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RESEARCH PAPER

Isoniazid Resistance Level and Associated Resistance Conferring-mutations in Rifampicin Resistant *Mycobacterium tuberculosis*.

Naomee Shareef[1], Shaheda Anwar[2], Abu Naser Ibne Sattar[2], ASM Rayahanul Hoque[3], Mohammad Jobayer[4], Ahmed Abu Saleh[2]

[1]Department of Microbiology, Mugda Medical College Hospital, Dhaka, Bangladesh, [2]Department of Microbiology and Immunology, Bangabandhu Sheikh Mujib Medical University, Dhaka, Bangladesh, [3]Department of Anesthesiology, Shaheed Suhrawardy Medical College Hospital, Dhaka, Bangladesh, [4]National Center for Control of Rheumatic Fever & Heart Disease, Dhaka, Bangladesh

Abstract:

Background: Multidrug resistant tuberculosis (MDR-TB) is a global public health problem causing treatment failure. Rifampicin (RIF) resistance has been used as a surrogate marker for MDR-TB diagnosis, but level of isoniazid (INH) resistance and associated resistance conferring mutations for INH in rifampicin-resistant TB cases are little known.

Objective: The objective of this study was to determine level of isoniazid resistance and associated resistance conferring mutations in rifampicin resistant Mycobacterium tuberculosis (MTB) in sputum samples.

Methods: A total 53 RIF resistant MTB isolates in sputum, detected by Xpert-MTB RIF assay were enrolled in the study. Culture positive samples were tested by BACTEC MGIT 960 system and level of isoniazid resistance was determined, defined as minimum inhibitory concentration of INH of >0.4 µg/mL and 0.1-0.4 µg/mL as high level and low level INH resistance respectively. Distribution of mutation in katG (codon 315) and inhA promoter (-5, -8, -15 and -16) genes by Real-time PCR among the different degrees of INH resistance was investigated.

Results: Among the growth positive isolates, 68.8% of the resistant isolates had high level INH resistance, where katG was found to be the prominent mutation, with or without combined with inhA mutation. Positive predictive value (PPV) of katG mutation was 84.6% in detecting a high level of INH resistance. Low level resistance was present in 31.3% isolates, conferring mutation in inhA and katG in equal percentage (40%), but no detectable mutations were found in 20% low level INH resistant MTB isolates. The PPV of inhA mutation was 33.3% in detection low level resistance.

Conclusion: Most of the INH resistant isolates conferred high level resistance and were associated with katG mutation. Evaluation of level INH resistance before using high dose INH will help to avoid dose dependent toxicity and to determine an appropriate treatment regimen.

Keywords: MDR-TB, INH resistance, katG, inhA, High level INH resistance, Low level INH resistance.

Introduction

Tuberculosis (TB) is the second deadliest infectious disease after COVID-19 worldwide.[1] Bangladesh is ranked fourteenth among the high multidrug-resistant TB (MDR-TB) burden countries.[2] *Mycobacterium tuberculosis* (MTB), resistant to at least isoniazid (INH) and rifampicin (RIF) require longer treatment, costly therapies with higher treatment failure and mortality rates.[3] In 2019, 3.3% of new TB cases and 18% of previously treated cases had multidrug or RIF resistant TB (RR-TB). In Bangladesh, 0.7% of new TB cases and 11% of previously treated cases are MDR/RR-TB, where the combined incidence is 5.8%.[2] Here, resistance to INH in previously treated cases is 49.9% and in new patients 10.8%; rate of INH mono-resistance is 3.5% in previously treated cases and 2.9% in newly diagnosed cases, whereas, prevalence of isoniazid resistance among RIF resistant TB cases is 82.1%.[4,5] RIF and INH resistance often occur concurrently as in MDR-TB strains, but can occur without resistance to the other as well.[3]

In molecular assay, INH resistance has been associated with multiple genes, most commonly katG

***Correspondence:** Naomee Shareef, Department of Microbiology, Mugda Medical College Hospital, Dhaka.*
Email: naomeeshareef987@gmail.com
Orcid ID: 0000-0001-5274-5600

and inhA.[6] Studies from different parts of the world show that, 40-95% of INH resistant isolates have mutations in katG, 75-90% of which are located in codon 315, while 8-20% have mutations in inhA promoter.[7] katG encodes catalase/peroxidase enzymes which causes activation of INH, that ultimately disrupts mycolic acid biosynthesis by inhibiting inhA, which is NADH-dependent enoyl-ACP reductase enzyme encoded by inhA gene.[8] inhA mutation results in over expression of isoniazid's target and tends to increase the minimal inhibitory concentration (MIC) of INH.[9] katG mutation, particularly at codon 315, results in an enzyme which do not have ability to activate isoniazid and thus confer high-level INH resistance, while some katG mutations that retain catalage-peroxidase activity may result in low-level INH resistance.[10] As mutations in inhA active site or promoter region cause reduced target affinity or over expression, respectively, inhA mutation is associated with low level resistance and higher doses may overcome this condition.[11] But 6.8% INH resistant isolates, which show low level resistance by phenotypic method, does not show detectable gene mutation, as other less common INH-resistance mutations may be responsible. On this background, WHO recommends that both phenotypic and genotypic drug sensitivity test should be performed for all isolates.[12]

Effective management of MDR-TB begins with early diagnosis of the cases.[13] Among the phenotypic methods, automated BACTEC Mycobacterial growth indicator tube (MGIT) 960 system can evaluate anti-tubercular drug susceptibility with high sensitivity and efficiency with a short turnaround time.[14] Molecular techniques as GeneXpert MTB/RIF targets the rpoB gene for detection of RIF resistance associated mutations using DNA probes.[15] Line probe assay and Real-time PCR can also detects mutations related to INH resistance, in addition to rpoB gene, completing the detection of MDR-TB.[16]

National Guidelines and for Programmatic Management of Drug Resistant TB recommends use of shorter all-oral bedaquiline-containing regimen for treatment of MDR/RR TB since 2020, where high-dose INH is used.[5] This recommendation is made under the assumption that treatment with high dose of isoniazid may be effective in MTB strains with low-level resistance due to mutation in inhA promotor region.[17] But, in this high TB burden setting, MDR-TB is mainly diagnosed using RIF resistance as a proxy and the genotypic or phenotypic testing for INH resistance are not performed. A high-dose of INH is unable to overcome a high-level INH resistance, and may result in drug toxicity.[18]

The aim of this study was to determine level of INH resistance by BACTEC MGIT 960 system in culture positive isolates and to detect the distribution of drug resistant genes among the different levels of INH resistance.

Materials and Methods

This cross sectional study was conducted in the Department of Microbiology and Immunology, Bangabandhu Sheikh Mujib Medical University, Dhaka from July 2021 to January 2022. Fifty-three RIF resistant MTB isolates, detected by Xpert MTB/RIF assay, in sputum, identified at five icddr,b TB Screening and Treatment Centers (TBSTC) in Dhaka city and 250 bedded TB Hospital, Shyamoli, Dhaka, were enrolled in this study.

The participants were interviewed individually and all relevant clinical history (history of tuberculosis, treatment history, previous treatment regimen, regularity of treatment) was documented in a predesigned data sheet.

Laboratory methods: All samples were decontaminated for DNA extraction and for inoculation in Lowenstein-Jensen (L-J) media for culture. In culture positive isolates, INH resistance was detected by BACTEC MGIT 960 system, using M960 SIRE kit, following the standard procedure of the manufacturer. According to Clinical and Laboratory Standards Institute (CLSI), low- and high-level isoniazid resistance in liquid media had to have an MIC of 0.1-0.4 and >0.4 µg/mL, respectively.[19] Real-time Quantitative PCR (qPCR) was carried out using a qPCR kit (TRUPCR Rif/INH MTB Drug Resistant Detection Kit, India) in Real-time PCR (Applied Biosystem 7300) to detect mutations in katG gene (codon 315) and inhA promoter gene (-5, -8, -15 and -16).

Data management: All the data were rechecked, coded, entered in a data base, and analyzed using SPSS software (Version-26). To observe the sensitivity, specificity, Positive Predictive Value (PPV), Negative Predictive Value (NPV) and diagnostic accuracy, Diagnostic validity test was performed. Chi-square test was used to analyze the categorical variables, shown

with cross tabulation. For statistical analysis p-value <0.05 was considered statistically significant.

Results

Among 53 sputum samples, BACTEC MGIT 960 assay was performed in 19 samples that yield growth in L-J media. Among culture positive RIF resistant samples, BACTEC MGIT 960 system detected 16(84.2%) isolates as INH resistant and 3(15.8%) as INH sensitive. Out of INH resistant isolates, 11(68.8%) of isolates had high level INH resistance with MIC of >0.4 µg/mL and 5(31.3%) had low level INH resistance, with MIC ranging 0.1–0.4 ìg/mL (Table I).

Table I: INH resistance profile by BACTEC MGIT 960 system in culture positive isolates (n=19).

INH sensitivity	Isolates n(%)
Sensitive	3 (15.8)
Resistant	16 (84.2)
High level resistance	11 (68.8)
Low level resistance	5 (31.3)

INH= Isoniazid

Mean time required for the BACTEC MGIT system to detect sensitivity along with level of resistance was 9.24 days.

A high level resistance was exhibited by 11 isolates, of which 7(63.6%) had katG mutation, 4(36.4%) had mutations in katG along with inhA (double mutation). Among five low level resistant isolates, 2(40%) had mutation in inhA and 2(40%) had mutation in katG. Although gene mutations were undetectable in 1(20%) isolate, it exhibited low level resistance (Figure 1).

katG mutation conferring isolates had high level resistance in 77.8% cases, whereas all double mutation conferring isolates had high level resistance. Isolates having mutation in inhA only had low level INH resistance in 100% cases (Table II)

Isolates with katG mutation (with or without inhA mutation), exhibited high-level resistance to INH, with sensitivity, specificity, positive predictive value and negative predictive value of 100%, 60%, 84.6% and 100% respectively. Isolates with inhA mutation exhibited low-level resistance to INH, with sensitivity, specificity, positive predictive value and negative predictive value of 40%, 63.6%, 33.3% and 70% respectively (Table III).

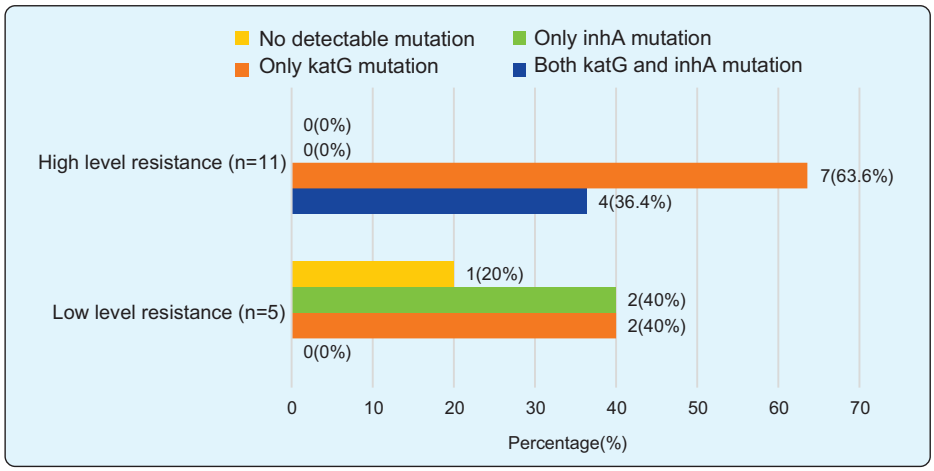

Figure 1: Distribution of mutation among high level and low level INH resistant samples

Table II: INH resistance profile detected by BACTEC MGIT 960 system and real-time PCR in culture positive INH isolates (n=19).

INH resistance profile by BACTEC MGIT 960 system	Mutation profile			
	Double mutation (Both katG and inhA) (n=4)	katG (n=9)	inhA (n=2)	No mutation in katG or inhA (n=4)
High level resistance	4(100%)	7(77.8%)	0(0.0%)	0(0.0%)
Low level resistance	0(0.0%)	2(22.2%)	2(100%)	1(25.0%)
INH sensitive	0(0.0%)	0(0.0%)	0(0.0%)	3(75%)

Table III: Diagnostic performance of katG mutation for detection of high level and inhA mutation for detection of low level resistance among INH resistant isolates detected by BACTEC MGIT 960 system.

Diagnostic performance	katG mutation for high level resistance	inhA mutation for low level resistance
Sensitivity	100.0%	40.0%
Specificity	60.0%	63.6%
Positive predictive value	84.6%	33.3%
Negative predictive value	100.0%	70.0%
Accuracy	87.5%	56.2%

Discussion

Bangladesh is one of the highest global MTB and MDR-TB burden countries with 3,300 MDR/RR-TB cases in 2019.[6] In the present study, among the growth positive isolates, majority of the resistant isolates had high level INH resistance, where katG was found to be the prominent mutation.

Conventional DST for INH on culture positive isolates by BACTEC MGIT 960 system found 15.79% isolates as INH sensitive and 84.2% isolates as resistant. Out of resistant isolates majority had high level resistance. Findings of this study coincided with reported proportions in other parts of world where 65.6% of all isoniazid-resistant strains showed high level resistance and 22.7% had low level resistance.[20] Riviere et al observed similar findings, where 21% isolates were INH susceptible, 62% had high level resistance and 17% had low-level resistance.[21] Prevalence of high level INH resistance in majority of the cases can be explained by molecular basis of INH resistance. Wide range of moderate to high-level isoniazid resistance, are found to be associated with mutations in katG. As mutations in katG are the most frequent (64%) cause of isoniazid resistance, INH resistant isolates predominantly conferred a high level resistance.[11]

INH resistant isolates in this study, conferring high level resistance were accompanied with mutation in katG, with or without inhA mutation. High level resistance was not found in any isolate carrying only inhA mutation. Rather, low level INH resistance was found in association of inhA and katG mutation at equal proportion (40%) and in an isolate, which was not associated with any detected mutations. These findings were consistent with other studies which revealed that, isoniazid-resistant *M. tuberculosis* isolates with katG315 mutation were strongly associated with high-level drug resistance. In contrast, isolates with inhA promoter-15 mutation exhibited low-level resistance along with other or undetected mutations.[20] Study in Bangladesh showed that moderate level resistance was accompanied with katG315 mutation in 94.4% cases and when in combination with inhA mutation, was responsible for 63.3% of high level resistance. inhA mutation alone was responsible for 50% of low level resistance.[9] All isolates in this study that had double mutations, were associated with high level INH resistance and all isolates with inhA mutation only, were associated with low level INH resistance. The significant parameter, which describes this phenotype of drug-resistance, including MDR strains, is the enzymatic activity of catalase-peroxidase system.[10] katG gene deletion or spontaneous mutations in katG gene as in S315 and some other regions may result in loss of enzymatic activity to an extent, where there is no activation of pro-drug isoniazid to its active form. In these conditions isoniazid will not be effective even when administered in higher dose, resulting in high level INH resistance.[22] On the other hand, mutations in the promoter region of inhA, which lead to over expression of isoniazid's target InhA, requiring higher doses of drug to achieve complete inhibition and high dose of INH can overcome this condition. So, mutations in the promoter region of inhA tend to result in low-level phenotypic resistance.[9] A substantial proportion (22.2%) of katG mutant isolates in this study also showed low level resistance. Otto-Knappa et al found similar findings in 36% of MTB strains.[23] Ando et al found some mutations weakly affect the enzymatic activity or binding affinity of INH and result in low level resistance.[22] Even the S315T mutation located at the INH binding pocket could block binding of INH without interfering with catalysis and results in low level INH resistance.[24]

A single isolate, that did not confer mutation in either katG or inhA gene, by real-time PCR, showed low

level resistance by BACTEC MGIT 960 assay, where real-time PCR detected this isolate as INH sensitive. Such finding was also reported in Thailand, where 6.8% of isolates had low levels of isoniazid resistance, and the mutant genes could not be detected.[12] Ramaswamy et al found that 10.5% isoniazid resistant MTB isolates did not show mutation in any commonly identified target genes. This may be due to the presence of less common isoniazid-resistance associated mutations such as in ahpC promoter, ndh or fabG1, which were not detected in this study.[12]

Isolates with katG mutation in this study, exhibited high-level resistance to isoniazid, with high sensitivity, PPV and NPV. Isolates with inhA mutation, exhibited low-level resistance to isoniazid, with less sensitivity, specificity and PPV. Data consistent to this, regarding katG was reported by Charoenpak et al, where katG mutation exhibited high-level resistance to isoniazid, with sensitivity, PPV and NPV of 98.2%, 100% and 93.8% respectively.[12] This was because, most of the isolates, conferring katG mutation, were associated with high level resistance and thus detection of katG mutation may help predict high level INH resistance in considerable proportion of cases. But, in the same study, isolates with inhA mutation exhibited low-level resistance to isoniazid, with sensitivity, specificity and PPV and NPV of 100%, 98.2%, 93.8% and 100%, respectively which was not in accordance with the present study.[12] Lempens et al reported that, this condition may arise due to wide variation of inhA among high and low-TB burden countries.[9] Therefore, low level resistance may not be always indicated by mutation of inhA gene and presence of inhA mutation does not always indicate low level resistance as well.[12]

WHO recommends short-course MDR-TB treatment regimen, containing high-dose INH in MDR-TB patients since 2016, which was included in National Guideline and Operational Manual for Drug-resistant TB of Bangladesh in 2020.[5] Results from this study strongly suggest that, samples should be evaluated for high-dose isoniazid sensitivity before starting standardized short-course treatment regimen for MDR-TB to avoid dose related toxicity. The presence of mutation in katG gene has been found to be a good marker of high-level INH resistance in most cases; therefore, Real-time PCR may help to determine high level INH resistance with short turnaround time.

Conclusions

Most of the isoniazid resistant isolates had high level resistance and was associated with mutation in katG.

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