ORIGINAL RESEARCH

Insights into the in-vitro Susceptibility and Drug–Drug Interaction Profiles Against Drug-Resistant and Susceptible *Mycobacterium tuberculosis* Clinical Isolates in Amhara, Ethiopia

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Background: In Ethiopia, tuberculosis (TB) is a major public health problem. The aim of the study was to determine the in vitro susceptibility level of drugs and drug interaction profiles against drug-resistant and susceptible *M. tuberculosis* clinical isolates. A laboratory-based cross-sectional study was conducted between January 2023 and August 2023. GenoType MTBDRplus v.2.0 was facilitated in genetic mutation detection. Minimum inhibitory concentration (MIC) was determined using resazurin microtitre assay (REMA), while fractional inhibitory concentration index (FICI) using resazurin drug combination microtitre assay (REDCA) for in vitro quantitative susceptibility and drug interaction prediction.

Results: Among 32 clinical isolates, a total of 14 (43.8%) RIF, 20 (62.5%) INH, 2 (6.3%) EMB-related resistant and 14 (43.8%) MDR isolates were identified. Five of RIF-resistant isolates (55.6%) carrying *rpoB* common mutations at codon S450L were associated with high levels of RIF-resistance with MICs of ≥ 2μg/mL, whereas 100% of isolates harboring *rpoB* substitutions at codons D435V and H445Y were linked with moderate or low-level RIF-resistance in the MIC ranges from 0.5 to 1μg/mL. A proportion of 81.8% of isolates harboring *katG* S315T mutations were associated with high-level INH resistance (MIC ≥ 1μg/mL), while the 18.2% of isolates with S315T *katG* mutations and 100% of isolates with *inhA* C-15T mutations were linked to the low-level of INH resistance with MIC variability from 0.25 to 0.5μg/mL. Our results indicated that most FICIs of the dual drugs INH+RIF and INH+LEV combination for 9 (28.1%) and 4 (12.5%) INH-resistant isolates, respectively, were ≤0.5, whereas triple drugs INH+RIF+EMB, INH+RIF+LEV and INH+EMB+LEV combination for 6 (18.8%), 11 (34.4%) and 8 (25%) INH-resistant isolates were from 0.62 to 0.75, all showed synergistic effect.

Conclusion: The study highlights that isolates with *rpoB* S450L and *katG* S315T substitutions were associated with high level of RIF and INH resistance. It is concluded that REDCA can quantitatively determine anti-mycobacterial synergy and that LEV being of potential use against INH-resistant isolates including MDR-TB when combined with RIF+INH and INH+EMB.

Keywords: drug combination, drug interaction, MIC, mutations, synergism

Introduction

Tuberculosis (TB) remains a major global health concern, and the emergence of multidrug- and extensively drug-resistant (MDR/XDR-TB) has further complicated its diagnosis, treatment and control.[1] The WHO advocates a standardized chemotherapy for drug-susceptible TB with generic, fixed-dose combinations (FDCs) and a daily dosing frequency. This is recommended for reasons of adherence, cost-effectiveness, and logistical simplicity.[2] The treatment plan involves the long-term combination of all four first-line drugs (FLDs) during a 2-month intensive phase, followed by a 4-month continuation phase with just rifampicin (RIF) and Isoniazid (INH) in order to prevent relapse and the development of

drug resistance.[2] However, this protocol is no longer appropriate for managing the high rates of drug resistance in TB cases. Some of the significant risk factors associated with drug resistance include mutations in genes that confer resistance,[3] the genetic background of the strain, TB/HIV co-infection, and environmental influences such as poor diagnostic capacity, improper prescription of drugs, insufficient patients' follow-up, lack of patient compliance, and high incidence of relapsed TB history.[4–6]

An early and rapid diagnosis of TB is important in determining the appropriate TB treatment and preventing the spread of drug resistant (DR) TB. The conventional phenotypic Mycobacterium Growth Indicator Tube (MGIT) 960 macro-dilution method, using a single critical concentration (CC) per drug, has been used to determine susceptibility profiles of *M. tuberculosis* isolates individually. However, it is time-consuming, expensive, and technically complex.[7] Although currently available molecular assays can overcome some disadvantages of the MIGT 960 CC method, they are expensive and cannot identify all genetic loci associated with drug resistance.[8,9] Treating TB not only benefits the individual patient but also the community as a whole. However, the level of drug resistance can impact treatment choices and outcomes. The treatment of DR-TB patients requires an individualized regimen that depends on the level of inhibitory for drug resistance, TB/HIV co-infection status, drug–drug interactions,[10] and the drug's toxicity to the patient that may lead to treatment interruptions and influence the treatment success.[11,12] This in turn could promote the development of drug resistance.[13]

However, in some situations, especially when the treatment fails, or in immunocompromised individual, the drug's minimum inhibitory concentration (MIC) determination could help to establish a new therapeutic scheme to treat patients with resistant TB. The susceptibility of drugs in vitro, as determined by MIC and the synergism of drug interactions, can be studied using various methods.[14] Among these, the colorimetric resazurin microtiter assay (REMA) and resazurin drug combination microtiter assay (REDCA) have gained greater attention due to its simplicity, lack of need for sophisticated equipment or highly trained personnel and relatively short turnaround time in detecting *M. tuberculosis* within 8–10 days in a 96-well microplate.[15–18] The REMA/REDCA method is based on the oxidation-reduction colorimetric indicator resazurin (also known as alamar blue) after exposing *M. tuberculosis* to various drugs in vitro. A change in the indicator's color is directly proportional to the number of viable mycobacteria in the medium.[15] Unlike MGIT based on single critical concentration, MIC testing a wider range of drug concentrations is a valuable tool for determining the significance of low-level resistance to RIF, which causes diagnostic and treatment challenges, is significant in our population, and may be underreported. The interactions of drugs in combination have not been routinely considered in therapeutic regimens, possibly due to the absence of in vitro markers indicating this combined effect. However, with the increasing prevalence of drug-resistant TB, it is essential to design new synergy-based therapeutic regimens to restore the antibiotic sensitivity of drug-resistant strains. This can be achieved by assessing the inhibitory activity when two or more drugs are combined.[18,19]

Investigating the susceptibility of *M. tuberculosis* clinical isolates in vitro, as well as their drug interaction profiles, is crucial for providing insights into the efficacy of drugs alone and in combination. It is also important for developing alternative treatment strategies. This information can help clinicians make informed decisions about treatment options and combat the growing challenge of drug resistance.[7,20,21] To our knowledge, the association of resistance-conferring gene mutations with the MIC levels of RIF and INH resistance is not investigated, and there is currently a lack of comprehensive data on drug interaction profiles in drug combinations. Therefore, the aim of this study was to determine the in vitro susceptibility and drug–drug interaction profiles of clinical isolates of *M. tuberculosis*.

Materials and Methods
Study Settings, Design and Period

A laboratory-based cross-sectional study was conducted between January 2023 and August 2023 in the Northwest Amhara region of Ethiopia. For the present study, among 168 phenotypic CCs MGIT 960 resulted isolates, a total of 32 stored clinical isolates of *M. tuberculosis* (20 resistant and 12 susceptible isolates) were used due to resource constraints.[22] A reference H37Rv drug-susceptible *M. tuberculosis* strain and molecular-grade water were used as positive and negative growth controls, respectively. The experiment was conducted at the University of Gondar

Comprehensive Specialized Hospital (UoGCSH) TB culture laboratory. The UoGCSH is a fully equipped and certified laboratory for the manipulation of TB cultures and the performance of phenotypic and genotypic susceptibility testing. It serves MDR-TB patients from neighboring woredas and zones in the Amhara regional state.

Inclusion and Exclusion Criteria

The clinical isolates of *M. tuberculosis* that had genotypic and phenotypic MGIT susceptibility results, along with the correct patient identification number and socio-demographic data, were included in this study. However, isolates that had no susceptibility results and non-tuberculosis mycobacteria (NTM) isolates were excluded from the study.

Study Variables

The socio-demographic and clinical determinant factors of the patients were considered independent variables, while the MIC and fractional inhibitory concentration index (FICI) drug interaction responses of RIF, INH, and EMB drugs in alone, double and triple drug combinations were considered dependent variables.

Phenotypic and Genotypic Susceptibility Testing of Clinical Isolates

Initially, sputum samples were collected from pulmonary TB patients who tested positive on the GeneXpert in the Amhara region of Ethiopia. Digestion and decontamination of specimens was performed using the N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) method and then inoculated onto Lowenstein-Jensen (LJ) egg medium slants. The slants were incubated at 37°C for up to 8 weeks and monitored weekly for growth.[23] Confirmation of the *M. tuberculosis* complex isolates was done through antigenic testing (SD Bioline TB Ag MPT64, Standard Diagnostics, Inc., South Korea).[24] The culture-positive *M. tuberculosis* clinical isolates were freshly sub-cultured on liquid medium and subjected to drug susceptibility testing using the rapid automated MGIT 960 SIRE method using CCs of 0.1 INH, 1.0 RIF, and 5.0 EMB in µg/mL.

The GenoType® MTBDRplus line probe assay (LPA) version 2.0 was used for detection of resistance-conferring mutations in the *rpoB, katG,* and *inhA* genes that confer resistance to RIF and INH according to the manufacturer's instructions (Hain Life Science, Nehren, Germany).[8,9] DNA was extracted from the culture isolates, and the amplified products were hybridized using specific oligonucleotide probes that were immobilized on the strip. The strip contained control zones (CC, AC, *rpoB, katG,* and *inhA*) and additional probes to detect specific mutations for *rpoB, katG,* and *inhA* genes. The *M. tuberculosis* clinical isolates were then sub-cultured and stored at −80 °C with the 7H9 broth base until further analysis was needed.

Mycobacterium tuberculosis Inoculum Preparation

To prepare the suspension of the tested *M. tuberculosis* clinical isolates for REDCA, a loopful of colonies was scraped off from a well-grown fresh LJ subculture that was 21–28 days old. The *M. tuberculosis* colonies were then transferred to a tube that contained 3–5 glass beads and 3 mL of M7H9 broth that was supplemented with OADC enrichment (Oleic Acid, Albumin, Dextrose, and Catalase) from (Becton Dickinson/BD Difco BBL in New Jersey, USA). The culture pellet was vortexed for at least 1 minute and then left for 5 minutes. The supernatant was transferred to a new sterile vial and allowed to settle for 15 minutes. The suspension was adjusted to the same turbidity as the 1.0 McFarland standards. The suspension was then further diluted with a 1:10 ratio in 7H9 broth that was supplemented with OADC at the time of inoculation into the 96-well microplates.[17,25,26]

Drug Stocks and Growth Indicator Reagent Preparations

The first-line INH, RIF, and EMB and second-line drug Levofloxacin (LEV) anti-TB drugs (Sigma-Aldrich Chemicals GmbH, Steinheim, Germany) were obtained in pure powder form and stored at −20°C as per the manufacturer's instructions. Stock solutions of the INH, RIF, and EMB drugs were made at concentrations of 1 mg/mL in sterile distilled water, 10 mg/mL in methanol, and 10 mg/mL in sterile distilled water, respectively.[16] Small amounts of each stock solution were kept at −20°C until needed. At the time of the experiment, a working solution for each drug concentration was prepared by diluting stock solutions in M7H9 broth that was supplemented with OADC (BD Difco

BBL, NJ, USA)[15] at four times (4x) the final highest concentration (4μg/mL for INH, 8μg/mL for RIF, and 32 μg/mL for LEV, and 64μg/mL for EMB). The final drug concentrations tested were 1μg/mL for INH, 2μg/mL for RIF,[27,28] 8 μg/mL for LEV and 16μg/mL for EMB in 96-well plates.[29,30] LEV was found to be more efficacious than other ofloxacin (OFX) when incorporated into multidrug regimens for treatment of MDR-TB.[15] The resazurin reagent was obtained in the form of resazurin sodium salt powder. A fresh working solution of resazurin sodium salt was prepared in sterile distilled water at a concentration of 0.02% (wt/vol) (0.02g/100mL in sterile distilled water) (Acros, Morris Plains, NJ, USA). The solution was filter-sterilized and stored at 4°C for up to 7 days.[25,31–33]

Resazurin-Based Colorimetric Microtiter Assay

In vitro susceptibility testing and synergistic effects of drug combinations were determined using the REMA[25,31–33] and REDCA[15,16] assays, respectively. The REMA and REDCA experimental setups used micro-dilution in 96-well microplates (BD Falcon, USA) to test the susceptibility of three first-line drugs RIF, INH, and EMB individually, as well as double drug combination (RIF+INH, INH+EMB, RIF+LEV, INH+LEV, and EMB+LEV) and triple drugs (RIF+INH+EMB, RIF+INH+LEV, and EMB+INH+LEV) interactions against clinical isolates of *M. tuberculosis* (Table 1).

Briefly, 100μL of M7H9 broth supplemented with OADC was aseptically dispensed into all-96-well microplates. One hundred microliters of the working RIF concentration (4x) was dispensed into wells of A1-A5 and A8-A11 containing 100μL 7H9-S broth, but A6 and A12 had an 8x final drug concentration. Serial two-fold micro-dilution of RIF was prepared from A1-A6 to G1-G6 and A8-A12 to G8-G12 wells using a multichannel pipette with a volume of 100μL concentration, and finally 100μL portions were discarded from the last row (G1-G6 and G8-G12). Moreover, 100μL working INH drug concentrations (4x) were delivered to A6-H6 and A12-H12 wells. Serial two-fold dilutions of INH were prepared from A6-H6 to A2-H2 and A12-H12 to A8-H8 wells, and the final volume of 100μL solutions from A2-G2 and A8-G8 wells were discarded. Also, the working solutions of EMB or LEV were dispensed into the remaining well A7, and its double serial dilution was performed from A7 to G7, and a 100μL solution was discarded to obtain the final-tested concentration. One-hundred microliters of inoculum were used as per well of microplates using a multichannel pipette, except for the H1, H7, and H8-H12 wells. H1 wells with only medium and sterile distilled water were used as a negative control as per each batch of tested *M. tuberculosis* clinical isolates. H7 was unused well. H8-H12 wells containing 7H9 medium without drugs and 10^{-2} bacterial inoculum dilution were used as a positive bacterial growth control in each plate.[15,16,25,31–33]

In the study, in a single 96-well microplate, wells A1-A6 and A6-H6 have the final highest concentrations of RIF and INH, respectively, in order to determine the drug susceptibility pattern and evaluate pairwise-drug interactions, while wells A8-A12, A12-H12, and A7 also have the highest concentrations of RIF, INH, and EMB, as well as LEV, respectively, for assessing the in vitro triple-drug interaction potential against each *M. tuberculosis* clinical isolate by microdilution-based REDCA assay. The triple drug combination (eg, RIF+INH+EMB) REDCA method was principally based on the standard two-drug (eg, RIF+INH and INH+EMB) combination, where dispensing the serially diluted drugs along the X-axis and Y-axis in a 96-well plate, respectively, while the third drug (LEV or EMB) was then dispensed on the A8-H8 to A12-H12 wells with one of the serial concentrations from the MIC range (eg, MIC of 0.5 for EMB and 0.25 for LEV).[18] Finally, 200μL of sterile distilled water was added to all outer perimeter wells to avoid evaporation during the incubation. The microplates were sealed with aluminum plate seals, placed on a plate shaker for 30 minutes, and incubated at 37 °C in a normal atmosphere for 7 days.[15,16,25,31–33]

After one week of incubation time, 30μL of freshly prepared 0.02% resazurin (Acros, Morris Plains, NJ, USA) working solutions were added to each REMA microplate well, and the microplates were incubated for additional 24–48 hours at 37 °C in a normal atmosphere. A color change from blue (the oxidized state) to pink/purple (the reduced state) indicated the reduction of the resazurin agent by bacterial metabolism. This reduction of resazurin is directly proportional to the presence of viable bacterial growth called resistance strains. A change in color of the growth control well from blue to pink indicated the proper growth of the isolate, and no color change in the sterile control well indicated the absence of contaminants.[25,31–33]

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Table 1 Preparation of Drug MICs (µg/mL) in 96-Wells Micro-Dilution Plate Assay

	1	2	3	4	5	6	7	8	9	10	11	12
A	RIF/2	RIF/2 INH/0.06	RIF/2 INH/0.125	RIF/2 INH/0.25	RIF/2 INH/0.5	RIF/2 INH/1	EMB/16	RIF/2 INH/0.06 EMB/0.5	RIF/2 INH/0.125 EMB/0.5	RIF/2 INH/0.25 EMB/0.5	RIF/2 INH/0.5 EMB/0.5	RIF/2 INH/1 EMB/0.5
B	RIF/1	RIF/1 INH/0.06	RIF/1 INH/0.125	RIF/1 INH/0.25	RIF/1 INH/0.5	RIF/1 INH/1	EMB/8	RIF/1 INH/0.06 EMB/0.5	RIF/1 INH/0.125 EMB/0.5	RIF/1 INH/0.25 EMB/0.5	RIF/1 INH/0.5 EMB/0.5	RIF/1 INH/1 EMB/0.5
C	RIF/0.5	RIF/0.5 INH/0.06	RIF/0.5 INH/0.125	RIF/0.5 INH/0.25	RIF/0.5 INH/0.5	RIF/0.5 INH/1	EMB/4	RIF/0.5 INH/0.06 EMB/0.5	RIF/0.5 INH/0.125 EMB/0.5	RIF/0.5 INH/0.25 EMB/0.5	RIF/0.5 INH/0.5 EMB/0.5	RIF/0.5 INH/1 EMB/0.5
D	RIF/0.25	RIF/0.25 INH/0.06	RIF/0.25 INH/0.125	RIF/0.25 INH/0.25	RIF/0.25 INH/0.5	RIF/0.25 INH/1	EMB/2	RIF/0.25 INH/0.06 EMB/0,5	RIF/0.25 INH/0.125 EMB/0.5	RIF/0.25 INH/0.25 EMB/0.5	RIF/0.25 INH/0.5 EMB/0.5	RIF/0.25 INH/1 EMB/0.5
E	RIF/0.125	RIF/0.125 INH/0.06	RIF/0.125 INH/0.125	RIF/0.125 INH/0.25	RIF/0.125 INH/0.5	RIF/0.125 INH/1	EMB/1	RIF/0.125 INH/0.06 EMB/0.5	RIF/0.125 INH/0.125 EMB/0.5	RIF/0.125 INH/0.25 EMB/0.5	RIF/0.125 INH/0.5 EMB/0.5	RIF/0.125 INH/1 EMB/0.5
F	RIF/0.06	RIF/0.06 INH/0.06	RIF/0.06 INH/0.125	RIF/0.06 INH/0.25	RIF/0.06 INH/0.5	RIF/0.06 INH/1	EMB/0.5	RIF/0.06 INH/0.06 EMB/0.5	RIF/0.06 INH/0.125 EMB/0.5	RIF/0.06 INH/0.25 EMB/0.5	RIF/0.06 INH/0.5 EMB/0.5	RIF/0.06 INH/1 EMB/0.5
G	RIF/0.03	RIF/0.03 INH/0.06	RIF/0.03 INH/0.125	RIF/0.03 INH/0.25	RIF/0.03 INH/0.5	RIF/0.03 INH/1	EMB/0.25	RIF/0.03 INH/0.06 EMB/0.5	RIF/0.03 INH/0.125 EMB/0.5	RIF/0.03 INH/0.25 EMB/0.5	RIF/0.03 INH/0.5 EMB/0.5	RIF/0.03 INH/1 EMB/0.5
H	NC	INH/0.06	INH/0.125	INH/0.25	INH/0.5	INH/1	UN	PC	PC	PC	PC	PC

Abbreviations: INH, Isoniazid; RIF, Rifampicin; EMB, ethambutol; UN, unused well; NC, Negative Control; PC, Positive Control.

Determination the Minimum Inhibitory Concentrations (MICs)

The MIC was determined in triplicate for anti-TB drugs against each isolate using REMA through visual reading of the color change in 96-well microplates.[25,31–34] The individual MICs for INH, RIF, LEV and EMB were determined for each isolate and H37Rv reference strain using a serial two-fold micro-dilution of each drug's working solution concentration with the final MIC ranges of 1–0.06μg/mL for INH, 2–0.03μg/mL for RIF,[27,28] 8–0.125μg/mL for LEV[15] and 16–0.25μg/mL for EMB.[29,30] A color change from blue to pink/purple was considered as bacterial growth by reducing of resazurin. The MIC was defined as the lowest drug concentration that inhibited visible bacterial growth by no resazurin color change from blue to pink in the micro-wells. Isolates were considered resistant if the MIC values were ≥0.25μg/mL for INH, ≥0.5μg/mL for RIF,[25,30,31] ≥2μg/mL for LEV and ≥4μg/mL for EMB, which were the cut-off breakpoint concentrations.[29] The MIC results for RIF classified as wild-type strains with *rpoB* mutations that are not associated with resistance were grouped with a MIC range of <0.25μg/mL, strains with *rpoB* mutations associated with low-level RIF resistance were included with a MIC range of 0.25 to 1.0μg/mL, and *rpoB* mutations associated with high-level RIF resistance were grouped with a MIC of ≥2μg/mL.

Prediction of Drug–Drug Interaction Profiles

The in vitro double and triple interactions between anti-TB drugs were evaluated in triplicate using the modified micro-dilution REDCA method, by employing resazurin as mycobacterial growth indicator agent.[15,16] The potential combinatorial effects of multiple drugs (drug–drug interaction responses) were determined by the FICI using the MIC results for each combination experiment of isolate. This evaluation was performed in triplicate. The FICI for pairwise interaction between two drugs was calculated using the following formula: $\sum FICI_2 = FIC_A + FIC_B = (MIC_{A+B}/MIC_A) + (MIC_{B+A}/MIC_B)$, where MIC_{A+B} represents the MIC of drug A in combination with drug B; MIC_{B+A}, the MIC of drug B in combination with drug A; and MIC_A, the MIC of drug A tested alone, and MIC_B, the drug B tested alone. The pairwise (two-drug) interaction response was interpreted based on the FICI result and being considered synergistic interaction (FICI ≤ 0.5), indifference or additive interaction (FICI > 0.50 ≤ 4), and antagonistic interaction (FICI >4).[15,18] Furthermore, the FICI for the triple-drug interaction was computed using the following formula: $\sum FICI_3 = FIC_A + FIC_B + FIC_C = (MIC_{A+B+C}/MIC_A) + (MIC_{B+A+C}/MIC_B) + (MIC_{C+B+A}/MIC_C)$, where MIC_{A+B+C} represents the MIC of drug-A in combination with drug-B and drug-C; MIC_{B+A+C}, the MIC of drug-B in combination with drug-A and drug-C; MIC_{C+B+A}, the MIC of drug-C in combination with drug-B and drug-A; and MIC_A, MIC_B and MIC_C, the MIC of drug-A, drug-B and drug-C, respectively, when tested alone. The FICI values were interpreted as synergistic when FICI ≤ 0.75, indifferent when 0.75 > FICI ≤ 4, and antagonistic when FICI > 4 for the three-drug interaction response.[14,18,27]

Statistical Analysis

All the data were recorded and entered into Epi-Data v.3.1. The susceptibility profile of each isolate was determined by measuring the MICs of each drug. Descriptive statistics were used to analyze the distribution of MIC and FICI responses in double and triple drug combination values of MDR, RIF, and INH resistant isolates. A p-value ≤0.05 was considered statistically significant. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the micro-dilution REMA assay for susceptibility testing of RIF, INH, and MDR-TB were evaluated in comparison to the gold standard MGIT 960. The kappa (k) statistic was used to assess the agreement between REMA and MGIT 960-determined resistance. All statistical analyses were performed using SPSS V. 23.0.

Results

In vitro Susceptibility Profiles of Clinical Isolates

Among 168 DST resulted isolates from pulmonary TB patients, a total of 32 clinical isolates of *M. tuberculosis* were used in the study. Of which, 20 (62.5%) of isolates were obtained from male participants and the age range of 18–44 years with the mean age of 31 years. The prevalence of 11 (34.4%) study patients had a history of previous TB treatment, whereas 9 (28.1%) were TB with HIV co-infected cases.

Table 2 Frequency of *M. tuberculosis* Isolates with Minimum Inhibitory Concentration (MIC) of Drugs (n = 32)

Phenotypic MIGT 960 and LPA Results		Frequency (n) and Proportion (%) of Isolates Based on MIC Level Using REMA Assay (in µg/mL)									
Variables	Category	0.03	0.06	0.125	0.25[a]	0.5[b]	1	2[c]	4[d]	8	16	N, %
CC MGIT 960 for RIF	RIF-susceptible	–	1(3.1)	5(15.6)	11(34.4)	3(9.4)	–	–	–	–	–	20(62.5)
	RIF–resistant	–	–	–	1(3.1)	2(6.3)	4(12.5)	5(15.6)	–	–	–	12(37.5)
rpoB gene mutations by LPA	ΔWT8/MUT3	–	–	–	–	–	–	4(12.5)	–	–	–	4(12.5)
	ΔWT3&4/MUT1	–	–	–	–	–	2(6.3)	–	–	–	–	2(6.3)
	ΔWT7/MUT2A	–	–	–	–	1(3.1)	1(3.1)	–	–	–	–	2(6.3)
	+WT/MUT3	–	–	–	–	–	–	1(3.1)	–	–	–	1(3.1)
	ΔWT7/UN	–	–	–	–	2(6.3)	–	–	–	–	–	2(6.3)
	ΔWT8/UN	–	–	–	–	1(3.1)	–	–	–	–	–	1(3.1)
	Resistance undetected	–	1(3.1)	5(15.6)	12(37.5)	2(6.3)	–	–	–	–	–	20(62.5)
CC MGIT 960 for INH	INH-susceptible	–	5(15.6)	6(18.8)	1(3.1)	–	–	–	–	–	–	12(37.5)
	INH- resistant	–	–	1(3.1)	5(15.6)	5(15.6)	9(28.1)	–	–	–	–	20(62.5)
katG/inhA mutations by LPA	*katG* ΔWT/MUT1	–	–	–	–	2(6.3)	9(28.1)	–	–	–	–	11(34.4)
	katG ΔWT/UN	–	–	–	1(3.1)	1(3.1)	–	–	–	–	–	2(6.3)
	inhA ΔWT1/MUT1	–	–	–	3(9.4)	1(3.1)	–	–	–	–	–	4(12.5)
	Resistance undetected	–	5(15.6)	7(21.9)	2(6.2)	1(3.1)	–	–	–	–	–	15(46.9)
	EMB-resistant level	–	–	–	–	16(50)	5(15.6)	9(28.1)	2(6.3)	–	–	32(100)
	LEV-resistant level	–	–	–	13(40.6)	3(9.4)	14(43.8)	2(6.3)	–	–	–	32(100)

Notes: [a,b,c,d]Breakpoint concentration in µg/mL used to define resistance to INH, RIF, LEV and EMB, respectively.
Abbreviations: INH, isoniazid; RIF, rifampicin; EMB, ethambutol; LEV, levofloxacin; MDR-TB, multidrug resistant tuberculosis; MIC, minimum inhibitory concentration, CCs, critical concentrations; LPA, line probe assay.

All the tested isolates of the MIC results using the REMA micro-wells were done on the 8–9 days of incubation. No contaminated isolate was observed. Eighteen isolates (56.3%) were found to be RIF-susceptible in REMA micro-plate (MIC < 0.5μg/mL); while 14 (43.8%) isolates were RIF-resistant, of which 5 isolates (35.7%) were high-level of resistance with MIC values of ≥2μg/mL. The most of the high-level RIF resistant isolates (4/5, 80.0%) analyzed in this study were linked with TB/HIV co-infected cases and (3/5, 60.0%) were recorded as retreated patients of TB cases. Twelve isolates (37.5%) were found to be INH-susceptible (MIC < 0.25μg/mL); whereas 20 isolates (62.5%) were INH-resistant, of which 9 isolates (45.0%) showed high-level of resistance with the MIC value of ≥1μg/mL. Three and one isolates were detected as resistant to RIF and INH using REMA plate MIC ≥ 0.5μg/mL and ≥0.25μg/mL, respectively, as previously defined as susceptible by CCs MIGT 960 method, whereas two and three isolates were MIC-resistance to RIF and INH that were resistance undetected to RIF and INH by MDRTBplus assay, respectively. Fourteen isolates (43.8%) were resistant to both RIF and INH and categorized as MDR-TB, where the MICs ≥0.5 and 0.25μg/mL for RIF and INH, respectively (Table 2). Among the MDR-TB isolates, the rate of TB/HIV co-infected patients and previously treated cases were 42.9% and 35.7%, respectively, whereas an equal proportion of 33.3% INH mono-resistant isolates were found from TB/HIV co-infected patients and previously treated cases.

Distribution of Mutation Patterns and Their Association with the MICs

The distribution of different drug resistance mutation patterns and their relationship with the MIC level of RIF and INH drugs was investigated. Interestingly, 55.6% (5/9) of isolates having *rpoB* common mutations at codon S450L (codon 531 in the *E. coli* numbering system) were developed a higher-level RIF-resistance with MICs of ≥2μg/mL, whereas four of isolates (100%) carried *rpoB* gene substitutions at codons D435V and H445Y (codons 516 and 526 in the *E. coli* numbering system) were linked with moderate and low-level RIF-resistance in the MIC ranges from 0.5 to 1μg/mL. A frequency of three isolates (100%) with unknown mutations in the *rpoB* gene was associated with low-level RIF-resistance with a MIC of 0.5μg/mL. All the isolates (100%) harboring *katG* and *inhA* mutations at codons S315T and C-15T by the MTBDRplus LPA were revealed with MICs of ≥0.25μg/mL and determined to be resistant to INH. A proportion of 81.8% (9/11) *M. tuberculosis* clinical isolates conferring known S315T mutations in the *katG* gene were revealed high-level INH resistance with the MIC of ≥1μg/mL. However, 18.2% (2/11) of isolates harboring specific S315T *katG* mutations and 100% of isolates (2/2) with unknown mutations at *katG* gene and 100% (4/4) of isolates carried common C-15T mutations at *inhA* gene were showed a low-level INH resistance with the MIC values of 0.25–0.5μg/mL (Table 2).

Prediction of Drug–Drug Interaction Profiles in *M. tuberculosis* Isolates

The synergistic, indifferent, or antagonistic effects of each drug combination on the clinical isolates of *M. tuberculosis* are presented in Tables 3 and 4. Among the 20 INH-resistant clinical isolates that had MICs ranging from 1 to 0.25μg/mL and 12 INH-susceptible isolates with the MIC values ranging from 0.125 to 0.06μg/mL for INH alone; the individual MICs of drugs in the dual combination of INH+RIF and INH+LEV were decreased from one to two dilutions (INH ranging from 0.25 to 0.06μg/mL), showing synergistic interaction with a FICI value of 0.5 against 9/32 (28.1%) and 4/32 (12.5%) INH-resistant isolates, respectively, while there was no clear synergism effect in the INH+EMB combination in a condition of certain concentration. However, FICI in any of tested dual-drug combinations for all INH-susceptible isolates were ranged from 1 to 2, all showing indifference, where the MICs decreased to one dilution or no dilution change (Table 3). The MICs of drugs in the triple-drug combination INH+RIF+EMB, INH+RIF+LEV, and INH+EMB +LEV against the 6/32 (18.8%), 11/32 (34.4%), and 8/32 (25%) INH-resistant isolates, respectively, were decreased the individual MICs from one to two dilutions (INH ranging from 0.25 to 0.06μg/mL) showing synergistic effect with a FICI value of ≤0.75 (Table 4).

Moreover, the REMA assay found that the MIC value of RIF alone ranged from 2 to 0.5μg/mL for 14/32 (43.8%) resistant isolates and from 0.25 to 0.06μg/mL for 18/32 (56.2%) susceptible isolates. Of which, the MIC values of drugs in the dual drugs combination RIF+INH and RIF+LEV were decreased from one to two dilutions against 5/32 (15.6%) and 4/32 (12.5%) MDR isolates, respectively, leading synergistic activity with a FICI value of 0.5. However, the most studied clinical isolates exhibited indifferent with the FICI values from 1 to 2 against the pairwise INH+EMB (32/32, 100%), EMB

Table 3 MICs and FICI of the INH, RIF, EMB and LEV in Double Drug Combination Against *M. tuberculosis* Clinical Isolates

Isolate ID	MIGT 960 Susceptibility Patterns	MIC of Drugs Alone (µg/mL)				REMA MIC Resistant Status	MIC in µg/mL and FICI in Double Drug Combination									
							RIF+INH		INH+EMB		RIF+LEV		INH+LEV		EMB+LEV	
		RIF	INH	EMB	LEV		MICs	FICI	MICs	FICI	MICs	FICI	MICs	FICI	MICs	FICI
A005	Susceptible	0.25	0.125	0.5	0.25	Susceptible	0.125/0.06	1	0.06/0.25	1	0.125/0.125	1	0.06/0.125	1	0.5/0.25	2
A026	INH-mono	0.25	1	2	1	INH-mono	0.06/0.25	0.5	0.5/1	1`	0.125/0.5	1	0.25/0.25	0.5	1/0.5	1
A036	Susceptible	0.125	0.06	0.5	0.25	Susceptible	0.06/0.06	1.5	0.06/0.5	2	0.06/0.125	1	0.06/0.25	2	0.5/0.25	2
A043	MDR	1	0.5	2	1	MDR	0.25/0.125	0.5	0.25/1	1	0.25/0.25	0.5	0.25/0.5	1	1/0.5	1
A047	MDR	0.5	1	1	1	MDR	0.25/0.5	1	0.5/0.5	1	0.25/0.5	1	0.5/0.5	1	0.5/0.5	1
A049	Susceptible	0.25	0.125	0.5	0.25	Susceptible	0.125/0.06	1	0.06/0.25	1	0.125/0.125	1	0.06/0.125	1	0.5/0.25	2
A053	MDR	2	0.25	4	1	MDR	0.5/0.06	0.5	0.125/2	1	0.5/0.25	0.5	0.125/0.5	1	2/0.5	1
A056	MDR	1	0.25	2	2	MDR/pre-XDR	0.5/0.125	1	0.125/1	1	0.5/1	1	0.06/0.5	0.5	0.5/0.5	0.5
A062	Susceptible	0.25	0.06	0.5	0.25	Susceptible	0.25/0.06	2	0.06/0.5	2	0.125/0.125	1	0.06/0.25	2	0.5/0.25	2
A074	INH-mono	0.125	0.5	1	0.5	INH-mono	0.06/0.25	1	0.25/0.5	1	0.06/0.25	1	0.25/0.25	1	0.5/0.25	1
A078	MDR	0.5	1	2	1	MDR	0.25/0.5	1	0.5/1	1	0.25/0.5	1	0.5/0.5	1	1/0.5	1
A082	Susceptible	0.25	0.125	0.5	1	Susceptible	0.125/0.06	1	0.06/0.25	1	0.125/0.5	1	0.06/0.5	1	0.25/0.5	1
A089	INH-mono	0.06	0.5	1	0.25	INH-mono	0.06/0.5	2	0.25/0.5	1	0.03/0.125	1	0.25/0.125	1	0.5/0.125	1
A097	INH-mono	0.25	0.5	1	0.25	INH-mono	0.06/0.125	0.5	0.25/0.5	1	0.125/0.125	1	0.25/0.125	1	0.5/0.125	1
A105	Susceptible	0.125	0.25	0.5	0.25	INH-mono	0.03/0.06	0.5	0.12/0.25	1	0.06/0.125	1	0.125/0.125	1	0.25/0.125	1
A108	INH-mono	0.5	1	2	1	MDR	0.25/0.5	1	0.5/1	1	0.25/0.5	1	0.5/0.5	1	0.5/0.25	0.5
A111	MDR	2	0.5	2	2	MDR/pre-XDR	1/0.25	1	0.25/1	1	1/1	1	0.125/0.5	0.5	1/1	1
A113	INH-mono	0.25	1	0.5	1	INH-mono	0.06/0.25	0.5	1/0.25	1.5	0.125/0.5	1	0.5/0.5	1	0.25/0.5	1
A122	Susceptible	0.25	0.06	0.5	0.25	Susceptible	0.25/0.06	2	0.06/0.5	2	0.125/0.125	1	0.06/0.25	2	0.25/0.125	1
A144	Susceptible	0.25	0.125	0.5	0.25	Susceptible	0.25/0.125	2	0.06/0.25	1	0.125/0.125	1	0.125/0.25	2	0.25/0.125	1
A152	MDR	2	1	1	1	MDR	0.5/0.25	0.5	0.5/0.5	1	0.5/0.25	0.5	0.5/0.5	1	0.5/0.5	1
A155	INH-mono	0.5	1	4	1	MDR	0.25/0.5	1	0.5/2	1	0.25/0.5	1	0.5/0.5	1	2/0.5	1
A172	MDR	1	0.25	0.5	0.5	MDR	0.5/0.125	1	0.12/0.25	1	0.5/0.25	1	0.125/0.25	1	0.25/0.25	1
A180	Susceptible	0.125	0.125	0.5	0.25	Susceptible	0.06/0.06	1	0.06/0.25	1	0.06/0.125	1	0.06/0.125	1	0.25/0.125	1
A190	MDR	2	0.25	2	1	MDR	0.5/0.06	0.5	0.125/1	1	1/0.5	1	0.125/0.5	1	1/0.5	1
A191	MDR	0.5	1	2	1	MDR	0.25/0.5	1	0.5/1	1	0.125/0.25	0.5	0.25/0.25	0.5	1/0.5	1
A225	Susceptible	0.25	0.06	0.5	0.25	Susceptible	0.25/0.06	2	0.06/0.5	2	0.125/0.125	1	0.06/0.25	2	0.25/0.125	1
A240	MDR	2	0.25	0.5	1	MDR	0.5/0.06	0.5	0.25/0.5	2	1/0.5	1	0.125/0.5	1	0.25/0.5	1
A249	INH-mono	0.5	1	2	1	MDR	0.25/0.5	1	0.5/1	1	0.25/0.5	1	0.5/0.5	1	1/0.5	1
A217	MDR	0.25	0.125	0.5	0.5	Susceptible	0.125/0.06	1	0.06/0.25	1	0.125/0.25	1	0.06/0.25	1	0.25/0.25	1
A087	Susceptible	0.25	0.06	0.5	0.25	Susceptible	0.25/0.06	2	0.06/0.5	2	0.125/0.125	1	0.06/0.25	2	0.25/0.125	1
A105	Susceptible	0.125	0.125	0.5	0.25	Susceptible	0.06/0.06	1	0.06/0.25	1	0.06/0.125	1	0.06/0.125	1	0.25/0.125	1
H37Rv	Susceptible	0.06	0.06	0.5	0.25	Susceptible	0.06/0.06	2	0.06/0.5	2	0.06/0.25	2	0.06/0.25	2	0.5/0.25	2

Abbreviations: MIC, Minimal Inhibitory Concentration; FICI, Fractional Inhibitory Concentration Index; INH, Isoniazid; RIF, Rifampicin; EMB, Ethambutol; LEV, Levofloxacin.

+LEV (30/32, 93.8%), RIF+INH (23/32, 71.9%), RIF+LEV and INH+LEV (28/32, 87.5%) drug combination, where the MICs of each drugs did not differ from the individual MICs or decreased the individual MICs up to one dilution (Table 3). The individual MICs in the triple drug combination of RIF+INH+EMB, RIF+INH+LEV and EMB+INH+LEV against the 5/32 (15.6%), 11/32 (34.4%) and 7 (21.9%) MDR isolates, respectively, were decreased from one to two dilutions ranging from 0.5 to 0.06μg/mL, showing synergistic interaction with the FICI values from 0.62 to 0.75; while most of the studied 23/32 (71.9%), 21/32 (65.6%) and 24/32 (75%) clinical isolates, in respective triple drug combination, did not differ from the individual MIC or decreased the individual MIC up to one dilution, indicating indifference with FICI values from 1.13 to 3 (Table 4). There was a significant difference in the synergistic effect for drug-resistant isolates against the combination INH+RIF vs RIF+LEV (p = 0.026), RIF+LEV vs RIF+INH+LEV (p = 0.002), INH+LEV vs EMB+INH+LEV (p = 0.013) and RIF+INH+EMB vs RIF+INH+LEV (p < 0.001). Nevertheless, no statistical difference was observed in the synergistic effect for RIF+INH vs INH+LEV (p = 0.887), RIF+LEV vs INH+LEV (p = 0.435), and RIF+INH vs RIF+INH+LEV (p = 0.469) combination against drug-resistant isolates (Table 5).

Evaluation Performance of Resazurin Microtitre Assay (REMA)

The study also showed the utility performance of REMA assay for in vitro susceptibility patterns compared to the gold standard phenotypic BACTEC MGIT 960 DST method as shown in Table 6. Of the 32 tested *M. tuberculosis* clinical isolates for susceptibility profile, both methods were revealed a total of 19 resistant isolates (59.4%) for INH. Two isolates were produced discordant result, where one isolate being INH-resistant at MIC of 0.25μg/mL in REMA assay but susceptible at CCs 0.1μg/mL in MGIT 960 system, while a single isolate was INH-susceptible at MIC of 0.125μg/mL that was INH-resistant to MGIT 960. The REMA assay's sensitivity, specificity, PPV, and NPV for the detection of INH-resistance were 95.0%, 91.7%, 95.0% and 91.7%, respectively, compared to the MGIT 960 system. Besides, 11 isolates (34.4%) were found to be RIF-resistant and 17 isolates (53.1%) were RIF-susceptible by both methods. However, four isolates produced discordant results; three isolates were determined to be low-level RIF-resistant to the REMA assay at MIC value 0.5μg/mL but RIF-susceptible to the MGIT 960 at 1.0μg/mL, while a single isolate was RIF-susceptible at MIC 0.25μg/mL but RIF-resistant in MIGT 960. The REMA assay was demonstrated a 91.7% of sensitivity, 85.0% of specificity, 78.6% of PPV and 94.5% of NPV for identifying RIF-resistant isolates, as well as MDR-TB isolates. The two susceptibility testing methods had excellent agreement of 87% in detecting INH and 75% for both RIF and MDR-TB.

Discussion

The emergence of MDR-TB is a serious public health problem.[34] The SLDs have to be used for treatment of MDR/XDR-TB patients but are consistently difficult with less effectiveness, less availability in low-income countries, more toxicity, and higher costs. Susceptibility testing using MIC instead of the CCs shows that some FLDs act inhibitory in slightly higher concentrations in vitro.[21] Thus, to our knowledge, this is the first study to investigate the distributions of MIC values and in vitro synergism effect in double and triple drug combinations against *M. tuberculosis* clinical isolates.

The present study showed that different drug resistance conferring mutations in the *rpoB, katG*, and *inhA* genes underlying resistance to RIF and INH were associated with different MIC ranges. Among the 14 (43.8%) RIF-resistant isolates with the MIC value ≥0.5μg/mL, the 5 (35.7%) isolates harbored high-level RIF-resistance at the MIC value ≥2μg/mL, while 9 (64.3%) isolates were exhibited a low-level RIF-resistance with MIC diversity ranges from 0.5 to 1μg/mL in REMA plate which previously defined as susceptible isolates by CCs MIGT 960 at 1μg/mL or that were no carried specific mutations in the *rpoB* gene underlying drug-resistance using LPA. This finding was slightly lower than the previous studies that showed a rate of 61.5% high-level RIF-resistance and 38.5% low-level RIF-resistance in Vietnam,[31] 73.2% high-level and 26.8% low-level RIF-resistance among Brazilian clinical isolates using the REMA assay,[32] and 100% MDR-TB isolates displaying high-level RIF-resistance with MICs ≥32μg/mL on LJ medium in Germany.[21]

Herein, five of nine RIF-resistant clinical isolates (55.6%) in this study, which carried common mutations at codons S450L in the *rpoB* gene, were found to have high-level resistance to RIF with a MIC value ≥2μg/mL, while four isolates (100%) that carried *rpoB* gene substitutions at codons D435V and H445Y were exhibited moderate and low-level RIF resistance, with MIC values ranging from 0.5 to 1μg/mL on the REMA plate. Interestingly, a similar finding was identified in previous study where RIF-resistant strains having mutations *rpoB* mutants at codons S450L (100%) were

Table 4 MICs and FICI of the INH, RIF, EMB and LEV in Triple Drugs Combination of the Tested *M. tuberculosis* Clinical Isolates

Isolate ID	MIGT 960 Susceptibility Patterns	MIC of Drugs Alone (µg/mL)				REMA MIC Resistant Status	MIC in µg/mL and FICI in Triple Drugs Combination					
							RIF+INH+EMB		RIF+INH+LEV		EMB+INH+LEV	
		RIF	INH	EMB	LEV		MICs	FICI	MICs	FICI	MICs	FICI
A005	Susceptible	0.25	0.125	0.5	0.25	Susceptible	0.125/0.06/0.5	2	0.125/0.06/0.25	2	0.25/0.06/0.25	2
A026	INH-mono	0.25	1	2	1	INH-mono	0.125/0.5/1	1.5	0.125/0.5/0.25	1.25	0.5/0.25/0.25	0.75
A036	Susceptible	0.125	0.06	0.5	0.25	Susceptible	0.125/0.06/0.5	3	0.125/0.06/0.25	3	0.5/0.06/0.25	3
A043	MDR	1	0.5	2	1	MDR	0.25/0.125/0.5	0.75	0.25/0.125/0.25	0.75	0.5/0.125/0.25	0.75
A047	MDR	0.5	1	1	1	MDR	0.25/0.5/0.5	1.5	0.125/0.25/0.25	0.75	0.5/0.5/0.25	1.25
A049	Susceptible	0.25	0.125	0.5	0.25	Susceptible	0.125/0.06/0.5	2	0.125/0.06/0.25	2	0.25/0.06/0.25	2
A053	MDR	2	0.25	4	1	MDR	1/0.125/2	1.5	0.5/0.06/0.25	0.74	2/0.125/0.25	1.25
A056	MDR	1	0.25	2	2	MDR/pre-XDR	0.25/0.06/0.5	0.74	0.25/0.06/0.25	0.62	1/0.125/0.25	1.13
A062	Susceptible	0.25	0.06	0.5	0.25	Susceptible	0.25/0.06/0.5	3	0.25/0.06/0.25	3	0.5/0.06/0.25	3
A074	INH-mono	0.125	0.5	1	0.5	INH-mono	0.06/0.25/0.5	1.5	0.06/0.25/0.25	1.5	0.5/0.25/0.25	1.5
A078	MDR	0.5	1	2	1	MDR	0.125/0.25/0.5	0.75	0.125/0.25/0.25	0.75	0.5/0.25/0.25	0.75
A082	Susceptible	0.25	0.125	0.5	1	Susceptible	0.125/0.06/0.5	2	0.125/0.06/0.25	1.23	0.25/0.06/0.25	1.23
A089	INH-mono	0.06	0.5	1	0.25	INH-mono	0.03/0.25/0.5	1.5	0.03/0.25/0.25	2	0.5/0.25/0.25	2
A097	INH-mono	0.25	0.5	1	0.25	INH-mono	0.03/0.06/0.5	0.74	0.125/0.25/0.25	2	0.5/0.25/0.25	2
A105	Susceptible	0.125	0.25	0.5	0.25	INH-mono	0.06/0.125/0.5	2	0.06/0.125/0.25	2	0.25/0.125/0.25	2
A108	INH-mono	0.5	1	2	1	MDR	0.25/0.5/0.5	1.25	0.25/0.5/0.25	1.25	1/0.5/0.25	1.25
A111	MDR	2	0.5	2	2	MDR/pre-XDR	0.5/0.125/0.5	0.75	0.5/0.125/0.25	0.63	0.5/0.125/0.25	0.63
A113	INH-mono	0.25	1	0.5	1	INH-mono	0.125/0.5/0.5	2	0.125/0.5/0.25	1.25	0.25/0.5/0.25	1.25
A122	Susceptible	0.25	0.06	0.5	0.25	Susceptible	0.25/0.06/0.5	3	0.25/0.06/0.25	3	0.5/0.06/0.25	3
A144	Susceptible	0.25	0.125	0.5	0.25	Susceptible	0.125/0.06/0.5	2	0.125/0.06/0.25	2	0.25/0.06/0.25	2
A152	MDR	2	1	1	1	MDR	1/0.5/0.5	1.5	0.5/0.25/0.25	0.75	0.25/0.25/0.25	0.75
A155	INH-mono	0.5	1	4	1	MDR	0.25/0.5/0.5	1.13	0.125/0.25/0.25	0.75	2/0.5/0.25	1.25
A172	MDR	1	0.25	0.5	0.5	MDR	0.5/0.125/0.5	2	0.5/0.125/0.25	1.5	0.25/0.125/0.25	1.5
A180	Susceptible	0.125	0.125	0.5	0.25	Susceptible	0.06/0.06/0.5	2	0.06/0.06/0.25	2	0.25/0.06/0.25	2
A190	MDR	2	0.25	2	1	MDR	1/0.125/0.5	1.25	1/0.125/0.25	1.25	0.5/0.06/0.25	0.74
A191	MDR	0.5	1	2	1	MDR	0.25/0.5/1	1.5	0.125/0.25/0.25	0.75	0.5/0.25/0.25	0.75
A225	Susceptible	0.25	0.06	0.5	0.25	Susceptible	0.25/0.06/0.5	3	0.25/0.06/0.25	3	0.5/0.06/0.25	3
A240	MDR	2	0.25	0.5	1	MDR	1/0.125/0.5	2	0.5/0.06/0.25	0.74	0.25/0.125/0.25	1.25
A249	INH-mono	0.5	1	2	1	MDR	0.125/0.25/0.5	0.75	0.125/0.25/0.25	0.75	0.5/0.25/0.25	0.75
A217	MDR	0.25	0.125	0.5	0.5	Susceptible	0.125/0.06/0.5	2	0.125/0.06/0.25	1.5	0.25/0.06/0.25	1.5
A087	Susceptible	0.25	0.06	0.5	0.25	Susceptible	0.25/0.06/0.5	3	0.25/0.06/0.25	3	0.5/0.06/0.25	3
A105	Susceptible	0.125	0.125	0.5	0.25	Susceptible	0.06/0.06/0.5	2	0.06/0.06/0.25	2	0.25/0.06/0.25	2
H37Rv	Susceptible	0.06	0.06	0.5	0.25	Susceptible	0.06/0.06/0.5	3	0.06/0.06/0.25	3	0.5/0.06/0.25	3

Abbreviations: MIC, Minimal Inhibitory Concentration; FICI, Fractional Inhibitory Concentration Index; INH, Isoniazid; RIF, Rifampicin; EMB, Ethambutol; LEV, Levofloxacin.

associated with elevated MICs of resistance in Romania using the MycoTB assay.[7] However, other studies demonstrated that the RIF-resistant strains having mutations *rpoB* mutants at different codons S450L, H445Y, H445D were linked with high levels of MICs resistance in China by microplate Alamar blue assay,[34] and isolates with harboring substitutions at codons S450L, H445Y, H445D, and D435G were associated with high levels of MIC resistance, while mutation at codon L430P was related to low-level RIF resistance using either MycoTB or REMA MIC testing.[35–39] Nevertheless, the MIC of a RIF-resistant strain increased up to 32μg/mL when it contained L430P mutation along with D435G mutation. This suggests that the L430P mutation alone is responsible for low-level RIF-resistance and only affects the MIC when it co-exists with additional mutations that are responsible for high RIF-resistance.[39] Earlier study has shown that RIF-resistant strains with double mutations in the *rpoB* gene are associated with high-level resistance of MIC with ≥16μg/mL, as determined by whole-genome sequencing (WGS) and MycoTB MIC testing.[36] Over 95% of RIF-resistant isolates have been found to have mutations in the *rpoB* gene within the RRDR region. Mutations at codons 450, 445 and 435 in the RRDR are generally linked to high levels of RIF resistance, while specific amino acid substitutions at codons 430, 433, 434, 437, 451, and 452 are associated with lower levels of resistance.[37,39] This suggests that the position and nature of the amino acid substitution strongly influences the MIC levels. The type and frequency of *rpoB* mutations related to RIF resistance may vary depending on the setting and level of resistance.[34] When compared to *M. tuberculosis* populations with isolates exhibiting high MICs, isolates with low-level resistance may have lower fitness in the presence of RIF. The cost of resistance has been identified as a crucial factor in the spread of DR-TB strains. The competitive fitness of *M. tuberculosis* strains is not only dependent on the specific *rpoB* mutation (eg, S450L versus H445Y), but also on the strain's genotype. Fitness varies significantly between lineages, even when the *rpoB* mutation and resistance level are identical.[40]

Moreover, three strains that initially appeared to be resistant to RIF with no common mutations in the *rpoB* gene were later found to be able to grow at a MIC of 0.5 to 1 μg/mL as determined by MIC assay in our study. This finding is consistent with earlier reports, where four strains with low-level RIF resistance had no mutations in the RRDR region,[34] and two strains with high-level resistance and six strains with low-level resistance had no mutations in the entire *rpoB* gene.[39] This suggests that MIC is important in detecting borderline RIF-resistance in *M. tuberculosis* strains, especially when it is associated with low-level resistance and difficult to detect using traditional phenotypic methods like MIGT 960 with a single concentration of 1μg/mL, as well as genotypic methods like LPA. Treatment outcomes for patients with borderline RIF-resistance have been linked to treatment failures.[20,41,42]

In addition, information on the level of resistance to INH is important to guide the treatment choice with high dose of INH for MDR-TB isolates with *katG* mutations.[21] The present study revealed that a proportion of 62.5% (20/32) isolates were INH resistance with the MIC values of ≥0.25μg/mL. Among INH-resistant isolates which carrying mutations in the *katG* gene by MTBDRplus assay, 81.8% (9/11) isolates were associated with high level of INH resistance at the MIC ≥ 1μg/mL and four of isolates (100%) with mutations at the *inhA* gene displayed low-level INH resistance with the MIC ranges from ≥0.25 to 0.5μg/mL. Previous studies described similar findings, a study in Ethiopia reported that a proportion of 88.5% MDR-TB isolates occurring mutations in the *katG* gene exhibited a moderate-level INH resistance (MIC ≥ 2 to 4 μg/mL) and 7.7% isolates with mutations in the *inhA* gene detected by the MTBDRplus showed a low-level INH resistance (MIC ≤ 0.5μg/mL) using MYCOTB MIC plates,[20] 84.2% high-level and 15.8% low-level resistance to INH,[32] the *katG* S315T mutation (100%) was associated with elevated MICs in Romania using MycoTB assay,[7] an equal proportion of 50% high-level and low-level INH-resistance in Vietnam using REMA MIC assay.[31]

The present study suggests that different resistance mutations lead to distinct MICs, some of which may still be overcome by increased dosing. Conversely, a study conducted in Germany reported that 88.6% and 83.3% MDR-TB strains showed low-level INH and EMB resistance with a MIC value of 1 to 8μg/mL for INH and 0.5 to 10μg/mL for EMB on LJ/7H10 medium, respectively; while 11.4% and 16.7% isolates revealed high-level INH and EMB resistance at a MIC above 8μg/mL for INH and 20μg/mL for EMB.[21] This suggests that high-dose INH (15–20 mg/kg daily) than the standard dose (3–5mg/kg daily) and EMB (up to 25 mg/kg) could be benefit in treating MDR-TB patients harboring a high proportion of *katG* gene mutations if low-level resistance MIC testing is detected. Different drug resistance genetic mutations inside the resistance determining region of *rpoB* and *katG* are associated with distinct MIC levels of resistance to RIF and INH.[43] The INH-resistant strains having mutation *katG* S315T was significantly associated with high-level of

Table 5 Distribution of Isolates and Comparison of FICI in Double and Triple Drug Combinations Using REDCA Assay (n = 32)

MIC Resistance Patterns	Numbers of Isolates in Double and Triple Drug Combinations (N/%) and its Range of MIC (in µg/mL)															
	RIF+INH[a]		INH+EMB[b]		RIF+LEV[c]		INH+LEV[d]		EMB+LEV[e]		RIF+INH+EMB[f]		RIF+INH+LEV[g]		INH+EMB+LEV[h]	
	n/%	FICI	n/%	FICI	n/%	FICI	n/%	FICI	n/%	FICI	n/%	FICI	n/%	FICI	n/%	FICI
Resistant	9(28.2) 11(34.4) –	≤ 0.5 0.50–4 >4	– 20(62.5) –	≤ 0.5 0.50–4 >4	4(12.5) 16(50) –	≤ 0.5 0.50–4 >4	4(12.5) 16(50) –	≤ 0.5 0.50–4 >4	2(6.3) 18(56.3) –	≤ 0.5 0.50–4 >4	6(18.8) 14(43.8) –	≤ 0.75 0.75–4 >4	11(34.4) 9(28.2) –	≤ 0.75 0.75–4 >4	8(25) 12(37.5) –	≤ 0.75 0.75–4 >4
Susceptible	– 12(37.5) –	≤ 0.5 0.50–4 >4	– 12(37.5) –	≤ 0.5 0.50–4 >4	– 12(37.5) –	≤ 0.5 0.50–4 >4	– 12(37.5) –	≤ 0.5 0.50–4 >4	– 12(37.5) –	≤ 0.5 0.50–4 >4	– 12(37.5) –	≤ 0.75 0.75–4 >4	– 12(37.5) –	≤ 0.75 0.75–4 >4	– 12(37.5) –	≤ 0.75 0.75–4 >4

Notes: p-value of in vitro synergism for a vs c, 0.026*; a vs d, 0.887; c vs d, 0.435; a vs f, 0.211; a vs g, 0.469; c vs g, 0.02*; d vs h, 0.013*; f vs g, 0.001*; f vs h, 0.001*; and *Statistically significant.
Abbreviations: INH, isoniazid; RIF, rifampicin; EMB, ethambutol; LEV, levofloxacin; MDR-TB, multidrug resistant tuberculosis; MIC, minimum inhibitory concentration.

Table 6 Diagnostic Utility of the REMA Assay for Susceptibility Testing as Compared with Gold Standard MGIT 960 (n = 32)

Frequency (n) and Proportion (%) of *M. tuberculosis* Clinical Isolates Profiles									
REMA Assay Using MICs		**BACTEC MGIT 960 Using CCs**		**Sensitivity (%)**	**Specificity (%)**	**PPV (%)**	**NPV (%)**	**k-value**
		Resistant (n/%)	**Susceptible (n/%)**					
INH	Resistant	19(59.4)	1(3.1)	95.0	91.7	95.0	91.7	0.87
	Susceptible	1(3.1)	11(34.4)					
RIF	Resistant	11(34.4)	3(9.4)	91.7	85.0	78.6	94.5	0.75
	Susceptible	1(3.1)	17(53.1)					
MDR	Resistant	11(34.4)	3(9.4)	91.7	85.0	78.6	94.5	0.75
	Susceptible	1(3.1)	17(53.1)					

Abbreviations: PPV, positive predictive value; k, kappa value; NPV, negative predictive value; INH, isoniazid; RIF, rifampicin; MDR-TB, multidrug resistant tuberculosis; MGIT, Mycobacteria Growth Indicator Tube; REMA, resazurin microtitre assay.

MICs in *M. tuberculosis*,[7,37] and *rpoB* S450L and *katG* S315T mutations of clinical *M. tuberculosis* isolates are known to be low-cost genetic variants and are associated with high-level of MIC resistance to RIF and INH, respectively,[44] while lower-levels of resistance are related with T-8A and C-15T mutations in the *inh*A region.[37] Commonly, high-levels (MIC≥1μg/mL) and low-levels (MIC<1μg/mL) of resistance have been linked with mutations in the *katG* and *inhA* genes, respectively.

The MICs in the double drugs INH+RIF, RIF+LEV, INH+LEV and EMB+LEV combination were decreased the individual MIC from one to two dilutions, showing synergistic effect (FICI = 0.5) against 9/32 (28.1%), 4/32 (12.5%) 4/32 (12.5%) and 2/32 (6.3%) INH-resistant isolates, respectively, but not in those which were drug-susceptible by the REDCA assay, where the synergistic difference of NH+RIF vs RIF+LEV for drug-resistant isolates was significant (p = 0.026). This finding is in agreement with previous studies that the RIF+INH combination showed a synergistic effect for 13.8% (4/29) clinical MDR-isolates[18] and EMB+LEV combination against two MDR of ten resistant *M. tuberculosis* clinical Brazilian isolates (20.0%) as determined by REDCA assay.[15] However, previous studies showed no synergism effect in RIF+INH combination against *M. tuberculosis* on checkerboard titration assay.[19,45] This might be due to the difference in the isolate and patient characteristics. INH+LEV against drug-resistant or susceptible isolates as shown by both checkerboard and REDCA assays,[15] and a combination of pasiniazid (Pa) with rifapentine (RFP) more likely to show in vitro synergism than that of the standard INH+RIF combinations (P < 0.0001), which can provide a new regimen for retreatment TB patients.[18]

The potential combined effect of triple drugs combination INH+RIF+EMB, INH+RIF+LEV, and INH+EMB+LEV in our study exhibited a synergistic activity with FICI value of ≤0.75 against 6/32 (18.8%), 11/32 (34.4%), and 8/32 (25%) INH-resistant isolates, respectively, where the individual MIC from decreased one to two dilutions. This finding is in agreement with previous studies that showed a synergistic effect for RIF+INH+EMB combination by 3D-chequerboard assay against two resistant isolates (20%) (FICI = 0.72),[45] RIF+INH+EMB for twelve (100%) INH-resistant isolates (FICI = 0.31–0.38) and ofloxacin (OFX)+RIF+EMB drug combination (FICI = 0.31–0.62) in 21 out of the 23 isolates (91.3%),[14] and the INH and RIF combined to LEV at ¼ MIC for 40% of resistant isolates and no antagonism was observed among the tested drugs.[17] In our study, the RIF+INH+LEV combination showed significantly more synergism than that of the RIF+INH+EMB for drug-resistant isolates (p < 0.001), which is consistent with a study revealed that the OFX+RIF+EMB combination showed significantly more synergism than that of the INH+RIF+EMB combination (p = 0.007).[14] This suggests that the FQs groups, either LEV or OFX, displayed better efficacy and potentially greater power than EMB or INH when used in triple combinations for drug-resistant and susceptible isolates. This combination may be an effective therapeutic option for the treatment of both the drug susceptible and resistant *M. tuberculosis* infections by restoring susceptibility against *M. tuberculosis* strains carrying genetic resistance to any one of the partner drugs. Rifamycin derivatives such as RIF, unfortunately, have the potential to cause drug–drug interactions through either enzymatic and/or transporter induction. On the other hand, EMB, which also affects mycobacterial cell wall synthesis, did not exhibit synergy with RIF.[46] This implies that the three-drug combinations might have synergistic activity when

compared to individual or two-drug regimens. In our study, during triple-drug combination of INH and RIF with 0.5μg/mL MIC of EMB, the MIC values of INH and RIF were decreased the individual MIC from one to two dilutions. This suggests that the synergistic or even antagonistic activity of a specific combination against a particular microorganism is not only a property of each drug but also depending on the dose of each drug in the combination. This assumption is supported by Santos et al.[17] Our finding is clearly inconsistent with the in vitro synergism activity of INH+RIF+EMB combination against two INH-susceptible isolates (18.2%) (FICI = 0.6).[14] The inconsistency of the synergism interaction profile in different studies with similar anti-TB drugs could be attributed to patient-specific characteristics, such as variations in drug-metabolizing enzymes (CYP). These variations can affect the rate at which drugs are broken down and eliminated from the body, which may potentially lead to interactions if one drug inhibits or induces the activity of these enzymes. A study of three drug combination using sub-MIC spectinomycin (SPT)+RIF+INH resulted in enhanced activity in vitro against RIF-mono-resistant *rpoB* S450L mutant, but similar effect was not observed for the INH-mono-resistant strain harboring *inhA* promoter -c15t mutation. The ability of INH to inhibit cytochrome P450 (CYP) may contribute to synergy in the RIF and INH with SPT combination when the active form of INH is not rapidly eliminated inside *M. tuberculosis* and when SPT acts by further reducing the activity of CYPs.[19] The elevated levels of CYP have been associated with drug resistance due to the enhanced rate of elimination of the drugs by metabolism.[47] Furthermore, the synergy of drug interactions and positive epistasis between low-cost drug resistance-conferring mutations in *rpoB* S450L and *katG* S315T may also be influenced by the phylogenetic background of *M. tuberculosis* strains.[48] Different regions could exhibit diverse strains that possess various resistance patterns, resulting in complex drug interactions and posing a challenge for "one-size-fits-all" regimens and predicting treatment outcomes.

One more finding in our study has revealed an excellent sensitivity and specificity of 95% and 91.7% for detecting INH-resistance, respectively, 91.7% and 85% for RIF-resistance/MDR-TB when compared to the phenotypic BACTEC MIGT960 as a reference method. This finding is consistent with previous studies conducted the sensitivity and specificity of REMA were 89% and 68% for identifying isolates resistant to RIF, respectively, and 95% and 96% for INH compared to the proportion method (PM) in South Africa;[25] 94.5% and 100% for RIF, and 92.7% and 98% for INH compared to PM in India;[30] 100% and 98.9% for RIF, 100% and 99.4% for INH, and 100% and 99.5% for MDR-TB strains compared to the MGIT 960 DST in Vietnam;[31] 95% and 100% for RIF, 100% and 100% for INH compared to MIGT 960 in Brazil;[32] 91.3% and 100% for RIF, and 100% and 90.4% for INH compared to MIGT 960 in Turkey;[49] colorimetric tetrazolium microplate assay (TEMA) was 100% and 86% in detecting RIF resistance, and 100% and 100% for INH compared to LJ in Ethiopia;[50] and MycoTB assay demonstrates improved performance with a sensitivity of 96.2% and specificity of 95%, using a MIC cutoff of 0.5μg/mL for low-level RIF-resistance in New York.[36]

In the present study, two strains with MICs of 0.5μg/mL would not be classified as RIF-resistant by genotypic LPA and phenotypic MIGT at 1.0μg/mL, but they exhibited an equal MIC to the *rpoB* wild-type strains. This implies that the low-level resistance mutations in *rpoB* underscore the value of genotypic methods for diagnosing RIF-resistance, particularly methods that interrogate the full-length *rpoB* gene. The absence of mutation in *rpoB* gene does not rule out the possibility of RIF-resistance by another mechanism, but no other genetic loci involved with RIF-resistance have been well characterized thus far. This might be due to the strains harboring such uncommon mutations within the RRDR in the *rpoB* gene when tested by LPA, results in false-positive predictions of resistance as well as the other mutations that are not targeted by LPA, which may underscore the value of genotypic LPA for identifying RIF-resistance. The relationship between the low-level RIF-resistance disputed variants of the *rpoB* gene and poor patient outcomes as well as the elevated MICs caused by these mutations has led to a recent change in the CCs recommended by the WHO for RIF from 1 to 0.5μg/mL in MGIT 960 DST to involve low-level resistant isolates.[51] However, it is important to appreciate that there are unknown resistance mechanisms, inadequate limits of detection, and epistatic interaction that could all play a role.[7] Therefore, there is a need for WGS with MIC testing that predicts drug susceptibility in a number of phenotypically susceptible isolates and finds disputed mutations conferring low-level drug resistance. Those methods overcame the limitations of CCs MIGT 960 and probe-based genotyping methods and are important to guide the clinical decision-making process and facilitate therapeutic drug monitoring, ultimately leading to a better treatment outcome. In the absence of WGS or MIC testing, most of the mutations that confer low-level RIF resistant isolates may have gone completely undetected, potentially leading to treatment failure and the acquisition of further resistance to drugs.[36]

Strength and Limitation of the Study

To the best of our knowledge, this study is the first to employ resazurin to assess the in vitro susceptibility and antimycobacterial synergistic activity of RIF, INH, EMB and LEV agents in drug-resistant and susceptible *M. tuberculosis* clinical isolates. The REMA/REDCA approach that performed in a single microplate experiment is cost-effective compared to MIGT 960 and checkerboard methods for testing of susceptibility and drug interaction profiles, respectively. Nevertheless, the study has its own limitations. First, the small number of *M. tuberculosis* clinical isolates were evaluated. Second, probe-based *MTBDRplus* assay is limited to common mutations, and there is a need of WGS data analysis. Third, limited classical anti-TB drug combinations and drug–drug interaction performed between the currently available classical FLDs and LEV but unable to incorporate new generation of drugs, second-line injectable agents and certain anti-retroviral drug combinations for TB/HIV co-infected patients. There are reasonable chances of clinically important drug–drug interactions between RIF (CYP enzyme inhibitor) and anti-retroviral drugs (ARVs) that may account for poor MDR-TB/HIV treatment outcomes.[47] Therefore, further investigations on WGS with MIC and FICI testing will be needed that include a large sample size, new anti-TB drugs, and ARVs to allow for personalized treatment of TB patients with HIV co-infection in low-income countries like Ethiopia.

Conclusion

Diagnosing all types of drug resistance patterns can be challenging, particularly when genotypic LPA and phenotypic MIGT 960 DST results are discordant. Detection of isolates with rare mutations that cause diagnostic and treatment challenges by both testing is significant. The MIC determination in REMA assay is powerful to understand the profiles of drug resistance when isolates were phenotypically susceptible and carried unknown mutations. Low-level RIF-resistance *M. tuberculosis* isolates cause diagnostic and treatment challenges and the data related to the low-level RIF-resistant of TB incidences are still lacking. Moreover, over half of MDR-TB isolates had moderate level INH resistant indicates the potential benefit of high-dose INH treatment in a high proportion of *katG* gene harboring MDR-TB isolates if aligned with MIC values. The in vitro synergistic effects of three-drug combination of RIF+INH with LEV showed better efficacy and being of potential use in INH-resistant isolates. The REDCA result makes us hopeful in future investigations to test the synergism of different combination of drugs before formulating new chemotherapy regimens against clinical isolates of *M. tuberculosis* which is inexpensive and the use of resazurin reagent makes the visual reading of the assay to be more reliable. It will be useful for designing and simplifying therapeutic decision-making to optimize regimen efficacy in resistant cases. Due to small sample size, it is necessary to do more research on this method to investigate the application value. Clinical practice should prioritize strategies that promote its reliability.

Abbreviations

DST, drug susceptibility testing; EMB, ethambutol; FICI, fractional inhibitory concentration index; HIV, human immunodeficiency virus; INH, isoniazid; LEV, levofloxacin; LJ, Lowenstein-Jensen; MDR-TB, multidrug-resistant tuberculosis; MIC, minimum inhibitory concentration; RIF, rifampicin; STR, streptomycin; TB, tuberculosis; WHO, World Health Organization; WGS, whole genome sequencing.

Data Sharing Statement

Datasets used in the study are available from the corresponding author upon reasonable request.

Ethical Approval and Consent

The study was ethically approved by the Institutional Review Board (IRB) of the University of Gondar (UoG) (Ref: VP/ RTT/05/829) after reviewing the study proposal. A written letter of support was obtained from the Amhara Public Health Institute (APHI), Bahir Dar branch (Ref: APHI/W/D/03/146). In addition, written informed consent was obtained from all study participants before starting of the study. The ethics was carried out in line with the ethical standards laid down in the Declaration of Helsinki.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors have declared that no competing interests exist.

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