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Research Paper

Rapid detection of Multidrug resistance by Line Probe Assay in 100 sputum positive Mycobacterium tuberculosis cases

Medical Science

KEYWORDS : MDR TB, Genotype MTBDRplus Line Probe Assay, Isoniazid (INH), Rifampicin (RIF)

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ABSTRACT

INTRODUCTION: Multidrug-resistant tuberculosis (MDR-TB) is defined as resistance to two major bactericidal drugs- Isoniazid (INH) and Rifampicin (RIF) with or without resistance to other first line drugs used for the treatment of tuberculosis. It is the result of random genetic mutations in particular genes conferring resistance.
METHODS: A total of hundred consecutive sputum smear positive samples of TB suspects attending the Designated microscopic centre (DMC) of a tertiary care hospital were included in the study. Decontaminated sputa were subjected to Genotype MTBDRplus line probe assay based on multiplex PCR combined with reverse hybridization on nitrocellulose strips, targeting common mutation , yielding results in 2 days.
RESULTS: Out of 100 smear positive sputum samples only 4 (4%)showed Multi Drug Resistance(new and retreated cases combined). Among 28 INH resistant isolates, katG mutation occurred in 15(53.57%) of isolates and in InhA gene occurred in 11 of 28 (39.28%) isolates Among 15% rifampicin resistant isolates, the most common mutation was encountered in codon S531L of rpoB gene. CONCLUSION: Accurate diagnosis and identification of resistant strains is essential for early and efficient treatment and control of MDR-TB. Line probe assay is a good tool for rapid detection of known mutations which could be reliable in predicting the response to therapy.

INTRODUCTION

In an age when we believe that we have the tools to conquer most diseases, the ancient scourge of tuberculosis (TB) still causes 2 million deaths a year worldwide—more than any other single infectious organism—reminding us that we still have a long way to go. In 2013, an estimated 9.0 million people developed TB and 1.5 million died from the disease, 360 000 of whom were HIV-positive[1].This means that someone somewhere contacts TB every four seconds and one of them dies every 10 seconds[2,3].

Globally, 5% of TB cases were estimated to have had MDR-TB in 2013 (3.5% of new and 20.5% of previously treated TB cases). Drug resistance surveillance data show that an estimated 480 000 people developed MDR-TB in 2013 and 210,000 people died[4].

Multidrug-resistant tuberculosis (MDR-TB) is a form of tuberculosis that is resistant to at least two major first line bactericidal drugs- Isoniazid (INH) and Rifampin (RIF) used for the treatment of tuberculosis. MDR-TB most commonly develops in the course of TB treatment. Inadequate treatment or improper use of these anti-tuberculosis medications remains an important cause of drug-resistant tuberculosis which is difficult to treat, costly and can be fatal.

Detection of drug resistance by conventional methods is inadequate due to slow growth rate of *M.tuberculosis*. Many methods have been developed in last few years to shorten the period of the Drug susceptibility testing for MTB.

Several molecular methods like line probe assays have been developed in recent years for the diagnosis of tuberculosis and rapid detection of drug resistance in clinical specimens. Rapid tests can provide results within days (even without culture, directly on samples) and thus enable prompt and appropriate treatment, decreased morbidity and mortality, and interrupt transmission. Line probe assays (based on reverse hybridization DNA strip technology) could potentially address this urgent need.[5]

Currently two types of line probe assays exist - the INNO-LIPA Rif TB assay (Innogenetics, Ghent, Belgium) and GenoType MTBDR assay (HainLifescience GmbH,Nehren, Germany). The LiPA test can simultaneously detect *Mycobacterium tuberculosis* and the presence of a mutation in INH and RIF genes which confers resistance to the drug.

The Genotype MTBDR *plus* assay(HainLifescienceGmbH,Nehren, Germany) is a novel kit based method for detection of the most common mutations in the *M.tuberculosis :rpoB*, *katG*, and *inhA* genes. The aim of the present study was to evaluate the performance of MTBDR*plus* assay for the rapid detection of *M. tuberculosis* and mutations causing Rif and high or low level INH resistance directly in smear-positive pulmonary specimens. So the present study was done to understand drug resistance and mutational patterns in sputum positive samples from MDR-TB suspected cases.

MATERIAL AND METHODS

The present study was conducted in Department of Microbiology, Sri Guru Ram Das Institute of Medical Sciences & Research, Vallah, Amritsar. A total of hundred consecutive sputum positive patients attending RNTCP laboratory with the history of cough for more than 15 days were included in this study. A proper counseling and thorough informed consent was taken from all patients. All newly detected sputum positive cases, Sputum positive cases not responding to ATT, Sputum positive defaulter cases were included. All sputum negative cases were not included. Two smears were prepared on separate slides – one smear was stained by fluorescent stain using Auramine and Rhodamine stains and examined under Light emitting diode (LED) microscope. Other smear was subjected to ZeihlNeelsen (ZN) staining and examined by light microscope. Acid fast bacilli are seen as red, beaded and slightly curved rod against a blue background. The smears were then graded depending upon the bacilli seen under 1000X magnification according to RNTCP grading[6].

Samples were decontaminated by standard N-acetyl-L-cyste-

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ine-sodium hydroxide (NALC-NaOH) method recommended by CDC. One part of sediment of the decontaminated sample were cultured on the Lowenstein- Jensen (LJ) media and other part was subjected to Genotype MTBDR*plus* line probe assay. This assay is based on multiplex PCR combined with reverse hybridization on nitrocellulose strips, targeting common mutations. The whole procedure was divided into three steps- DNA extraction from clinical specimens, a multiplex amplification with biotinylated primers, a reverse hybridization.

The GenoType MTBDR*plus* line probe assay was carried out according to the manufacturer's specifications. Briefly, 500 µl of the decontaminated smear positive pulmonary sample was used. Heat killing was done at 95°C for 20 min followed by sonication for 15 min. The samples were then centrifuged at 13000g for 5 min and 5 µl of the supernatant was used for the PCR. Amplification mixture consisted of 5 µl of DNA solution per sample, 10 µl amplification solution A, 35 µl of amplification solution B(Total volume 50 µl). Amplification was done in a thermal cycler (MyCycler, Bio-Rad Laboratories) using the amplification profile: denaturation of 15 min at 95°C, followed by 20 cycles of 30 sec at 95°C and 2 min at 65°C, and 30 cycles of 25 sec at 95°C, 40 sec at 50°C and 40 sec at 70°C and the extension step of 8 min at 70°C.Hybridisation was performed using a pre-programmed TwinCubator (Hain Lifescience GmbH, Nehren, Germany). After denaturation, the biotin-labelled amplicons were hybridised to the single stranded membrane-bound probes. After a stringent washing, as streptavidin-alkaline phosphate conjugate was added to the strips, an alkaline phosphate mediated staining reaction was observed as bands where the amplicon and the probe had hybridised.

Strips were pasted on the evaluation sheet by aligning the bands CC and AC with their respective lines on the sheet. The nitrocellulose strips were then interpreted according to manufacturer's guidelines.

Each strip of LPA had 27 reaction zones (bands), including six controls (conjugate, amplification, M. tuberculosis complex (TUB), rpoB, katG and inhA controls), eight rpoB wild-type (WT1–WT8) and four mutant probes (rpoB MUT D516V, rpoB MUT H526Y, rpoB MUT H526D, and rpoB MUT S531L), one katG wild-type and two mutant probes (katG MUT S315T1 and katG MUT S315T2), and two inhA wild type and four mutant probes (inhA MUT1 C15T, inhA MUT2 A16G, inhA MUT3A T8C, inhA MUT3B T8A) . Either missing of wild-type band or the presence of mutant band was taken as an indication of a resistant strain.

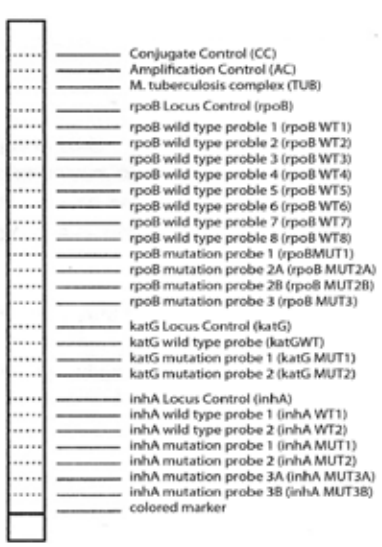

Figure 1: GenoTypeMTBDR*plus* strip
(HainLifescience, Nehren,Germany)

Table no. 1:
Pattern of gene mutations in isoniazid resistant *M. tuberculosis* strains form clinical specimens using Genotype MTBDR*plus*assay

I/D	katGpattern	inhApattern	Result
1.	S315T 1	WT	R
2.	S315T 1	C15T	R
5.	WT	C15T	R
12.	WT	C15T	R
15.	WT	WT	R
16.	S315T1	C15T	R
17.	WT	C15T	R
24.	WT	C15T	R
25.	S315T1	#WT 1	R
26.	S315T1	WT	R
28.	WT	C15T	R
31.	WT	WT	S
32.	WT	WT	S
34.	WT	C15T	R
36.	S315T1	WT	R
42.	S315T1	WT	R
52.	WT	C15T	R
53.	S315T1	WT	R
60.	WT	WT	S
61.	S315T1	WT	R
62.	S315T1	WT	R
67.	S315T1	WT	R
68.	S315T1	WT	R
71.	WT	C15T	R
73.	S315T1	WT	R
76.	S315T1	WT	R
83.	WT	WT	S
97.	S315T1	WT	R

Table no. 2:
Pattern of gene mutations in rifampicin resistant *M. tuberculosis* strains form clinical specimens using Genotype MTBDR*plus* assay

I/D	Rifampicin	
	rpoBmutation detected	Result
9.	S531L	R
12.	D516V	R
25.	S531L,#WT3,4	R
31.	#WT8	R
32.	#WT8	R
34.	S531L	R
39.	S531L	R
43.	#WT8	R
48.	S531L	R
56.	#WT8	S
60.	S531L	R
71.	D516V	R
83.	S531L	R
91.	S531L	R
99	#WT8	R

WT= all wild type probes resent, #WT=missing wild type probe
S=sensitive, R= resistant

RESULTS

A total of hundred consecutive patients attending RNTCP laboratory with the history of cough for more than 15 days was included. In this study the mean age of patients was 38.51 years and about 94% of the patients belonged to 14-69 years of age group which is the economically productive age group. In the present study males patients (62%) outnumbered the females (35%). Children less than 12 years were only 3 in number.58% patients belong to poor socio- economic status whereas 29% patients to middle class and only 13% to the upper middle class. The most common symptom with which the patients presented was cough (96%) followed by anorexia (92%), weight loss (92%) and fever 86%. Haemoptysis was only seen in 18% of patients. Out of the 100 isolates 71% of patients had a past history of tuberculosis and 64% of the patients had past history of ATT.

In our study 49% of patients attending the RNTCP laboratory were taking treatment under category I and 51% belong to category II. Only 9 out of 100 patients (9%) had family history of tuberculosis. There was significant relation (p value0.002) between fluorescent staining which shows 100% results in comparison to ZN staining (91%). Culture was also done on LJ medium. All sputum samples which were positive by microscopic examination gave positive results with GenoType MTBDR*plus* assay (100%). 72% samples were sensitive to isoniazid and 28% showed resistance in either of the two genes responsible for Isoniazid resistance. RIF resistance is caused by altered beta-subunit of DNA dependent RNA polymerase, caused by missense mutation commonly found in 81-bp hot-spot region of the rpoB gene. Resistance to INH is most frequently associated with a specific mutation S315T in katG gene and/ or C15T, A16G, T8A and T8C in the inhA gene. Among 28 INH resistant isolates, *kat*G mutation occurred in 15(53.57%) of isolates with Specific mutations in codon S315T1 of *kat*G gene. Mutations in *Inh*A gene occurred in 11 of 28 (39.28%) INH resistant isolates. Specific *Inh*A mutations were found in 10 of 28 (35.71%) INH resistant isolates with mutation in codon C15T and one isolate showed no specific mutation. None of the confirmed mutation was found in other regions such as A16G, T8C and T8A of inhA gene. Among 15% rifampicin resistant isolates, the most common mutation was encountered in codon S531L of rpo B gene. Specific mutation could be detected rpoB gene in 15 out of 100 15% RIF resistant isolates. Of these, 8 (53.3%) had mutation in codon S531L, 2 (13.3%) in D516V and 5 of them showed missing of wild type bands. In this study only 4(4%) among 100 isolates exhibited to be MDR by showing mutations in both RIF and INH. These included both new and retreated cases.

DISCUSSION

The present study has evaluated the performance of MTBDR*plus* molecular assay for rapid detection of multidrug resistant *M. tuberculosis* directly from smear positive pulmonary samples.

In our study, Flourescent and ZN staining method results were compared which corresponds to the various studies[7,8] as all sputum samples (100) were positive by fluorescent LED microscopy whereas only 82 samples were positive by ZN staining method showing significant comparable sensitivities(p value0.002).

Many recent studies have demonstrated the feasibility of MTBDR*plus* assay as an effective tool in early detection of MDR TB and have good concordance with phenotypic drug susceptibility results In these studies, the rates of valid test results for smear positive and smear negative clinical specimens have been reported to be 91.7%-100%and 46.1-76%[9,10,11] .Our result with smear positive clinical samples revealed 100% positive test results with GenoType MTBDRplus Assay .

Among 28 INH resistant isolates, *kat*G mutation occurred in 15(53.57%) of isolates with specific mutations in codon S315T1 of *kat*G gene. In a study by Min Zhang et al[12] shows 94.3% INH-resistant isolates had mutations in the katG gene, with the katG Ser315T as predominant mutation (55.2%).

Mutations in *Inh*A gene occurred in 11 of 28 (39.28%) INH resistant isolates. Specific *Inh*A mutations were found in 10 of 28 (35.71%) INH resistant isolates with mutation in codon C15T and in one isolate, no specific mutation band could be detected. The south African study reported high prevalence of inhA mutations among all INH resistant cases(41.7%).[13] None of the confirmed mutation was found in other regions such as A16G, T8C and T8A of inhA gene.

Concerning Rif resistance, specific mutation could be detected in rpoB gene in 15 out of100 (15%) RIF resistant isolates. Of these, 8 (53.3%) had mutation in codon S531L, 2 (13.3%) in D516V and 5 of them showed missing of wild type bands. Mutation S531L was detected in 53.3% of RIF resistant cases in the present study, similar to other Indian studies; 59.8% and 84.6% cases. Internationally the rate varies from 47 to 70.5%[14]. Our study is in concordance with other reports from India and abroad which have found S531L mutation to be commonest [15,16,17,18,19]

In 5 of 15(33.3%) RIF resistant isolates, one or more wild type probes were missing with no gain in mutant probes. These isolates are depicted as unknown (#WT). Among these, maximum isolates had missing WT8 (14.28%)band. In various studies from France(29%)[20], New Dehli (11.1%)[21] and South Vietnam(66.7 %)[16] isolates did not have any known mutation.

In this study only 4 among 100(4%) isolates exhibited to be MDR (combined new and retreated cases) by showing mutations in *rpoB* gene(S531L) and in one of the *inhA(* C15T)or *katG* gene(S315T) responsible for RIF and INH resistance respectively. Resistance to both the drugs was detected as appearance of mutant bands at respective probes for different genes responsible for mutations. The prevalence of multi drug resistant (MDR) TB has been believed to be at a low level in most regions of the country. Prevalence of MDR-TB in different studies of India shows that MDR-TB found in new cases was 2. 4% and 17.4% in retreated cases respectively[22].

CONCLUSION

The emergence of antitubercular resistance in mycobacterium is an increasing public health problem, so it is essential to develop quicker and reliable methods for sensitivity testing on tubercle bacilli as an aid and guide to treatment. Diagnostic methods to identify resistant cases and provision of suitable and effective treatment to prevent further spread of MDR-TB is the need of the hour. Now WHO also recommends the use of molecular line probe assay (LPA) GenoTypeMTBDR assay for the diagnosis of MDR-TB. Accurate diagnosis and identification of resistant strains is essential for early and efficient treatment and control of MDR-TB. Line probe assay is a good tool for rapid detection of known mutations which could be reliable in predicting the response to therapy.

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