ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 15, Issue 10, 2022

Online - 2455-3891
Print - 0974-2441

Research Article

STUDY OF RESISTANCE TO FIRST-LINE DRUGS IN MULTIDRUG RESISTANCE TUBERCULOSIS SUSPECTS FROM SPUTUM SAMPLES BY GOLD STANDARD – STANDARD ECONOMIC VARIANT 1% PROPORTION METHOD IN NORTH COASTAL ANDHRA PRADESH

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Received: 14 June 2022, Revised and Accepted: 19 July 2022

ABSTRACT

Objectives: The objectives of the study were to culture *Mycobacterium tuberculosis* from sputum samples of multidrug resistance (MDR) suspects on Lowenstein-Jensen (LJ) media, to test for susceptibility and resistance to first-line antitubercular drugs rifampicin (RMP), isoniazid (INH), streptomycin (SM), and ethambutol (EMB) by conventional DST on LJ media by standard economic variant proportion (1%) method and to calculate the rate of MDR and pattern of drug resistance in MDR tuberculosis (MDR-TB) suspects.

Methods: A prospective, laboratory-based study was done on a total of 80 samples from cases of suspected pulmonary and extra-pulmonary TB. Identification of culture isolates as *M. tuberculosis* was done by susceptibility to p-nitrobenzoic acid (PNB), niacin test, and catalase activity at 68°C/pH 7. Drug sensitivity testing (DST) of *M. tuberculosis* cultures on LJ medium for drugs was performed by a standard economic variant of the 1% proportion method.

Results: Among MDR suspects, the maximum is in the age group of 45–60 years (35%) with a mean age of 42.75±15.93 years. The sex ratio of male to female is 2:1. Out of 80 samples, 36 samples (45%) were graded 1+, 31 samples (38.7%) 2+, and 13 samples (16.3%) 3+. All 80 sputum samples showed growth on LJ media within 8 weeks. When colonies obtained on LJ medium were subjected to biochemical tests for identification, all were positive for *M. tuberculosis*. Out of 80 *M. tuberculosis* isolates tested for sensitivity against first-line drugs (except pyrazinamide), 45 (56.3%) were found to be sensitive to all four drugs (INH, RMP, EMB, and SM), and 35 (43.7%) were resistant to at least one drug. Nine isolates (11.3%) showed resistance to a single drug (monoresistance). The various MDR patterns were INH+RMP seen in 6 strains (7.5%) and INH+RMP+SM seen in 4 strains (5%).

Conclusion: Drug resistance trends must be closely monitored to evaluate the efficiency of existing therapies and their effect on pulmonary tuberculosis epidemic. To design the new drug regulations, more extensive data on drug sensitivity with standardized testing protocols which is quality assured are required.

Keywords: *Mycobacterium tuberculosis*, Drug resistance, Multidrug resistance-tuberculosis, First-line drugs.

 DOI: http://dx.doi.org/10.22159/ajpcr.2022v15i10.45521. Journal homepage: https://innovareacademics.in/journals/index.php/ajpcr

INTRODUCTION

Tuberculosis (TB) by *Mycobacterium tuberculosis* is a disease that has tormented humans for more than 5000 years and continues to be the largest cause of ill-health and death, despite the knowledge that it is curable [1]. In recent years, the multidrug resistance tuberculosis (MDR-TB) strain has risen as a dangerous and life-threatening disease [2]. MDR-TB is a big challenge for program managers around the world. Although India's MDR-TB rate is low, the country has a substantial absolute number of cases, with an estimated yearly incidence of 131,000 MDR-TB patients in the country [1]. MDR-TB is mostly a self-inflicted human problem that develops because of poor treatment of tuberculosis that can be cured by available drugs. Previous TB therapy is the utmost factor for MDR-TB progress [3]. MDR-TB suspects (those who fail Category I therapy and test positive for tuberculosis after 4 months of Category II treatment) have previously been treated with antituberculosis drugs. They have heightened chances of harboring strains resistant to multiple antituberculosis drugs. As a result, knowing the rate of MDR-TB and the pattern of treatment resistance among MDR-TB suspects are critical. The main objectives of the study are to culture *M. tuberculosis* from sputum samples of MDR suspects on Lowenstein-Jensen (LJ) media, to test for susceptibility and resistance to first-line antitubercular drugs, namely, rifampicin (RMP), isoniazid (INH), streptomycin (SM), and ethambutol (EMB) by conventional drug susceptibility testing on solid media by standard economic variant proportion (1%) method and to calculate the rate of MDR and pattern of drug resistance in MDR-TB suspects.

METHODS

This study was performed at Culture and Drug Susceptibility Testing Laboratory (Intermediate Reference laboratory for Tuberculosis), Government Hospital for Chest and Communicable Diseases, Visakhapatnam, under the Department of Microbiology, Andhra Medical College, Visakhapatnam, during a period of 2 years (2010–2012). Sputum samples from four districts of North Coastal AP, that is, Srikakulam, Vizianagaram, Visakhapatnam, and East Godavari were included in this study. Sputum samples from 80 MDR TB suspects were studied. Sputum samples were digested and decontaminated by the NALC-NaOH method. Sediment obtained was resuspended and used for smear preparation and Ziehl–Neelsen staining and culture on LJ medium. Usually, incubation at 37°C for 2–4 weeks is required to obtain visible growth from smear-positive specimens. Identification of culture isolates as *M. tuberculosis* was done by susceptibility to p-nitrobenzoic acid (PNB), niacin test, and catalase activity at 68°C/pH 7. Isolates sensitivity testing for drugs was performed by a standard economic variant of the 1% proportion method on LJ media. The latter method

determines the % of resistant bacteria in isolates. To obtain countable colonies on media, two appropriate bacilli dilutions, 10^{-2} and 10^{-4} dilutions (undiluted = 10^6–10^8 CFU/mL), were inoculated on media with drugs (INH – 0.2 μg/mL, EMB – 2 μg/mL, SM – 4 μg/mL, and RMP – 40 μg/mL) and media without drugs (both prepared in-house). Bottles of media used for testing one isolate were: Six LJ slopes (two for neat, two for 10^{-2}, and two for 10^{-4} suspensions), eight LJ media slopes containing drugs, that is, two each of drugs H, R, E, and S (one each for 10^{-2} and 10^{-4} suspensions) and one PNB slope. A total of 15 LJ slopes were inoculated. The proportion of bacilli resistant to drugs present in the strain is the ratio of colonies counted (on the 28th day and on the 42nd day for the sensitive strain only) on the media containing drug to media without drug. The critical proportion is 1%. Below 1% proportion, the strain is classified as sensitive and above1% as resistant. The $H_{37}RV$ (control) strain (standard) of *M. tuberculosis* was tested with each new medium batch.

RESULTS

A total of 80 sputum samples from MDR suspects were included in the study. The frequency distribution of MDR suspects concerning age group and sex is shown in Table 1. Among MDR suspects, the maximum are in 45–60 years (35%) with a mean age of 42.75±15.93 years. Fifty-six (70%) are males and 24 (30%) are females. The sex ratio is 2:1. The distribution of resistance to any one of first-line drugs among MDR suspects concerning age group and sex is shown in Table 2. Maximum cases with resistance are in 45–60 years (20%) with a mean age of 41.1±11.7 years. Resistance was seen only in males. Cases exhibiting MDR patterns were all males in 15–45 years age. Table 3 depicts the grading of sputum samples according to AFB count. All sputum samples were positive for AFB by microscopy. Thirty-six samples (45%) were graded 1+, 31 samples (38.7%) 2+, and 13 samples (16.3%) 3+. Readable GenoType MTBDRplus assay results were obtained from DNA extracts of all the 80 sputum samples. All MTBDRplus assay strips had TUB bands indicating the presence of *M. tuberculosis* complex. All 80 sputum samples showed growth on LJ media within 8 weeks. When colonies obtained on LJ medium were subjected to biochemical tests for identification, all were positive for *M. tuberculosis*. Hence, all the 80 sputum samples from MDR suspects were included in the study. The sensitivity pattern in MDR suspects (determined by conventional DST, i.e., 1% proportion method) is depicted in Table 4. Among 80 *M. tuberculosis* isolates tested for sensitivity against first-line drugs (except pyrazinamide), 45 (56.3%) were found to be susceptible to four drugs (INH, RMP, EMB, and SM), and 35 (43.7%) were resistant to at least one drug. The outline of acquired drug resistance is shown in Table 5. Isoniazid resistance was most common (43.7%), SM (25%), RMP (12.5%), and EMB (5%). Nine isolates (11.3%) showed single-drug resistance. The later type was seen with INH only. A total of 26 (32.5%) strains were resistant to >1 drug. The most frequent resistance style seen among the cultures was INH+SM (15%) followed by INH+RMP (7.5%). Resistant to three drug pattern was INH+RMP+SM (5%) and INH+EMB+SM (5%). MDR-TB was observed in 10 (12.5%) isolates from MDR suspects. The various MDR patterns were INH+RMP seen in 6 strains (7.5%) and INH+RMP+SM seen in 4 strains (5%). The distribution of *M. tuberculosis* isolates according to the number of drugs resistant to is shown in Table 6. Two drug pattern resistances were the most common. None of the isolates showed resistance to all four drugs.

Table 1: Distribution of MDR suspects with respect to age group and sex

Age group (years)	Males (%)	Females (%)	Total (%)
15–30	12 (15%)	12 (15%)	24 (30%)
30–45	12 (15%)	4 (5%)	16 (20%)
45–0	24 (30%)	4 (5%)	28 (35%)
>60	8 (10%)	4 (5%)	12 (15%)
Total	56 (70%)	24 (30%)	80

MDR: Multidrug resistance

Table 2: Distribution of resistance to any one of first-line drugs among MDR-TB suspects concerning age group and sex (N = 80)

Age group (years)	Males (%)	Females (%)	Total (%)
15–30	12 (15%)	-	12 (15%)
30–45	3 (3.75%)	-	4 (3.75%)
45–60	16 (20%)	-	16 (20%)
>60	4 (5%)	-	4 (5%)
Total	35 (43.75%)	-	35 (43.75%)

MDR-TB: Multidrug resistance tuberculosis

Table 3: Concentration of AFB in sputum specimens (n=80)

Sputum AFB grading	Number (%)	Readable GenoType MTBDRplus pattern	Culture positive
1–9 AFB/100 fields	-	-	-
1+	36 (45%)	36 (45%)	36 (45%)
2+	31 (31.7%)	31 (31.7%)	31 (31.7%)
3+	13 (16.3%)	13 (16.3%)	13 (16.3%)

DISCUSSION

The mean age of MDR suspects having resistance to at least one drug was 41.1 years in the present study. All MDR suspects resistant to at least one drug were males and maximum males were in the reproductive age. Bhatt *et al.* [4] performed an epidemiological analysis of MDR-TB. In the study population, 83.7% of individuals were in the reproductive age of 16–45 years (mean age of 33.64+11.03 years). About 68.5% were males

Table 4: Sensitivity pattern in MDR suspects

Sensitivity pattern	Study population (n=80)	Percentage
Sensitive to all four drugs (INH, RMP, EMB, and SM)	45	56.3
Acquired drug resistance	35	43.7

MDR: Multidrug resistance, RMP: Rifampicin, INH: Isoniazid, SM: Streptomycin

Table 5: Pattern of acquired drug resistance in previously treated pulmonary tuberculosis patients

Pattern of resistance	Study population (n=80)	% of a resistant population (n=35)
Any resistance	35 (43.7%)	-
Isoniazid (INH)	35 (43.7%)	100% (35)
Rifampicin (RMP)	10 (12.5%)	28.6% (10)
Ethambutol (EMB)	4 (5%)	11.4% (4)
Streptomycin (SM)	20 (25%)	57.1% (20)
Monoresistance	9 (11.3%)	25.7% (9)
Isoniazid (INH)	9 (11.3%)	25.7% (9)
Rifampicin (RMP)	-	-
Ethambutol (EMB)	-	-
Streptomycin (SM)	-	-
MDR	10 (12.5%)	28.6% (10)
INH+RMP	6 (7.5%)	17.1% (6)
INH+RMP+EMB	-	-
INH+RMP+SM	4 (5%)	11.4% (4)
INH +RMP+EMB+SM	-	-
OTHER PATTERNS	16 (20%)	45.7% (16)
INH+EMB	-	-
INH+SM	12 (15%)	34.3% (12)
INH+EMB+SM	4 (5%)	11.4% (4)
RMP+EMB	-	-
RMP+SM	-	-
RMP+EMB+SM	-	-
EMB+SM	-	-

Table 6: Number of drugs resistant

Number of drugs	Study population (n=80)	% of resistant population (n=35)
0	45 (56.3%)	-
1	9 (11.3%)	9 (25.7%)
2	18 (22.5%)	18 (51.4%)
3	8 (10%)	8 (22.9%)
4	0	0

and 31.5% were females. Although not quite reaching significance, a study from rural Uganda showed more rate of resistance in female patients (6/50; 12%) than males (2/75; 2.7%). This could be linked to health-seeking behavior, with longer wait times for female individuals probably because of the absence of financial power at the domestic level [5].

When colonies obtained on LJ medium were subjected to biochemical tests for identification, all were positive for *M. tuberculosis*. In a study conducted by Raveendran *et al.* at New Delhi, out of 34 smear-positive pulmonary specimens, three were non-tuberculous Mycobacteria (*Mycobacterium intracellulare, Mycobacterium fortuitum, and Mycobacterium kansasii*) [6]. In a study from Vietnam by Huyen *et al.*, one MDR strain was identified as *Mycobacterium avium intracellulare* [7]. The isolation of NTM in the present study was nil. The total rate of acquired drug resistance was 43.7% to 1 or >1 antitubercular drugs, similar to Vijay *et al.* and Rai *et al.* [8]. Rawat *et al.* [9] and Vasanthakumari *et al.* [10] stated acquired drug resistance to at least one antitubercular drug as 62.2% and 63%, respectively. Andhra Pradesh has started the 2nd line antituberculosis treatment, that is, the DOTS plus program in the year 2006 [11].

In the present study, maximum resistance was to INH (43.7%), followed by SM (25%), RMP (12.5%), and EMB (5%), and was in agreement with Vijay *et al.* [3]. Between 1994 and 2002, nearly 90,000 samples from around the world were analyzed, confirming that more bacteria were isoniazid resistant rather than to other anti-TB treatment drugs (0-42%). Resistance to INH and SM was more common than RMP/EMB [11]. Sharma *et al.* [12] reported RMP resistance to be most common than INH resistance, and Rawat *et al.* [9] said that RMP resistance was second most after INH resistance. INH-resistant strains in the present study were encountered in 43.7%, which was high. Rawat *et al.* [9] reported an even higher rate of 62.2%. In the present study, acquired resistance to RMP was 12.5% and was almost similar to that reported by Vijay *et al.* [3] (15.5%). RMP is a highly effective bactericidal and sterilizing drug, making it a crucial component of the DOTS program. Resistance to RMP could lead to the DOTS program's demise. In the current investigation, RMP resistance was invariably linked to INH resistance. As a result, it can be stated that in Andhra Pradesh, RMP resistance is a strong predictor of MDR. Rawat *et al.* [9] made similar discoveries and came to similar conclusions. The findings of this study are significant because there have been little current data on the prevalence of acquired medication resistance, and none from North Coastal AP. Because drug resistance is ever-changing, it is critical we track its progression on a regular basis [13].

The present study showed an MDR rate of 12.5% among MDR-TB suspects and similar to MDR-TB rates reported by Vijay *et al.* [3]. According to RNTCP annual report (2012), MDR-TB prevalence among previously treated cases in India was 17.2% (DRS survey – 2007–2008 carried out in Maharashtra, AP, and Gujarat). In the present study, strains labeled MDR had the following resistance patterns, INH+RMP (12.5%) and INH+RMP+SM (5%). A similar resistance pattern for MDR was seen in the study by Sharma *et al.* [12]. INH and SM are the main gateways to acquiring additional resistance [11]. Unlike studies by Vijay *et al.* [3] and Rawat *et al.* [9], resistance to all first-line drugs was not seen here. The resistance patterns other than MDR seen were 15% for INH+SM and 5% for INH+EMB+SM. Vijay *et al.* [3] reported the following additional resistance pattern excluding MDR, namely, INH+SM (5.8%), INH+EMB+SM (0.4%), RMP+SM (0.8%), and EMB+SM (0.4%).

The most typical pattern observed in the present study was INH+SM (15%) followed by monoresistance to INH (11.3%). The most typical resistance pattern observed by Sharma *et al.* [12] and Rawat *et al.* [9] were INH+RMP (18.4% and 33.8%). Vijay *et al.* [3] reported monoresistance to SM (9.3%) as the most common pattern followed by INH+SM (5.8%).

The individual mono-drug resistance pattern observed in the present study was 11.3% and was seen with INH only. Mono-drug resistance to RMP was seen in Rawat *et al.* (5%) and Sharma *et al.* [12] (1.5%). Vijay *et al.* [3] showed individual mono-drug resistance patterns as 9.3% for SM, 8.4% for INH, and 1.8% for RMP. EMB monoresistance was non-existent in the present study. EMB monoresistance was not seen in any of the other studies also. Two drug resistance patterns were most common in the present study. A similar observation was seen in Rawat *et al.* [9] and Sharma *et al.* [12]. The investigation by Sophia *et al.* [3] showed that resistance to one drug was the most common pattern.

The inclusion of the molecular test in the National Tuberculosis Program represents a significant step forward in the quick detection of MDRTB among suspected PMDT patients. Applying the molecular test directly to clinical material containing sufficient bacteria will reduce the time it takes to diagnose MDR-TB. Individualizing treatment regimens for drug-resistant TB and detecting XDR-TB will require continued surveillance using standard culture and DST approaches.

CONCLUSION

Drug resistance trends must be closely monitored for assessing the effectiveness of present therapies and their influence on tuberculosis epidemic. Irrational use of second-line medications with inadequate tailored regimens based on unreliable laboratory data, as well as the use of an empirical line of treatment in drug-resistant TB in an unspecialized setting, should be discouraged and denounced. To design the new drug regulations, more extensive drug susceptibility data with standardized testing protocols which are quality assured are required.

AUTHORS' CONTRIBUTION

Author Nitin Mohan contributed conceptual design, performed the work, and wrote the first draft of manuscript. Author I. Jyothi Padmaja guided the work and corrected the manuscript. Author Ratna Harika Dusi collected the literature and data. Author K. Suresh performed statistical analysis.

CONFLICTS OF INTEREST

The authors declared no conflicts of interest.

AUTHORS' FUNDING

The work was not supported by any kind of funds.

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