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Rapid and Economical Detection of Multidrug Resistant (MDR) Tuberculosis: Can Rifampicin Resistance (RMP^r) be a Surrogate Marker for MDR-TB ?

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Research Article

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ABSTRACT

Successful treatment of multidrug resistant tuberculosis (MDR-TB) relies on prompt laboratory detection of drug resistance. The study was aimed to investigate utility of GenoType MTBDR*plus* (Line probe assay: Genotypic) as rapid method for MDR-TB detection and for rapid economical detection of MDR-TB, Direct Sensitivity Test (Phenotypic-Direct) was evaluated. Further an attempt also made to investigate the importance of Rifampicin resistance (RMP^r) as a surrogate marker for multidrug resistance (MDR) tuberculosis. Forty-three sputum (acid fast bacilli smear positive =1+ & above) specimens constituted the material for this study. RMP^r /MDR detection was done by Line Probe Assay(LPA) and Direct Sensitivity(DS), which was further evaluated with Proportion method (PM: phenotypic) which was considered as gold standard for statistical calculations. LPA as well as DS was found to be 100% sensitive for detection for MDR. LPA results analysis further revealed S531L (rpoB) + S315T1 (kat G) to be the commonest mutation pattern. The present study found RMP^r to be a good surrogate marker for identifying MDR strains. However, our retrospective analysis of 5-years published data based on proportion method showed the percentage of MDR would increase from 47.54% to 74.44% if we consider rifampicin monoresistance as surrogate marker for MDR.This study found that the LPA is good rapid technique which can be used for rapid detection of MDR/RMP^r. Direct Sensitivity is another suitable option for resource constraint settings. Both the techniques were well comparable with the gold standard proportion method. Larger studies are required to call RMP^r as surrogate marker of MDR-TB. LPA is definitely a good tool for rapid detection of MDR provided one has a requisite infrastructure and trained man-power. The results of DS test were really encouraging which made us to think to replace proportion method where a lot of technical expertise required. Larger studies are required to call RMP^r as surrogate marker of MDR-TB.

INTRODUCTION

More than a century after the discovery of the tubercle bacillus by Robert Koch, Tuberculosis (TB) remains one of the major causes of global death from a single infectious agent and constitutes a serious public health problem worldwide [1]. Global surveillance has shown that drug resistant tuberculosis is widespread and is now a threat to tuberculosis control programs in many countries [2]. MDR-TB defined as resistance to at least isoniazide (INH) and rifampicin (RMP) is an increasing threat to effective TB control in both industrialized and developing countries [3]. Early detection of MDR-TB is of primary importance for both patient management and infection control. Optimal methods for identifying drug resistance in TB in a timely and affordable way in resource-limited settings are certainly the need of the hour.

The most commonly used method for drug sensitivity testing (DST) is Proportion method (PM) on Lowenstein- Jensen medium requires 4-8 weeks to yield result. With the purpose of detecting drug resistance in a shorter period of time, and for rapid screening of MDR markers, such as resistance to rifampicin (RMP ^{r}) in certain populations, several molecular approaches have been proposed in the last

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few years. The reliability of rapid tests for RMP^r detection as a surrogate marker of MDR-TB largely depends on the prevalence of RMP-monoresistance in the study population [4]. As this needs to be validated in each local situation, an attempt also has been taken to analyze our last 5 years DST data of *M. tuberculosis*.

MATERIALS AND METHODOLOGY

This study was undertaken in the Department of Microbiology, Grant Govt Medical College & Sir J J Hospital, Mumbai, India after obtaining necessary ethical permissions and patient consent. The study period was for 2 months (June-July), however some of the results could be obtained only in Sep/Oct. Forty-three sputum (smear positive =1+ & above) specimens constituted the material for this study. Sputum negative cases of TB & sputum showing presence of atypical mycobacteria were excluded from the study.

Smears were made from thick purulent part of sputum specimens & subjected to Ziehl Neelsen staining [5]. The slides were graded according to RNTCP guidelines. Specimens which were graded as 1+ & above using RNTCP guidelines were further subjected to N-acetyl- L- Cysteine (NALC) -NaOH Concentration Method [6]. Inoculation was done on pair of slants of L.J. media. The same concentrate was used for LPA and also for Direct Sensitivity test. The slants were incubated for 8-12 weeks at 35° C-37°C. After sufficient growth, the culture was further subjected to Drug sensitivity testing using economic variant of proportion method. Proportion method results were considered as gold standard fro statistical calculations. The details of the three techniques used for MDR detection are as follows:

Line Probe Assay (LPA) : GenoType MTBDR*plus* [7]

The GenoType MTBDR*Plus* detects resistance to INH and RMP in clinical isolates and sputum samples based on the detection of the most common mutations in katG, inhA and rpoB genes.MDR detection was done using GenoType MTBDR*plus*(Hain Lifescience) as per manufacturer's instructions which constitutes DNA Extraction Amplification, post amplification procedures – reverse hybridization and sequencing. . Results were read by lining strips up to code provided with kit. In order for results to be valid, CC (conjugate control) and AC (amplification control) bands appeared for every sample. The presence of TUB band indicated that *M. tuberculosis* complex is present in the sample. A mutation in the relevant gene (and resistance to the relevant drug) was signified by either an absent wild type band and/or the presence of a mutant band for each gene cluster. The rpoB, katG and inhA each had a control band which was present in order to interpret the results. rpoB predicted RIF resistance, katG predicted high level INH resistance, inhA predicted low level INH resistance. For results to be valid the bands were of intensity equal to or greater than the intensity of the AC band. In order for a batch of results to be valid, the negative control strip had a CC and AC band present, but no other bands were visible. The product insert was referred for interpretation of banding patterns and troubleshooting.

Direct Sensitivity Test (DS) [8]

The sputum deposits obtained by N-acetyl- L- Cysteine (NALC) -NaOH method was inoculated with a 5 mm loop (27 SWG) onto a pair of plain L-J and drug containing L-J media (INH 0.2 µg/ml; R-40 µg/ml). The slopes were incubated and examined weekly for 8 weeks and the growth was recorded each week.

The definition of resistance is based on the amount of growth seen on the drug-free medium. Thus, when the growth on the drug-free medium is 2+ or more, growth of 1+ or more on the drug containing medium was defined as resistance to the drug. When growth on the drug-free medium is 1+ or less (i.e. < 100 colonies), any growth on the drug-containing medium was considered to be an indication of resistance to that particular drug. For this purpose, the higher growth observed on the paired slopes were considered for interpretation.

Proportion method [9]

After sufficient growth, drug susceptibility testing was carried out against primary anti-tuberculosis drugs by economic variant of proportion method19 against Isoniazide : 0.2µg/ml, Ethambutol: 2µg/ml, Streptomycin: 4µg/ml and Rifampicin: 40µg/ml.All isolates resistant to isoniazid and rifampicin was taken as multi drug resistant (MDR). The opacity/turbidity of inoculum was matched to MacFarland no 1, against a black background. The slopes were incubated at 37°C. The growth was read at 28 days and again at 42 days. (Growth recorded as - Confluent growth = 3+; More than 100 colonies = 2+; Record actual number of colonies = 1-100 cols.)First reading was taken at 28th day after inoculation. The colonies were counted only on the slopes seeded with the inoculum that had produced exact readable counts or actual counts (up to 100 colonies on the slope). The average number of colonies obtained for the drug-containing slopes indicated the number of resistant bacilli contained in the inoculum. Dividing the number of colonies in drug containing slopes by that in drug free slopes gave the proportion of resistant bacilli existing in the strain. Below a certain value – the critical proportion – the strain is classified as sensitive; above that value, it is classified as resistant. The proportions were reported as percentages. For each batch of testing, a culture of H37Rv (or a known all sensitive strain) was tested and validated.

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RESULTS

A total of 43 sputum (smear positive 1+ and more) specimens were subjected to line probe assay and then the cultures from the same specimens were further subjected to economic variant of proportion method. Table 1 shows the comparison of the results by these two techniques. In 38(88.37%) cases both the techniques showed concordance. However, there were 5(11.62 %) discrepant results. The discrepancy was seen with rifampicin in 1 case & with INH in 4 cases (Table 2). Line probe assay results were further evaluated considering proportion method as gold standard. LPA was found to be 100% sensitive for detection of MDR as well as RIF^r (Table 3). Additional information yielded by LPA was different banding patterns. Table 4 shows different patterns of the MDR strains obtained in the present study. There were three different patterns observed amongst MDR strains. Commonest (91.6%) pattern seen was S531L(rpoB)+ S315T1(kat G).

Table 1: LPA Vs Proportion

Result	No.
MDR by both techniques	27
Rifampicin sensitive , INH sensitive by both techniques	09
Rifampicin sensitive, INH resistant by both techniques	01
Rifampicin resistant, INH sensitive by both techniques	01
Discrepant results	05
Total	43

Table 2: Showing details of discrepant results

LPA RESULT(RIF & INH)	PROPORTION RESULT (RIF & INH)	NO
RR	RS	1
RR	SR	1
SR	SS	2
SS	SR	1
TOTAL		05

Table 3: Evaluation of LPA for detection of MDR, Rif^r & INH^r

Detection	Sensitivity	Specificity	Positive predictive value	Negative predictive value
MDR	100 %	87.5%	93.1%	100 %
RIF^r	100 %	92.3 %	96.6 %	100 %
INH^r	96.6 %	76.92 %	90.6 %	90.9 %

Table 4: Banding patterns amongst MDR (LPA) strains

19	S531L WT 8 missing, MUT3 present.	S315T1 WT missing, MUT1 present.	------
01	S531L WT 8 missing, MUT3 present	Unidentified WT missing	------
01	S531L WT 8 missing, MUT3 present	S315T2 WT missing, MUT2 present	T8C WT2 absent, MUT3 present.
01	S531L WT8 missing, MUT3 present.	S315T1 WT missing, MUT1 present.	T8C WT2 absent, MUT3 present.
02	S531L WT8 missing, MUT3 present.	S315T1 WT missing, MUT1 present.	C15T WT 1 absent, MUT1 present
01	S531L WT 8 missing, MUT3 present	S315T1 WT missing, MUT1 present.	Unidentified WT2 absent
01	S531L ,WT 8 missing, MUT3 present.	----------	C15T, WT 1 absent, MUT1 present.
01	WT 8 missing, MUT2 present	S315T1 WT missing, MUT1 present.	----------
01	Unidentified, WT3, 4 Missing	S315T1, WT missing, MUT1 present	C15T, WT 1 absent, MUT1 present.
01	D516V WT3, 4 , MUT1 present	S315T1 WT missing, MUT1 present	

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Table 5: Comparison of LPA, DS and Proportion method.

	LPA	DS*	Proportion
Turn-around-time	48-72 hours	2-4 weeks	8-10 weeks
MDR(RR)	14	14	14
MONO RIF (RS)	0	0	0
MONO INH(SR)	2	1	2
SENSITIVE TO RIF, INH.	6	6	6
CONTAMINATION	0	1	0
TOTAL	22	22	22

Another technique which was evaluated in the present study was direct sensitivity test. Because of time constraint, this test was performed on 22 samples. Table 5 shows the comparison between these three techniques used for MDR detection. There was significant difference observed in turn-around-time. Molecular methods are rapid but there was also a significant difference seen in turn-around-time of two phenotypic methods i.e. DS (2-4 weeks) and PM (8-10 weeks). Out of 22 Direct sensitivity put, one showed contamination. Only 21 were available for analysis. Three results were available at the end of 2nd week, seven at 3rd week, four at 4th week and seven at 8th week. Table 5 also shows that RMPr can be taken as a surrogate marker for MDR by all the three tests. However, our retrospective analysis of 5-years DST data based on proportion method (see the published paper) showed that if we consider rifampicin monoresistance as marker for MDR, then the percentage of MDR will increase from 47.54% to 74.44%.

DISCUSSION

The main aim of the study was rapid and economical evaluation of MDR detection. For studying this aspect, genotypic method (LPA) and phenotypic (DS) was evaluated. The results of both techniques were further compared with the gold standard proportion method. The study further was also aimed to evaluate the utility of RMP^r as surrogate marker of MDR-TB.

LPA Vs Proportion

In recent years substantial progress has been made in our understanding of the molecular basis of Mycobacterium tuberculosis drug resistance. Molecular based assays are potentially the most rapid and sensitive methods for the detection of drug resistance. These assays detect all common drug resistance mutations. Some of these techniques include direct sequencing of PCR products, SSCP analysis, heteroduplex analysis, dideoxy fingerprinting, an RNA/RNA duplex, base-pair mismatch assay, luciferase mycobacteriophage strategy, a rRNA/DNA-bioluminescence-labelled probe method, a reverse hybridization -based line probe assay, and other strategies [10]. This study evaluated the performance of GenoType MTBDR*plus* (Line probe assay) which detects resistance to INH and Rif in clinical isolates and sputum positive samples based on the detection of the most common mutations in rpoB, katG and inhA genes. It uses PCR and reverse -hybridization to probes immobilized on a plastic strip and improves a previous version of the same test, MTBDR that detected mutations in rpoB and katG genes [11].

The performance of the GenoType MTBDR*plus* used for LPA test directly from smear positive sputum correlated very highly with proportion method. Out of 43 sputum positive specimens tested, 38(88.37%) results were found to be in concordance with the gold standard proportion method (Table 1). Discrepancy was noted with five specimens i.e.1 in RIF and 4 in INH results (Table 2). Thus the sensitivity for MDR, Rif and INH detection was found to be 100%, 100% and 96.6% respectively. Barnard *et al* [12] performed the same assay directly on 536 smear positive specimens in Cape Town, South Africa. Results were compared with conventional liquid culture and drug susceptibility testing on solid medium. Ravindran *et al.* [13] also used the same assay for smear positive pulmonary specimens and culture isolates and both concluded that this assay have good concordance with phenotypic drug susceptibility results. Details of these studies are shown in Table No. 6.

Rifampicin resistance (RMP^r)

Rifampicin resistance arises due to mutations in rpo B gene DNA dependant RNA polymerase [14]. The nature and frequency of mutations in the rpoB gene of Rif resistant isolates vary considerably according to geographical location [15]. Analysis of approximately 500 rifampicin resistant strains from global sources has found that 96% of rifampin resistant clinical isolates of Mycobacterium tuberculosis have mutations in the 81-bp core region of *rpoB* gene, which encodes the B subunit of RNA polymerase [10,16]. Detection of mutations in the 81 bp-region of the rpoB gene correlated (100%) very highly in their study. Similar findings have been reported in the literature [12,13]. However, the sensitivity of RMP^r can be lower in other setting where mutations outside the 81-base-pair region of rpoB gene which are not detected by this assay. In fact, our study had one false positive (RMP^r by LPA but sensitive by proportion). This can be attributed the

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difference in the principle of these two methodologies where one is genotypic (LPA) and other one is phenotypic (proportion). We found a higher (89.65%) proportion of RMP resistance due to S531L mutations. Barnard *et al.* [12] also found higher proportion (70.5%0 RMP resistance due to S531L in their study. Similar to both above mentioned studies, Ravindran *et al.* [13] also found codon 531 of the rpoB was the most frequently encountered (84.6%). Other codons affected in all these three studies were 526 & 516.

Table 6: Showing comparison of results of studies based on MTBDR Plus

Study	Technique used	MDR Sensitivity & Specificity	Rif Sensitivity & Specificity	INH Sensitivity & Specificity	Mutations Rf	Mutations INH
Barnard *et al.* 2008	MTBDR Plus	98.8%, 100%	98.9%, 99.4%	94.2%, 99.7%	531, 526, 516, S531L	S315T1, S315T2:Kat G C15T : inh A
Ravindran *et al.* 2012	MTBDR Plus	----------	100%, 97.3%	91.9%, 98.4%	531, 526, 516 S531L	S315T1: Kat G C15T: inh A
Present study	MTBDR Plus	100%, 87.5%	100%, 92.3%	96.6%, 76.92%	531, 526, 516, S531L	S315T1: Kat G C15T: inh A

INH resistance

INH is also used as a first line drug for TB is a bactericidal agent. Mutations leading to INH resistance have been identified in different gene targets including Kat G, inhA, ahpc and other genes that remain to be established [17]. In the present study, kat G mutations were found in 96.55% (KatG alone in 75.86% and with inh A in 20.668%) and inh A in 3,44 %. Barnard *et a.* [12] & Ravindran *et al.* [13] reported 97% & 86.5% mutations in kat G. In inh A region the percentage of resistance was low as compared with kat G (Barnard *et al.* [12]: 24% & Ravindran *et al.* [13]: 5.4%). However, they did not find the mutations in both regions simultaneously as well as in inh A region alone which we encountered in our study. The prevalence of mutations in the inhA and the katG genes seems to vary widely in different geographical locations with more mutations in kat G region [18,19,20]. However, our study noted S315T1 (Kat G) &C15T (inh A) as the commonest mutation region which was also true with other studies [12,13].

Telenti *et al.*[21] analysed MTB isolates by PCR and found that the mutation frequencies were as follows for INH resistant strains(Kat G:36.8%, inhA: 31.6%, Kat G-inhA:2.6%, ahpc: 13.2%, Kat G-ahpc: 2.6%). Mutations in inh A were rarely reported in Germany [11]. A high prevalence of kat G mutations has been reported to account for a high proportion of INH resistance in high TB- prevalence countries and for a much lower proportion in lower TB prevalence settings presumably due to ongoing transmission of these strains in high burden settings [19]. Additional information yielded by this assay is about the mutation patterns. Commonest (65.51%) pattern seen was S531L (rpoB) + S315T1 (kat G). Similar observations have been reported [12,13].

Sharma *et al.* [22] analyzed 36 strains of Mycobacterium tuberculosis by INNO-LiPA Rif TB and compared with the results of conventional susceptibility method. They found that the Mycobacterium tuberculosis probe was 100% specific. The most commonly observed mutation was His-526-Tyr in the rpoB gene. Lingala *et al.* [23] from Hyderabad studied the geographical profile of rpoB mutations of *Mycobacterium tuberculosis* clinical isolates using PCR and DNA sequencing. They found that 28 out of 38(74%) rifampicin resistant isolates showed commonly occurring mutations such as 531, 526 and 516. Multiple silent mutations between 145-184(out side the hot spot region) were also reported in their study.

Rifampicin resistance as surrogate marker for MDR-TB

Resistant to rifampicin is a relatively rare event and leads to selection of mutants that are already resistant to other components of short course chemotherapy. Therefore, RMP^r is often regarded as an excellent surrogate marker [24]. However the correlation between RMP mono resistance and MDR TB may vary across population depending on a number of factors. As a result in some populations RMP^r may not be an accurate marker of MDR TB. Our results (Table 3) shows that RMP^r can be taken as a surrogate marker for MDR by all the three tests. However, our retrospective analysis of 5-years published DST data [25] based on proportion method showed that out of 673 isolates, 578(85.88%) isolates were resistant to one or more drugs. Out of 578, 501(86 67%) were rifampicin resistant [40(7.98%): monoresistant, 461(92.01%): in combination with other drugs]. 320(47.54%) were MDR. If we consider even rifampicin monoresistance as MDR, then the percentage of MDR would have increased to from 47.54% to 74.44%. Larger studies are required to reach to conclusion. What is of graver concern at this point is the increasing incidence of primary incidence of MDR -TB. Patients infected with RMP^r strain of MTB generally have a poor prognosis, particularly because RMP^r is often associated with resistance to other first line drugs. It also found that a majority of RMP^r Indian isolate to be resistant to at least one or the other anti TB drugs, supporting the idea of using RMP^r as a surrogate marker for MDR TB [14].

The direct tests described here would serve the purpose well in countries with limited resources. Their performance characteristics suggest that assay is equivalent to conventional Lowenstein Jensen medium based proportion. The direct sensitivity tests were set for 22 smear positive sample among which one got contaminated. Table 3 shows comparison of LPA, direct sensitivity (DS), and proportion method. Sensitivity tests for INH and RMP by DS were available for the end of 2nd week in three cases, seven at 3rd week, four at 4th week and seven at 8th week. The agreement with the results of proportion and direct sensitivity was found to be 100%. Though the number of strains tested was small, DS seems to be a good alternative for rapid detection of MDR. The advantage of the direct tests over the indirect tests is that in most samples it gives sensitivity results at the same time as that of primary culture. This process not only reduces the turn around time by four weeks, but also contamination by eliminating the step of making a sub culture. Most importantly, the results of the direct tests are more closely the representative of the bacterial population in the given sputum sample, unlike in the indirect test, which can suffer from errors of selection when drug susceptibility test was set up from a primary culture. In the direct tests, resistance can be reported if adequate growth is seen on the drug slopes even when the plain medium is contaminated. Mathew *et al.* [8] also have reported the utility of DS in MDR detection. Their results also indicate that resistance could be detected with growth as low as 10 colonies and with total agreement with the result of the indirect test. Hence, it is recommended that such results be accepted provisionally and confirmed with indirect test on subculture.

The disadvantage of LPA and DS is that both the tests need to be performed on smear positive (1+ or more) pulmonary specimens where conventional culture and proportion method gets an upper hand. To further assess the feasibility, impact and cost effectiveness of this study, it is necessary to establish large scale demonstration projects in high burden TB setting to provide further evidence for policy change and further adoption in resource limited settings.

CONCLUSION

Direct Sensitivity method can be used as a good alternative for rapid drug susceptibility testing of *M tuberculosis* in resource constraint settings. The results of this test were really encouraging which made us to think to replace proportion method where a lot of technical expertise required. Similarly, DS also can compete with LPA in terms of turn around time (as cheaper technique and less turn around time compared to proportion). Line probe assay is definitely a good tool for rapid detection of MDR provided one has a requisite infrastructure and trained man-power. Proportion still remains the gold standard when it comes to false-positives of genotypic techniques. One query remains in the mind that whether this false-positives are real false positives?? Or is it due to the difference in detection methods?? Phenotypic Vs Genotypic?? This was a short study which needs to a lot of strengthening before we actually reach to any conclusions!

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