

Prevalence of Drug Resistance in *Mycobacterium tuberculosis* in a Teaching Hospital of Kanchipuram District, Tamilnadu, South India

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Abstract Objectives: To study the prevalence of drug resistance in *Mycobacterium tuberculosis* isolated from patients with pulmonary and extrapulmonary tuberculosis. **Materials and Methods:** A total of 178 extra pulmonary clinical specimens were collected from patients suspected with extrapulmonary tuberculosis (EPTB) and again 2,048 sputum samples from 1030 patients with clinical evidence of pulmonary tuberculosis (PTB) were collected in SRM Hospital and Research Centre of Kanchipuram District. Both pulmonary and extrapulmonary specimens were stained by Ziehl-Neelsen (ZN) method. All sputum AFB smear positive specimens and 178 extrapulmonary clinical specimens were cultured in Lowenstein Jensen (LJ) medium. All Mycobacterial isolates were identified by standard protocols and were tested by conventional drug susceptibility method using absolute concentration method. **Results:** Out of 1030 clinically suspected pulmonary tuberculosis patients, 125 (12.13%) patients were smear positive for AFB. Among these 125, 76 (54.4%) were positive for Mycobacterium species cultured on LJ medium. Out of 76 mycobacterial isolates, 68 (89.4%) were *M.tuberculosis* and 8 (10.52%) were non tuberculous mycobacteria (NTM). Of 178 extrapulmonary clinical specimens, 10 (5.6%) were direct AFB smear positive and 6 (3.37%) were positive for *M. tuberculosis* on LJ medium. All 74 *M.tuberculosis* isolates (68 pulmonary isolates and 6 extrapulmonary isolates) were tested for first line anti-TB drugs by conventional drug susceptibility method. Three (4.05%) *M.tuberculosis* isolates were resistant to rifampicin, isoniazid and ethambutol. All the three multidrug resistance tuberculosis (MDR - TB) strains were isolated from pulmonary tuberculosis patients. **Conclusion:** In this hospital area, 4.05 per centage of MDR - TB isolates were reported in culture positive PTB cases.

Keywords: prevalence of drug resistance, pulmonary and extra pulmonary tuberculosis, absolute concentration method, Middle brook 7H10 agar, first line anti-tb drugs

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1. Introduction

Multi drug resistance in *Mycobacterium tuberculosis* (MTB) is a serious threat in developing countries [1]. The average prevalence of multi drug resistant tuberculosis (MDR-TB) in new cases of tuberculosis was 1.1% (range 0-14.2%). Among the previously treated cases median prevalence of resistance to any drug was 33.4% (range 0-93.8%). High levels of resistance have been reported in certain regions of the world, particularly in Asia and parts of Africa [2-8]. India is contributing to nearly one third of the world's tuberculosis (TB) cases and has the highest rate of new cases [9]. Prevalence of MDR-TB cases is on the rise in India, being about 1.1% to 5.3%. The mean average of MDR TB among previously treated patients varied from 8% to 67% [10]. MDR-TB most commonly

develops due to inappropriate treatment, or patients missing doses or failing to complete their treatment [11].

Drug resistance in mycobacteria is defined as a decrease in sensitivity to a drug to be reasonably certain that the strain concerned is different from a sample of wild strains of human type that have never come in contact with the drugs [12]. The probability of incidence for drug resistant mutant is 10^{-8} for rifampicin, while for isoniazid and some of the other commonly used drugs it is 10^{-6} . Therefore, the probability for resistance to be developed for a combination isoniazid and rifampicin is 10^{-14} , which is much higher than the number of organisms present in a medium sized cavity in a patient with open pulmonary TB. Although for several years, drug resistant strains of *M. tuberculosis* were considered to be less infectious than the drug susceptible ones, recent studies have demonstrated that the drug resistant mutants are equally infectious and

can cause severe disease in an individual exposed to the same [13].

For both guidance of therapy and surveillance of drug resistance, accurate drug susceptibility testing (DST) for *Mycobacterium tuberculosis* complex is considered highly important [14]. In 1950s Cannetti *et al.*, described the first DST method for *M. tuberculosis*, involving the preparation of a serial dilution of drug against *M. tuberculosis* complex in Lowenstein-Jensen (L-J) medium and inoculation of the bacterial cultures on the slants, to the inhibition of growth by drugs at different concentrations [15]. The World Health Organization (WHO) recommended that drug susceptibility testing be done preferably by the method on L-J medium, but other media, such as Middle brook 7H10, 7H11, 7H12 (BACTEC 460 TB) including the absolute concentration and resistance ratio methods, may also be used [16].

The present study was undertaken to find the prevalence of drug resistance in *Mycobacterium tuberculosis* isolated from sputum AFB smear positive pulmonary and clinically suspected extrapulmonary tuberculosis patients in a teaching Hospital of Kanchipuram district, Tamilnadu, South India.

2. Materials and Methods

A total of 178 extra pulmonary clinical specimens were collected from patients suspected with extrapulmonary tuberculosis (EPTB) during the period of May 2008-May 2009 and again 2,048 sputum samples were collected from 1030 patients with clinical evidence of pulmonary tuberculosis (PTB) during the period of May 2009 to May 2010 in a teaching Hospital of Kattankulathur, Kanchipuram district, Tamilnadu, South India.

Out of 1030 suspected patients, 125 patients were sputum AFB smear positive PTB included and were categorized according to the Revised National Tuberculosis Control Programme (RNTCP) guidelines such as; those who had never received anti-TB drugs previously, patients who had history of treatment for pulmonary tuberculosis in the past and patients with history of treatment for pulmonary tuberculosis for more than one month during illness. The study was approved by the Institutional Ethical Committee (IEC) of the SRM Medical College Hospital and Research Centre (SRM MCH & RC) and informed consent was obtained from each patient.

2.1. Sample Collection

According to the WHO guidelines [17], two consecutive sputum samples namely one "spot" and another one "early morning" sputum sample were collected from 1030 patients who had clinical evidence of pulmonary tuberculosis (PTB). One hundred and seventy eight non-repeated extrapulmonary clinical specimens were collected under sterile condition from patients who were clinically suspected to have extrapulmonary tuberculosis (EPTB). The clinical specimens included; sputum samples, body fluids, pus, liver abscess, semen, biopsy and tissue specimens collected in sterile universal container.

2.2. Specimens Processing

All pulmonary and extrapulmonary specimens were processed and stained by Ziehl-Neelsen (Z-N) method and examined for Acid Fast Bacilli (AFB). Direct AFB smear positive sputum samples and pus samples were processed for culture by petroff's (4% NaOH) method. Other sterile body fluids (synovial fluid, cerebrospinal fluid (CSF), pleural fluid, ascetic fluid, pericardial and pancreatic cyst fluid) were centrifuged at 3000 g for 15 minutes. Three consecutive early morning urine samples were collected and centrifuged at 3000 g for 15 minutes. The deposit was decontaminated with equal volume of 5 percent H₂SO₄. Omental biopsy and skin tissue samples were ground well with 5ml of sterile distilled water. The samples were centrifuged and the deposit was decontaminated with 5 percent H₂SO₄. Fine needle aspiration (FNA) samples were directly inoculated on to a pair of L-J medium. The decontaminated deposits of clinical specimens were inoculated into another pair of L-J medium and monitored for growth. Once growth appeared, it was tested by Ziehl-Neelsen (Z-N) staining for Acid-Fast Bacilli (AFB). Mycobacterial species identified by standard conventional protocol [18] such as; based on slow growth rate, absence of pigmentation, Niacin test positivity and absence of growth on L-J medium with ρ -nitrobenzoic acid, semi-quantitative Catalase test and Catalase test at 68°C /P^H 7.0.

2.3. Culture for Primary Isolation and Drug Susceptibility for *M. tuberculosis*

2.3.1. Preparation of Lowenstein-Jensen (L-J) Medium

Lowenstein-Jensen (L-J) medium was prepared for primary isolation of *M. tuberculosis* from clinical specimens. Approximately 15 ml of medium was poured into sterilized 25 ml Bijoux (*McCartney*) Bottle, secured with sterilized cap and kept at 85°C in slanting position for 45 min. Once the medium solidified, it was kept at 115°C for 10 min, cooled, labeled and stored at 2-8°C. Every batch of L-J medium was checked for quality control before specimen inoculation.

2.3.2. Preparation of Middle Brook (MB) 7H10 Agar Medium

To prepare 500 ml medium solution, the amount of Middle brook (MB) 7H10 powder as recommended by the manufacturer (*HiMedia, India*) was added to 450ml distilled water. Subsequently, 2.5 ml of glycerol was added and the solution was placed in a water bath at 100°C until the agar was completely dissolved. This solution was sterilized for 10 min at 121°C. After cooling to 50°C in a water bath, 50 ml freshly prepared filtered (by using seize filter for sterilization of OADC enrichment) sterile Oleic acid-Albumin-Dextrose Catalase (OADC), preheated to the same temperature, was added. Two cocktail antibiotics namely carpenicillin (15 µg/ml), and cyclohexamide (50 µg/ml) were added in the Middle brook 7H10 agar, to avoid bacterial and fungal contamination. Finally the agar was solidified and final P^H of the medium was adjusted to 6.6 ± 0.2. Every batch of Middle brook 7H10 medium was checked for quality control before drug sensitivity procedure.

2.3.3. Susceptibility Testing of *Mycobacterium tuberculosis*

The drug susceptibility method was standardized as per WHO and International Union against Tuberculosis and Lung Diseases (IUATLD) guidelines [19,20] and work was carried out in coordination with Department of Immunology laboratory, National Institute for Research in Tuberculosis (NIRT), [Formerly Tuberculosis Research Centre (TRC)], Indian Council of Medical Research (ICMR), Chennai. The drug susceptibility testing was performed within 1-2 weeks after obtaining the growth of *M. tuberculosis*. Along with 80 DST plate, two drug free plates were used as control and two more were used for maintaining the culture of *M. tuberculosis*. A volume of Middle brook 7H10 medium, supplemented with OADC, was prepared. Antituberculosis drug were incorporated in the following concentrations: 16, 32, 64 and 128 µg/ml of Rifampicin (RMP), 0.1, 0.2, 1 and 5 µg/ml of Isoniazid (INH); 8, 16, 32 and 64 µg/ml of streptomycin (SM) and 1, 2, 4 and 8 µg/ml of ethambutol (EMB); (*Sigma Chemicals, St. Louis, USA*) by absolute concentration method.

2.4. Preparation of Inoculums

Two to three loopful (3 mm of Nichrome wire [22SWG]) of *M. tuberculosis* colony was taken from primary culture and placed into McCartney vials containing 1.0 ml of sterile Phosphate Buffer Solution (PBS) and 5 sterile glass beads. This was homogenized by vortex mixing for 1-3 minutes and kept in biological safety cabinet II (BSC II). The opacity of the *M. tuberculosis* culture suspension was adjusted to McFarland standard No.5.

2.5. Inoculation Procedure

Ten microlitres (10µl) of *M. tuberculosis* culture suspension was transferred to each of the four quadrants of Middle brook 7H10 agar plate containing different concentrations of anti-TB drugs; rifampicin, isoniazid, ethambutol and streptomycin.

2.6. Incubation and Reading

All inoculated and control (drug free) media were properly labeled, sealed by parafilm and incubated at 37°C for 28 days. Readings were taken on 28th day. The lowest concentration of the drug which inhibits the growth of *Mycobacterium tuberculosis* was considered as minimum inhibitory concentration (MIC). The control (drug free) media maintained that showed good growth without any contamination. *Mycobacterium tuberculosis H₃₇R_V* reference strain was used as a quality control for both culture and susceptibility testing for every batch. A strain was considered as MDR-TB if the cut off MIC value was more than 64 µg/ml for rifampicin, 1 µg/ml for isoniazid, 4 µg/ml for ethambutol and 32 µg/ml for streptomycin.

3. Results and Discussion

Among 178 extra-pulmonary clinical specimens (included 59 ascetic fluids, 54 pleural fluids, 25 cerebrospinal fluids (CSF), 12 fine needle aspirations (FNA), 8 urine samples, 7 pus samples, 6 synovial fluids,

2 skin tissues, one each of pericardial fluid, liver abscess, pancreatic cyst fluid, omental biopsy and semen), 10 samples (5.61%) were AFB smear positive (included FNA [5], urine [3], pus [1], and synovial fluid [1]) and six (3.37%) were LJ culture positive from 10 AFB smear positive cases (included FNA [2], urine [2], pus [1], synovial fluid [1]). Among 1030 clinically suspected PTB patients, 125 were AFB smear positive cases, among which 106 (84.8%) were male and 19 (15.2%) were female. Maximum number of patients belonged to the age group of 41-60 (Table 1). Out of 125 AFB smear positives, 76 specimens yielded mycobacterial growth. In this, 68 (54.4%) were *Mycobacterium tuberculosis* and 8 (6.4%) were nontuberculous mycobacteria (NTM). Among these NTM strains, 2 were scotochromogens and 6 were photochromogens by Runyon's classification [21]. All 68 *M. tuberculosis* strains were slow growers, positive for niacin test, failed to grow on PNB containing L-J media and negative for Catalase test at 68°C /P^H 7.0. They were weakly positive for semi-quantitative Catalase test. In this study, one NTM isolate was a slow grower, produced visible colonies on PNB containing media on 21st day, Catalase positive, niacin negative and was reported to be photochromogens. The other NTM isolates were scotochromogens which produced pigment in the dark with confluent growth on a pair of L-J medium on 14th day. They produced visible colonies on PNB medium and were niacin negative and Catalase positive.

Table 1. Age group distribution of sputum AFB smear positive pulmonary tuberculosis patients (N = 125)

S. no.	Age group	No of Male	No. of Female
1.	20 - 40	29	7
2.	41 - 60	52	8
3.	> 60	25	4
	Total	106	19

In this study revealed, resistance to three anti-TB drugs was found in three (4.41%) patients from 68 culture positive PTB patients. Six strains isolated from EPTB patients were susceptible to four anti-TB drugs tested. Out of 99 sputum AFB positive new cases, 46 were *M. tb* culture positive. Of these, two strains were resistant to RMP, INH and EMB which are considered as primary drug resistance. Out of 26 patients who had taken anti-TB drugs previously, 22 were *M. tuberculosis* culture positive (Table 2). Of these 22, one strain was resistant to RMP and INH. In addition, resistant to EMB was considered as acquired drug resistance. None of the strains showed resistance to streptomycin. Streptomycin is widely used for treatment of MDR-TB patients and is a core component of the standard and DOTS regimens. It is a relatively more affordable drug with a vital role in the treatment of tuberculosis.

Table 2. Correlation of LJ culture results for *M.tuberculosis* with different categories of sputum AFB positive pulmonary tuberculosis patients under the RNTCP (N = 125)

S.No	Categories of pulmonary tuberculosis patients	No. of <i>M.tb</i> culture positive cases	Frequency (%)
1	New case (99)	46	46.5%
2	Defaulter (19)	17	89.5%
3	Treatment Failure (4)	3	75.0%
4	Relapse (3)	2	66.7%
5	Total (125)	68	54.4%

The overall MDR-TB percentage is reported in this hospital area to be 4.05%. Among these, resistance to

three drugs was observed in 4.34% of new cases and 4.54% of patients previously treated with anti-TB drugs in culture positive PTB cases. None of the *M. tuberculosis*

strains showed single (mono) resistance. The details of MDR-TB patients, MIC value of anti-TB drugs are shown in Table 3.

Table 3. Details of three MDR-TB patients

Diagnostic criteria	Patient 1	Patient 2	Patient 3
Age/gender	58/M	40/M	73/F
Marital status	Married	Married	Married
Literacy status	Illiterate	Illiterate	Illiterate
Smoking habit	Chain smoker	Occasionally	-
Alcoholic habit	Yes	-	-
District	Kanchipuram	Kanchipuram	Kanchipuram
Type of case	Treatment failure	New case	New case
History of contact	Family member	Nil	Nil
Previous history of PTB	yes	-	-
Co morbid conditions	Nil	Nil	COPD
Chest X-ray findings	Bilateral PTB	Lung fibrosis	Consolidation
HIV status	Negative	Negative	Negative
Direct AFB smear status (grading as per the RNTCP guidelines)	3 +, ... 3 +	1 +, ... 3 +	2 +, ... 1 +
L-J Culture grading from primary isolation of <i>M.tb.</i>	3 +	3 +	2 +
Rifampicin (Con., 16,32,64,128 µg/ml)	≥ 128	≥ 128	≥ 128
Isoniazid (Con., 0.1, 0.2, 1, 5 µg/ml)	≥ 5	≥ 5	≥ 5
Ethambutol (Con., 1,2,4,8 µg/ml)	≥ 8	≥ 8	≥ 8
Streptomycin(Con., 4,8,16,32 µg/ml)	Sensitive	sensitive	sensitive
Follow up result			
Constitutional symptoms	Positive	Positive	Positive
Sputum AFB microscopy result after 6 month treatment completed under the DOTS programme	Positive	Positive	Positive
MDR-TB treatment started under the DOTS plus programme	Yes	Yes	Yes

Key: PTB: Pulmonary tuberculosis, COPD: Chronic obstructive pulmonary disease, HIV: Human immuno deficiency virus, *M.tb* : *Mycobacterium tuberculosis*, RNTCP: Revised National Tuberculosis Control Programme, LJ: Lowenstein-Jensen medium, AFB: Acid fast bacilli, MDR-TB: Multi drug resistant tuberculosis, DOTS: Directly observed treatment short course

A similar study from Bangladesh, Dhaka, reported MDR-TB of the same order (3.92%) [22]. Tatar *et al.*, study reported multi drug resistant pattern to be 6% for INH+RMP+EMB [23]. Nagaraja *et al.*, study reported a resistance of 6.14% to INH+RMP+EMB [24]. A study from Calicut Medical College, Kerala reported 2.2% MDR-TB and single drug resistance to INH in 17.7%, RMP in 8.8%, EMB in 11.1%, and SM in 8.8% [25].

In a large scale study, among 64,104 TB cases from 58 geographical settings, WHO found drug resistant TB to be between 2.9% and 40.8% [26]. A relatively lower frequency of MDR-TB has been reported in Bangladesh (0.23% for new cases and 5.56% for previously treated patients) [22]. In a study conducted in nine centers of Indian Council of Medical Research (ICMR), MDR-TB ranged from 0.6% to 3.2% with respect to primary drug resistance and 6% to 30% with respect to acquired drug resistance [27].

Santha *et al.*, study reported resistance to both INH and RMP as 1.7% among newly diagnosed patients and 12% among previously treated patients [28]. Jain reported 18.5% resistance to INH and 0.6% to rifampicin [29]. The global level surveillance of drug resistant tuberculosis conducted by WHO and IUATLD between 1994 to 1997 showed a single drug resistance of 9.95% and multidrug resistance of 1.4% [30]. Reports from other developing countries, reflect resistance to one or more anti-tuberculosis drugs ranging from 3.4 to 37.0%; for instance 18.7% in Korea [31], 7.3% in South Africa [32], 5.2% in India [33], 30.5% in Taiwan [34] and 30.5% in Central Asia [35].

Possible causes of drug resistance included inadequate treatment provided by health services, poor case holding, poor drug supply, poor quality of drugs and non adherence of patients to the prescribed drug regimens and indiscriminate use of anti-tuberculosis drugs in the private sector. However the most important cause of drug

resistance may be the error of health care workers in not providing the correct regimens [36]. Mutations in the genome of *M. tuberculosis* conferring resistance to anti-TB drugs may occur spontaneously with an estimated frequency of 3.5×10^{-6} for INH and 3.1×10^{-8} for RMP. Since the chromosomal loci responsible for resistance to various drugs are not linked, the risk of a double spontaneous mutation is extremely low i.e., 9×10^{-14} for both INH and RMP [37].

INH resistance is considered to be very important, because it is a potential bactericidal drug, and is an important component of short course of anti-tuberculosis regimen. It is capable of killing 90% of the bacillary population in the patient's lesions during the first few days of chemotherapy. Ethambutol (EMB) is combined with RMP and INH in directly observed treatment short course (DOTS) programme. It is bacteriostatic but in large dose can be bactericidal [38]. So resistance against INH and EMB leads to treatment failure. RMP is the most important drug in DOTS programme. It is also gradually becoming ineffective due to development of resistance. RMP is a potent bactericidal drug which acts on dormant and persistent bacilli on short exposure and resistance to RMP may lead to the failure of DOTS programme [39].

All three MDR-TB patients were further managed with Category-IV regimen of anti-tuberculosis drugs in DOTS plus Centre of Government Chest Thoracic Hospital, Tambaram Sanatorium, Kanchipuram District, Tamilnadu under RNTCP programme. The limitation of this study is exploited MDR-TB only reported in culture positive *M. tb* isolates, recovered from AFB smear positive pulmonary and suspected extra-pulmonary tuberculosis patients. The accurate diagnosis of MDR-TB requires a positive culture isolates and conventional drug susceptibility testing. Susceptibility testing of *M. tb* isolates is, therefore, imperative for therapy selection and prevention of spread of multi-drug resistant organisms. From the public health

perspective, treatment is focused on MDR-TB cases, which are the reservoir of infection in the community.

4. Conclusion

Persons excreting tubercle bacilli in their sputa are considered dangerous to the community and those who excrete bacilli resistant to multiple drugs are even more dangerous. Hence routine surveillance of drug resistant profile found in a particular population of newly diagnosed and previously treated sputum AFB positive pulmonary tuberculosis patients would provide useful information for adapting strategies for efficient treatment under DOTS programme with RNTCP and is essential for the detection of the source of MDR-TB infection in a particular population. Conventional drug susceptibility testing is still considered as “gold standard” method and an essential core diagnostic component of continuous monitoring of drug resistance trends, in order to assess the efficacy of current programme and epidemiological surveillance for planning.

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Conflict of Interest

None declared

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