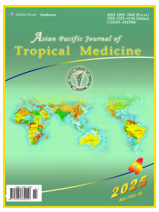

Letter to Editor

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Frequency of mutations in drug resistant *Mycobacterium tuberculosis* causing extrapulmonary tuberculosis

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Detection and treatment of drug resistance in extrapulmonary tuberculosis (EPTB) is a major challenge worldwide. Drug resistance in EPTB has not been studied extensively. However, patients with drug-resistant EPTB have been reported to have poor outcomes[1].

Rifampicin and isoniazid are the cornerstone drugs in the management of EPTB. Resistance in *Mycobacterium (M.) tuberculosis* to these drugs commonly arises due to mutations in the '*rpo*B' gene and '*kat*G & *inh*A' genes, which confer resistance to rifampicin and isoniazid, respectively. Treatment outcomes are affected by the presence of these mutations. In addition, anatomical and physiological barriers impede the effective delivery of drugs to the affected extrapulmonary site[1]. An analysis of the frequency of mutations in drug resistant *M. tuberculosis* strains causing EPTB in our region can help identify patterns of drug resistance. This, in turn, can provide inputs that may be used for modifying standard treatment regimens to make them more effective. The present study aims to identify the frequency and pattern of mutations in the '*rpo*B' gene and '*kat*G & *inh*A' genes in *M. tuberculosis* strains isolated from EPTB samples.

Ethics approval was obtained from the Institutional Ethics Committee of Bhopal Memorial Hospital and Research Centre, Bhopal (approval No.: IEC/42/Micro/18, approval date: 23.01.2019). A waiver of consent was provided for this study.

A total of 7507 extrapulmonary (EP) samples were received at the National Reference Laboratory (NRL) from January 2015 to December 2023. All EP samples were inoculated in Mycobacterial Growth Indicator Tube (MGIT) tubes for culture. Of 7507 samples, MTB grew in 1010 (13%) samples. All positive MGIT tubes were examined for smear microscopy for the presence of acid-fast bacilli after staining with Ziehl-Neelsen stain. The presence of acid-fast bacilli indicated culture positivity. The immunochromatographic test was used for confirmation of the growth of *M. tuberculosis*.

DNA was extracted from the culture isolates using GenoLyse® kit (Hain Lifescience GmbH, Nehren Germany) as per the manufacturer's instructions. Line probe assay (LPA) was performed on DNA samples in accordance with the manufacturer's guidelines using Genotype MTBDRplus kit ver 2.0. The results generated from the LPA were interpreted as per the World Health Organization guidelines for the global laboratory initiative[2].

LPA strip consisted of twenty-one reaction zones indicating wild type (WT) and specific mutation (MUT) bands for *rpo*B, *kat*G and *inh*A genes. The detection of any mutation band in the *rpo*B, *kat*G or *inh*A genes or missing of at least one of the wild-type band was considered as the presence of drug-specific resistance. When one or more wild-type bands were absent and no mutation band/s were present, it indicated that the resistance was inferred to a specific drug. When all wild-type bands were present and no mutation band was present, it indicated susceptibility to rifampicin and isoniazid. The test was considered valid based on the presence of five control zones that comprised conjugate control (CC), an amplification control (AC) and three different locus control zones for *rpo*B, *kat*G and *inh*A genes along with the presence of a master mix and extraction negative control bands. H37Rv was included as a known positive control in each run.

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In LPA, mutations in the *rpo*B gene between the codons 505 to 533 are indicated by mutations at any of the codons D516V (*rpo*B MUT1), H526Y (*rpo*B MUT2A), H526D (*rpo*B MUT2B) and S531L (*rpo*B MUT3). The mutations in the *kat*G gene are indicated by the presence of mutations at any of the codons S315T1 (*kat*G MUT1) and S315T2 (*kat*G MUT2). The mutations in the *inh*A gene is indicated by mutations at any of the codons C15T (*inh*A MUT1), A16G (*inh*A MUT2), T8C (*inh*A MUT3A) and T8A (*inh*A MUT3B). The absence of any wild-type band along with the absence of mutation band/s represent unknown mutations.

Out of a total of 1 010 DNA isolated from 1 010 EP samples, 86.7% (876/1 010) of isolates were sensitive to isoniazid and rifampicin and 13.3% (134/1 010) isolates were resistant to isoniazid and/or rifampicin.

Of the 134 resistant isolates, 3.7% (5/134) had mutations conferring resistance only to rifampicin (rifampicin monoresistance), 56.7% (76/134) of isolates had mutations conferring resistance only to isoniazid (isoniazid monoresistance) and 31.3% (42/134) of isolates had mutations conferring resistance to both isoniazid and rifampicin [multidrug resistance (MDR)]. Resistance was inferred in 11 (8.2%) isolates which included 9 isolates in which resistance to rifampicin was inferred and 2 isolates in which resistance to isoniazid was inferred due to absence of wild type band, with concomitant absence of detectable mutations.

Of the 134 resistant isolates, the most frequent mutation in the *rpo*B gene was seen at the codon S531L (MUT3), which was detected in 24.6% (33/134) of isolates. The second most prevalent mutation was at the codon D516V (MUT1), and was identified in 6.7% (9/134) of isolates, followed by mutation at the codon H526D (MUT2B) which was seen in 2.2% (3/134) of isolates. The mutation at the codon H526Y (MUT2A) was detected in 0.7% (1/134) of isolates. Simultaneous mutations were observed at the codons H526Y (MUT2B) and S531L (MUT3) in 0.7% (1/134) of isolates.

Resistance to rifampicin was inferred (due to an unknown mutation in the *rpo*B gene) in 6.7% (9/134) of isolates, where one or more wild-type bands were absent but no MUT band/s were present. Out of nine isolates that had rifampicin resistance inferred, 1.5% (2/134) isolates had missing WT2 band and 1.5% (2/134) of isolates had missing WT8 band. There were 5 isolates that showed simultaneously missing multiple wild-type bands, which included 3.0% (4/134) of isolates with missing WT3 and WT4 bands; and 0.7% (1/134) of isolates with missing WT1 and WT2 bands (Figure 1).

Of the 134 resistant isolates, the most frequent mutation in the *kat*G gene was seen at codon S315T1 (MUT1), which was detected in 75.4% (101/134) of the isolates. There were 1.5% (2/134) of isolates that showed a MUT2 band indicating a mutation at the codon S315T2. There were 1.5% (2/134) of isolates which showed the absence of WT as well as MUT bands (MUT1 & MUT2) indicating the presence of an unknown mutation. In these isolates, the resistance in the *kat*G gene was inferred (Figure 1).

Of the 134 resistant isolates, the most frequent mutation in the *inh*A gene was seen at the codon C-15T (MUT1), which was detected in 15.7% (21/134) of the isolates. No other mutations were observed in the *inh*A gene (Figure 1).

The frequency of mutations in *M. tuberculosis* can vary across

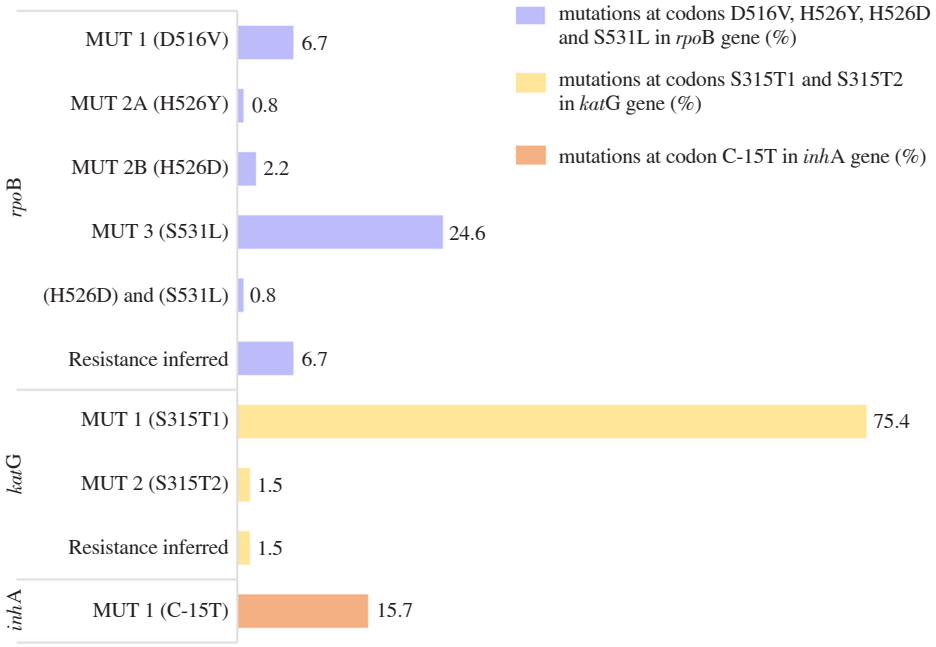

Figure 1. Percentage of mutations in drug resistant *Mycobacterium tuberculosis* samples (*n*=134). MUT: mutation.

different geographic regions. Despite variations, the mutation in the S531L codon has consistently been reported to be the most frequent mutation in the *rpo*B gene across the world[3–5]. In the present study, we also found that mutation in the S531L codon was prevalent, detected in 24.6% resistant isolates of *M. tuberculosis* causing EPTB. The strains of *M. tuberculosis* that harbour the S531L mutation typically exhibit high level resistance to rifampicin which presents significant challenges in the therapeutic management of EPTB[5]. An earlier study from this region reported a predominance of S531L mutation in drug resistant *M. tuberculosis* strains causing pulmonary tuberculosis as well[6].

The second most common mutation was found at the codon D516V in the *rpo*B gene. This mutation was detected in 6.7% of strains of *M. tuberculosis* isolated from the EPTB samples. An earlier study from this region reported similar findings in pulmonary tuberculosis as well[6].

The third prevalent mutation was detected at the codon H526D (2.2%), followed by a mutation at codon H526Y (0.8%). This is different from earlier findings in pulmonary TB, where H526Y mutation was the third most predominant mutation in this region, followed by the H526D mutation[6].

In this study, we found 6.7% unknown mutations in which resistance to rifampicin was inferred. Such isolates failed to hybridize with one or more *rpo*B wild type probes or any mutation probe on the LPA strip. An earlier study on pulmonary tuberculosis in this region, however, found a significantly higher proportion of unknown mutations[6].

Isoniazid is another critical first line drug used in the treatment of tuberculosis. Mutations in the *kat*G and *inh*A genes lead to resistance to isoniazid. Mutation at the codon S315Tl in the *kat*G gene is known to cause a high level resistance which alters the structure of the enzyme catalase-peroxidase and reduces its ability to convert isoniazid into its active form, thus conferring resistance[7]. In the present study, mutation at codon S315Tl was prevalent in the *kat*G gene, detected in 75.4% of *M. tuberculosis* strains isolated from EPTB samples. Similar findings were seen in an earlier study in this region in pulmonary samples[6]. We found that 1.5% of the *M. tuberculosis* strains isolated from EPTB samples had an unknown mutation in the *kat*G gene. However, an earlier study in this region on pulmonary samples showed 6.7% unknown mutations[6].

Another significant genetic locus is the promoter region of the *inh*A gene in *M. tuberculosis*. Mutations in the *inh*A promoter gene result in overexpression of the enzyme enoyl-ACP reductase which reduces the effectiveness of the drug isoniazid[8]. In this study, the commonest mutation was found at the codon C-15T in the *inh*A gene, which was present in 15.7% of the *M. tuberculosis* strains isolated from EPTB samples. Many studies have reported the predominance of this mutation[9,10]. Interestingly, no other mutations, including unknown mutations, were found in the *inh*A gene. This was similar to findings in an earlier study in this region on pulmonary samples[6].

This study was carried out under programmatic settings on the samples received from the patients suspected of having EPTB, from their culture isolates. Given the low sensitivity of culture method, many EPTB cases may not have been detected, leading to a relatively smaller cohort for analysis of the mutation frequencies. Our findings, based on the analysis of the culture isolates, indicate that the most predominant mutations in *rpo*B, *kat*G and *inh*A genes are generally similar across various geographic regions in strains of *M. tuberculosis* in pulmonary as well as extrapulmonary samples. However, there is a difference in the frequencies of the less predominant mutations in these genes between strains causing pulmonary and extrapulmonary tuberculosis in this region. Although these mutations are less frequent, they continue to play role in developing resistance to key first line anti TB drugs. The less frequent or rare mutations might have less competitive advantage than the predominant ones but still contribute to the burden of drug resistance. Further studies on a larger cohort of EPTB samples would help understand the implication of these differences in the frequencies.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Authors' contributions

P.D. designed the study, analyzed data, critically revised the manuscript, approved final version. A.R. critically revised the manuscript. N.P. wrote the manuscript, acquired, analyzed and interpreted the data. R.P.P. acquired, analyzed and interpreted the data. S.A. critically revised the manuscript for important intellectual inputs.

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