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ORIGINAL ARTICLE

Multi-drug resistant gene mutation analysis in *Mycobacterium tuberculosis* by molecular techniques

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ABSTRACT

Background and Objectives: Rifampicin (RIF) and isoniazid (INH), two most potent antibiotics, are prescribed to cure tuberculosis. *Mycobacterium tuberculosis*, the causative agent of multidrug-resistant tuberculosis (MDR-TB), is resistant to these first-line drugs. Here, two molecular techniques were demonstrated such as PCR sequencing-based and GeneXpert assay for rapidly identifying MDR-TB.

Materials and Methods: Pulmonary samples (sputum) were collected from 55 MDR-TB suspected patients from the National Tuberculosis Reference Laboratory (NTRL), Dhaka where the research work was partially accomplished and continued in the department of Microbiology, University of Dhaka, Bangladesh. We strived for sequencing technique as well as GeneXpert assay to identify mutations in *rpo*B and *kat*G genes in MTB strains and sputum directly. Culture-based drug susceptibility testing (DST) was performed to measure the efficacy of the molecular methods employed.

Results: When analyzed, *rpo*B gene mutations at codons 531 (54.54%), 526 (14.54%), and 516 (10.91%) were found by sequencing in 80% of the samples. Nucleotide substitution at *kat*G315 (AGC→ACC) was spotted in 16 (76.19%) out of 21 samples. When comparing the sequencing results with DST, sensitivity and specificity were investigated to determine drug-resistance (rifampicin-resistance were 98 and 100% whereas isoniazid-resistance were 94 and 100% respectively). Additionally, as a point of comparison with DST, only 85.45% of RIF mono-resistant TB cases were accurately evaluated by the GeneXpert assay.

Conclusion: This research supports the adoption of PCR sequencing approach as an efficient tool in detecting MDR-TB, counting the higher sensitivity and specificity as well as the short period to produce the results.

Keywords: Drug susceptibility testing; GeneXpert assay; Multidrug-resistant tuberculosis; Multidrug-resistant; Mycobacteria; Pulmonary tuberculosis

INTRODUCTION

Each year, 1.6 to 2.2 million people die due to tuberculosis (TB), especially the drug resistant one which emerged as a prime public health problem globally (1, 2). Multidrug-resistant tuberculosis (MDR-TB) has reached in an epidemic situation in several countries. The emergence of multidrug-resistant *Mycobacterium tuberculosis* isolates has been increasing worldwide. MDR-TB is explicated as resistant to at least two drugs, rifampicin (RIF) and isoniazid (INH), the mainstay of short-course TB chemotherapy (3, 4).

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Gene mutations in restricted regions of the genome causes drug resistance in *M. tuberculosis*. Mutations in codons Ser531Leu, His526Tyr and Asp516Val were observed in the *rpo*B gene in about 96% of the isolates of rifampicin resistant MTB (5, 6). In addition, INH resistance takes place due to a single mutation at codon 315 in the *kat*G gene in 60-80% of cases (7), with the rest occurring in other genes.

This deadly disease is an emerging threat in Bangladesh. The lack of prompt and proper management is the root cause for the overall drug-resistant TB situation in this country. The persistent rise in the multidrug-resistant TB cases often heightens difficulties in TB control (8). Despite the fact that MDR-TB patients present a harsh challenge for medications, treatment is often possible with rapid detection of resistance and an appropriately designed regimen (2).

Albeit microscopic methods (auramine O and Ziehl–Neelsen staining) are simplest, rapid and economical means for TB diagnosis, these techniques require 5,000 to 10,000 bacilli per ml to be identified (9, 10). On the contrary, culture-based conventional methods (considered to be the gold standard for TB diagnosis) have high sensitivity but they take 4 to 8 weeks for isolating slow-growing MTB and even more time for DST (8). To address such diagnostic delay as well as to discretely improve MTB gene mutation detection, molecular aspects need to be regarded. Sequence-based approaches have one advantage: the interpretation of the data are not ambiguous after analysis in view of the fact that the resistance-associated mutation is found in two options-either present or absent (11). Therefore, molecular techniques were evaluated in this study for rapidly detecting multidrug resistant strains. We assessed the efficacy of the PCR sequencing strategy for early screening of MDR-TB strains directly from sputum and culture to guide treatment regimens. We concentrated on evaluating the performance (sensitivity and specificity) of this approach to detect drug resistance in MTB. We also concentrated on identifying highly prevalent mutations associated with RIF and INH resistance.

The GeneXpert method has been broadly reported as a type of nucleic acid assay over the years. It showed higher sensitivity and accuracy than conventional smear and culture techniques and can detect MTB even in the presence of negative sputum smears. It allows early interference in TB patients to avert grave consequences of this illness without delaying the optimal treatment of first diagnosed patients (12). On that account, we evaluated the efficiency of GeneXpert assay that can rapidly identify MDR-TB.

MATERIALS AND METHODS

Selection of patients. We included sputum samples from 55 clinically diagnosed patients with pulmonary TB in the National Tuberculosis Reference Laboratory (NTRL), Dhaka which provides quality lab services for the detection of tuberculosis. Suspected patients were selected based on a patient health questionnaire who didn't respond to anti-TB drugs after a sufficient duration of treatment over three months period in 2018 (March to May). The age of the patients ranges from 21-30 years (40%), 11-20 and 31-40 years (16.4%), 41-50 years (14.5%), 51-60 years (7.3%) and 61-80 years (5.4%). Additionally, male patients (52.7%) were slightly higher than female counterparts (47.3%).

Sample collection and decontamination. For screening *M. tuberculosis* directly in sputum specimens, Auramine O fluorescent staining shows high sensitivity but less specificity than Ziehl Neelsen staining. Lack of specificity is owing to false positivity obtained by mycobacterium other than tuberculosis and weakly acid fast bacteria. In our study, Auramine O fluorochrome acid-fast staining was performed for microscopic examination directly in sputum samples. Consecutive early morning sputum was coughed up by the patients into the plastic cup and shifted to the laboratory as early as possible. Smear were prepared, stained and examined properly using a LED fluorescent microscope (Primo Star iLED) after fluorescent staining. Decontamination of the sputum was done according to N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) method (10).

GeneXpert assay. Two hundred microliter (200-µl) of the sputum sample (decontaminated) was suspended in 500 µl phosphate-buffered saline. 1.5 ml of sample reagent (1.5 ml) was then added with the sample test. According to test instructions, the mixture was shaken by hand and vortexed for 30 seconds. It was left to stand for 15 minutes with recurrent manual shaking. Using a Pasteur pipette, the solution was then transferred to the XpertMTB/RIF cartridge, which was loaded onto the GeneXpert machine. Finally, the results were accepted after 2 hours (13).

Detection of Mycobacteria by rapid Mycobacteria growth indicator tube (MGIT) (BACTEC MGIT960). 800 µl of MGIT growth supplement/ PANTA was added to the MGIT tube. Then 0.5 ml of the decontaminated and concentrated sputum sample was put in the tube and mixed properly. It was placed in the MGIT 960 instrument which automatically tests the sample at 37ºC ± 1ºC for 42 days. The tubes were taken out after appearance of the positive signal and observed carefully (14).

Isolation of Mycobacteria by conventional culture technique. Egg based Lowenstein-Jensen (L-J) medium was prepared in a test tube and an aliquot of 100 µl of the digested sample was spread evenly over the entire surface of the medium. It was incubated at 37°C for up to eight weeks in a vertical position. The result was observed weekly for any growth of mycobacteria. Ziehl-Neelsen staining confirmed the growth of acid-fast bacilli in the L-J medium (15).

Conventional DST. DST was performed by the standard proportion method (16). LJ media with antibiotics which are mixed in various concentrations (0.2 ug/mL isoniazid, 40 ug/mL rifampicin, 4 ug/mL streptomycin and 2 ug/mL ethambutol) and LJ medium without antibiotics (for control) were prepared. The percentage resistance (R) was determined as follows: the ratio of the number of colonies on media containing antibiotics to those on the control medium.

***M. tuberculosis* (MTB) gene amplification**

DNA extraction from MTB strains and directly from sputum samples. Using an InstaGene matrix kit (Bio-Rad, USA), DNA was extracted from strains grown on solid culture. Using RTP® Mycobacteria Kit (STRATEC molecular, Germany), DNA extraction was accomplished directly from processed sputum samples according to the provided instructions (17).

PCR amplification assay. PCR amplification of IS6110 gene which contains a 123-bp fragment was performed for initial detection of MTB (18). The amplification reactions: primer IS1 (5'-CCT GCG AGC GTA GGC GTC GG-3') and IS2 (5' CTC GTC CAG CGC CGC TTC GG-3') 0.5 µL (each) (19), dNTP 2.0 µL, $MgCl_2$ 3.8 µL, template DNA 5 µL, PCR reaction buffer 2.5 µL in a total volume of 25 µL. The following thermocycler parameters were applied with an initial denaturation at 95°C (10 min), followed by 30 cycles of 95°C (30s), 68°C (30s), 2°C (30s); and a final extension at 72ºC (7 min).

Only the primer pair and $MgCl_2$ concentration were different for the *rpo*B gene. In a 25 µL reaction mixture, the final concentration of each primer and $MgCl_2$ were 0.2 pmol/µL and 4 mM respectively. The primers PR1 (5'-CCG CGA TCA AGG AGT TCT TC-3') and PR2 (5'-ACA CGA TCT CGT CGC TAA CC -3') were designed to amplify 315-bp PCR products (20). Amplification conditions were initial denaturation at 95°C (10 min), followed by 30 cycles of 95°C (30s), 63°C (30 s), 72°C (50s); and a final extension at 72ºC (7 min).

The amplification mixture for the *kat*G gene was the same as the *rpo*B gene, except the primers RTB59 (5'-TGCCCGCGGCGGTCGACATT-3') and RTB36 (5'-TCGGGGTCGTTGACCTCCCA-3'), which yielded 808-bp PCR products (21). Amplification conditions were initial denaturation at 94°C (10 min) followed by 35 cycles of denaturation at 94°C (1 min), annealing at 64°C (1 min), and extension at 72°C (1 min); and a final extension at 72°C (10 min). Amplicons were visualized in 2% (IS6110 and *rpo*B gene) and 0.8-1.0% (*kat*G gene) agarose gel in 1x TBE buffer.

Sequencing of *rpo*B and *kat*G genes. Purification of the PCR products were executed using the AccuPrep® PCR Purification Kit (Bioneer, Korea) according to the instructions provided and sequenced with the same primer used in the amplification of the genes (12). Using (MEGA)-6 software (22), we made an analogy between MDR strain's nucleotide and amino acid sequences with reference-sensitive strain-H37Rv (GenBank accession no. NC_000962.3). Using BLAST (www.ncbi.nlm.nih.gov/blast) algorithm, sequences were analysed with those from the reference resistant strain *M. tuberculosis* H37RV.

RESULTS

55 sputum samples from the patients were registered and analysed for microscopic examination, bacteriological culture, drug susceptibility testing (DST) and the GeneXpert assay (Table 1).

Mycobacteria detection through microscopic and bacteriological culture methods. 46 (83.64%)

Table 1. Microscopic identification, the GeneXpert, rapid culture, conventional bacteriological culture (solid media) and drug susceptibility test results of sputum samples along with patient ID, age and sex.

Sample ID	Age	Sex	Microscopic identification	Gene Xpert	Rapid culture	Solid culture	Drug susceptibility test			
							RIF	INH	SM	EMB
1	40	F	Negative	RR	Negative	2+	R	R	S	S
2	75	M	2+	RR	Positive	1+	R	R	R	R
3	30	M	2+	RR	Positive	3+	R	R	S	S
4	21	M	3+	RR	Positive	3+	R	R	R	R
5	22	M	3+	RR	Positive	3+	R	R	R	R
6	25	M	2+	RR	Positive	3+	R	R	R	R
7	25	M	1+	RR	Positive	3+	R	R	R	R
8	49	M	2+	RS	Positive	3+	R	R	R	S
9	19	F	2+	RR	Positive	3+	R	R	R	R
10	42	F	2+	RR	Positive	3+	R	R	R	S
11	60	F	Negative	RR	Positive	1+	R	R	R	R
12	21	F	2+	RR	Positive	3+	R	R	R	R
13	18	M	3+	RR	Positive	2+	R	R	R	S
14	23	M	Negative	RS	Positive	3+	R	R	R	R
15	25	F	Negative	RR	Positive	2+	R	R	R	S
16	35	M	Negative	RR	Positive	2+	R	R	R	R
17	40	M	1+	RR	Positive	3+	R	R	S	S
18	24	F	2+	RS	Positive	3+	R	R	R	R
19	13	M	Negative	RR	Negative	1+	R	R	S	S
20	26	M	3+	RS	Positive	3+	S	S	S	S
21	25	M	1+	RR	Positive	3+	R	R	S	R
22	28	F	3+	RR	Positive	1+	R	R	R	R
23	40	F	3+	RR	Positive	3+	R	R	R	S
24	27	F	2+	RR	Positive	3+	R	R	R	R
25	18	F	Negative	RR	Negative	2+	R	R	R	R
26	50	M	Negative	RR	Negative	2+	R	R	R	R
27	47	M	1+	RR	Positive	2+	R	R	S	S
28	25	M	1+	RS	Positive	2+	R	R	S	S
29	43	F	2+	RR	Positive	2+	R	R	R	R
30	37	M	1+	RR	Positive	1+	R	R	R	R
31	21	F	2+	RR	Positive	2+	R	R	R	S
32	34	M	1+	RS	Positive	2+	R	R	S	S
33	75	M	2+	RR	Positive	1+	R	R	R	R
34	21	M	3+	RR	Positive	3+	R	R	R	R
35	25	M	2+	RR	Positive	2+	R	R	R	R
36	42	F	2+	RR	Positive	3+	R	R	R	S
37	16	F	Negative	RR	Negative	1+	R	S	S	S
38	11	F	Scanty	RR	Negative	3+	S	S	S	S
39	45	F	3+	RS	Positive	3+	R	R	S	S
40	30	F	3+	RR	Positive	3+	R	S	S	S
41	40	M	2+	RR	Positive	2+	R	S	S	S
42	30	F	2+	RR	Positive	2+	R	R	R	R
43	18	F	2+	RR	Positive	2+	R	R	R	R
44	22	M	2+	RR	Positive	2+	R	R	R	R
45	22	F	2+	RR	Positive	2+	R	R	S	S

Table 1. Continuing...

46	55	F	2+	RR	Positive	2+	R	S	S	S
47	60	F	3+	RR	Positive	3+	R	R	S	S
48	26	M	3+	RS	Positive	3+	R	S	S	S
49	45	M	2+	RR	Positive	2+	R	R	S	R
50	38	M	3+	RR	Positive	3+	R	R	R	R
51	35	F	3+	RS	Positive	3+	S	S	S	S
52	20	F	2+	RR	Positive	3+	R	R	S	S
53	13	F	scanty	RR	Negative	3+	S	S	S	S
54	65	M	3+	RR	Positive	3+	S	S	S	S
55	55	M	3+	RS	Positive	3+	R	R	S	S

M = Male, F = Female, R = Resistant, S = Sensitive, RR = Rifampicin-resistant, RS = Rifampicin-sensitive

The table showed that all samples were positive for *M. tuberculosis* complex by solid culture growth whereas 87.27% were positive by rapid culture method. DST results show that 91% of samples were resistant to rifampicin and isoniazid. The positive rate, sensitivity, specificity, positive (PPV), and negative (NPV) predictive values were 81.8%, 90%, 100%, 100%, and 50% of the GeneXpert assay as compared to the results of phenotypic DST which is regarded as the gold standard for MTB diagnosis.

samples were AFB positive by observing rod-shaped, bright yellow, fluorescent-colored microorganisms after Auramine O staining. All samples were found positive for *M. tuberculosis* complex by conventional culture. Preliminary identification was made by observing buff colored, rough and waxy colonies on L-J slant. Additionally, growth confirmation was done by Ziehl-Neelsen staining from mycobacterial colonies. 48 (87.27%) samples were tested positive by the MGIT 960 in six weeks when the mycobacteria settled at the bottom of the tube and usually appear granular and not very turbid.

Drug susceptibility testing (DST). A majority of the samples turned out to be resistant to at least one drug: rifampicin-monoresistant- 91% (50/55); rifampicin and isoniazid- 91%, rifampicin, isoniazid and one more drug (streptomycin or ethambutol)- 60%. 43.6% of the patients were found to be resistant and 9.1% were sensitive to all four drugs. The analysis depicts identification of rifampicin resistance alone can be employed for detecting MDR-TB.

Rifampicin resistance detection by the GeneXpert assay. Considering DST as the reference standard where rifampicin and isoniazid resistance was 91% (91% patients were also rifampicin-monoresistant), the positive rate, sensitivity, specificity, positive (PPV) and negative (NPV) predictive values were 81.8% (45/55), 90%, 100%, 100% and 50% of the GeneXpert assay. Eight samples (14.5%) showed sensitivity to rifampicin detected by the GeneXpert assay, but not on DST.

Results of PCR amplification of MTB genes. All samples showed 123 bp PCR product in agarose gel using IS6110 primers (Fig. 1). For amplification of *rpo*B gene, 55 samples were subjected to PCR and all of them yielded 315 bp products (Fig. 2). In case of 36 samples (ID: 1-36), DNA extraction was completed from *M. tuberculosis* strains grown on solid culture media with the antibiotic rifampicin; for 19 samples (ID: 37-55), DNA was directly extracted from sputum samples. For *kat*G gene, 21 samples out of 55 were

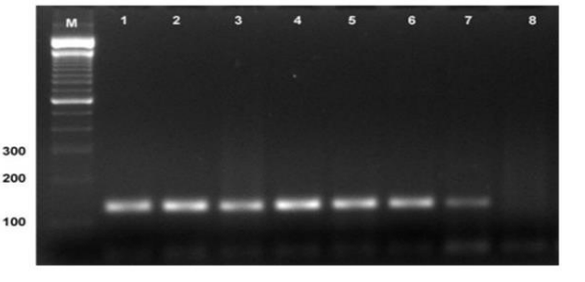

Fig. 1. 123 bp band was revealed in PCR products corresponding to IS6110 sequence in MTB

Agarose gel electrophoresis detected 123 bp band corresponding to IS6110 sequence amplified in PCR in *M. tuberculosis*. Lane M: 100 bp DNA ladder; Lane 1: Positive control (MTB positive); Lane 2-7: Sample ID- 5, 9, 22, 27, 36, 37; Lane 8: Negative control.

subjected to PCR and yielded 807bp PCR products (Fig. 3). For the rest of the 12 samples (ID: 1-3, 5-7, 9-13, 26), DNA extraction was accomplished from MTB strains grown on solid culture with the antibiotic isoniazid, and directly from sputum samples for 9 samples, ID-47-55.

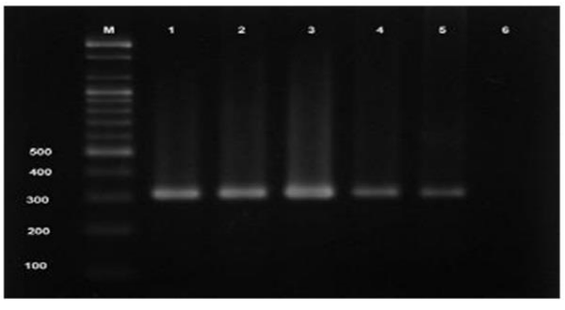

Fig. 2. 315 bp band in *rpo*B gene
PCR Analysis showed 315 bp band in *rpo*B gene in *M. tuberculosis*. Lane M: 100 bp DNA ladder, Lane 1: Positive control (MTB positive); Lane 2-5: Sample ID: 37-40; Lane 6: Negative control.

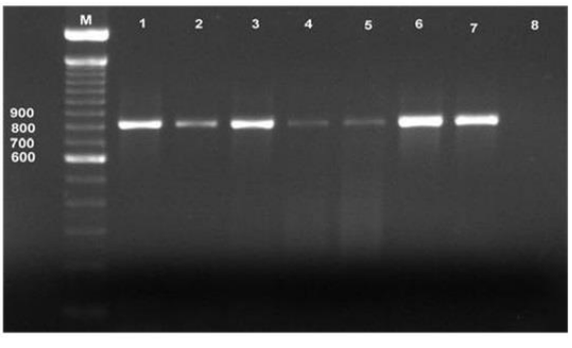

Fig. 3. 807 bp band in *kat*G gene in amplified PCR products in MTB
807 bp band was spotted in *kat*G gene after gel electrophoresis. Lane M: 100 bp DNA ladder, Lane 1: Positive control (MTB positive); Lane2-7: Sample ID: 50-55; Lane 8: Negative control

DNA sequence analysis. Sequencing revealed mutations in 83.6% samples in the *rpo*B gene (Table 2). The most frequent mutation was substitution of TCG→TTG at codon 531 (Ser531 Leu), accounting for 54.5%. Changes of His→Asp (CAC→GAC) and His →Tyr (CAC→TAC) occurred at codon 526 (14.5%). Mutations at *rpo*B516 occur less frequently (11%), mostly changing aspartate to valine (GAC GTC) or tyrosine (GAC→TAC). Other resistance associated mutation occurred at *rpo*B511 (3.6%), changing Leu→Pro (CTG→CCG). Moreover, 2 samples had both mutated and wild type sequences (ID- 24 and 28) whereas DST results showed that both were multidrug-resistant. Out of 21, sixteen samples (76.2%) showed mutations at the *kat*G315 (Table 2), changing serine to threonine (AGC→ACC).

Comparative analysis of phenotypic DST and DNA sequencing. 97.2% (35/36) samples were RIF resistant on DST media, while 91.7% (33/36) samples had mutations showing RIF-resistance by sequencing. In case of 19 samples which were analysed by sequencing assay directly from sputum samples, 15 (79%) of them were rifampicin resistant by DST, whereas 13 (68.4%) of them contained RIF associated mutations by sequencing. Positive rate (83.6%), sensitivity (92%), specificity (100%), positive and negative predictive values (100% and 55.5%) of sequencing for Rif- resistance were detected compared to DST (rifampicin resistance was 91%) which is considered as "gold standard" (Table 3).

In case of *kat*G gene, 12 isolates had INH resistance on DST and gene mutation showing INH resistance was also revealed in 12 isolates by sequencing. For 9 samples, PCR amplification was done directly from sputum samples. Among them, INH resistance was observed in 5 samples on DST, whereas 4 carried gene mutation on sequence analysis. Compared to the DST result (isoniazid-resistance, 81%), the positive rate, sensitivity, specificity, positive and negative predictive values of sequencing for isoniazid-resistance were 76.2%, 94%, 100%, 100% and 80% respectively (Table 4).

DISCUSSION

Expeditiously identifying the antimicrobial susceptibility pattern of MTB is requisite for the treatment of MDR-TB which causes high death rates of 50% to 80%. This research work was designed to adopt molecular techniques to identify drug-resistant MTB rapidly from TB-suspected patients in Bangladesh.

PCR analysis requires less than 4 hours to detect MTB from clinical specimens which is the major advantage of this method. It is reported that the *rpo*B gene mutations account for more than 95% of rifampicin resistance. Similarly, the *kat*G gene mutations in MDR-TB strains at codon 315 presents high-level resistance to isoniazid in up to 75% (23, 24). Rifamp-

Table 2. Determination of *rpo*B and *kat*G gene mutation pattern by sequencing assay

DNA sequence results			No. (%) of
Position of mutation	**Mutation in nucleotide**	**Mutation in amino acid**	**Samples with mutation**
*511	CTG→CCG	Leu→Pro	2 (3.6)
516	GAC→GTC/TAC	Asp→Val/Tyr	6 (11)
526	CAC→GAC	His→Asp	8 (14.5)
531	TCG→TTG	Ser→Leu	30 (54.5)
315	AGC→ACC	Ser→Thr	16 (76.2)

* two of the samples contained mixed sequences

This table revealed mutations by DNA sequencing in 83.6% samples at codon 531 (54.5%), 526 (14.5%) and 516 (11%) in the RRDR of the *rpo*B gene. 76.2% samples showed mutations at *kat*G315, changing serine to threonine.

Table 3. Analysis of rifampicin-resistance by phenotypic and genotypic techniques

	Sample size	**Samples (%) showing RIF- resistance on phenotypic DST**	**Samples (%) carrying RIF-associated gene mutation on DNA sequencing**
Isolates grown on culture	36	35 (97.2)	33 (91.7)
Sputum samples	19	15 (79)	13 (68.4)
Total	55	50 (91)	46 (83.6)

Sensitivity, specificity, positive and negative predictive values of sequencing for Rif- resistance detection were 92%, 100%, 100% and 55.5% respectively

Table 4. Isoniazid-resistance detection by phenotypic DST and DNA sequencing assay

	Sample size	**Samples (%) showing INH- resistance on DST**	**Samples (%) carrying gene mutation to INH on sequencing**
Isolates grown on culture	12	12 (100)	12 (100)
Sputum samples	9	5 (55.5)	4 (44.4)
Total	21	17 (81)	16 (76.2)

Sensitivity, specificity, positive and negative predictive values of sequencing for the detection of isoniazid resistance were 94%, 100%, 100% and 80% respectively

These tables (3, 4) showed comparative analysis between phenotypic DST and DNA sequencing. Positive rate (83.6%), sensitivity (92%), specificity (100%), positive and negative predictive values (100% and 55.5%) of sequencing for Rif- resistance were detected compared to DST (rifampicin resistance-91%) whereas isoniazid resistance were 76.2%, 94%, 100%, 100% and 80% respectively (isoniazid resistance-81% on DST).

in-resistance serves as a surrogate marker for MDR-TB detection as 91% isolates of rifampin-resistant are also isoniazid-resistant. Previously, frequent mutations in the *rpo*B gene were found in the short 81-bp region (codons 507 to 533) and mutations take place in codons 531, 526 and 511 in 95% of rifampin-resistant strains (25-27).

When investigated, prevalence of gene mutations associated with RIF (81%)- and INH (76.2%)- resistance were observed in this research based on DNA sequencing and the performance was highly correlated with phenotypic DST.

Mutations in codons 511, 516, 526, and 531 of the *rpo*B gene were detected in sequence analysis. We spotted most prevailing mutations at codon 531 (54.54%) which is in consonance with reports from

other countries: India, 54.5% (28), Brazil, 54% (29), Turkey, 56.1% (30) and South India, 54.4% (31). However, dissimilarities were also manifested with some countries, including Hungary, 31% (32), Mexico, 47% (33) with a lower frequency; and India, 63.3% (34), Beijing and non-Beijing isolates (61.9% and 66.9%, respectively) (35), the Republic of Moldova, 86.8% (36) with a higher frequency.

We found mutations in *rpo*B526 at a rate of 14.54% which is in agreement with findings from Germany, 13.6% (37). Other studies also mentioned low frequencies in countries like: the Republic of Moldova, 0.9% (36), Hungary, 6.9% (32) and India, 8.56% (38); and high frequency in country like: China, 40%% (39) and Thailand, 32.3% (40).

The least mutation was found in *rpo*B516 (10.91%) which was comparable with the mutation rate in South Africa, 9.6% (41), but higher than in Republic of Moldova, 4.4% (36), India, 6.42% (38), much smaller than in Thailand, 35.5% (40) and Hungary, 37.9% (32).

Resistance was also marked at codon 511 (42) from previous reports. Mutations were discovered in codon L511 (3.64%) in 2 samples in our findings. On top of that, DNA sequencing results of these 2 samples contained both mutated and wild-type sequences. DST results showed that both were multidrug-resistant. There is a probability that these samples contained both wild-type and mutated alleles in the *rpo*B gene, which leads to the corresponding wild-type PCR product while amplified. In comparative analysis with drug susceptibility testing, eight samples had no mutation in the targeted locations investigated within the *rpo*B gene which could be explicated by the presence of rare mutations in MTB strains.

In the enzyme catalase-peroxidase coding the *kat*G gene, most mutations take place at codon 315 (43). We were in accordance with other reports showing vast involvement of this codon in isoniazid-resistance globally where we showed 76.2% resistance mutations to isoniazid in codon 315. Some researchers also pinpointed efficient mutations in isoniazid-resistance in 315 codon region, such as 95.8% in Ethiopia (44), 91.7% in Petersburg, 62.2% in New York, 59.2% in South Korea, 34.6% in Madrid, 32% in Barcelona and 0% in AkvatvryalGvynh (45, 46).

Owing to the close association of MDR-TB and rifampicin resistance, the GeneXpert assay can mark mutations in the *rpo*B gene only. In this research, eight samples were inconsistent with DST results where they had resistance to RIF on DST but were sensitive to GeneXpert assay. Discrepancies between the results concerning rifampin-resistance (false resistance or sensitivity) have also been published earlier (33, 34). As DST takes long incubation period for culturing MTB, there might be an apparent switch from a susceptible to a resistant phenotype (47). In 2011, Teo et al. analysed two amplification methods in respiratory and non-respiratory specimens where they found 90.9% sensitivity of GeneXpert assay and 5.5% invalid reads were generated (48). This result highly correlates with our findings where we found 90% sensitivity and 14.5% equivocal result in respiratory samples by this technique. Hillemann et al. also investigated the performance of GeneXpert system in the same year in non-respiratory samples and reported 77.3% sensitivity and 98.2% specificity; 3.8% specimens showed no interpretable result (49). Zetola et al. concentrated on the feasibility of this method in mixed MTBC infections detection. The interpretations revealed an increased false-negative rate for identifying RIF-resistance (50). On that account, further confirmation of the results generated by GeneXpert are needed in community where these mixed infections are frequent.

CONCLUSION

In conclusion, if we are looking for a prompt and sensitive alternative to conventional diagnostics, molecular tools may pick up the baton. We mainly paid attention to PCR-sequencing assay which successfully isolated and identified drug-resistant MTB and their gene mutations rapidly with high efficacy from the growth culture and from the patient's sputum directly. Though GeneXpert assay elicits rapid detection of rifampicin-resistance in clinical specimens, it is not a suitable method to follow up the diagnosis and treatment of TB in respiratory and non-respiratory specimens. Consequently, it can be used as a reliable diagnostic tool in detection of MTB along with other conventional methods.

However, the potential for false positives or negatives can impede the vast application of molecular techniques. Moreover, Our research work has some limitations as the sample size was insignificant. This is because we only selected clinically diagnosed and treated patients for three months who were resistant to anti-TB medications. Since the rate of MDR-TB in

Bangladesh varies from 2.5-13% depending on the age, this number represents a much larger number of TB-infected cases.

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