Original Article

Study of Genetic Mutation Exhibiting Resistance to Rifampicin and Isoniazid in the Tuberculosis Cases of Eastern Region of Bihar

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Studies regarding epidemiology of mutations associated with Anti-TB drug resistance in Bihar are sparse. The present study analyzes the presence and prevalence of different genetic mutations associated with resistance to Rifampicin and Isoniazid. Specimens from presumptive MDR TB patients were received and LPA were performed to study the mutation patterns. Samples of 3322 patients were incorporated in this study. Processing of samples followed by DNA extraction, PCR and reverse hybridization were carried out and results were interpreted and analyzed. The prevalence of rifampicin resistance observed was 4.54% which includes 119 MDR TB cases and 32 rifampicin mono resistant cases. Similarly, the prevalence of isoniazid resistance observed was 7.6%. The mutation pattern depicts the presence of S531L, S315T1and C15T as the most prevalent mutations for *rpoB*, *katG* and *inhA* promoter region respectively. These findings resemble with other national and international reports.

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Key words **: Tuberculosis (TB), Multi-drug resistant (MDR) TB, Rifampicin, Isoniazid, *rpoB*, *katG*, *inhA*.**

Tuberculosis (TB) is predominantly an infectious disease caused by a group of mycobacterial species called *Mycobacterium tuberculosis* complex. One-third of the global population has been l infected with latent TB[1]. India carries 30% of the global TB burden[2]. The incidence of drug resistance against the key Anti-TB drugs is the leading challenge for the global TB control[3]. The combination therapy with Anti-TB drugs was a brilliant initiative to prevent the emergence of drug-resistant TB[4]. Unsuccessful treatment and dissemination of resistance have emerged as two of the biggest threats for the fight against drug resistant TB[5]. This situation could lead to multiply the number of Multi drug resistance (MDR) TB cases, where the bacilli resist to both rifampicin and isoniazid, which has been reported in all settings. The second-line anti-TB drug treatments for MDR-TB cases, however, are comparatively less effective with more side effects[6]. The estimated global MDR/RR TB burden was 4.1% in new cases and 19% in previously treated cases for the year 2016[7]. The rapid diagnostics of drug resistant TB plays an important role both in suppressing emergence of resistance with other prescribed drugs and better patient management. The WHO has endorsed the use of GenoType MTBDR*plus* assay (Hain Lifescience, Nehren, Germany) also called as line probe assay (LPA) for molecular based rapid diagnostics of drug resistant TB[8]. The principle of LPA is based on the amplification of genetic regions that bears resistance associated mutations[9]. It is a DNA strip based technology and method includes processing of samples, DNA extraction, master-mix preparation, amplification with biotinylated primers and detection with reverse hybridization process. The LPA for rifampicin and isoniazid can confirm the presence of MTB with presence or absence of the most common mutations associated with resistance with Rifampicin and Isoniazid. *rpoB* gene was the target for identification of rifampicin resistance. However, *katG* and promoter region of *inhA* gene have been identified to confer resistance against isoniazid. There is not enough

Editor's Comment :

- Rapid Diagnostics of Drug resistant TB play an important role both in suppressing emergence of resistance and better patient management.
- It is of great significance to attain the knowledge of mutation patterns of key anti TB drugs Rifampicin and Isoniazid by Line Probe Assay in all TB patients.
- rpoB gene is the target of Rifampicin resistance while KatG and inhA genes are associated with INH resistance.
- Detection of mutation patterns associated with Rifampicin and INH resistance helps in formulation of accurate different Regimens for mono resistance , MDR resistance and different level of mutation for INH resistance.
- Effect of common and uncommon mutations associated with these anti TB drugs may pave way for prospect of future research.

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published epidemiological studies available for mutations associated with anti-TB drug resistance in Bihar. The present study analyze the presence and prevalence of different mutations associated with resistance to Rifampicin and Isoniazid in the Eastern Bihar.

Materials and Methods

Setting :

The tuberculosis culture and drug susceptibility testing (DST) laboratory functioning in the premise of Jawaharlal Nehru Medical College and Hospital, Bhagalpur, has been certified by National Mycobacteriology Certification System of Central TB Division, New Delhi, for performing line probe assay based molecular DST as well as liquid culture based DST for first and second line Anti-TB drugs. The laboratory is serving nine districts of Bihar, *ie*, Araria, Banka, Bhagalpur, Begusarai, Khagaria, Kishanganj, Katihar, Munger and Purnia. The study was performed on clinical samples received from July, 2016 to June, 2020.

Study design and sample size :

Specimens from presumptive MDR TB patients were received and LPA were performed to study the mutation patterns. Samples of 3322 patients were incorporated in this study.

Sample processing :

The specimens were processed by using the N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) method[9,10]. Equal volumes of NALC-NaOH solution were gently added to the samples and allow the samples to liquefy properly by intermittent vortexing. The reaction time for NALC-NaOH was 15 minutes. After 15 minutes, phosphate buffer saline (PBS) was added to the 45 ml mark of the 50 ml falcon tube to stop the action of NaOH. After that, samples were centrifuged at 3000x g for 15 minutes at 4°C. After the centrifugation, the pellets were saved for further DNA extraction.

DNA extraction :

Further steps of DNA extraction, PCR and reverse hybridisation were carried out as per the manufacturer's instructions[11]. The pellets were resuspended with 2 ml PBS and 500 µl processed specimens were transferred into cryovials. These processed specimens were centrifuged at 10000xg for 15 minutes. After centrifugation, pellets were saved and 100 µl of lysis buffer were added to each of the samples and mixed properly. Samples were then incubated at 95°C for five minutes inside hot air oven to disrupt the cell wall. Again, 100 µl neutralization buffers were added to each sample and centrifuged at 13000 x g for 5 minutes after proper mixing. Finally, supernatant containing DNA was isolated in a fresh cryovial for each sample.

Multiplex PCR :

45 µl master-mix were prepared for each sample as per the kit instructions and aliquoted in PCR tubes. 5 µl of extracted DNA were added into the master mix prepared and PCR were performed with following reaction conditions - initial denaturation at 95°C for 15 minutes, followed by 20 cycles of denaturation at 95°C for 30 seconds and elongation at 65°C for 2 minutes, followed by 30 cycles of denaturation at 95°C for 25 seconds, annealing at 50°C for 40 seconds, elongation at 70°C for 40 seconds, followed by a final extension at 70°C for 8 minutes.

Reverse hybridization and result interpretation :

The amplicons were denatured and hybridized with probes attached on the LPA strips and following a series of washing, conjugate and substrate treatment, bands develop on the strips. Interpretations of bands were done and results were analyzed.

Results

A total of 3322 LPA tests have been performed in the study. Out of which, 3037 (91.42 %) detected as susceptible for both rifampicin and isoniazid. 119 (3.5%) cases have been detected as MDR TB. However, apart from MDR cases, additional 32 (0.96%) cases have been detected as resistant with Rifampicin and susceptible with Isoniazid. On the other hand, 134 (4.03%) cases have been identified as resistant with Isoniazid and susceptible with rifampicin. The details of mutation patterns for Rifampicin and Isoniazid are depicted in Table 1 & 2 respectively.

When the mutation patterns were analyzed, it was observed that out of 151 (4.54%) rifampicin resistant cases, 105 (69.5%) cases had missing wild type 8 probe with presence of mutation 3 probe for *rpoB* gene. 14 (9.2%) cases had missing wild type 3 and wild type 4 probe with presence of mutation 1 probe. 10 (6.6%) cases had missing wild type 7 probe with no mutation band present. 6 (3.9%) cases had missing wild type 3 and wild type 4 probe with no mutation band present. 3 (1.9%) cases had missing wild type 7 probe with presence of mut 2B probe. 2 (1.3 %) cases had missing of wild type 2 probe with no presence of mutation band. In 2 (1.3 %) cases had missing wild type 2 and wild type 3 with no mutation band present. 2 (1.3%) cases had missing wild type 4 with mutation 1 band present. 2 (1.3%) cases had missing wild type 4 probe with presence of mutation 1 band. 2 (1.3%)

Table 1 — *Mutation pattern associated with Rifampicin resistance*

Rifampicin resistance					
Failing of wild type band	Codon analysed	Presence of mutation band	Specific mutation	Number of cases observed	Percent shared
wt 2	510-513	no common mutation		2	1.32%
wt 2 and wt 3	510-517	no common mutation		2	1.32%
wt 2 and wt 3	510-517	mut 1	Aspartate516Valine	1	0.66%
wt 3 and wt 4	513-519	mut 1	Aspartate516Valine	14	9.27%
wt 3 and wt 4	513-519	no common mutation		6	3.97%
wt 4	516-519	mut 1	Aspartate516Valine	2	1.32%
wt 7	526-529	no common mutation		10	6.62%
wt 7	526-529	mut 2A	Histidine 526 Tyrosine	2	1.32%
wt 7	526-529	mut 2B	Histidine 526 Aspartate	3	1.99%
no wt absent		mut 2B	Histidine 526 Aspartate	2	1.32%
no wt absent		mut 1	Aspartate516Valine	1	0.66%
no wt absent		mut 3	Serine 531Leucine	1	0.66%
wt 8	530-533	mut 3	Serine 531Leucine	105	69.54%

Table 2 — *Mutation pattern associated with Isoniazid resistance*

Isoniazid resistance										
katG					ihhA				Cases	Percent share
Locus	Failing of wild type band	Codon analysed	Presence of mutation band	Specific mutation	Failing of wild type band	Analysed nucleic acid position	Presence of mutation band	Specific mutation		
Present	1	315	1	Serine 315 Threonine	Mutation not detected				194	76.68%
Present	1	315	0		Mutation not detected				6	2.37%
Absent					Mutation not detected				4	1.58%
Present	1	315	1	Serine 315 Threonine	1	-15	1	Cytosine15 Thymine	5	1.98%
Present	1	315	1	Serine 315 Threonine	2	-8	3A	Thymine 8 Cytosine	1	0.40%
Present	Mutation not detected				1	-15	1	Cytosine15 Thymine	37	14.62%
Present	Mutation not detected				2	-8	3B	Thymine 8 Adenine	3	1.19%
Present	Mutation not detected				0		1	Cytosine15 Thymine	3	1.19%

cases had missing wild type 7 probe with presence of mutation 2A probe. 2 (1.3%) cases had no missing wild type with mutation 2 B band present. 1 (0.6%) case had no wild type probe absent with mutation 1 band present. 1 (0.6%) case had no wild type probe absent with mutation 3 probe was present.

Out of 253 Isoniazid resistance cases, 194 (76.6%) had missing *katG* wild type probe with presence of mutation 1 probe. 6 (2.3%) had missing *katG* wild type probe with no mutation probe present. However, 4 (1.5%) had missing *katG* locus with no wild type and mutation bands present. 37 (14.6%) had missing of *inhA* wild type 1 band with presence of mutation 1 band. 3 (1.1%) cases had missing *inhA* wild type 2 band with presence of mutation 3B band. 3 (1.1%) cases had no missing wild type probe with presence of mutation 1 band. However, 6 (2.3%) cases had both *katG* and *inhA* mutation. For *katG*, all of them had missing wild type probe and presence of mutation 1 probe but for *inhA* region, 5 cases had missing wild type 1 probe and presence of mutation 1 band and 1 case had missing *inhA* wild type 2 probe and presence of mutation 3A probe was observed.

Discussion

India shares 26% of the global TB burden and 27% of the global Rifampicin resistance TB burden[12]. The global TB data 2020 states the presence of Rifampicin

resistant TB in 3.3% new and 17.7 % previously treated cases[12]. Rapid diagnostics of drug resistance by detecting the genes associated with resistance plays a very important role in management of TB cases. Resistance with rifampicin has been identified as one of the main reasons behind the treatment failure of TB cases[13]. Rifampicin has bactericidal effects on both metabolically active as well as semi -dormant *M tuberculosis* Bacilli[14]. This effect of Rifampicin, in addition with the effectiveness of Pyrazinamide, has allowed reducing the TB treatment from one year to six months[14]. Rifampicin, basically targets the DNA dependent RNA polymerase of mycobacteria which inhibits the bacterial transcription. The 81 base pairs (27 codons) central region of the gene that encodes the β-subunit of RNA polymerase (*rpoB)* constitutes the resistance determining region. Mutation in this region may results to resistance with Rifampicin. Isoniazid, on the other hand, is a prodrug and it gets activated inside the mycobacterial cell. It enters the mycobacterial cytoplasm through passive diffusion and can kill only actively dividing bacilli[15,16]. It blocks the synthesis of mycobacterial cell wall mycolic acids. An interesting effect of isoniazid on M.TB was identified as loss of acid Fastness[17]. The prodrug isoniazid is activated by catalase-peroxidase enzyme (*katG*) and specific mutation in the *katG* gene confers high level resistance to Isoniazid. However, mutation in the promoter region of *inhA* gene, which codes for one of the main targets of Isoniazid - enoyl acyl carrier protein (ACP) reductase, confers low level resistance with Isoniazid. The mutation in promoter region of *inhA* gene results in over expression of this protein which generally counter-balances the effect of Isoniazid. The present study incorporate a huge sample size of 3322 LPA tests. The prevalence of Rifampicin resistance was 151 (4.54%) which includes 119 MDR TB cases and 32 Rifampicin mono resistant cases. Similarly, including the 119 MDR and 134 Isoniazid mono resistant cases, the prevalence of isoniazid resistance reached 7.6%.

When the mutation patterns were analyzed, it was observed that a huge 70.20% of specific mutation was detected in the 531 codon region of the *rpoB* gene, which was S531L missence mutation. However, 11.26% D516V mutation appears as the second most prevalent mutation in *rpoB* followed by 3.31% H526D and 1.32% H526Y. Similarly, for isoniazid, *katG* S315T1 covered 79.05% of total isoniazid resistance detected. These specific mutations were observed to be the most prevalent mutations detected by LPA across different regions. Singhal *et al* (2015) observed 59% prevalence of S531L mutation in *rpoB* gene and 88.3% prevalence of S315T1 in *katG* gene in New Delhi, India[18]. Aparna *et al* (2010) observed 40% of such mutation in *rpoB* locus in their study based in Hyderabad and Koraput in India[19]. Maurya *et al.* (2013) showed 62.3% and 93.3% prevalence of such mutations for Rifampicin and Isoniazid respectively in Northern India[20]. International studies showed mutation in the 315 region of *katG* was present in 93.3% of isoniazid resistant cases predominantly reported from Germany, Russia and other countries[21-24]. Sinha *et al.* (2020) reported 63.3% S531L mutation followed with 21.4% of D516V and 12.2% H526Y mutation for *rpoB* gene in a study carried out at Varanasi, India[25].

The presence of *inhA* mutations were observed in 19.37% of total Isoniazid resistance cases detected. In these, 2.37% have both *katG* and *inhA* mutation present. Singhal *et al* (2015) and other studies have similar findings over prevalence of *inhA* mutation[18]. The most prevalent mutation in *inhA* gene was observed as C15T and it covers 14.62% of total isoniazid resistant cases.

Conclusion

In the present era of precision medicine, it is of great importance to attain the knowledge of mutation patterns of Isoniazid and Rifampicin for all microbiologically confirmed TB patients. Different regimen could be prescribed based on the information of high and low level resistance of Isoniazid. The WHO has estimated that the COVID-19 pandemic and its associated effects could increase the TB burden by one million per year in the period 2020 to 2025. Both research and robust implementation of new findings are important for the National TB elimination programme to fight against TB. The present study is the first one to analyse the epidemiology of mutations associated with Rifampicin and Isoniazid resistance in the Eastern Bihar. With a huge sample size, the study concluded with the presence of S531L, S315T1and C15T as the most prevalent mutations for *rpoB*, *katG* and *inhA* promoter region respectively. These findings resemble with other National and International reports. However, the effect of common and uncommon mutations on the treatment with that particular drug with different doses could be the future prospect of research.

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