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Analyzing Antibiotic Resistance in Clinical *Mycobacterium tuberculosis* Isolates using Microplate Alamar Blue Assay

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ABSTRACT

Introduction: Tuberculosis, caused by *Mycobacterium tuberculosis*, is one of the most common infectious diseases worldwide. Epidemiological studies of *M. tuberculosis* drug resistance are critical for improving patient treatment approaches and controlling the spread of tuberculosis. The present study aimed to determine antibiotic resistance among *M. tuberculosis* clinical isolates using the Microplate Alamar Blue Assay (MABA). **Methods**: In this descriptive cross-sectional study, 25 *M. tuberculosis* isolates from clinical samples were identified and confirmed using standard microbiological and biochemical tests. Then, the MIC for the antibiotics Bedaquiline, isoniazid, rifampin, ethambutol, ofloxacin, moxifloxacin, capreomycin, and streptomycin was determined using the MABA method. The results were analyzed using SPSS version 16 software. **Results**: Among the 25 investigated isolates, the frequencies for MDR, Pre-XDR, and XDR isolates were 20%, 8%, and 32%, respectively. The highest rate of drug resistance was to isoniazid (80%), rifampicin, and ethambutol (76%), and the highest rate of sensitivity was to moxifloxacin (68%). The frequency of isoniazid mono-resistance and rifampicin mono-resistance was 5 cases (50%) and 4 cases (40%), respectively. **Conclusion:** Our study revealed an alarming rate of MDR and XDR *M. tuberculosis* strains, indicating that current first-line treatments may be ineffective for a significant number of patients. The bedaquiline resistance among the isolates with no history of previous exposure to this drug suggests unexplored resistance mechanisms. Molecular techniques to accurately identify these mechanisms may contribute to developing more effective treatment strategies to combat drug-resistant tuberculosis.

INTRODUCTION

Tuberculosis (TB) is a globally prevalent infectious disease caused by *M. tuberculosis* [1, 2]. Approximately one-third of the world's population is affected by this disease [3, 4]. According to the WHO report, the number of tuberculosis patients in 2022 was approximately 10.6 million. Of these, 1.4 million and 187,000 were among HIV-negative and HIV-positive people, respectively. Most cases and deaths from tuberculosis occur in developing countries [5, 6]. Tuberculosis is a leading cause of infectious disease death worldwide, including among people with HIV [7-9]. Despite advances in medicine and the efforts of international organizations, tuberculosis remains one of the most life-threatening diseases in the world [10]. In 2019, tuberculosis incidence in Iran was 14 (10-17) per 100,000 people. In some countries bordering Iran, including Afghanistan and Pakistan, the prevalence is two to three times higher than this figure [11]. Drug-resistant tuberculosis is a major global public health problem, fueling the ongoing tuberculosis epidemic and increasing tuberculosis mortality and morbidity worldwide. One of the essential features of *M. tuberculosis* is a thick, lipid-rich cell wall, which limits the penetration of antibiotics into bacterial

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cells, making it inherently resistant to many antibiotics [12].

The current treatment regimen for drug-sensitive tuberculosis includes the four first-line medications, i.e., isoniazid, ethambutol, rifampin, and pyrazinamide for two months, followed by the combination of isoniazid and rifampin for at least four months [13]. One of the essential factors in anti-tuberculosis treatment failures is the patient's non-adherence to treatment plans, which is due to the extended treatment duration and the complexity of the treatment regimen, along with the many adverse effects of drugs [14, 15]. Multidrug-resistant TB (MDR-TB) is resistant to at least two major first-line drugs (INH and RIF), pre-XDR-TB is resistant to both drugs and fluoroquinolones, and XDR-TB is the MDR-TB, which is also resistant to all fluoroquinolones and at least one other Group A drug (levofloxacin, moxifloxacin, bedaquiline, linezolid) [16, 17]. Worldwide, 3.5% of new TB cases and 18% of previously treated TB cases are MDRs. Currently, multidrug-resistant TB (MDR-TB) cases are reported from all countries worldwide. Since 2005, Extensive resistant tuberculosis (XDR) has been reported in some parts of the world, especially among AIDS patients. Various methods are used to test the drug susceptibility of *M. tuberculosis*, including the Lowenstein Jansen (LJ) ratio method, the BACTEC 460 system, and Alamar blue (a colorimetric method). The proportional method and the BACTEC system are both labor-intensive and expensive methods. Alamar blue is a redox indicator that is blue in its oxidized state and turns pink when reduced by bacteria. Therefore, when bacteria grow in Middle Brook 7H9 liquid medium containing Alamar blue and antibiotics, the color of the nutrient medium changes from blue to pink, indicating the bacteria's resistance to antibiotics. Otherwise, when the bacteria are sensitive to the drug, there is no change in the color of the nutrient medium [18, 19].

Due to growing concern over antibiotic resistance amongst TB cases and the urgent need for more effective treatment regimens, this study aims to elucidate the MICs of diverse antibiotics against *M. tuberculosis* clinical isolates. The results will allow us to adopt efficacious treatment strategies to combat resistant *M. tuberculosis* isolates.

MATERIAL AND METHODS

Bacterial strains. This retrospective study examined 25 clinical isolates from the Tuberculosis and Lung Research Department of the Pasteur Institute from 2017 to 2022. Strain H37Rv (ATCC27294) was used as the reference strain. The Ethics Committee of the Pasteur Institute of Iran (IR.PII.REC.1399.054) approved this study, and written consent was obtained from participants. All clinical strains were cultured on Lowenstein-Johnson medium and stored at 37°C for 4–6 weeks. The strains were identified using standard microbiological and biochemical methods, including Ziehl-Neelsen staining, niacin, catalase, and nitrate tests.

Determination of MIC of investigated antibiotics. A microplate Alamar Blue assay (MABA) was performed to determine the MICs for clinical isolates. Alamar Blue was purchased from AbD Serotec (Oxford, UK). Bedaquiline (BDQ), isoniazid (INH), rifampin (RIF), ethambutol (EMB), ofloxacin (OFX), moxifloxacin (MFX), capreomycin (CAP), and streptomycin (STR) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All solutions were prepared on the day of the experiment. MIC was defined as the lowest drug concentration that prevented color change. According to the results of other studies, the antibiotics concentration range used in this study included 0.125–128 μg/mL for BDQ, EMB, RIF, INH, STR, and CAP, and 0.0078–16 μg/mL for MFX and OFX. Isolates with MICs of INH <0.25 μg/ml, RIF <1 μg/ml, BDQ ≤0.25 μg/ml, EMB ≤2.5 μg/ml MFX and OFX ≤ 2 μg/ml, STR and CAP ≥2.0 μg/ml were defined as susceptible. All the tests were performed at least in duplicate to calculate the mean MIC for each strain. Initial stock solutions of antibiotics were prepared in dimethylsulfoxide (DMSO), with a final concentration of <0.1% to avoid bacterial toxicity. Briefly, fresh bacterial clones were picked from Löwenstein–Jenson slants. The turbidity of each suspension was adjusted to match the McFarland standard of 1.0 (3×10^8 CFU/ml). After dilution with Middlebrook 7H9 culture medium (Sigma Aldrich, St.Louis, MO, USA), supplemented with 10% albumin dextrose catalase (Becton Dickinson, Oxford, UK), amounts of 100 μL were added to the 96 wells of a microplate plate containing corresponding drugs. Each microplate included a positive control well containing the bacterial suspension and a negative control well containing each antibiotic and culture medium. After incubation for seven days, Alamar Blue reagent was added to wells. After 24 h of incubation at 37°C, bacterial growth was evaluated by the color change.

Statistical analysis. The results were analyzed using SPSS version 16 software.

RESULTS

Of the 25 investigated isolates, 15 were MDR and XDR, and 10 were antibiotic-sensitive. The frequency of MDR, Pre-XDR, and XDR isolates was 20% (n=5), 8% (n=2), and 32% (n=8), respectively. Of the 25 isolates, only 10 (40%) were sensitive to bedaquiline, and the other 15 (60%) isolates were resistant to this medication with MICs ≥ 0.25 μg/mL. (Table 1). Among the remaining ten isolates, the highest resistance levels were to streptomycin (60%), isoniazid (50%), and bedaquiline (50%), and the highest sensitivity among the isolates was to moxifloxacin (90%) (Table 2). The frequency of isoniazid mono-resistance and rifampicin mono-resistance was 50% (n=5) and 40% (n=4), respectively. The MIC results of some of the studied antibiotics are shown in Figure 1.

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Table 1. MIC results for antibiotics used against MDR *M. tuberculosis* isolates.

No.	INH (µg/mL)	RIF (µg/mL)	EMB (µg/mL)	MFX (µg/mL)	OFX (µg/mL)	BDQ (µg/mL)	STR (µg/mL)	CAP (µg/mL)
S-1	1	32	16	0.5	2	1	2	4
S-2	8	16	4	0.25	1	0.25	1	2
S-3	32	32	8	1	0.5	0.125	2	1
S-4	8	64	4	2	1	8	1	1
S-5	16	64	32	1	8	0.5	4	16
S-6	1	64	16	0.5	1	1	2	2
S-7	8	32	64	4	0.5	0.125	1	4
S-8	64	128	64	4	4	8	8	32
S-9	64	64	32	1	1	16	16	1
S-10	64	128	16	1	4	8	4	4
S-11	8	4	8	0.25	16	0.125	8	16
S-12	32	32	8	4	1	2	2	32
S-13	8	32	32	0.5	1	0.125	1	1
S-14	4	64	32	4	0.25	0.25	4	8
S-15	16	64	32	1	4	8	32	4
H37Rv	0.25	0.5	0.5	0.25	0.5	0.125	1	1

BDQ: Bedaquiline, INH: Isoniazid, RIF: Rifampsin, EMB: Ethambutol, OFX: Ofloxacin, MFX: Moxifloxacin, CAP: Capreomycin, STR: Streptomycin

Table 2. MIC results for antibiotics used against non-MDR *M. tuberculosis* isolates.

No.	INH (µg/mL)	RIF (µg/mL)	EMB (µg/mL)	MFX (µg/mL)	OFX (µg/mL)	BDQ (µg/mL)	STR (µg/mL)	CAP (µg/mL)
S-16	0.25	4	1	0.5	2	0.0312	0.5	0.5
S-17	0.125	2	4	2	1	0.125	1	2
S-18	4	0.5	0.5	1	2	0.25	0.5	0.25
S-19	0.25	2	2	2	1	0.0625	1	1
S-20	4	0.125	8	2	0.5	0.5	4	1
S-21	1	0.5	16	0.5	4	1	0.5	2
S-22	0.125	2	0.5	0.25	2	0.0625	4	0.25
S-23	2	0.25	1	4	4	8	8	1
S-24	8	0.5	4	1	0.25	0.125	16	2
S-25	0.125	0.25	2	0.5	4	0.0625	0.5	0.5
H37Rv	0.25	0.5	0.5	0.25	0.5	0.125	1	1

BDQ: Bedaquiline, INH: Isoniazid, RIF: Rifampsin, EMB: Ethambutol, OFX: Ofloxacin, MFX: Moxifloxacin, CAP: Capreomycin, STR: Streptomycin

DISCUSSION

Antibiotic-resistant *M. tuberculosis* has become a significant challenge worldwide due to the limited available effective drugs. Here, we investigated 25 *M. tuberculosis* clinical isolates, of which 15 had multidrug resistance, and the remaining showed varying degrees of drug resistance. The highest drug resistance rates belonged to isoniazid (80%), rifampicin, and ethambutol (76%), while the highest susceptibility rates were to moxifloxacin (68%) and ofloxacin (60%). Among the 25 isolates, the frequencies for MDR, Pre-XDR, and XDR isolates were 20% (n=5), 8% (n=2), and 32% (n=8).

In Isfahan, Iran, the resistant rates to isoniazid, rifampicin, and ethambutol among the clinical isolates were 66.7%, 54.5%, and 12.1%, respectively [20]. In another study in Tehran, the resistance rates to isoniazid and rifampin were 27.3% and 22.7%, respectively, with an MDR rate of 22.7%, consistent with our results (20%) [21]. In a survey in Isfahan, Iran, more than 71% of examined isolates were resistant to isoniazid, similar to our results. However, the rifampin level of resistance in our study was higher (76%) than their report (40.6%) [22]. Although all isolates tested in our research were naïve to the antibiotic bedaquiline, approximately 60% were resistant to bedaquiline with a MIC ≥0.25 µg/ml.

This high level of drug resistance can be seen as a severe problem and a warning for future tuberculosis treatment. Other studies have reported varying rates of antibiotic resistance to bedaquiline. In the study by Ghajavand *et al*. (2019), more than 62% of isolates of this bacterium were resistant to bedaquiline, which was consistent with our results [23]. Drendinger *et al*. (2022) reported bedaquiline resistance in 47% (24/51) of drug-

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resistant tuberculosis patients in South Africa [24], while this rate among XDR isolates in China was 16.7% [25].

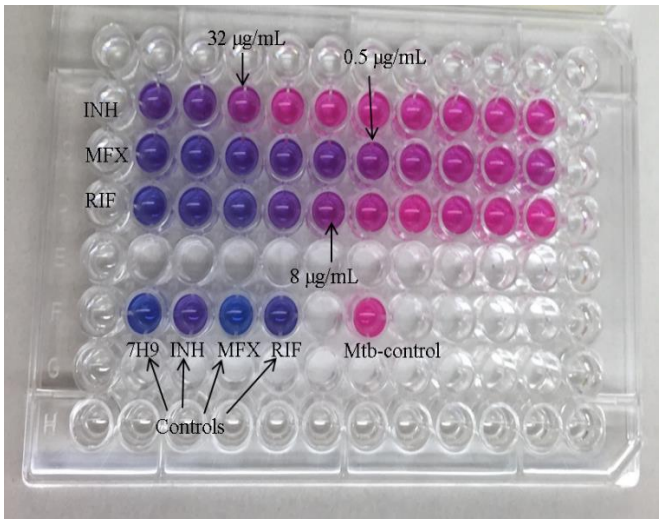

Fig. 1. MIC results for isoniazid (INH), rifampin (RIF), and moxifloxacin (MFX) antibiotics against *M. tuberculosis* isolates.

Fluoroquinolones are among the most widely used antibiotics to treat bacterial infections and are still used as anti-tuberculosis drugs. However, their indiscriminate use has led to the development of resistance in *M. tuberculosis* clinical isolates. One of the characteristics of drug-resistant tuberculosis is resistance to fluoroquinolones [26]. In our study, the resistance rate to fluoroquinolones was 36% (MFX: 32%, OFX: 40%). Other studies have reported variable rates of fluoroquinolone resistance among *M. tuberculosis* isolates. In a Sayadi et al. (2020) survey, 4.8% of Mtb isolates were resistant to fluoroquinolones [27]. In Pakistan, Kabir *et al*. (2020) reported that the frequency of Mtb isolates resistant to fluoroquinolones was 18.5% [28]. In China, Pang *et al*. (2017) reported a fluoroquinolone-resistant rate of 87.5% among Mtb isolates [29]. However, in Ethiopia, only 2 (5.3%) of 38 MDR isolates were resistant to fluoroquinolones [30].

The limited *M. tuberculosis* isolates were the restriction of this study. The differences in the results of the drug resistance patterns of these bacteria include geographical differences and specific characteristics of drug susceptibility patterns in each region. The variation in drug resistance patterns in geographical areas may be due to differences in TB monitoring, prevention, and control programs. Our results showed high MDR and XDR rates among *M. tuberculosis* isolates. One exciting aspect of our study was a high bedaquiline-resistant rate among mycobacterial isolates without previous exposure to this antibiotic. Given the limitations of phenotypic studies to identify mechanisms driving bacterial resistance to antibiotics, molecular techniques are needed to identify these resistance mechanisms accurately.

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AF supervised the project. AF, SDS, and FV designed the project. KA and NM-G wrote the manuscript. KA, NM-G, and MM performed the work in the laboratory. NM-G and MN performed the statistical analysis. All authors have read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare no conflicts of interest associated with this manuscript.

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