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Original Research Article

Drug resistance in tuberculosis- resurvey in Wardha district, India after implementation of revised national TB control program

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ABSTRACT

Level of drug resistance among new TB patients indicates the efficacy of any control programme. A drug resistance survey, as a part of global study in new pulmonary tuberculosis (NPTB) patients, was conducted in Wardha district, India under WHO /IUTLD in 2001, before the implementation of the Revised National Tuberculosis Control Programme (RNTCP) which was implemented in the district in 2002.

Objective: The present study was conducted in Wardha district in 2014-2015, thirteen years after the implementation of RNTCP, and the drug resistance in *Mycobacterium tuberculosis* isolates from NPTB patients was compared to the results of 2001 survey. The methodology used was same in both the surveys.

Material and Methods: In addition to 132 isolates from Wardha, the study also included 112 isolates from adjoining city, Nagpur and total of 244 sputum isolates were subjected to drug sensitivity by standard 1% proportion method on Lowenstein Jensen's medium. In addition molecular typing of resistant strains was done.

Results: In Wardha, compared to 2001 survey, overall susceptibility to first line drugs was higher (94.7% vs 80.2%); and resistance to streptomycin (3% vs 7.6%) and isoniazid (2.2% vs 15.2%) were significantly lower ($p \leq 0.05$). MDR was 0.75% against 0.50% in the earlier study but the difference was statistically not significant. Only two MDR isolate were recovered, of which only one was from Wardha.

Conclusion: After the implementation of RNTCP in Wardha District, drug resistance in new PTB patients has shown a decline and MDR continues to be low reflecting upon the efficiency of the program.

1. Introduction

Tuberculosis (TB) remains a major global health problem, responsible for ill health among millions of people each year resulting in devastating social and economic impact.[1] The development of drug resistance in *Mycobacterium tuberculosis* (MTB), specially multidrug resistance (MDR) and extensive drug resistance has further dealt a major blow to tuberculosis control programmes leading to increased morbidity and mortality. MDR-TB is a man-made problem mainly related to poor case management in the earlier stages of treatment after which accurate and rapid detection of the resistant strains is critical for providing appropriate treatment to the patient and to intercept the transmission of drug resistant

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tuberculosis in the community.

The Revised National Tuberculosis Control Programme (RNTCP) was launched in India in 1997 in a phased manner as the ongoing National Tuberculosis Programme (NTP), started in 1962, had many lacunae. RNTCP adopted the internationally recommended Directly Observed Treatment Short Course (DOTS) strategy where the first line anti TB drugs (isoniazid H, rifampicin R, pyrazinamide Z and ethambutol E) were given thrice weekly under direct supervision New patients received a total of 6 months therapy with 2H3R3Z3E3 in the intensive phase followed by 4H3R3 in the continuation phase.[2] The old National TB control program followed daily regimen for a duration of 18 months.

In Wardha district RNTCP was introduced in 2002.[3] In order to assess the impact of any intervention it is necessary to generate data and compare it with the relevant data already available from previous studies. The Department of Microbiology at the Mahatma Gandhi Institute of Medical Sciences (MGIMS), Sewagram Wardha, India was involved with the Tuberculosis Research Centre (currently National Institute for Research in Tuberculosis), Chennai, India, in conducting the WHO/IUTLD global project on anti-tuberculous drug resistance surveillance in 2000-2001[4] when sputum isolates from new pulmonary tuberculosis (NPTB) patients were subjected to first line anti tuberculosis drug susceptibility testing (DST) by 1% proportion method on Lowenstein-Jensen (LJ) medium.[5] Level of multidrug resistance (MDR ie., resistance to isoniazid and rifampicin) reported at that time was 0.5%.[4] In a recently concluded study by the Indian Council of Medical Research on recurrence of TB among NPTB cases,[6] of which the same department was a part of, *Mycobacterium tuberculosis* (MTB) isolates from sputum samples of new pulmonary TB patients from the same region were tested by 1% proportion method on LJ medium in the Mycobacteriology laboratory which is certified under the RNTCP for DST using solid media. The aim of the present study was therefore to conduct first line anti TB drug sensitivity on all the *Mycobacterium tuberculosis* isolates from Wardha district and compare the sensitivity results with the previous pre RNTCP data generated in the WHO/IUTLD study. These results could give us the status of drug resistance in the study area and an insight into any change in prevalence of drug resistance after the introduction of RNTCP.

Both the studies followed the same well established protocols and in both only new cases were recruited. In addition to Wardha, samples from NPTB patients were also collected from the adjoining city of Nagpur and drug sensitivity was also put up for ofloxacin.

The secondary objective was to subject the resistant isolates to line probe assay (LPA) in order to substantiate the phenotypic results of rifampicin and isoniazid resistance and to get the mutational patterns prevalent in this region of central India.

2. Material and Methods

This prospective cross sectional study was conducted from 2014 -2016 after obtaining due ethical clearance from the Institutional Ethics Committee and informed consent from the patients. A new PTB patient was defined as a case of pulmonary TB positive for acid fast bacilli (AFB) in the sputum and who had either never taken anti-tuberculosis drugs or had taken them for less than one month.

Sputum samples were collected in sterile containers from new smear positive pulmonary tuberculosis patients reporting to our hospital in Wardha district and at Wardha and Nagpur Tuberculosis Units (TUs). A TU is the subdistrict level managerial unit responsible for RNTCP implementation in its geographical jurisdiction inhabited by 2,50,000-5,00,000 population. During the study period, at all sites, all consecutive new patients were screened by the RNTCP technician for acid fast bacilli and those who gave written consent were inducted into the study. The sputum samples were collected by the employed social worker and brought on motorbikes to the laboratory on the same day. The sample coming directly to the laboratory from the hospital were also included after takeing due consent. In the laboratory the samples were cultured as per RNTCP protocol.[7] The sputum was first concentrated by modified Petroff's method and then cultured on two Lowenstein Jensen (LJ) slopes and incubated for 8 weeks before reporting as negative.[8] The isolates were identified as *M. tuberculosis* (MTB) by rapid immune-chromatographic MPT64Ag test (SD Bioline, Korea). All MTB isolates were subjected to drug susceptibility testing by 1% proportion method on LJ medium (LJ-DST) for streptomycin, isoniazid, rifampicin, ethambutol and ofloxacin as per the standard recommendations and interpretations.[5] All the drugs were procured from Sigma laboratories. The final concentrations of drugs used were: streptomycin (dihydrostreptomycin sulfate) 4μg/ml, isoniazid 0.2 μg/ml, ethambutol 2 μg/ml, rifampicin 40 μg/ml and ofloxacin 2 μg/ml.[9] The slopes were incubated at 37°C and read at 4 weeks and then at 6 weeks. The test was considered as invalid if no growth was seen on drug free medium even after 8 weeks of incubation.

Isolates resistant to isoniazid and rifampicin by proportion method were subjected to Line Probe Assay (LPA) in order to compare the results with LJ-DST and to see the mutational patterns.

2.1. Line probe assay

The WHO approved GenoType® MTBDR*plus*, (Hain's Life Science Nehren, Germany) was used for confirmation of resistance to rifampicin and isoniazid and to elucidate, as per the kit protocol, the mutational patterns for these two

drugs in the clinical isolates. The genes investigated were *katG, inhA*, for isoniazid and *rpoB* for rifampicin. This part of the study was carried out at the RNTCP certified Intermediate Reference Laboratory for Tuberculosis at Nagpur.

An isolate was considered a) Susceptible when there was presence of wild-type bands and absence of mutant bands; b) Resistant when there was absence of one or more wild-type bands with or without presence of mutant bands and c) hetero-resistant when both the wild type bands and mutant bands were present, due to hybridization of both wild-type and the corresponding mutant probes indicating heterogeneous population or mixed infection with sensitive and resistant strain.[10]

The H37Rv (ATCC 27294) laboratory strain was used as the susceptible control both for the proportion method as well as LPA. In addition, extraction negative controls and master mix negative controls were included with every batch.

3. Results

A total of 244 *M. tuberculosis* isolates, from 280 new sputum smear positive patients from Wardha (132) and Nagpur TU (112), were subjected to drug susceptibility test by LJ-DST. Sixteen (6.55%) isolates were resistant to various drugs. Any resistance was maximum to streptomycin (4.5%), followed by isoniazid (2.45%), rifampicin (0.81%) and ethambutol (0.4%) Prevalence of drug resistance in both the TUs was similar for all drugs except for streptomycin which was more in Nagpur, but the difference was statistically not significant. (Table 1)

No mono resistance to rifampicin was detected and only 2 isolates were MDR (0.81%), one each from Wardha and Nagpur (Table 1). Resistance to ofloxacin among the 244 isolates was low (0.81%) with only 2 resistant strains, one each from Wardha and Nagpur.

To achieve the secondary objective all the 16 isolates resistant to any drug by proportion method were subjected to LPA for the detection of isoniazid and rifampicin resistance. Results for both the drugs were in line with the phenotypic results and no additional isolate resistant to either rifampicin or isoniazid was detected. The two isolate detected as MDR by LJ–DST were also detected as such by LPA and for rifampicin, one of the isolate showed the mutation at *S531L* while the other had mutational pattern with unusal mutational pattern with all missing codons in *WT4,WT6,WT7* and *WT8* bands bands.(Table 2)

The mutation patterns of isolates that were resistant to isoniazid by LJ-DST are also shown in (Table 2). Three isoniazid mono resistant strains showed *katG* mutation at *S315T1* while the fourth depicted low level resistance with mutation in *inhA* gene at *C15T*. Out of the two MDR strains, one of them showed high level isoniazid resistance with mutation in *katG* at *S315T2* while the other isolate showed low level resistance with mutation in *inhA* at C15T. In all, four out of six isolates showed high isoniazid resistance while two had low isoniazid resistance.

4. Discussion

The main objective of the study was to find out the level of drug resistance among new PTB patients in the two study areas of Wardha and Nagpur and to compare the results of the current study with the data of 2001 available for Wardha district under the WHO/IUATLD surveillance project.[4]

The present study has been conducted thirteen years after the previous study[4] and twelve years after the implementation of RNTCP in the Wardha district in July 2002.[3] The comparison of Wardha figures with the earlier study show a significant fall in the resistance to streptomycin and isoniazid (Table 3). Drug resistance for isoniazid has fallen from 15.2% to 2.27% ($p< 0.05$) and streptomycin has decreased from 7.6% to 3.03% ($p< 0.05$). Resistance to rifampicin shows a slight increase from 0.5% to 0.75% but the difference is not statistically significant. The figure 0.75% with ethambutol is also not significant. There is also a significant fall in mono resistance to streptomycin and isoniazid ($p< 0.05$)(Table 3).

Though no earlier surveillance data from Nagpur TU is available for comparison, where again RNTCP was implemented in May 2002, the current study found that drug resistance per se was low in the region. In the WHO/IUATLD project,[4] a total of 197 MTB isolates from NPTB patients were processed for drug susceptibility and 80.2% were reported to be sensitive to all the four first line drugs tested. In the present study 244 isolates from similar patients from Nagpur and Wardha were tested and 93.44% were sensitive to all the four drugs. Whereas any resistance to streptomycin was 7.6%, isoniazid 15.2%, ethambutol 1% in the earlier study, the similar figures for the Wardha and Nagpur together were 4.5%, 2.45%, and 0.40% respectively which are considerably low (Table 3). No mono resistance to rifampicin was detected in either study and MDR was 0.5% (1/197) in the previous survey compared to 0.81% (2/244) in the present study (Table 3) which is again not statistically significant ($p>0.05$). The low level of resistance in NPTB patients has great implication as it not only means that the RNTCP control programme with DOTS has been running well, it also means that even in the absence of laboratory report on drug susceptibility, majority of newly diagnosed patients in the area can be started on the first line regimens resulting in minimal risk of treatment failure.

A study from Delhi in 2008-2009 using same 1% proportion method (LJ-DST) on 177 isolates from 218 NPTB cases, has also reported on similar lines.[11] They have found 1.1% prevalence of MDR TB with 6.2% resistance to isoniazid, 1.1% to rifampicin, 3.4% to ethambutol, 2.3% to streptomycin and none to pyrazinamide. The author have also stated that after the implementation of RNTCP and

Table 1: Drug resistance pattern of *M. tuberculosis* culture isolates from Wardha and Nagpur when tested by 1% proportion method on LJ medium.

Pattern of Drug Resistance	Number resistant (tested n=244)		Number resistant		Number resistant	
		%	Wardha (tested n=132)	%	Nagpur (tested n=112)	%
S	7	4.37	2	1.51	5	4.46
H	2	0.81	1	0.75	1	0.89
OF	1	0.40	1	0.75	0	0
SH	2	0.81	1	0.75	1	0.89
SE	1	0.40	1	0.75	0	0
SOF	1	0.40	0	0.00	1	0.89
HR	2	0.81	1	0.75	1	0.89
Sensitive to all	228	93.44	125	94.69	103	91.96

S- streptomycin, H-isoniazid, R- rifampicin, E-ethambutol, OF-ofloxacin

Table 2: Pattern of rifampicin and isoniazid resistant isolates as detected by Geno Type MTBDRplus

S.No	Gene	Band	Codon region	Mutations	No of isolates
Isoniazid monoresistant					
1.	*katG*	WT	315	S315T1 (MUT-1)	3
2.	*inhA*	WT-1	-15	C15T (MUT-1)	2
Multidrug resistant (MDR)					
	rpoB	WT-8	530-533	S531L (MUT-3)	1
	Kat G	WT	315	S315T2(MUT2)	
2.		Missing bands WT3/WT4	513-519		1
	rpo B	WT5/WT6	518-525		
		WT-7	526-529		
		WT-8	530-533		
	inhA	WT-1	-15	C15T (MUT-1)	

Table 3: Comparison of drug resistance in the present study (2015-2016) with WHO/IUATLD (2001) study from Wardha)

Any resistance Drugs	WHO/IUATLD 2001 figures for resistance (A) (n=197)	Wardha 2015-2016 figures for resistance (B) (n=132)	p value Between (A) and (B)	Nagpur plus Wardha 2015-16 resistance (n=244)	Nagpur 2015-16 Resistance (n=112)
S	7.60%	3.03%	p<0.05	4.50%	6.25%
H	15.20%	2.27%	P<0.05	2.45%	2.67%
R	0.50%	0.75%	p>0.05	0.81%	0.8%
EB	1.00%	0.75%	p>0.05	0.40%	0%
Overall susceptible	80.20%	94.69%	p<0.05	93.44%	91.96%
HR(MDR)	0.50%	0.75%	p>0.05	0.81%	0.89%
Mono S resistant	4.60%	1.51%	p<0.05	4.37%	4.46%
Mono H resistant	10.70%	0.75%	p<0.05	0.81%	0.89%
Two drugs resistance	4.60%	3.0%	p>0.05	2.42%	1.78%

DOTS, MDR-TB prevalence in Delhi has not risen over the years and continues to be low among NPTB cases.

The recent report on the first national anti-tuberculosis drug resistance survey 2014-2016[12] using liquid culture (MGIT 960) has reported MDR-TB to be 2.84% (CI 95%, 2.27-3.5%) among the new pulmonary TB patients. Any resistance to streptomycin, isoniazid, rifampicin and ethambutol was 6.88% (6.01-7.84%), 11.06% (9.97-12.22%), 2.84% (2.28-3.49%) and 2.28% (1.78-2.88%) respectively. Similar values in our study area are even lower than the lowest range quoted in this national survey reflecting on the efficacy of the control programme in the area.

Earlier to the implementation of RNTCP in India the range for MDR-TB in new smear positives was described as 0-5%.[13] It was reported as 4.4% in North Arcot in 1989-1998 and 5.3% in Wardha district 1982-89.[14] Even any resistance to rifampicin was respectively 11.8% and 8% in the two districts and isoniazid was 21% and 19%. Later when WHO/IUATLD study was conducted in Wardha, the rifampicin resistance in the district was reported as 0.5%.[4] The reason for high resistance reported earlier from many centres can be attributed probably to the use of non standardized methods with no quality control measures.

The present study in Wardha and the WHO/IUTLD study have both been carried out in certified laboratories. Thus the data from the present study, using the same methodology and comparing the population in the same area does reveal that drug resistance continues to be low and there has been no significant change in the multidrug resistance, if at all, the mono resistance among the NPTB patients has shown significant fall (Table 3).

Data on fluroquinolone (FQ) resistance in new cases is limited. Any resistance to ofloxacin, the only fluroquinolone tested in the present study, was 0.85% which is much lower than 10.4% reported in new cases from Tamil Nadu%.[15] A drug resistance surveillance (DRS) study from Gujarat in 2009, reported FQ mono-resistance to be around 24% with no significant difference between new and retreatment TB cases.[16] The national survey[12] has reported it to be 3.72% (3.08-4.45) among the new smear positives and 6.29 (5.23-7.48) among the previously treated patients and has expressed concern over the developing high resistance rate of fluoroquinolones among the MDR cases (21.82%). Recently in 2018 Rohini Sharma et al from All India Institute of Medical Sciences, Delhi[17] have reported using MGIT 960 liquid media, only 1.8% mono resistance in their new TB cases (13/728) and among re-treated drug sensitive TB cases it was 5.9% (22/371). The overall FQ mono-resistance among drug sensitive TB cases including new and re-treated TB cases was 3.1%, Mono resistance in our study was only 0.4%. The use of this drug in our district, which is mostly rural in nature, is probably less than that in metropolis where the drug is easily available and very frequently recommended by the private practitioners. Fluoroquinolones and now bedaquiline and other upcoming drugs like delamanid, should be used with extreme care and reservation as they will essentially be the life line drugs for the survival and cure of MDR cases.

Isolates differ from one geographical area to another and molecular epidemiology is used to trace the prevalence and source of infection. There is no published data on the mutational pattern of the resistant isolates from our study area. The commonest mutational pattern reported from other areas for rifampicin resistance[18–22] has been *MUT-3* band for codon *S531L*, accounting for 36-56% of all *rpoB* gene mutations which was the same as one of our MDR isolate. The second most prevalent strain all over is with mutation in *WT-7* codon 526 of the *rpo B gene*. Our second isolate showed a different pattern with all 513 to 531 codons missing in all the four bands *WT4, WT6, WT7* and *WT8* which is an unusual.

The studies on previously treated patients from India ie., Delhi.,[23,24] Gujarat,[25] Himachal Pradesh[26] and a multicentric study conducted in Ahemdabad,[27] have all reported *MUT-3 S531L* mutation in *rpoB* gene to be the most prevalent circulating strains for rifampicin resistance. The frequency of mutation in the *rpoB* gene with *S531L* pattern reported in MDR-TB isolates was 48.9%, 58.2%, 74%, 62.22% and 47%, respectively in the studies mentioned above. The second prevalent strain had mutational pattern *MUT-2 H526D*.

The resistance to isoniazid is of two types –the high level resistance due to mutation in the *katG* gene and low level resistance due to mutation in *inhA* promoter region. High level resistance to isoniazid is most prevalent in India[28,29] the pattern being mutation in codon *S315T*, less common are strains showing low level resistance with mutation in *inhA* C15T promoter region. Among six of our isoniazid resistant strains, four showed high level resistance with mutation in *katG* at S315T and two isolates showed low resistance in *inhA* at C15T of the promoter region, one of which was an MDR isolate. Being a study only on newly detected cases, the number of isolates detected are few. However, future mutational studies should be undertaken on all previously treated patients as the isolates generated by them are the actual strains circulating in the community and responsible for initial drug resistance. It is expected that even in them the predominant detected mutations would be the ones mentioned above.

5. Conclusion

In order to monitor the efficacy of the national control programmes repeat drug resistance surveys have been advocated so as to detect the trends in the development of resistance and institute steps if necessary, to reverse the situation. Though India has just completed its first such survey at National level, this exercise is operationally

difficult. Small studies like the present one can act as great contributors since follow up studies available are very few. The present resurvey has brought out that the drug resistance among new pulmonary TB cases in Wardha district continues to be low and that the *RNTCP* has done well. Similarly the present figures for Nagpur are also very reassuring and, though the numbers are very small, the mutational patterns as derived from LPA are much in line with those reported from other parts of the country.

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7. Conflict of Interest

The Authors declare that they have no conflict of interest.

8. Source of Funding

None.

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