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Patterns of Initial Drug Resistance of *Mycobacterium tuberculosis* Isolates from Kashmir Valley, India

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Authors' contributions

This work was carried out in collaboration between all authors. Authors TW, VMK, DKK, AS, IAB and MMM designed the study and managed literature searches. Author GB performed the statistical analysis, wrote the first draft of the manuscript, and managed the analyses of the study and literature searches. Authors TW and RL wrote the protocol and performed the statistical analysis. All authors read and approved the final manuscript.

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ABSTRACT

Aims: We carried out this study to determine the patterns of initial drug resistance in *Mycobacterium tuberculosis* isolates and prevalence of MDR-TB among category-I pulmonary TB patients in Kashmir Valley. MDR-TB was defined as tuberculosis caused by bacilli showing resistance to at least isoniazid and rifampicin.
Study Design: Prospective study.
Place and Duration of Study: Department of Microbiology, Sher-i-Kashmir Institute of Medical Sciences, Srinagar, Kashmir, J&K, India between May 2007 and April 2010.

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Methodology: This study involved 300 category-I pulmonary TB patients attending DOTS clinics in different districts of Kashmir Valley. AFB positive sputum samples were randomly collected in 1% cetylpyridinium chloride from such patients and were subjected to repeat AFB staining and mycobacterial culture in Department of Microbiology, SKIMS. Drug susceptibility testing (DST) to the first line drugs; isoniazid, rifampicin, ethambutol and streptomycin was performed on cultures identified as *Mycobacterium tuberculosis* using the indirect proportion method on LJ medium.
Results: Out of 300 samples, culture results were available only for 207 samples. Out of 207 samples, 134 (64.73%) were culture positive, 52 sterile (25.12%) and 21(10.14%) showed contamination. Out of the 134 isolates, 123 were identified as MTB and 11 as mycobacteria other than tuberculosis (MOTT). Of the 101 DST results available, 74.25% were sensitive to all four first line drugs, 17.82% showed monoresistance, 7.92% showed polyresistance and 3.96% were MDR.
Conclusion: Resistance to any one drug was 39.60% with a high streptomycin resistance of 20.79%. Since most of these patients harboured organisms susceptible to isoniazid and rifampicin, standard short-course chemotherapy is likely to remain highly effective among the great majority of new TB patients in Kashmir Valley. Prevalence of MDR was relatively low but with a high rifampicin resistance of 6.93% there is a need for restricting use of rifampicin (supervised therapy only for TB and leprosy). It is important to strengthen the capacity of laboratories in Kashmir Valley for TB culture and DST for correct management of TB patients and to prevent emergence of drug resistance. Also, continuous monitoring of resistance in both new and previously treated TB cases needs to be done to know the changing trend of drug resistance in future.

Keywords: Category-I patients; initial drug resistance; proportion method; Mycobacterium tuberculosis; MDR-TB; Kashmir Valley.

1. INTRODUCTION

Tuberculosis (TB) remains a major global health problem. In 2013, an estimated 9.0 million people developed TB and 1.5 million people died from the disease (1.1 million deaths among people who were HIV-negative and 360 000 among people who were HIV-positive) [1]. The number of TB deaths is unacceptably large given that most are preventable. Globally in 2013, an estimated 480 000 people developed MDR-TB and there were an estimated 210 000 deaths from MDR-TB. Most of the estimated number of cases in 2013 occurred in Asia (56%) and the African Region (29%); India and China alone accounted for 24% and 11% of global cases, respectively [1].

Although standard anti-tubercular regimens have been established for decades, low success rates continue to hamper TB control, resulting in an increased prevalence of MDR-TB, mostly due to incorrect regimens and poor patient adherence [2]. One of the aims of ensuring effective management of TB is to minimize the development of drug resistance, which results from inadequate therapy. Surveillance of anti-TB drug resistance is therefore an essential tool for monitoring the effectiveness of TB control programs and improving control efforts. The emergence of drug resistance in recent years has highlighted the importance of an effective control strategy for tuberculosis [3].

Revised National Tuberculosis Control Programme (RNTCP) was launched in 1997 by Government of India, and by March 2006, entire country was covered under the programme. To formulate a treatment policy for the nation, reliable and periodic information on the prevalence of drug resistance in the country as a whole is needed. National surveys of drug resistance are however, expensive and logistically difficult considering the many constraints faced, including the large size of the country [4]. Every geographical area has some inherent differences due to local factors including genetics, coverage of the programme, adherence to proper regimens and other social problems. For this purpose we need to have regional / area specific data.

There have been several studies on prevalence of initial drug resistance in tuberculosis in India, however data from Kashmir Valley which belongs to the state of Jammu and Kashmir, India, is limited with only two published reports available [5,6]. It would be important to establish some meaningful baseline data about drug resistance for monitoring the effectiveness of RNTCP in Kashmir Valley. The aim of the present study was to determine the pattern of initial drug

resistance in *Mycobacterium tuberculosis* isolates and prevalence of MDR-TB among category-I pulmonary TB patients in Kashmir Valley.

2. MATERIALS AND METHODS

2.1 Settings, Patients and Samples

This prospective study was conducted in the Department of Microbiology, SKIMS. Sputum samples of 300 smear positive cases who gave written consent to participate in the study were randomly collected over 3 years (May 2007 - April 2010) from designated microscopy centres (DMCs) of various district tuberculosis centres (DTCs) of Kashmir Valley and DMC of Department of Microbiology, SKIMS. These pulmonary TB cases belonged to Cat I-previously untreated category. Detailed history related to intake of antitubercular therapy in past was taken.

2.2 Exclusion Criteria

Patients with extra pulmonary tuberculosis, immune-compromised patients, retreatment and relapse cases and those who did not consent were excluded from the study.

2.3 Case Definitions

2.3.1 Initial drug resistance

It was defined as resistance to anti tubercular drugs in patients who gave a history of never having received chemotherapy in the past. It included primary resistance and resistance to previous treatment concealed by the patient or of which the patient was unaware.

2.3.2 MDR-TB

It was defined as tuberculosis caused by bacilli showing resistance to at least isoniazid and rifampicin.

The study protocol was approved by the Ethics Committee of the Institute. Due to lack of systemic sampling in our study because of unrest in the Valley during the study period, it is difficult to interpret our study sample in terms of representative of the whole region, hence it is arbitrarily designated as a “convenience sample.”

2.4 Isolation and Identification of Mycobacteria

Samples which were positive on smear microscopy were transported in equal volume of 1% cetylpyridinium chloride (CPC) [7] to the Mycobacteriology laboratory of SKIMS where after decontamination [7], they were subjected to repeat Ziehl Neelsen (ZN) staining and culture for AFB. Culture was done on two bottles of Lowenstein Jensen (LJ) media with glycerol and one bottle of LJ medium with sodium pyruvate which were incubated at 37°C and read weekly for 8 weeks. No growth after 8 weeks of incubation was treated as negative. Positive cultures were identified as *Mycobacterium tuberculosis* on the basis of growth characteristics, susceptibility to p-nitrobenzoic acid (500 ug/ml) and results of standard biochemical tests viz. niacin, nitrate reduction, catalase at 68°C, tween 80 hydrolysis [8].

2.5 Drug Susceptibility Testing for *Mycobacterium tuberculosis*

For all isolates identified as *Mycobacterium tuberculosis*, drug susceptibility test by the indirect proportion method on LJ media was performed at the following final drug concentrations: isoniazid (INH) at 0.2 µg/ml, rifampicin (RIF) at 40.0 µg/ml, streptomycin (STM) at 4.0 µg/ml and ethambutol (EMB) at 2.0 µg/ml [9]. Briefly, two appropriate dilutions of the bacilli, 10^{-2} and 10^{-4} dilutions (undiluted = 10^{6} to 10^{8} CFU/ml), were inoculated on drug-containing and drug-free media, in order to obtain countable colonies on both media. One set of media bottles for testing one culture consisted of five LJ slopes, one for neat, two for 10^{-2} and two for 10^{-4}; eight LJ drug containing slopes, two each for drugs INH, RIF, EMB & STM (one each for 10^{-2} and 10^{-4} suspensions) and one for PNB slope, total 14 LJ slopes were required. The reference strain H37Rv which is susceptible to all standard antituberculosis drugs, was used as susceptible control in each batch of tests. Slopes were put in a stand at a very slight angle from the horizontal and placed in the incubator at 37°C.

2.5.1 Reading of tests

The inoculated slopes were evaluated for growth after 28 and 42 days of incubation. The results were read for the first time on the 28th day. The colonies were counted only on the slopes seeded with the lowest inoculum that produced growth.

The average number of colonies obtained for the 2 control slopes indicated the number of culturable particles contained in the inoculum. The average number of colonies obtained for the drug-containing slopes indicated the number of resistant bacilli contained in the inoculum. The ratio of number of colonies observed on the drug-containing media to drug-free medium indicated proportion of resistant bacilli present in the strain.

2.5.2 Criteria of resistance

Drug resistance was defined as growth on a drug containing medium greater than or equal to 1% of that recorded on the drug-free control medium of the same experiment. If, the result of the reading made on the 28th day was "resistant", no further reading of the test for that drug was required: the strain was classified as resistant. If the result at the 28th day was "sensitive", a second reading was made on the 42nd day: this provided the definitive result.

Training in DST was received at NJIL & OMD, Agra who also provided external quality control for DST for the project.

3. RESULTS

Of the 300 AFB positive sputum samples processed, culture results were available only for 207 samples. Out of 207 samples, 134 (64.73%) were culture positive, 52 sterile (25.12%) and 21(10.14%) showed contamination. Out of the 134 isolates, 123 were identified as *Mycobacterium tuberculosis* and 11 as mycobacteria other than tuberculosis (MOTT) on the basis of culture characteristics and biochemical tests. MOTT were not further categorized/ tested. Of the 123 *Mycobacterium tuberculosis* isolates, DST results were available only for 101 as 22 either failed to grow or were lost to contamination during drug susceptibility testing.

Of the 101 patients finally included in analysis, age ranged between 10-90 years with a median age of 38 years. 54 were males and 45 females with a male to female ratio of 1.14. Most of the patients (77.22%) belonged to rural areas.

Seventy five (74.25%) of the 101 isolates were sensitive to all the four first line antitubercular drugs. Four (3.96%) of 101 isolates were MDR. Two of the MDR strains were also resistant to streptomycin whereas 2 were resistant to all the four drugs. Of the 101 isolates 4(3.96%) strains were resistant to INH and STM; 13(12.87%) showed monoresistance to STM; 3(2.97%) to RIF and 2(1.98%) to INH. No monoresistance was seen to EMB (Tables 1 and 2).

4. DISCUSSION

Tuberculosis is the world's leading curable cause of infectious diseases mortality with a disproportionate burden of the disease falling on low and middle income countries [10]. Most of these countries do not have mycobacterial culture and drug susceptibility testing capabilities leading to inadequate information on drug resistance in tuberculosis. At the time of inception of this study, no mycobacterial culture / DST facility was available in Kashmir. This prompted us to conduct a study on initial drug resistance in *Mycobacterium tuberculosis* in Kashmir Valley. However, because of lack of systematic sampling and less number of positive results, our data does not reflect the exact prevalence of drug resistance in Kashmir Valley.

CPC-NaCl method is considered an inexpensive and effective alternate decontamination method for the isolation of tubercle bacilli from sputum samples that must be in transport for more than 24 hours. This method has been seen to produce a lower contamination rate than NALC- NaOH method with an equal recovery of tubercle bacilli [7]. In our study culture negativity and contamination rates were around 35% of the total samples. This higher loss as compared to other studies with a loss of 21.3% and 12% respectively [11,12], can be attributed to poorly followed instructions in adding equal amount of CPC immediately to the sputum specimen, leakage and drying of specimen due to delay in transit.

In our study the overall observed initial resistance to four first line drugs was 39.6% which is higher than the high initial resistance of 21-29.8% reported from some centres in India [3,11,13,14]. However most studies have reported a prevalence between 5-20% [11,12,15,14,17]. Most frequent resistance in our study was against STM (20.79%) followed by INH (9.90%) and RIF (6.93%). Strains that were resistant to only one drug were mainly resistant to STM (12.87%). High resistance to STM and RIF could be the contributors to the high initial resistance seen in our study. The lowest resistance was against EMB (1.98%).

Table 1. Drug susceptibility profile of *Mycobacterium tuberculosis* isolated from sputum of Cat-I patients from various districts of Kashmir Valley

S. no	Districts of Kashmir valley	Rural (R) Urban (U)	DST results of isolates available	Sensitivity pattern							
				Fully sensitive n (%)	Resistant to N (%)						
					HRSE	HRS	HS	H	R	S	E
1	Srinagar	U	23	18 (78.26)	1 (4.34)	0	1 (4.34)	0	0	3 (13.04)	0
2	Anantnag	R	22	15 (68.18)	1 (4.54)	0	0	1 (4.54)	2 (9.09)	3 (13.63)	0
3	Baramulla	R	15	10 (66.66)	0	2 (13.33)	2 (13.33)	1 (6.66)	0	0	0
4	Kupwara	R	10	7 (70)	0	0	0	0	1 (10%)	2 (20)	0
5	Budgam	R	6	6 (100)	0	0	0	0	0	0	0
6	Kulgam	R	5	4 (80)	0	0	0	0	0	1 (20)	0
7	Pulwama	R	8	5 (62.5)	0	0	1 (12.5)	0	0	2 (13.33)	0
8	Ganderbal	R	4	4 (100)	0	0	0	0	0	0	0
9	Bandipora	R	4	2 (50)	0	0	0	0	0	2 (50)	0
10	Shopian	R	4	4 (100)	0	0	0	0	0	0	0
	Total (N)		101	75 (74.25)	2 (1.98)	2 (1.98)	4 (3.96)	2 (1.98)	3 (2.97)	13 (12.87)	0 (0)

** Kashmir Valley with an area of 16,000 square kms is divided into 10 districts. HRSE- Resistant to INH, RIF, STM and EMB; HRS- Resistant to INH, RIF and STM; HS- Resistant to INH and STM; H- resistant to INH; R- resistant to RIF; S- resistant to STM; E- resistant to EMB*

Table 2. Patterns of Initial drug resistance of *Mycobacterium tuberculosis* to first line drugs (n=101)

Resistance	N (%)	95% CI
Any resistance	40 (39.60)	30.06- 49.14
Any INH	10 (9.90)	4.08- 15.72
Any RIF	7 (6.93)	1.98- 11.88
Any STM	21(20.79)	12.88- 28.7
Any EMB	2(1.98)	-0.74- 4.7
Monoresistance	18 (17.82)	10.36- 25.28
INH	2 (1.98)	-0.74- 4.7
RIF	3 (2.97)	-0.34- 6.28
STM	13 (12.87)	6.34- 19.4%
EMB	0	
Polyresistance (resistance to more than one drug)	8 (7.92)	2.65- 13.19
INH and RIF resistances (MDR)	4 (3.96%)	0.16- 7.76
INH + RIF only	0	
INH + RIF + EMB only	0	
INH + RIF + STM only	2 (1.98)	-0.74- 4.7
INH + RIF+ EMB + STM	2 (1.98)	-0.74- 4.7
INH and other resistances	4 (3.96)	0.16- 7.76
INH + STM only	4 (3.96)	0.16- 7.76
INH + EMB only	0	
INH + EMB + STM only	0	
RIF and other resistances	0	
RIF + EMB only	0	
RIF + STM only	0	
RIF + EMB + STM only	0	
No. of drugs patients were resistant to		
0	75 (74.25)	65.72- 82.78
1	18 (17.82)	10.36- 25.28
2	4 (3.96)	0.16- 7.76
3	2 (1.98)	-0.74- 4.7
4	2 (1.98)	-0.74- 4.7

RIF= Rifampicin, INH=Isoniazid, EMB=Ethambutol, STM= Streptomycin, MDR=Multi-Drug Resistance, CI=Confidence Interval

Primary resistance to INH (9.90%) as a single agent in our study is close to that reported (10-15%) in various studies across India [11-13,15,16] and is within the range (0-16%) quoted by WHO [18]. However, high resistance rates of more than 20% have been reported by Jain et al. [3]. High INH resistance can not only compromise the standard DOTS regimen (in which INH plays an integral part) but also the INH preventive therapy in areas where it is prevalent.

High STM monoresistance in our study is similar to that reported by Jain et al. [3] and Sofia et al. [13] from Bangalore and Lucknow, India. Since most of these patients harboured organisms susceptible to INH and RIF they were expected to respond to category I regimen. Most other centres from India have reported a lower prevalence [11,12,14-17] with the global range between 0.1-23.5% [18]. The probable reason for this high level could be the indiscriminate use of STM containing regimens for treatment of tuberculosis under non-DOTS situations both in the government and the private sectors [11]. Observations from various studies indicate that resistance to both INH and STM increases the risk of selection for MDR-TB during the intensive phase of treatment [19,20]. Also, in some strains of *Mycobacterium tuberculosis* monoresistance to STM is related to development of MDR [21].

Resistance to RIF as a single agent in our study is high to the tune of 6.93% (Table 2).This is higher than the global range of 0-3% [18] but lower than that of 12.5% quoted by Jain et al. [3]. As 90% of all the RIF resistant isolates are also resistant to INH, a positive result for RIF resistance can be taken as a strong indicator of MDR-TB [22-24]. Hence, high initial RIF

resistance can be considered a threat to the ongoing TB control programme. Restricting use of RIF (supervised therapy only for TB and leprosy) and continuous monitoring of resistance in both Cat I and Cat II cases needs to be done to keep a close look on rifampicin resistance in future.

Our EMB resistance of 1.98% is low and well within the range of 0.5-3.4% reported by most Indian studies [4,11,13,15,17] and 0-4.2% globally [18].

The treatment outcome of patients harboring *Mycobacterium tuberculosis* strains resistant to INH and RIF (MDR) has been poor with a high mortality rate. Their chance of being cured is very low and they require significant expenditure of healthcare resources. Moreover, these patients remain infectious for a prolonged period and may therefore be more likely to infect others [25]. The prevalence of MDR of 3.96% among new cases of pulmonary tuberculosis in Kashmir Valley is consistent with the figures (0.14%-3.4%) from different parts of India [11-17,25] and also correlate well with the global estimates of 3.6% [18]. High prevalence of MDR-TB among new cases has been reported from Indian cities of Lucknow (13.2%) and Mumbai (24%) [3,26]. Bias in patient selection and differences in methodology may account for such high prevalence of MDR-TB noted in these studies. Globally, Eastern European and especially central Asian countries continue to have the highest levels (19% to 42%) of MDR-TB [1].

Prevalence of MDR-TB mirrors the functional state and efficacy of tuberculosis control programs and realistic attitude of the community towards implementation of such programs [27]. The performance of RNTCP in Kashmir Valley appears to be similar to other parts of country indirectly reflected by the magnitude of initial MDR in the present study. The findings from this study and the consistency with previously reported results reflects the success of DOTS in effective treatment of drug-susceptible TB in Kashmir Valley. They also support the interpretation that standard short-course chemotherapy is likely to remain highly effective among the great majority of new TB patients in Kashmir Valley.

Our study has several limitations. The major limitation is the small sample size which is not representative of the population at large. In fact, this limitation was observed in most previous studies on MDR-TB. Due to unrest in Kashmir Valley in the summers of 2008- 2010, transport of samples from various districts became difficult, many samples reaching the central laboratory after 7 days of collection in CPC. This lead to increased contamination and decreased recovery of *Mycobacterium tuberculosis* from these samples. Of the 300 samples processed, 93 samples in various phases of incubation were lost due to fault in an incubator which went unchecked for a few days because of complete shutdown in the Valley. These samples were excluded from the study. For logistic reasons, sputum samples were collected only from patients who were smear positive at a DMC located in the public health system. Smear negative and patients diagnosed outside RNTCP were not included. The biggest single risk factor for developing tuberculosis is HIV, however, in view of its low prevalence in Kashmir Valley and logistic reasons, HIV testing of patients was not undertaken. Well-designed studies are needed in future which will take care of these limitations and give a true representative data from Kashmir Valley.

5. CONCLUSION

In our study, resistance to any one drug was 39.60% with a high STM resistance of 20.79%. Since most of these patients harboured organisms susceptible to INH and RIF standard short-course chemotherapy is likely to remain highly effective among the great majority of new TB patients in Kashmir Valley. Prevalence of MDR was relatively low but with a high RIF resistance of 6.93% there is a need for restricting use of RIF (supervised therapy only for TB and leprosy). It is important to strengthen the capacity of laboratories in Kashmir Valley for TB culture and DST for correct management of TB patients and to prevent emergence of drug resistance. Also, continuous monitoring of resistance in both new and previously treated TB cases needs to be done to know the changing trend of drug resistance in future.

CONSENT

All the sputum positive patients gave written consent to participate in the study.

ETHICAL APPROVAL

The study protocol was approved by the Ethics Committee of Sher-i-Kashmir Institute of Medical Sciences, Srinagar, Kashmir.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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