as Tuberculosis, which is caused by the strains of Mycobacterium tuberculosis that are resistant at least to isoniazid (INH) and Rifampicin (RIF), the most powerful first line anti tuberculosis drugs. Extensive Drug Resistant Tuberculosis (XDR-TB) is defined as disease caused by strains of Mycobacterium tuberculosis that are resistant to not only INH and RIF (i.e, MDR-TB) but also to any Fluoroquinolones and any of second-line anti-TB injectible drugs like amikacin, kanamycin or capreomycin. These forms of TB do not respond to the standard six-month treatment with first –line anti-TB drugs and can take up to two years or more to treat with drugs that are less potent, more toxic and much more expensive [1]. Extremely or Totally drug resistant Tuberculosis (XXDR-TB/TDR-TB) is defined as tuberculosis caused by strains of Mycobacterium tuberculosis that are resistant to all the existing anti-tuberculosis drugs. All these drug resistant forms of TB are the emerging threats to the success of anti-TB programs.

According to WHO estimates, India has the world's largest tuberculosis epidemic. WHO statistics for 2013 giving an estimated incidence figure of 2.2 million cases of TB for India out of a global incidence of 8.6 million cases. It is estimated that about 40% of the Indian population is infected with TB bacteria, the vast majority of which have latent rather than active TB. Most of the statistics for India is from the government Revised National Tuberculosis Control Programme (RNTCP) which was started in 1997. Tuberculosis is the biggest health issue as all the existing forms of drug-resistant strains of Mycobacterium tuberculosis- MDR, XDR and TDR are prevalent in the country. Dr Udwadia ZF reported TDR-TB in Jan 2012 from Hinduja hospital, in Mumbai (Maharashtra). In 2012, India declared TB to be a notifiable disease- meaning that with immediate effect all private doctors, caretakers and clinics treating TB patients had to report every case of TB to the government [2]. According to WHO-2012 report, India ranks 2nd among 27 MDR-TB high burden countries worldwide after China (66000 cases emerge annually). The prevalence of XDR-TB has been reported from India, which varies between 2-4% to as high as 33.3% among HIV infected persons suffering from MDR-TB [3].

Maharashtra's current burden of drug-susceptible TB is 1, 37,320 lakh cases and there are 4,397 MDR-TB cases apart from the 115 XDR-TB cases, which are more difficult to treat. It is reported that in last 3 years state has recorded 165 XDR-TB cases. Out of these 115 were recorded last year in 2013. Barring nine cases, 123 are from Mumbai, 25 from Navi Mumbai and 8 from Pune [4].

According to RNTCP-DRS report, 2012; in Gujarat, Maharashtra and Andhra Pradesh, MDR-TB prevalence is low i.e less than 3% among new cases and 12-17% in re-treatment cases. Drug resistance is largely man-made and is a consequence of suboptimal regimens and treatment interruptions [5]. While TB is 100% curable, MDR-TB is difficult to treat.

Resistance to antimicrobial agents is an innate characteristic of M. tuberculosis. It is related to genetic mutations that occur naturally in large populations of microorganisms. These mutations are thought to be associated with loss of fitness so that, in the wild state, where specific antimicrobial agents have never been used, this resistance has no clinical significance. Clinically significant drug resistance constantly has its origins in the incorrect use of antimicrobial agents and is in this sense a 'man-made' phenomenon [6].

Globally in 2012, an estimated 4, 50,000 people developed MDR-TB and there were an estimated 1, 70,000 deaths from MDR-TB. According to WHO-2012 report, India ranks 2nd among 27 MDR-TB high burden countries worldwide after China (66000 cases emerge annually).

Materials and Methods

1. Source of data: The patients at Bharati hospital; Pune, with clinically suspected Tuberculosis as per World Health Organization (WHO) forms the source of data.

2. Sample collection: Early morning expectorated sputum samples were collected in wide mouthed disposable sterile containers on three successive days. The samples were stained to confirm the presence of *Mycobacterium tuberculosis* and detection of its resistance pattern to first-line anti-tuberculosis drugs ([7], [8], [9]).

Microscopic examination to detect *M. tuberculosis* - Acid-fast bacilli (AFB)

The smears were screened for AFB by Ziehl-Neelsen staining techniques and positive smears were graded as per revised national tuberculosis control program (RNTCP) guidelines [123].

Homogenization and Decontamination: Petroff's method

5ml of sputum was transferred to centrifuge tube and double the volume of sterile 4% NaOH solution was added aseptically. Cap was tightened and incubated at 37°c for 30 minutes. Then 15ml sterile distilled water was added, centrifuged at 3000g for 15 minutes and the supernatant was discarded slowly into container with 5% phenol solution. Again equal quantity of sterile distilled water was added, centrifuged at 3000g for 15 minutes, supernatant was discarded and sediment was used for the isolation of *M. tuberculosis* [10].

Isolation of *tuberculosis* by using Lowenstein Jensen's (LJ) media:

Inoculation method: From the sediment, one loopful was inoculated on the surface of slope of LJ media and incubated at 37°c for 48-72 hours.

Examination schedule: Inoculated LJ media was examined after 48-72 hours to detect gross contaminants. Thereafter, culture was examined weekly once for 8 weeks on a specified day of week. Contaminated cultures were discarded.

Reading of cultures: Typical colonies of *M. tuberculosis* were rough, crumbly, waxy, non-pigmented (buff colored) and slow growers i.e appearing 2-3 weeks after inoculation.

Identification of *M. tuberculosis* was done by performing specific biochemical tests Niacin test, Nitrate reduction test and catalase test.

Drug sensitivity testing by Proportion method

For the detection of MDR strains of isolates, 1st line drugs isoniazid (0.2µg/ml), rifampicin (40µg/ml), ethambutol (2.0µg/ml), Pyrazinamide (200µg/ml) and streptomycin (4.0µg/ml) were used.

Formula to be used to calculate the percentage of resistant cells:

No. of colonies on the Drug medium

X 100 = %

No. of colonies on the control medium

Accordingly, results are interpreted as:

1. Resistant: when >1%

2. Sensitive: when <1%

3. Intermediate: when =1%

Molecular methods for detection of drug resistance by line probe assay (LPA) [7]- GenoType MTBDRplus assay (Hain's Lifescience, Germany)

This molecular method was used to detect the mutation pattern in the genes of *M.tuberculosis*, which are responsible for the resistance of the specific anti-tuberculosis drugs.

Results

1. AFB Smear positivity rate: Out of 1186 clinically suspected cases, sputum smear for AFB was positive in 130 samples (10.96%). (Table-1)

Table 1 AFB Smear positivity

ZN-smear	No. of cases	Percentage (%)
Positive	123	10.96%
Negative	07	89.04%
Total	130	100%

2. Culture positivity rate: Out of 130 cases, 123 cases could be tracked during this study period. The smear positive samples were cultured on L.J. medium. The sensitivity of L.J. in comparison with smear is 94.61%. Comparison of results of Microscopy versus Culture on L.J. is shown in table no.-2.

Table 2: **Result of Cultures of M.tuberculosis isolates on L.J.**

AFB smear positive	L.J. +VE	L.J.-VE	Total
130	123 (94.61%)	07(5.38%)	130(100%)

The average turnaround time for culture of M. tuberculosis on L.J. was 21-28 days

3. Antibiotic Resistance pattern of tuberculosis isolates

The H37RV strain and total 123 AFB and culture positive isolates were tested for drug susceptibility by **Proportion method on L.J.** medium to detect drug resistance pattern (whether MDR /XDR). For detection of MDR-TB strains, 1st line anti-tubercular drugs- Streptomycin(S), Isoniazid (I), Rifampicin (R) and Ethambutol (E) were used.

As per the formula to be used to calculate the percentage of resistant cells i.e

The resistance / susceptible pattern of the isolates against the, 1st line anti-tubercular drugs are depicted in table no.3 and 4.

Table 3: Resistance pattern and rate

Drug combination	No. of drug resistant isolates	Percentage of drug resistance
SIRE	10	8.3%
SIR	0	0%
SIE	8	6.5%
IRE	0	0%
SI	4	3.25%
IR	0	0%
SE	12	9.75%
RE	6	4.87%
S	2	1.62%
I	9	7.3%
R	3	2.43%
E	7	5.69%

Table 4: Number of Sensitive and Resistant isolates of M. tuberculosis for each drug

Drug		No. of isolates of M.tuberculosis	Percentage of drug susceptibility
Streptomycin	Sensitive	87	70.73%
	resistant	36	29.26%
Isoniazi	Sensitive	92	74.79%
	resistant	31	25.20%
Rifampicin	Sensitive	104	84.55%
	resistant	19	15.44%
Ethambutol	Sensitive	80	65.04%
	resistant	43	34.95%

Interpretation

- 23% were sensitive to streptomycin.
- 26% were resistant to streptomycin.
- 79% were sensitive to Isoniazid.
- 20% were resistant to Isoniazid.
- 55% were sensitive to Rifampicin.
- 44% were resistant to Rifampicin.
- 04% were sensitive to Ethambutol.
- 95% were resistant to Ethambutol.

In our study, out of 123, only 10 strains are resistant to both rifampicin and isoniazid, therefore the MDR-TB rate observed is 8.13%.

LPA (MTBDRplus-assay) of the 10 isolates exhibited different band formations. Appearance of control bands validated the assay. The intensity of bands was compared with that of amplification control for positivity identification.

Mutations associated with Drug Resistance

Among Mycobacterium tuberculosis isolates, 8.13% were MDR-TB strains showing resistance to both INH and RMP (first-line anti-tuberculosis drugs). All these strains were subjected to line-probe assay (LPA) to detect mutation pattern in the respective genes. Table no. – 5 show different patterns of mutations in the genes of these 10 MDR-TB strains.

Table 5: **Pattern of gene mutations detected by line probe assay (LPA) - MTBDRplus in Mycobacterium tuberculosis isolates**

Sample no.	Banding patterns of MDR-TB isolates
1	Negative control
2	rpoB WT8 missing and rpoB MUT3, katG MUT1 present
3	rpoB WT8 missing and rpoB MUT3, katG MUT1 present
4	rpoB WT8 missing and rpoB MUT2A, inhA MUT3A present
5	rpoB WT8 missing and rpoB MUT3, inhA MUT3B present
6	rpoB WT7and8 missing and rpoB MUT3, inhA MUT3A present
7	rpoB WT4 and5 missing and rpoB MUT3, katG MUT1 present
8	rpoB WT8 missing and rpoB MUT3, katG MUT1 present
9	rpoB WT8 missing and rpoB MUT3, katG MUT3B present
10	rpoB WT8 missing and rpoB MUT3, katG MUT1 present
11	rpoB WT8 missing and rpoB MUT3, katG MUT1 present

In eight strains, RMP resistance was associated with the missing of rpoB WT8 gene while missing of rpoB WT7, 8 and rpoB WT4, 5 was observed in two different strains.

High level INH resistance was observed in 5 strains showing presence of katG MUT1 band while low level INH resistance was observed in 5 strains; showing presence of inh MUT3A band in 2 strains and inh MUT 3B band in 3 strains.

Discussion

1. Antitubercular drug susceptibility : The main objective of this research is to detect the drug resistance pattern and mechanisms in Mycobacterium tuberculosis isolates. In the present study for the detection of drug- resistant pattern of 1st-line anti-tubercular drugs: Streptomycin, Rifampicin, Isoniazid, Ethambutol, gold standard conventional Proportion method on L.J. is used to detect the susceptibility/resistance to each of the 1st-line drugs. (Table-3 and 4)

Susceptibility pattern to 1st-line drugs was:

- Ethambutol and Streptomycin resistance was seen in majority of the isolates (34.95% and 29.26%).
- **Monodrug** resistance was observed for all 1st-line drugs like Streptomycin (1.62), Isoniazid (7.3%), Rifampicin (2.43%), and Ethambutol (5.64%).
- **Resistance to two** 1st-line drugs was observed for Streptomycin and Isoniazid (3.25%), Streptomycin and Ethambutol (9.75%), Rifampicin and Ethambutol (4.87%).
- **Resistance to combination of three** 1st-line drugs was observed for Streptomycin, Isoniazid and Ethambutol (6.5%).
- **Resistance to all 4** 1st-line drugs was observed by 8.13% cases.
- In this study, the resistance pattern to first line drugs was INH (25.20%), Rifampicin (15.44%), Streptomycin (29.26%) and Ethambutol (34.95%).
- Resistance to INH was seen in 25.20% in our study which is much lower than studies reported by Verma et al. (40.5%), Sureshkumar et al. (42%), Menon et al. (53.2%) and Barat et a (80%) ([11],[12],[13],[14]).
- Resistance to Rifampicin was seen in 15.44% in our study which is much lower than studies reported by Verma et al. and Bhatt e al. (18%), Sureshkumar et al. (32%), Menon et al. (74.4%) and Barat et al. (76%) ([11],[12], [13],[14],[15]).
- Resistance to Streptomycin was seen in 29.26% in our studies which is much more lower than the studies reported by Bhat et al. (62%) and Menon et al. (70%) ([14],[13]).
- Bhat et al. (12%), Menon et al. (21.7%) and Verma et al. (27%) saw resistance to Ethambutol in 34.95% in our study, which is much higher than studies ([14],[16],[11]).

The present overall rate of MDR-TB in India is around 18-20%. In the present study, the MDR-TB rate is observed to be 8.13%. Rate of MDR-TB was higher in other studies of Filho et al. (78.8%), Barat et al. (72%), Verma et al. (66.6%) and Menon et al. (47.54%) ([17], [14], [15], [16]). Lesser MDR resistance was reported by Mulenga et al. (5%), 2.9% by Therese et al., 4% by Bhat et al. ([18], [19], [14]).

Overall drug resistance pattern shows moderate rate of MDR-TB (8.13%), which indicates the effective management of DOTS through RNTCP in the area.

2. Genotyping of Drug-Resistant strains of Mycobacterium tuberculosis by LPA:

This study aimed to provide preliminary data on the rate of resistance to first-line anti-tuberculosis drugs, especially INH and RMP and pattern of mutations in the genes responsible for drug resistance. For studying this aspect, the phenotypic Proportion method and the GenoType MTBDRplus-Hain Life science method of LPA was used.

3. Genotypic (LPA) Vs Phenotypic (proportion) methods:

It is reported that in recent years substantial progress has been made in our understanding of the molecular basis of Mycobacterium tuberculosis drug resistance. Molecular based assays are potentially the most rapid and sensitive methods for the detection of drug resistance. These assays detect all common drug resistance mutations. Some of these techniques include direct sequencing of PCR products, SSCP analysis, heteroduplex analysis, dideoxy fingerprinting, an RNA/DNA duplex, base-pair mismatch assay, Lucifer's mycobacteriophage strategy, a rRNA/DNA- bioluminescence-labled probe method, a reverse hybridization- based line probe assay and other strategies.(31-35,20)

In the present study, GenoType MTBDRplus (LPA) was used which detects resistance to INH and RMP in clinical isolates based on the detection of the most common mutations rpoB, katG and inhA genes. It uses PCR and reverse-hybridization to probes immobilized on an assay strips ([20],[22], [23],[24],[25]).

All the 10 MDR- strains detected by phenotypic methods have shown the mutations in the rpoB gene (RMP- resistance) and katG/inhA (INH-resistance) by GenoType MTBDRplus molecular method (Table no.5). Thus, the sensitivity for MDR, RMP, and INH detection was found to be 100%.

Kembhavi et al. [36] reported the same 100% sensitivity for MDR-TB detection by this method while Farroqui et al. and 97.7% by Maurya et al. [30] reported lower sensitivity (92.5%). The commonest pattern seen was missing of WT8 and presence of MUT3 in rpoB gene in nine (90%) strains while presence of rpoB MUT2A was seen in one strain. Low level INH resistance was observed in 50% strains while high-level resistance was observed in 50% strains. Kembhavi et al., Farroqui et al., and Maurya et al. (Table No.6) reported the similar pattern of mutation

Table 6.

Pattern of mutations in rpoB and inhA gene of M.tuberculosis by MTBDRplus assay in various studies:-

Study	Technique used	MDR sensitivity	Mutations in RMP	Mutations in INH
Farroqui et al. 2012	MTBDRplus	92.5%	531,533,S531L	S315T:katG C15T:inhA
Maurya et al 2013	MTBDRplus	97.7%	S531L	S315T1:katG
Kembhavi et al	MTBDRplus	100%	531,526,516.S531L	S315T1:katG C15T:inhA
Present study	MTBDRplus	100%	S531L	S315T:katG C15T:inhA

Rifampicin resistance (RMP)

Rifampicin is a powerful bactericidal agent used as a first line anti-tubercular drug. Rifampicin resistance arises due to mutations in rpoB gene- producing DNA dependent RNA polymerase. The nature and frequency of mutations in the gene of RMP resistant isolates vary considerably according to geographical locations.

Analysis of approximately 500 rifampicin resistant strains from global sources has found that 96% of rifampicin resistant clinical isolates of Mycobacterium tuberculosis have mutations in the 81- bp core region rpoB gene, which encodes the β-subunit of RNA polymerase. Detection of mutations in the 81- bp core region correlated (100%) very highly in their study. Similar findings have been reported by Barnard et al and Ravindran et al. ([26], [27]). The sensitivity of RMP can be decreased if mutations are outside the 81-bp region of rpoB gene which cannot be detected by this assay. A higher (90%) proportion of RMP resistance due to S531L mutations was observed. Kembhavi et al. also found higher proportion (89.65%) of RMP resistance due to mutations in S531L in their study but lower proportion (62.3%) and (62%) of RMP resistance was reported by Maurya et al. and Farroqui et al. respectively ([22],[28]).

Isoniazide resistance (INH)

INH is also a bactericidal agent used as a first line drug for TB. Resistance to INH arises due to mutations in different genes including katG, inhA, ahpc and other genes that remain to be established. In this study, katG mutations were found in 50% and 50% in inhA genes. Kembhavi et al. had reported higher (75.86%) katG mutations and lower (20.66%) inhA mutations. Maurya et al. had also reported commonest INH mutations in katG (93.3%) and lower (28.9%) in inhA genes. Similarly Farooqi et al. had reported the higher proportion (66.1%) of mutations in katG genes and lower proportion (1.9%) of mutations in inhA genes. High prevalence of katG mutations has been reported to confer resistance in high prevalence countries [29] and for a much lower proportion in lower TB prevalence settings presumably due to ongoing transmission of these strains ([22],[23],[24]).

Geographical profile of rpoB mutations in Mycobacterium tuberculosis was studied by Lingala et al. [23] reported that 74% of RMP resistant isolates in their study includes common mutations at 531,526 and 516 region of rpoB gene. They have also reported the multiple silent mutations between 145-184 (outside the hot spot region) in their study ([20], [21], [22]).

LPA is an appropriate tool for rapid screening for MDR-TB and has the potential to substantially reduce the turnaround time of DST results.

Conclusion

Use of MTBDRplus molecular assays will help in detection of drug-resistant cases and helps to detect mutations. However, these systems should be made affordable, as the prices are higher than the conventional tests.

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