STUDIES ON DRUG-RESISTANCE PATTERN BY PHENOTYPIC METHODS IN *Mycobacterium tuberculosis* ISOLATES IN A TERTIARY CARE HOSPITAL

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Abstract-

Background: Tuberculosis is a major health problem in India. The situation has been made worse because of the emergence of drug resistance. Tuberculosis has affected more than one third of the world's population and India contributes to ¼ of the global annual incidence. With ¼ of the drug resistant forms coming from India. With this context the present study was undertaken to assess the magnitude and pattern of drug resistance among the isolates of *Mycobacterium tuberculosis*.

Aims & Objective: To detect the drug-resistance pattern of the isolates of *Mycobacterium tuberculosis* from a tertiary care hospital. The objective was achieved by screening of sputum smears for acid-fast bacilli (AFB), culture of AFB positive samples on Lowenstein-Jensen's (L.J.), identification of isolates of *Mycobacterium tuberculosis* and drug susceptibility testing (DST) against first-line and second-line anti-tuberculosis drugs.

Methods: Standard procedures were followed for the isolation & identification of *Mycobacterium tuberculosis*. DST was carried out by proportion method on L.J. to detect drug resistance pattern.

Results: Out of 1186 samples studied, 123 were AFB & culture positive strains of *Mycobacterium tuberculosis*. Of these 123 strains 10 isolates were Multi-drug resistant (MDR-TB) showing resistance to both Isoiniazide and Rifampicin. Out of these 10 strains two are Extensively drug-resistant (XDR-TB strains -one is resistant to kanamycin, ethionamide, ciprofloxacin and rifabutin while the other is resistant to kanamycin, D-Cycloserine, ciprofloxacin and rifabutin. Mono-drug resistance to D-cycloserine was observed in one isolate while resistance to two drugs like ethionamide, P-amino salicylic acid; ethionamide, ciprofloxacin and ethionamide, D-Cycloserine was observed in three isolates.

Conclusion: The present work helps to study & monitor antibiotic susceptibility test results. Moreover its documentation will make us aware of any change in resistance pattern as and when it occurs.

Keywords- *Mycobacterium tuberculosis*, drug-resistance, phenotypic methods

Introduction

It has been more than a century since Robert Koch had discovered the causative agent of tuberculosis and more than 5 decades since the introduction of first anti-tuberculous drug, the elimination of this disease remains a dream. The problem of tuberculosis has worsened because of increasing drug resistance which has started being reported since 1993 [1]. Tuberculosis is a contagious disease, spreads by airborne route. The disease is more common in developing countries, the problem being compounded by poverty, urbanization and overcrowding. It is one of the most important cause of increasing morbidity and mortality in India [2].

According to the recent Revised National Tuberculosis Control Programmed report the prevalence of Tuberculosis in India is 3.1 million with 0.32 million mortality [3]. With the introduction of anti- tuberculous drugs in the late 1940's and early 1950's, there was a decrease in the incidence of Tuberculosis, with Tb being effectively controlled with the 4 drug regimen of Isoniazid (INH), Rifampicin (RMP), Streptomycin and Ethambutol [4]. In 1990, Multidrug resistant Tuberculosis (MDR) was reported, the organism exhibiting resistance to INH and RMP, the two most effective first line anti-tuberculous drugs [5]. In 2006, extensively resistant Tuberculosis (XDR) was reported i.e. Strains of *Mycobacterium tuberculosis* that

are resistant not only to INH and RMP but also to at least 3 out of 6 classes of second line anti- tuberculous drugs (Aminoglycosides, polypeptides, Fluoroquinolones, Thioamides, Cycloserine, & Para-amino salicylic acid) [6]. This was followed by the emergence of totally drug resistant strains in 2007 where in the organisms are resistant to all available anti-tuberculous drugs [7]. The possible cause of emergence of drug resistant forms being incorrect regimens, poor compliance & related factors contributes to the increase in resistance [5]. One of the barriers in Tuberculosis control is the delay in the diagnosis of the disease. Accurate and rapid diagnosis is the key component in prevention of transmission. For the early detection of *Mycobacterium tuberculosis* Ziehl-Neelsen staining method is most commonly employed but it is rather insensitive and fails to detect large cases [7]. Lowenstein Jensen (L.J.) is the most commonly used medium for the cultivation of *Mycobacterium tuberculosis*. The reported rate of isolation of tubercle bacilli on LJ medium ranges from 70-85% [8]. Anti-mycobacterial sensitivity test by agar Proportion method on L.J. and Middlebrook 7H10 or 7H11 are considered to be gold standard but takes 10-12 weeks [5]. An access to appropriate diagnostic & therapeutic facilities is required to meet the challenge of drug-resistance. This study was undertaken to know the magnitude of infection and the resistant pattern in Tuberculosis.

Aims and Objectives

To detect the drug resistance pattern of *Mycobacterium tuberculosis* by Proportion method on L.J. & to identify the number of MDR-TB & XDR-TB cases in this region.

Methodology

Study Design

A cross-sectional study was conducted in the period of June, 2011 to June, 2012 in a tertiary care centre in Western Maharashtra. Patients enrolled in this study were patients attending Out Patient department and those who were admitted in the Tuberculosis ward.

Study Population

All the patients attending the Pneumology units with symptoms suggestive of TB were included. These patients were considered as: 1) as new cases, if never previously treated and 2) retreatment cases. Inclusion criteria were cough for more than 2 weeks or blood stained sputum; or suggestive radiological signs, irrespective to HIV infection.

Specimen Processing

Sputum samples were collected and processed according to the standard guidelines [9-12]. Two sputum samples were collected for each patient, the first during the consultation, the second taken the next morning upon awakening. Screening for acid-fast bacilli (AFB) was performed on Ziehl-Neelsen stained smears. Sputum specimen was digested and decontaminated by modified Petroff's method, then inoculated on two slants of Lowenstein-Jensen medium & incubated at 37°C. Cultures were examined once a week for 80 days. Any suspect colony confirmed as AFB by microscopy was identified by biochemical tests and tested for antibiotic resistance by 'Proportion method'. All the isolates which are AFB and Culture positive and H37RV strain were tested for drug susceptibility by proportion method on L.J. To detect drug resistance pattern (whether MDR /XDR), both the first line anti-tubercular drugs- (streptomycin-S, isoniazid-I, rifampicin-R and ethambutol-E) and second line anti-tubercular drugs (Kanamycin, Amikacin, Ethiona-

mide, D-Cycloserine, Clarithromycin, Ciproflixacin, P-Amino salicylic acid, rifabutin) were tested. The concentrations of the first-line and second-line drugs used for DST are given in [Table-1].

Table 1- concentrations of first-line & second-line drugs used for DST

First-line drugs	Concentration (µgs/ml)	Second-line drugs (µgs /ml)	Concentration (µgs/ml)
Isoniazide	0.2	Kanamycin	30
Ethambutol	2	Amikacin	700
Pyrazinamide	200	Ethionamide	20
Rifampicin	40	d-Cycloserine	30
Streptomycin	4	Clarithrimycin	8
		Ciprofloxacin	125
		P-amino salicylic acid	25
		Rifabutin	0.5

Results

Total 1186 sputum samples were screened for the presence of *Mycobacterium tuberculosis* by the Ziehl-Neelsen Staining technique. Out of that 123 were positive for AFB which consists of 119 new cases 5 retreatment cases and 4 were HIV positive cases.

Age and Gender Wise Distribution

Majority of the patients of the patient's i.e. 22% were found in the age groups of 21-30 years [Table-2] & [Fig-1]. In this study it was seen that four (40%) MDR isolates are susceptible to all the drugs tested. Two out of ten (20%) MDR isolates were XDR-TB strains; One was resistant to kanamycin, ethionamide, ciprofloxacin and rifabutin while the other was resistant to kanamycin, D-Cycloserine ciprofloxacin and rifabutin. monoresistance to D-Cycloserine was observed in only one isolate. Resistance to 2 drugs *i.e* ethionamide, P-amino salicylic acid and ethionamide, D-Cycloserine was observed in 2 isolates. All the MDR isolates are susceptible to amikacin and clarithromycin. Maximum resistance (40%) was observed to ethionamide. All the 123 smear positive sputum samples were cultured successfully on L.J. media. The average turnaround time for culture on L.J. was 21-28 days.

Table 2- Age & gender wise distribution of cases

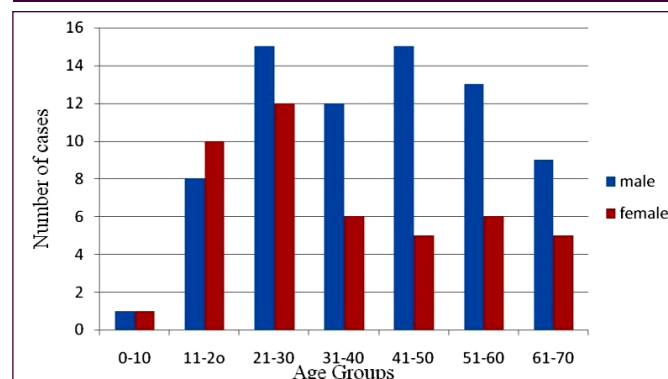| Age group	Male	Male %	Female	Female %
0-10	1	1	1	3
10-20	8	14	10	22
21-30	15	20	12	27
31-40	12	15	6	12
41-50	15	20	6	12
51-60	13	19	6	12
61 onwards	9	11	6	12
Total	78	100	45	100

Fig. 1- Age-wise distribution of male & female patients

The disease was found to be more common in male population and the male to female ratio was 1.7:1.

Antibiotic Resistance

All the 123 isolates which are AFB & Culture positive were tested for drug susceptibility to the first line drugs by Proportion Method on L.J. [Table-3] depicts the resistance pattern of 123 isolates against the four, first line anti-tubercular drugs. The number of strains resistant to each drug were 29.26% were resistant to streptomycin, 25.20% were resistant to isoniazid, 15.44% were resistant to rifampicin and 34.45% were resistant to ethambutol [Table-3]. In the present study the MDR rate was 8.13%. All these 10 MDR strains were then tested for drug susceptibility of 2nd line drugs by proportion method on L.J. [Table-4] depict the resistance pattern of these 10 MDR isolates against the 2nd line anti-tubercular drugs.

Table 3- Number of resistant isolates for each drug

Drug	No. of samples	Percentage
Streptomycin	36	29.26%
Isoniazid	31	25.20%
Rifampicin	19	15.44%
Ethambutol	43	34.95%

Table 4- Resistance pattern to the second line anti-tuberculosis drugs

Drug	Sample number									
	1	2	3	4	5	6	7	8	9	10
Kanamycin	-	+	+	-	-	-	-	-	-	-
Amikacin	-	-	-	-	-	-	-	-	-	-
Ethionamide	+	+	-	+	+	-	-	-	-	-
D-Cycloserine	-	-	+	-	+	+	-	-	-	-
Clarithromycin	-	-	-	-	-	-	-	-	-	-
Ciprofloxacin	+	+	+	-	-	-	-	-	-	-
P-amino salicylic acid	+	-	-	-	-	-	-	-	-	-
Rifabutin	-	+	+	-	-	-	-	-	-	-

Discussion

Tuberculosis is a major preventable communicable disease in developing countries like India. According to WHO report 2.3 million cases out of the global 9 million annual cases are from India and 64,000 out of 3,10,000 notified cases of drug resistant tuberculosis in a year are from India. Long duration of treatment, poor socioeconomic condition and poor quality of the drugs are responsible for the failure of Tuberculosis control programmed in developing countries. In 1993 WHO was forced to declare TB as a "Global Emergency" and instructed countries to take up drastic effective steps to control tuberculosis. Recently in May 2012, TB is declared as a notifiable disease in India [3]. Incomplete treatment or usage of only one or two anti-tubercular drugs has led to the emergence of drug resistant strains of *M. tuberculosis*. This has further complicated the management & control of TB. In this regard we have made an attempt to compare the drug resistance pattern of *M. tuberculosis* isolates in this area by using conventional Proportion method on L.J. medium.

In the present study, 123 clinically suspected Pulmonary Tuberculosis cases admitted at a tertiary Centre in Western Maharashtra were studied. Patients enrolled in our study were from all age groups. The disease was found to affect predominantly the patients in 21-30 years (22%) age group. Our findings are similar to the findings of other studies who have reported that most of the patients in this age group [13,14]. The reasons that make this age group vulnerable to TB are many. They are socially more active and are more exposed to an open case of TB than others. In our study, out of 123 patients there were 78 males and 45 females and Male to female ratio was 1.7:1. Male Preponderance of the disease in also been reported by others. Eriki, et al [15] and Fandinho, et al [16] have reported male to female ratio of 1.8:1 and 1.6:1 respectively. Likely reasons for male preponderance could be that in a male dominated society, usually he is the earning member and as he goes out for work, he is more likely to come in contact with an active TB case. Men are more likely to acquire habits like smoking and alcoholism which can predispose to TB. There are various modalities for the diagnosis of Tuberculosis. Direct smear microscopy after ZN staining is the most commonly employed diagnostic tool in many countries. It is only the laboratory facility available in many laboratories. It is well known that smear for AFB has a lower sensitivity. A number of technical and patient related factors can affect the sensitivity of direct smear microscopy. Also the sensitivity is affected by the nature of the specimen. In the present study sputum smear positivity rate was less (13.7%). Most of the other studies have reported the sensitivity range 28-65% [17-21]. *Mycobacterium tuberculosis* can be cultured by conventional L.J. medium. In the present study the rate of isolation of *Mycobacterium tuberculosis* was 94.61%. which is much higher than that seen in other studies, wherein the rate of isolation was 29-85% [17,20,22,23]. Isolation of tubercle bacillus by culture on L.J. generally takes 6-8 weeks. In our study, the mean duration of isolation of *Mycobacteria* on L.J. media was 24.5 days. The shortest time taken was 17 days and longest time taken was 41 days. Many authors have reported similar time period required for isolation of *Mycobacteria* on L.J. media. Negi, et al [17] and Rishi, et al [20] have reported 24.03 days and 28.81 days as mean duration of isolation respectively. Conventionally isolates are identified as *Mycobacterium tuberculosis* by putting up different conventional identification tests like nitrate reduction test, catalase test, niacin test. These tests were employed in the present study for identification of culture isolates of *Mycobacterium tuberculosis.* The main objective of the present study was to detect the drug resistance pattern of the *Mycobacterium tuberculosis* isolates from the Tuberculosis cases. The standard conventional Proportion method on L.J. was used to detect the susceptibility/resistance to each of the 1st and 2nd-line drugs. In the present study for the detection of drug- resistant pattern 1st-line anti-tubercular drugs: Streptomycin, Rifampicin, Isoniazid, Ethambutol and 2nd-line antitubercular drugs: Kanamycin, Amikacin, Ethionamide, D-Cycloserine, Clarithromycin, Ciproflixacin, P-amino salicylic acid & Rifabutin were carried out. In the present study, 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 al (80%) [24-27].

Resistance to Rifampicin was seen in 15.44% in our study which is much lower than studies reported by Verma, et al and Bhatt, et al (18%), Sureshkumar, et al (32%), Menon, et al (74.4%), and Barat, et al (76%) [24-28]. Resistance to Streptomycin was seen in 29.26% in our study which is much lower than studies reported by Bhat, et al (62%) and Menon, et al (70%) [27,26]. Resistance to Ethambutol was seen in 34.95% in our study which is much higher than studies by Bhat, et al (12%), Menon et al (21.7%) and Verma, et al (27%) [24,26,28]. While the present overall rate of MDR-TB in India is around 18-20% [3], multidrug resistance (i.e. resistance to Rifampicin and INH) observed in our study was 8.13%. MDR re-

sistance was higher in other studies like Filho, et al (78.8%), Barat, et al (72%), Verma, et al (66.6%), Menon, et al (47.54%) [26,28,29, 31]. Lesser MDR resistance than our study was reported are 5% by Mulenga, et al, 2.9% by Therese, et al and 4% by Bhat, et al [28,30,31]. In the present study pattern of drug resistance to second -line drugs was Kanamycin (20%), Ethionamide (40%), D-Cycloserine (30%), Ciprofloxacin (30%), P-amino salicylic acid (10%),Rifabutin (20%) and for Amikacin & Clarithromycin (0%). Resistance to Kanamycin was seen in 20% in our study which is much lower than the studies by Zimenkov, et al *(*52%), Bonnet, et al (31.4%) [32,33]. Resistance to Ethionamide was seen in 40% in our study which is much higher than studies by Kim, et al (23.6%), Sureshkumar, et al (32.26%), Bonnet, et al (37.3%) [34, 25, 33]. The present overall rate of XDR-TB in India is about 3.5% [3] while 1.6% (20% of MDR) was observed in our study. Much lower XDR rate was reported in studies by Bonnet, et al (4.5% of MDR), Mondal, et al (7.4% of MDR), Jain, et al (8% of MDR), Ajbani, et al (9.1% of MDR) [33,35-37]. Overall drug resistance pattern shows moderate rate of MDR (8.13%) and XDR-TB (1.6%) in our study, which indicates the effective management of DOTS through RNTCP in our area.

Conclusion

Detection of drug resistance pattern by phenotypic methods are easy to perform for routine diagnosis as compare to genotypic tests which are very costly and requires more sophisticated infrastructure. These phenotypic tests will help in detection of resistance independent of the underlying mechanisms unlike molecular tests which can detect only those mutations which are screened for. This study will help us to initiate the control measures by studying the antibiotic resistance pattern of *Mycobacterium tuberculosis* isolates and also by documentation of it, we will come to know any change in resistance pattern when it occurs.

Conflicts of Interest : None declared.

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