MUTATIONS IN THE RPOB GENE OF MYCOBACTERIUM TUBERCULOSIS IDENTIFIED BY SEQUENCING METHOD

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ABSTRACT

Objective: To identify the mutation in the rpoB gene of Mycobacterium tuberculosis (MTB), using by sequencing method from pulmonary specimens of presumptive TB patients belonging to the districts of Tamil Nadu.

Methods: A total of 8697 clinical specimens of presumptive MTB patients were collected from various districts of Tamil Nadu. Smear microscopy was performed by light emitting diode fluorescent microscopy and all the smear positive samples were tested using line probe assay (LPA) to detect the percentage of drug resistance pattern and to identify the missing mutation in LPA by the sequencing method.

Results: Among 4897 smear positives subjected to LPA method; 407 (8.3%) MTB was not detected and 16 (0.3%) showed invalid result; 4473 (91.4%) strains showed MTB positive; 3695 (82.6%) were sensitive for both rifampicin (RIF) and isoniazid (INH) drugs; 502 (11.2%) were resistance for INH; 73 (1.6%) resistant for RIF; 203 (4.5%) were resistance for RIF and INH. Totally, 52 (1.2%) strains results cannot be confirmed by LPA and reported as sensitive for RIF, because of the faint and the missing bands in both wild type and mutation. These strains were sequenced and 39 (75%) strains showed resistant to RIF.

Conclusion: Hence LPA may be the molecular technology for the rapid, feasible and reliable method for the detection of multidrug resistant mutation but few confusion bands cannot be reported as resistance, which should be confirmed by either conventional phenotypic drug susceptibility testing or by sequencing method.

Keywords: Line probe assay, Sequencing, Mutation, Multidrug resistant tuberculosis.

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INTRODUCTION

In 2013, more than 9 million people fell ill with tuberculosis (TB) and 4,80,000 people developed multidrug-resistant TB (MDR-TB) globally and there were an estimated 2,10,000 deaths from MDR-TB [1]. In worldwide, the TB disease remains the most dangerous infectious diseases. Providing treatment and control of TB is more complicated due to emergence of drug resistant even MDR strains. RIF resistance can be defined as resistant to both isoniazid (INH) and rifampicin (RIF) with or without other anti-TB drugs [2]. Around 500 new smear-positive MDR-TB cases were estimated to occur per year in the country [3].

Conventional culture and drug susceptibility testing (C & DST) by solid or liquid is a slow process which may take 2-4 months, during this time a patient is often treated according to the standard regimen for drug-susceptible TB. This results delay in proper treatment, adversely affect the outcome of the treatment and transmission of the drug resistant TB [4]. The financial, infrastructural and human resource requirements, widespread implementation of culture-based DST is challenging in such settings. The specimen transport and the specimen contamination issues may also present further challenges [5], hence early diagnosis of TB and rapid detection of RIF resistance is important for proper management of drug-resistant TB [6]. Even though major efforts are being taken and implemented by the government to increase case detection, one-third of new TB cases are still missed due to nonavailability of rapid, low-cost, and accurate diagnostic facilities in high-TB-burden countries like India. Over the last many years, efforts were taken to improve and develop rapid diagnostic tools for diagnosis and DST of TB. The World Health Organization (WHO) issued 10 policy statements for improving diagnosis of TB, including the use of commercial and noncommercial DST methods and implementation of molecular methods such as the line probe assay (LPA) and Xpert Mycobacterium TB (MTB)/RIF assay [7].

In INH-resistant strains, the most common mutations were occurred in either the gene (katG) encoding catalase-peroxidase, which is required for the activation of prodrug INH, or in the promoter region of the inhA, encoding enoyl-acyl reductase, which is involved in Mycobacterium cell wall biosynthesis [8,9]. RIF resistance strains were serve as a surrogate marker for MDR-TB detection because 96% RIF-resistant strains are also resistant to INH [10]. Resistance in RIF was attributed to mutations within an 81-bp RIF resistance-determining region (RRDR) of the rpoB gene, corresponding to codons 507-533 in 96% of RIF resistance strains [11,12]. Mutations outside of RRDR were also reported with a frequency of <2% [13]. More than 50 mutations were characterized within this region by deoxyribonucleic acid (DNA) sequencing but only point mutations at codons 526 or 531 are known to cause high levels of RIF resistance [14]. In contrast, mutations in codons 511, 516, 518, 522, and 533 cause low-level resistance to RIF. Mutations conferring RIF resistance occur rarely in other regions of the rpoB gene [15]. Of the two recently introduced molecular diagnostic methods for RIF resistance detection, LPA technology is based on reverse hybridization of DNA on the strip, when the Xpert MTB/RIF assay is based on real-time polymerase chain reaction (PCR). The strip-based DNA hybridization, i.e., the genotype MTBDRplus (Hain Lifescience, Nehren, Germany) referred as to LPA. Both LPA and Xpert MTB/RIF assays show good performance (98% sensitivity) for RIF resistance detection when compared with the gold standard phenotypic DST. As per the WHO guidelines, the standard turnaround time for reporting the LPA results is 2-3 days,
depending on the timings of sample receiving and reporting of the result. It is mainly advocated for using with smear-positive samples. Where its sensitivity is reported to be 98%. In smear-negative/culture-positive samples its detection rate is low (72.5-76.9%) [16], though its accuracy may vary from region to region due to variation in the circulating MTB strains [17]. Recently, the WHO recommended the use of molecular LPAs for rapid screening of MDR-TB in low and middle income settings [18]. These new assays were developed to detect resistance faster using genotype, rather than phenotype. The GenoType® MTBDRplus test is a DNA strip assay which uses multiplex PCR and amplification and reverse hybridization to identify MTB complex and mutations to detect mutations in the inhA, katG, and genes rpoB genes that confer Rif and INH resistance [19].

LPAs use PCR. LPA is performed directly from acid-fast bacilli (AFB) smear-positive sputum, or from culture isolates, and provide results in 1-2 days. A recent systematic review concludes that LPA are highly sensitive and specific for the detection of Rif resistance (≥97% and ≥99%) and INH resistance (≥90% and ≥99%) on culture isolates and smear-positive sputum. Overall agreement with conventional DST for detection of MDR-TB was 99% [20].

METHODS

Ethics and settings

This laboratory is accredited as an Intermediate Reference Laboratory (IRL) for LPA testing by Revised National TB Control Programme (RNTCP), India, and certified by the National Reference Laboratory (NRL), National Institute of Research in TB, Chennai. Since the observations were made as a part of national TB control program, a separate ethics clearance was not required [21].

Specimens and the study population

A total of 8697 specimens of presumptive MDR-TB cases were received for C & DST from the district TB center (DTC) of Tamil Nadu from January to December 2015. These specimens were collected and packed with ice gel pack and transported through courier as per standard operating procedure from DTC’s to the laboratory. The smear microscopy was performed by fluorescent microscopy method (Auramine-O)-light emitting diode FM [22]. Based on smear microscopy results, the smear positive specimens 4897 were subjected to LPA genotypic method. The GenoType® MTBDRplus assay (LPA) (Hain LifeScience GmbH, Nehren, Germany) was carried out according to the manufacturer’s instructions [23].

Laboratory work-up of sputum specimens

Sample processing

All sputum samples were processed using the N-acetyl-L-cysteine-sodium citrate-NaOH method and neutralized with phosphate buffer solution pH 6.8. [24]. Samples were decontaminated by the following centrifugation, and these sediments were resuspended for 2 ml of phosphate buffer solution. Aliquots were prepared from the processed sample per quantity of the original sample.

LPA

The LPA was performed according to the manufacturer’s protocol [25]. The test is based on DNA strip technology and has three steps: DNA extraction, multiplex PCR amplification, and reverse hybridization (Fig. 1), all the three steps were performed as per the WHO recommendations as follows (Fig. 2) [26].

DNA extraction

The LPA diagnostic technique is based on the PCR amplification of specific regions of DNA. DNA must, therefore, be extracted from the specimens under investigation to make a genotypic diagnosis of the drug susceptibility pattern. The extraction of the DNA from the AFB in the specimen is done by chemical denaturation after the partial cell lyses during the heat-killing step.

DNA amplification

Target DNA was amplified and it is the template to the amplification. Primers (short oligonucleotides of between 18 and 26 base pairs) that will recognize their corresponding sequence on the single stranded DNA and initiate the reaction, and the DNA precursors (the four deoxynucleotide triphosphates, dATP, dCTP, dGTP, and dTTP) these are used as building blocks for the elongation of the single stranded DNA. A thermo stable Thermus aquaticus DNA polymerase (Hot Start Taq) which will elongate the DNA molecule by facilitating the incorporation of the free nucleotides onto the end of the primer, according to the complementary base on the single stranded target DNA.

These reactions are fundamental in ensuring that sufficient DNA is amplified and will be easily detected in the hybridization process, after 30 cycles of amplification, the DNA products will be in the order of 2^n, with sufficient product to be easily visualized as a distinct band when bound to probes on the LPA strip.

Hybridization

Hybridization was performed by automated GT blot 48. This chemical denaturation of the amplified products were hybridization of the single-stranded-biotin-labeled amplicons to membrane-bound probes and stringent washing of the nonspecifically bound amplicons with the addition of a streptavadin/alkaline phosphatase (AP) conjugate. The banding patterns are produced when the substrate (hydrogen peroxide) reacts with the AP, these turns and visualized the bound amplicons purplish-brown (Fig. 3) [27].

Sequencing

Few strains results cannot be confirmed by LPA and reported as sensitive for Rif, because of the faint and the missing bands in both wild type and mutation. These strains were sequenced to identify the changes in the amino acid sequence and mutations were detected in rpoB gene of MTB for Rif resistance.

RESULTS

A total of 4896 AFB smear positive pulmonary specimens were tested with LPA method. Out of 4896 samples 407 (8.3%) samples were MTB not detected and 16 (0.3%) invalid results on the LPA. Of the remaining 4473 samples, 3695 (82.6%) were susceptible for both Rif and INH, 203 (4.5%) had MDR, 502 (11.2%) showed INH mono resistance, and 73 (1.6%) shows Rif mono resistance as shown in Table 1. Among 4473 MTB detected by LPA method, 52 (1.2%) were reported as sensitive with the faint and the missing band in WTB and also in MTU 3 but out of these 52 specimens, 39 (75%) specimens showed resistant by sequencing method Tables 1 and 2.

DISCUSSION

Rapid diagnosis for the resistant associated with the drug resistant strains due to the mutation is a major challenge for the successful treatment of the drug resistance TB. At present available methods such has LPA method (GenoType® MTBDRplus), which offers limited detection capabilities, particularly when novel or uncommon amino acid substitutions and in the silent mutation due to changes within the known drug resistance regions or even in the undiscovered amino acid mutation impact drug resistance [28]. Hence, the sequence for doubtful and the faint bands strains to identify the changes or the mutations in the genes to diagnose the drug resistance TB even rapidly are done in this study.

In this study, the drug resistant TB is about 273 (6.2%), this is comparatively less than other study of 2.3% [29]. LPAs are currently validated only for using directly from smear-positive specimens, although the reasonable performance in a small sample of smear-negative specimens was demonstrated by Barnard and Somoskovi. Although smear-positive TB cases are the most infectious [30], as part of its role in the development and evaluation process of new diagnostic
Fig. 1: Illustration of the DNA-strip technology by Hain Life Science. The GenoType® MTBDRplus molecular line probe assay is divided into three procedures: (1) DNA extraction from NaOH-N-acetyl-L-cysteine-sodium citrate decontaminated smear positive specimens or from cultured isolates (solid or liquid media), (2) a multiplex polymerase chain reaction amplification, and (3) the reverse hybridization (including detection and evaluation).

Fig. 2: An illustration of the work flow for the ideal physical design layout (4 room polymerase chain reaction [PCR] facility) and a basic (3 room PCR facility). Reagent preparation in the pre-amplification room must always be performed before any other procedure can be done.

Fig. 3: (a) The bands (except CC) should be compared to the AC control band for density, (b) The TUB zone is negative, the tested bacteria does not belong to the *M. tuberculosis* complex and the presence or absence of any other bands (except CC and AC) cannot be considered for evaluation, (c) if the locus control zones are negative, the respective mutation-specific positive bands cannot be considered for evaluation, (d) absence of the signal with wild type probes may predict resistance, while positive hybridization signal with a mutation-specific capture probe (for common mutations only) may predict the resistance, (e) presence of the rare mutations may only be indicated by the lack of hybridization with one or more wild type probes.

technologies, FIND negotiated with the manufacturing partner to obtain significant price reductions for equipment and reagents for LPA testing for the public health sector in high burden countries to facilitate widespread access to the WHO-approved technologies [46].

The MTB *rpoB* gene encodes the 1178 amino acid beta subunit for a DNA-dependent RNA polymerase enzyme, the mutations within an 81 base pair called “Core region” of the *rpoB* gene are responsible for approximately 95% of the RIF resistance in MTB strains [31]. It is found that about most of the resistant strains harbor mutations in 531 codon of the *rpoB* region. As reported widely elsewhere, RIF resistance was highly associated with mutation in the 81 base pair region of the *rpoB* gene [32,33].

The MTBDRplus assay is easy to perform and it has the capability for the rapid detection of RIF and INH-resistant MTB [34-37], the rarity of RIF-resistance-associated mutations in codons other than the *rpoB* 81-bp hot spot region and the rarity of silent mutations in the hot spot region are responsible for the high rate of detection of RIF resistance by investigation of this region [37-40]. In this study, the S531L/W mutation in *rpoB* is the most frequent 32 of 52 strains (61.5%), followed by mutations in codon 526, 7 of 52 strains (13.5%) as shown in Table 2. This is comparable to the frequencies reported in other studies [37], even though the distribution can also vary in few settings [47].

The detection of mutations in the hot spot 81-bp region of the *rpoB* gene was analyzed and compared with the sequence results. The confusion results such as the faint band in S531 codon WT8 or missing bands in both WT8 and MUT3 were selected and sequenced to identify the changes in amino acid sequence and nucleotide changes. When the WT8 band is weaker than that of AC and the corresponding mutation band *rpoB* MUT3 is not developed and it is considered as sensitive as shown in the Table 2 [41]. In this study, three of these mutations, yielding amino acid substitutions at positions S531 (Ser→Leu/Trp) and in S526 (His→Tyr/Asp/Arg/Leu) (Fig. 4) and also
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it is found that, out of 52 strains reported as sensitive the sequence results show amino acid changes in 531 codon, i.e., Ser531Leu for 30 strains and Ser531Trp for 2 strains, interestingly identified that additional changes in 526 codon, i.e., His526Tyr for 1 strain, His526Asp for 3 strains, His526Arg for 1 strain, and His526Leu for 2 strain, since both mutant and wild-type bands are absent (Table 2). The Ser531Trp mutation occurs less frequently than Ser531Leu, similarly uncommon amino acid substitution that are missed by LPA also is identified at position 526 (Table 2). However these sequencing reveals that 39 of 52 isolates are resistant and LPA is shown as the faint band or absent in wild type and missing of corresponding mutation band (Fig. 4).

When the isolate is RIF resistant, it is more likely that it is also INH resistant, thus making RIF resistance a surrogate marker for the identification of MDR-TB [40]. It is also well established that isolates harboring mutations between codons 526 and 531 shows high-level resistance to RIF and that these genetic markers carry very high accuracy in RIF resistance detection [42-45]. Molecular technologies like LPA is the most promising technology to detect these mutations but the recommendation in these cases is either to control the result by DNA sequencing or confirm the result by the conventional DST.

CONCLUSION

The analysis of 52 strains which was reported as sensitive due to the confusion, i.e., the faint band or the missing band in WT8 and also missing band in specific probe MUT 3 it is found that, out of 52 strains reported as sensitive the sequence results show amino acid changes in 531 codon, i.e., Ser531Leu for 30 strains and Ser531Trp for 2 strains, interestingly identified that additional changes in 526 codon, i.e., His526Tyr for 1 strain, His526Asp for 3 strains, His526Arg for 1 strain, and His526Leu for 2 strain, since both mutant and wild-type bands are absent (Table 2). The Ser531Trp mutation occurs less frequently than Ser531Leu, similarly uncommon amino acid substitution that are missed by LPA also is identified at position 526 (Table 2). However these sequencing reveals that 39 of 52 isolates are resistant and LPA is shown as the faint band or absent in wild type and missing of corresponding mutation band (Fig. 4).

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REFERENCES

1. WHO: Report on Global Tuberculosis Control; 2014. Available from: http://www.who.int/tb/publications/global_report/2014/en.

2. Yew WW. Management of multidrug-resistant tuberculosis and extensively drug-resistant tuberculosis: Current status and future prospects. Keikaku 2011;86:9-16.

3. World Health Organization: Global Tuberculosis Control - Epidemiology, Strategy, Financing. WHO Report, WHO/HTM/TB/2009.411. Available from: http://www.who.int/tb/publications/global_report/2009/en/index. html. [Last accessed on 2009 Nov 19].

4. Jessy S, Mahmood S. Drug-resistant tuberculosis, recent approach in polymer based nanomedicine. Int J Pharm Pharm Sci 2016;8(10):1-6.
46. Foundation for Innovative New Diagnostics: FIND Prices for Genotype® MTBDRplus and Country List. Available from: http://www.finddiagnostics.org/programs/find-negotiated-prices/mtbdrplus.html. [Last accessed on 2010 Feb 02].

47. Bártfai Z, Somoskővi A, Ködmön C, Szabó N, Puskás E, Kosztolányi L, et al. Molecular characterization of rifampin-resistant isolates of Mycobacterium tuberculosis from Hungary by DNA sequencing and the line probe assay. J Clin Microbiol 2001;39(10):3736-9.