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A Study on Molecular Detection of Mutations Associated with Second Line Anti-tuberculosis Drug Resistance

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Authors' contributions

This work was carried out in collaboration among all authors. Author AG designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors HG and BA managed the analyses of the study. Author SM managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Background: Efficient tuberculosis (TB) control is based on an early diagnosis followed by the rapid identification of drug resistance, in order to treat patients adequately, break the chain of transmission, and avoid the spread of resistant strains. Multidrug-resistant (MDR) *Mycobacterium tuberculosis* have emerged worldwide and seriously threaten TB control and prevention programs. At the same time, the emergence of extensively drug-resistant tuberculosis (XDR TB) has also become an important global health problem.
Objectives: To detect common gene mutation pattern associated with drug resistance against second line anti-tuberculosis drugs in TB patients by SL-LPA as a rapid and early diagnostic test.
Materials and Methods: A total 652 sputum samples were received from 30 districts at culture district laboratory, Jamnagar from October 2018 to December 2018, and were included in the study. Second line Second line- line probe assay (SL-LPA) was used to detect mutations associated with resistance for anti-tuberculosis drugs.

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Results: Out of the 652 samples analyzed for mutations associated with second line anti-tuberculosisdrug resistance, 43% of the samples exhibited various forms of mutations. Out of these samples, mutations associated with gyrA gene were detected in 36.6% samples, gyrB gene mutation in 0.7% samples, rrs gene mutation in 4.4% samples and eis gene mutation in 1.2% samples.

Conclusion: The present study provide information on the mutation pattern of drug resistant strains present in the geographical area and help to provide the basis for effective strategies for control of drug resistant TB in this region. It also re-emphasizes the importance of second line LPA which can diagnose TB and drug resistance in a single day and allows earlier administration of appropriate treatment as compared to culture result which take 1-2 months.

Keywords: Second line-line probe assay; extensively drug resistant tuberculosis; mutations.

1. INTRODUCTION

Tuberculosis (TB) remains one of the major health problems around the globe. India is the highest tuberculosis (TB) burden country in the world [1]. According to WHO estimate in 2018 almost 7 million new cases and around 1.5 million deaths occur every year due to tuberculosis [2,3], making it the world's leading cause of death by a single infectious agent [4]. Early diagnosis helps in initiating optimal treatment which would not only enable cure of an individual patient but also will curb the transmission of drug resistance in the community [1]. The standard treatment regimen combines four first line drugs, isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA) and ethambutol (EMB). Inadequate treatment with these drugs can cause bacilli to become drug resistant (acquired resistance) & these resistant organisms can be transmitted to other individuals (primary resistance). Main reasons for development of resistance are (i) cellular mechanisms, for example, inefficiency of mismatch repair, microsatellites, inadequate translations and error-prone DNA polymerases and (ii) external stress factors, including absence of rapid diagnostic facilities, improper anti-TB drugs prescribing practices, addition of single drugs to failing treatment regimens, inadequate drug supply, quality of the anti-tuberculosis drugs (a very relevant reason in many settings), differences in pharmacogenomics, and the pharmacodynamic and kinetic properties of the drugs administered [5,6], host environment and exposure to smoking or pollution [7].

The emergence of multi andextensively drug resistant tuberculosis is a significant obstacle to the control of this disease because treatment has become more complex and expensive. Multidrug-resistant (MDR) TB refers to *Mycobacterium tuberculosis* strains that are resistant at least to isoniazid (INH) and rifampin (RIF), which are two of the main first line anti-TB drugs, have emerged worldwide and seriously threaten TB control and prevention programs. MDR TB complicates treatment because inefficacious first-line antibiotics must be replaced with second-line drugs that are more costly and that can lead to more adverse side effects. These drugs include fluoroquinolone (FQ) compounds as well as the injectable drugs amikacin (AMK), capreomycin (CAP), and kanamycin (KAN). In 2005, the Centres for Disease Control and Prevention (CDC) and WHO defined extensively drug-resistant (XDR) TB as MDR TB that is also resistant to any FQ and one of the second-line injectable drugs [2,8].

Beside the emergence of drug resistant strains other studies revealed that a prolonged time of diagnosis of six to eight weeks taken by the conventional culture and drug susceptibility testing (DST) leads to the patients being treated with an inappropriate drug regimen, which results in the selection of drug-resistant mutant strains and their continuous spread in the community [9].

Spontaneous chromosomal point mutations are the main mechanism underlying drug resistance in TB and a limited number of mutations accounts for majority of the phenotypic resistance to first- and second-line anti-TB drugs [10]. Mutations related to resistance to FLQ, the most effective second line anti-TB drugs is associated with mutations in a short discrete region of the *gyrA* gene and less frequently *gyrB,* commonly referred to as the quinolone-resistance determining region (QRDR) [11,12,13]. Most of the FQ resistance is attributed to mutations in *gyrA*/QRDR mainly in codons 88, 90, 91 and 94 [13] and AMK, KAN and CAP resistance has been associated with mutations in the *16S rRNA* gene (*rrs*), between

nucleotide positions 1400 and 1500 particularly mutations in positions 1401, 1402 and 1484; up to 87% of KAN and AMK resistance is attributed to these mutations (especially a1401g) [14,15]. Additionally, KAN and CAP resistance can also be mediated by mutations in the *eis* and *tlyA* genes, respectively [16]. However, despite the advances in the detection of drug resistance gene regions in *M. tuberculosis*, a current limitation of molecular assays is that they do not accommodate all mutations conferring resistance to anti-TB agents [17].

The newly developed shorter regimens for the treatment of drug resistant TB requires prompt diagnosis of patients, however, it has been identified that the traditional. DST is not suitable for this purpose and as it takes weeks to give the result. The latest molecular techniques have been shown to be more accurate, rapid and cost effective tool for diagnosis of resistance against anti-tuberculosis drugs [18]. Among these, line probe assay (LPA) has been developed for the rapid detection of *M. tuberculosis* complex, its resistance to anti-tuberculosis drugs and also detect mutations associated with anti TB drug resistance. Hence, the study aims at identifying the mutation pattern of drug resistant strains of *M. tuberculosis* against second line anti-tuberculosis drug in the studied area using the LPA.

2. MATERIALS AND METHODS

This study was conducted in the TB laboratory, Guru Gobind Singh Hospital, Jamnagar. A total of 652 samples received from30 districts at culture district laboratory, Jamnagar during the time period from October 2018 to December 2018 were included in the study. GenoTypeMTBDRsl was used to detect mutations associated with second line anti-tuberculosis drug resistance. The test was performed as per manufacturer's guidelines.

Line probe assays are a family of DNA strip-based tests that determine the drug resistance profile of a MTBC strain through the pattern of binding of amplicons (DNA amplification products) to probes targeting the most common resistance associated mutations to first- and second-line agents and to probes targeting the corresponding wild-type (WT) DNA sequence. LPAs are WHO approved tests for rapid detection of drug resistance to first and second line agents. They can be used for testing of culture isolates (indirect testing), as well as direct testing of acid fast bacilli (AFB) smear microscopy positive specimens (FL-LPA), and both smear positive and smear negative sputum specimens (SL-LPA) [19].

2.1 Procedure

There are 3 Steps: 1. DNA extraction from decontaminated samples; 2. Amplification by PCR; 3. Reverse hybridisation.

2.2 DNA Extraction

Decontaminated sputum samples were used as starting material for DNA extraction.

2.3 Amplification

All reagents needed for amplification such as polymerase and primers are included in the Amplification Mixes A and B (AM-A and AM-B).

Following were prepared for each sample:

– 10 µl AM-A
– 35 µl AM-B
– 5 µl DNA solution

Final volume: 50 µl

The number of sample was determined (number of samples to be analyzed plus control samples). The numbers of tubes needed were prepared. Master mix containing AM-A and AM-B was prepared and mixed carefully. Aliquot 45 µl was added into each of the prepared PCR tubes and 5 µl water (molecular biology grade) was added to one aliquot (negative control). In a separate working area, 5 µl DNA solution was added to each aliquot (except for negative control).

2.4 Hybridization

2.4.1 Preparation

Solutions HYB and STR were warmed to 37-45°C before use. The remaining reagents with the exception of CON-C and SUB-C were brought to the room temperature. Using a suitable tube, Conjugate Concentrate (CON-C) and Substrate Concentrate (SUB-C) 1:100 with the respective buffer (CON-C with CON-D, SUB-C with SUB-D) were diluted in the amounts needed. For each strip, 10 µl concentrate was added to 1 ml of the respective buffer.

1. 20 µl of Denaturation Solution was dispensed in a corner of each of the wells used.

2. 20 µl of amplified sample was added to the solution; it was mixed well and was incubated at room temperature for 5 minutes.
3. 1 ml of prewarmed Hybridization Buffer was added to each well.
4. A strip was placed in each well.
5. Tray in shaking water bath and incubated for 30 minutes at 45°C.
6. Hybridization Buffer was aspirated completely.
7. 1 ml of Stringent Wash Solution was added to each strip and incubated for 15 minutes at 45°C in shaking water bath.
8. Work was done at room temperature from this step forward. Stringent Wash Solution was completely removed.
9. Each strip was washed once with 1 ml of Rinse Solution (RIN) for 1 minute on shaking platform.
10. 1 ml of diluted Conjugate was added to each strip and incubated for 30 minutes on shaking platform.
11. The solution was removed and each strip was washed twice for 1 minute with 1 ml of Rinse Solution and once for 1 minute with 1 ml of distilled water on shaking platform.
12. 1 ml of diluted substrate was added to each strip and incubated protected from light without shaking.
13. The reaction was stopped as soon as bands were clearly visible by briefly rinsing twice with distilled water.
14. Using tweezers, strips were removed from the tray and were dried between two layers of absorbent paper.

2.5 Evaluation and Interpretation of Results

Strips were pasted and stored away from light. The resistance status was determined and noted down in the respective column.

The second version of GenoTypeMTBDRsl includes the quinolone-resistance determining region (QRDR) of *gyrA* (from codon 85 to 96) and of *gyrB* (from codon 536 to 541) genes for detection of resistance to fluoroquinolones and the *rrs* (nucleic acid position 1401, 1402 and 1484) and the eis promoter region (from -37 to -2 nucleotides upstream) for detection of resistance to second line injectable (SLI) drugs. The WT reactions zones comprise regions of the genome with known resistance mutations. The MUT reaction zones correspond to probes that identify the most common resistance mutations of the gene interrogated. If a WT band was missing or if a MUT band was present, it was taken as an indication of a resistant strain. The DNA obtained from the standard MTB-H37RV strain (used as positive control) and one negative control was also tested in each batch in order to check the cross-contamination during hybridization and other quality parameters. The test was considered valid, only when the hybridization bands were obtained on MTB complex control (TUB), conjugate controls (CC) and the amplification controls (AC) along with the targeted gene loci controls [1].

Although LPA can detect the mutations that are most frequently identified in resistant strains, some mutations that confer resistance are outside the regions covered by the test and therefore resistance cannot be completely excluded even in the presence of all WT probes [20].

3. RESULTS

A total of 652 samples were analyzed for mutations associated with second line anti-tuberculosis drug resistance and utations were found in 43% of the samples. Mutations associated with *gyrA* gene was detected in 36.6% samples, *gyrB* gene mutation in 0.7% samples, rrs gene mutation in 4.4% samples and *eis* gene mutation in 1.2% samples.

4. DISCUSSION

Increasing trends of MDR-TB and XDR-TB requires development and implementation of rapid diagnostic techniques [3]. In recent years, a significant progress has been made in our understanding for the molecular basis of *M. tuberculosis* drug resistance. In May, 2016 the world health organization issued new recommendations on the use of a rapid diagnostic test- a line probe assay to detect resistance to second line anti-TB drugs (SL-LPA) [21], following which SL-LPA has been used routinely in different countries. Genetic characterization and identification of mutations that cause resistance will allow the selection of most efficient molecular methods to detect such mutations in order to optimize an effective antibiotic treatment [22]. In the present study it was found that maximum number of mutations associated with second line anti-tuberculosisdrug resistance are in gyrA gene (36.6%), similar to findings in study conducted by Hu et al. [23],

Table 1. Resistance pattern of floroquinolones and second line injectable drugs

Number of samples studied	Resistance pattern of fluoroquinolones against second line drugs (%)		
	Fluoroquinolones	Second line injectable	Fluoroquinolones & second line injectable
652	220(33.7%)	13(1.9%)	24(3.6%)

Table 2.Types of mutation patterns of drug resistant strains of *M. tuberculosis* against second line anti-tuberculosis drugs

Number of samples studied	Number of samples identified with mutations (%)	Types of mutation patterns (%)			
		gyrA gene	gyrB gene	rrs gene	eis gene
652	281(43%)	239(36.6%)	05(0.7%)	29(4.4%)	08(1.2%)

Kateete et al. [24] and Syed et al. [25], followed by rrs gene (4.4%), eis gene (1.2%) and least number of mutations in gyrB gene (0.7%), similar to findings in the study conducted by Gardee et al. [17].

Detection of any second-line resistance by the SL-LPA means that MDR-TB patients should not be enrolled on the shorter regimen as this could compromise their treatment outcome and will cause the development of XDR-TB [26]. These patients therefore need to be put on conventional MDR-TB regimens according to existing WHO policy guidance.

One limitation of this study is that phenotypic drug resistance testing (liquid culture and solid culture) was not performed. So, the performance of SL-LPA was not assessed as compared to culture methods. More studies with complete resistance profile to all second line drugs are required, given the fact of high fluoroquinolone resistance present in India.

5. CONCLUSION

In the present study, it was found that one third of the studied isolates exhibited resistance to fluoroquinolones and mutations in *gyrA* gene are the most prevalent in and around Jamnagar region of Gujarat, whereas mutations in *gyrB* gene are least prevalent.

This study helps us to understand the mutation pattern of drug resistant strains in this geographical area and help to provide the basis for effective strategies for control of drug resistant TB in this region. With the help of line probe assay we can diagnose TB and drug resistance in a single day and this will allow us to start appropriate treatment. The implementation of this assay will also reduce laboratory costs, thereby reducing the financial burden of the national tuberculosis control programs.

India has set a goal of TB elimination by 2025. The large burden of drug resistant TB will limit progress towards that goal. We believe that a strategy focusing on improving diagnostic capacity, guaranteeing appropriate treatment will be essential for meeting the challenge of DR-TB in India.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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