



Evaluation of Antibiotic Resistance in *Mycobacterium tuberculosis* Clinical Strains by Culture-Based Antibiogram, Target Gene Sequencing, and Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) Assay

Samer Montazeri¹, Fariba Sharifinia^{1*}, Ahmad Majd², Alireza Ghasempour³, Abbas Akhavan²

Abstract

Objectives: The identification and characterization of *Mycobacterium tuberculosis* isolates is very time-consuming, which causes a delay in timely treatments. Apart from diagnosis, the classical culture-based antibiogram for the characterization of bacterial drug resistance is very time-consuming, resulting in the development of alternative rapid, easy, and reliable techniques.

Materials and Methods: Matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) has been recently introduced as a novel technique for identifying microbial pathogens. However, the characterization of bacterial drug resistance by MALDI-TOF MS assay has rarely been studied. This work was performed to characterize drug-resistant *M. tuberculosis* strains using classical antibiogram, molecular, and MALDI-TOF MS assays. Pathogenic *M. tuberculosis* strains were isolated by culturing clinical specimens in Löwenstein–Jensen medium and identified by amplification of IS6110. Drug resistance was characterized using classical antibiogram, sequencing of the *rpoB*, *gyrA*, *katG*, and *rrs* genes, and analyzing the MALDI-TOF MS spectrograms of bacterial cell wall components.

Results: The highest resistance was observed for capreomycin and ciprofloxacin (48%), followed by isoniazid (42%). Sequencing of drug target genes showed that point mutations at codons 507 of *rpoB*, 315 of *katG*, 284 of *gyrA*, and 1260 of *rrs* genes were the major molecular causes of resistance to Rifampin, Ethambutol, Ciprofloxacin, and Kanamycin, respectively.

Conclusions: The MALDI-TOF MS assay revealed that bacterial strains have different spectrograms based on their drug resistance phenotypes. Therefore, this work revealed that the MALDI-TOF MS assay could be considered a novel and rapid technique to characterize the drug resistance pattern of *M. tuberculosis*.

Keywords: MALDI-TOF, *Mycobacterium tuberculosis*, Drug resistance, Antibiogram

Introduction

Tuberculosis (TB) is a global health concern caused by an airborne infection of *Mycobacterium tuberculosis*. The disease has been a global health threat since ancient times, and after the emergence of AIDS in 1981, the annual morbidity and mortality of TB increased. With 10.6 and 1.6 million morbidity and mortality cases in 2021, TB is regarded as a major health problem worldwide (1). Apart from the virulence of *M. tuberculosis*, the emergence of drug-resistant strains has increased the complexity of current anti-TB treatments, which results in therapeutic failure. Many mechanisms are associated with the drug-resistant phenotype in *M. tuberculosis* strains; however, gene mutations and low drug permeability, which arise under selective pressure, have been introduced as the main causes of drug resistance (2).

The resistance mechanisms of *M. tuberculosis* to the common anti-TB drugs can be divided into two categories, including intrinsic and acquired drug resistance.

Although acquired resistance through genetic alterations is an important factor in the emergence of drug resistance, intrinsic drug resistance mechanisms play a key role in the therapeutic failure of *M. tuberculosis* infections. Intrinsic drug resistance mainly relies on the low permeability of the bacterial cytoplasmic membrane and cell wall to uptake antimicrobial drugs. Phenotypic resistance to common anti-TB therapeutic agents is caused by a combination of factors, including the overexpression of transporter proteins that aid in the extrusion of antibiotics from the bacterial cytoplasm and low drug permeability associated with the characteristics of the bacterial cell wall, which contains a complex structure of mycolic acid (3).

There are several approaches for identifying drug-resistant *M. tuberculosis*. *Phenotypic identification* is an accurate and easy method based on isolating the tubercle bacilli and performing drug susceptibility testing (4). However, isolation and identification of *M. tuberculosis* and characterization of its antibiotic susceptibility pattern



Key Messages

- ▶ Conventional methods for rapid detection of mycobacterium cells are time-consuming and have a sensitivity of 90-85% in the molecular field.
- ▶ MALDI-TOF through protein extraction is a fast, economical for fast detection of MTB, to prevent the spread of drug-resistant strains.
- ▶ Conventional methods for rapid detection of mycobacterium cells are time-consuming and have a sensitivity of 90-85% in the molecular field.
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take several weeks due to the slow growth rate of the microorganism (5). Clinical results for *M. tuberculosis* can take up to six to eight weeks to produce, delaying patient management and therapeutic approaches. In addition, molecular detection of drug resistance could be investigated by replication and sequencing of candidate target genes to seek any possible mutations (6). Although this method could detect the main cause of drug resistance at the molecular level, the assay is time-consuming, expensive, and difficult to adapt to clinical laboratories.

Matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) provides a simple, reliable, and rapid way to identify or characterize large groups of bacterial pathogens, including *M. tuberculosis* (7). This technique is a Mass Spectrometry analysis in which a unique mass spectral fingerprint of the microorganisms is produced based on the detection of the mass-to-charge ratio (m/z) of microbial components and the generation of specific and characterizing spectra (8). The mass spectra consist of a spectrogram of m/z values determined by laser sample spot scanning. Bacterial identification could be achieved by comparing the mass fingerprint with a database including spectra from all relevant microorganisms (9).

MALDI-TOF MS provides a short and reliable approach for identifying bacterial species, which has considerable advantages over the classical culture methods. This method also applies to clinical specimens and can be used for bacterial culture. MALDI-TOF could also be adapted to characterize bacterial virulence traits, including antibiotic resistance (10). The current study aims to characterize *M. tuberculosis* cell wall components using the MALDI-TOF MS assay and associate it with bacterial drug resistance compared to classical antibiogram and gene sequencing assays.

Materials and Methods

Samples and Bacterial Isolation

Clinical specimens (sputum, gastric lavage, urine) were obtained from those suspected of *M. tuberculosis* infection and referred to clinical centers in different cities of Iran for three years (2018-2021). Consent forms were obtained

from the individuals prior to participation in the study. Smears of clinical specimens were prepared on laboratory slides, decontaminated using N-acetyl-L-cysteine (NALC)-NaOH solution, and subjected to Ziehl-Neelsen staining. Also, the specimens were inoculated to Löwenstein-Jensen medium and incubated for 45 days. Bacterial growth was monitored by measuring fluorescent rays at 365 nm with the BACTEC MGIT320 device. Any positive bacterial growth was further examined by Ziehl-Neelsen staining and then subjected to a screening assay using BD MGIT™ TBc ID test to differentiate *M. tuberculosis* from non-TB mycobacteria. Biochemical identification of the isolates was performed by catalase, detection of niacin in medium, and nitrate reduction assay (11).

Molecular Identification of the Isolates

Bacterial DNA was extracted using a Roche DNA extraction kit, according to the manufacturer's protocol, and then quantified using a Nanodrop spectrophotometer. Molecular identification was performed based on the detection and amplification of the insertion sequence 6110 (IS 6110). According to the manufacturer's instructions, the amplification was performed using the DNA technology PCR kit (Russia). The presence of IS6110 fragments was confirmed by detecting 113 bp products of PCR by agarose gel electrophoresis.

Antibiogram

A drug susceptibility test was performed: suspensions of bacterial strains with an approximate population equivalent to 0.5 McFarland solution were prepared. The suspensions were inoculated in the growth medium containing different antibiotics. The studied antibiotics include isoniazid (0.2 µg/mL), rifampicin (40 µg/mL), ethambutol (2 µg/mL), kanamycin (30 µg/mL), amikacin (30 µg/mL), capreomycin (40 µg/mL), ciprofloxacin (2 µg/mL), and ofloxacin (2 µg/mL). The tubes were incubated for 4-5 weeks at 37°C and monitored for bacterial growth. *M. tuberculosis* H37Rv and XDR were used as standard susceptible and resistant strains, respectively, and tube without antibiotics was considered negative control. The resistance to the studied antibiotics was determined using the following formula:

$$\text{Drug resistance (\%)} = \frac{\text{number of colonies in the medium containing antibiotic}}{\text{number of colonies in control medium}} \times 100$$

Amplification and Sequencing of Bacterial Target Genes Associated With Drug Resistance

Bacterial DNA was extracted, and the *rpoB*, *katG*, *gyrA*, and *rrs* genes were amplified using gene-specific primers in a PCR assay. The sequence of the primers used in this study is presented in Table 1. The PCR products were subjected to agarose gel electrophoresis to confirm gene amplification, and finally, the amplified fragments were sequenced by the Sanger method.

Table 1. Sequence of the Primers Used in This Study

Primer	Sequence (5'-3')	Product Length (bp)
<i>rpoB</i> -forward	TACGGTCGGCGAGCTGATCC	411
<i>rpoB</i> -reverse	TACGGCG TTTCGA TGAACC	
<i>katG</i> -forward	GAAACAGCGCGCTGGATCGT	209
<i>katG</i> -reverse	GTTGTCCCATTCGTCGGGG	
<i>gyrA</i> -forward	CCCTGCGTTCGATTGCAAAC	530
<i>gyrA</i> -reverse	CTTCGGTGTACCTCATCGCC	
<i>rrs</i> -forward	GTCCGAGTGTTCCTCAGG	516
<i>rrs</i> -reverse	GTCAACTCGGAGGAAGGTGG	

Protein Extraction

Bacterial proteins were extracted using an ethanol-formic acid method with a MycoEX 3.0 commercial kit, following the instructions by the manufacturer. In brief, a suspension of bacterial cells was prepared in 300 μ L of distilled water, incubated for 30 minutes to inactivate bacteria, and 900 μ L of 96% ethanol was added. After centrifugation for 2 minutes, Bartlesville BioSpec product (0.5 mm in diameter) and 20 μ L acetonitrile were added to the bacterial pellet. Next, 70% formic acid (Sigma-Aldrich) was added, vortexed for 2 seconds, and finally, the suspension was centrifuged for 2 minutes.

MALDI-TOF MS Assay

One microliter of extracted protein was placed on the sample loading plate of the MALDI-TOF MS device (Applied Biosystems 4800, Nd: YAG, 200-Hz laser) and left to dry. Then, 1 μ L of matrix solution containing beta-Cyano-4-hydroxycinnamic acid in 50% acetonitrile was added and left to air-dry. An *Escherichia coli* extract (Bruker Daltonics) was used to calibrate the device and as control. Finally, the spectra were obtained in a mass range of 800-4000 Dalton and were analyzed by the Data Explorer software (Applied Biosystems). The assay was performed in two replicates.

Results

Bacterial Strains

A total number of 3985 individuals participated in this study. A total of 60 positive samples were identified, wherein certain strains resisted multiple drugs. Finally, 23 drug-resistant strains were used for molecular and MALDI-TOF MS assays.

The identity of bacterial strains was confirmed by growth characteristics and Ziehl-Neelsen staining that indicated the presence of acid-fast bacilli (Figure 1a, b). Furthermore, the molecular identification assay based on the amplification of IS6110 revealed the identity of isolates as *M. tuberculosis*. Figure 1c shows agarose gel electrophoresis of the amplified IS6110 fragments.

Classical Antibigram Test

Antibiotic susceptibility profile of the strains for the 1st line treatment drug (isoniazid, rifampin, and ethambutol)

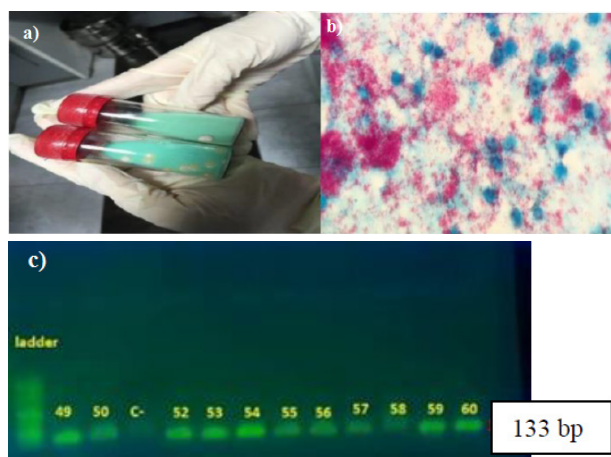


Figure 1. (a) Bacterial growth on Löwenstein-Jensen medium, (b) Ziehl-Neelsen staining, (c) amplification of IS6110. The presence of 133 bp fragments associated with IS6110 confirms the identity of *M. tuberculosis*.

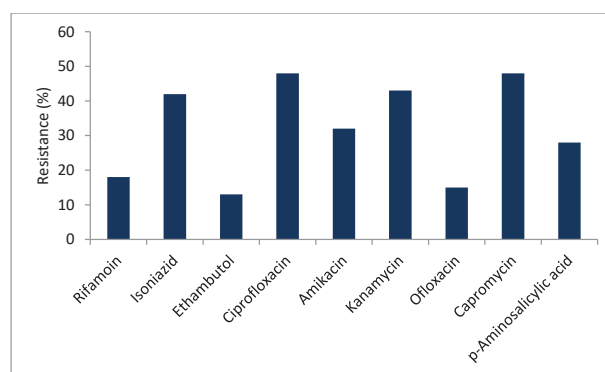


Figure 2. Antibiotic resistance of *M. tuberculosis* strains.

and 2nd line treatment drugs (ciprofloxacin, kanamycin, amikacin, capreomycin, ciprofloxacin, and ofloxacin) was determined according to the WHO guideline.

The highest resistance was observed for ciprofloxacin (48%) and capreomycin (48%), followed by isoniazid (42%). In contrast, most strains were susceptible to ofloxacin (85%). Figure 2 displays the overall antibiotic susceptibility percentage of the isolated bacteria.

Point Mutations Associated With Drug-Resistance

The frequency of mutations causing drug resistance within the *gyrA*, *katG*, *rpoB*, and *rrs* genes was investigated by PCR assay and subsequent sequencing of the amplified fragments. The results are presented in Table 2. Mutations in the *rpoB* gene could be associated with resistance to Rifampin. According to the results, out of 60 *M. tuberculosis* strains, 12 strains had a nucleotide substitution at codons 507, 521, and 523 of the *rpoB* gene, and mutation at codon 507 was the most prevalent mutation observed in seven strains. Point mutations at codon 521, a silent mutation, were observed in two strains, and three strains showed point mutation at codon 523.

Mutations in the *katG* gene may result in resistance to ethambutol. Considering the sequence of the *katG* gene

Table 2. Point Mutations Associated With Drug Resistant

Codon	Frequency	Nucleotide Substitutions	Amino Acids Substitutions	Strain Code
rpoB				
507	7	GGC→AGC	Gln→Ser	A1/3-6, A1/7-10, B2/4-6, C18/7-8, B5/6-7, B13/10 C17/1-2
521	2	CTG→TTG	Leo→Leo	B15/5, B13/1-5
523	3	GGG→GCG GGG→GGC GGG→GGA	Gly→Ala Gly→Gly Gly→Gly	C12/1-4, A2/4-6, C18/5-6
katG				
315	14	AGC→ACC	Ser→ Thr	A1/2-1 A2/4-6 B2/1-3 B2/4-6 B13/1-5 C18/5-6
		AGC→AAC	Ser→ Asn	B4/1-6 D1/2-5 C12/1-4 C18/7-8
		A→G	Ser→ Gly	B15/4 B16/10
		A→G	Ser→ Arg	B16/8 B15/10
		G→C, G→C	Gly→Ala, Ser→Thr	B5/8 B9/4-5
		G→T, T→C, G→C	Gly→ Cys, Phe, Ala, Ser→ Thr	B13/10 C18/1-2
314,315	1	C→A, G→C	Thr→Asn, Ser→Thr	B22/1-4
311,315	2	AC→TTC, TA, G→C	Asn→Phe, Tyr, Ser→Thr	B5/1-5 C17/1-2
315,316	1	AGC→ACC, AGG, GGC→AGC	Ser→ Thr, Arg, Gly→ Ser	C6/5
357,463	1	G→CAC, G→T	Asp→His, Arg→Leu	C6/9
357,454	1	G→A, GAG→CGA	Asp→ Asn, Glu→ Arg	C8/5-7
309,311, 315	1	G→C, GAC→TTC, G→C	Gly→ Ala, Asn→ Phe Ser→ Thr	B6/3-4
307,309, 315	1	G→C, T→G G→C	Gly→Arg, Gly→Gly Ser →Thr	D1/6-8
305,315, 321	1	G→C, G→C G→T	Gly→ Ala, Ser→ Thr Trp→ Leu	B15/5
gyrA				
284	11	G→C	S→T	A2/4-6, B2/1-3, B2/4-6 B5/9, B3/1-4, B5/6-7 B15/4, B9/1-3, B9/4-5 B15/5, B13/7
61	6	G→C	E→Q	B6/3-4, D1/9-10, B12/7, B12/5-6, B12/3-4, B11/7-8
281	2	A→G	-	B15/7, B13/1-5
262	3	G→T	-	B15/9, B15/8 B6/1-2
84	1	C→A	-	B15/10
107	1	T→A	D→Y	B16/8
109	1	G→C	-	B16/10
118	1	G→C	-	B13/8
55	1	A→G	-	C1/3-4
57	1	C→A	-	B5/1-5
rrs				
1260	4	G→A	-	C8/5-7, B6/1-2 C11/1-5, C17/1-2
1278	3	A→T	-	C17/3-4, D1/6-8 B22/1-4
1279	3	A→T	-	B13/10, B16/10 B16/8
1300	3	C→T	-	B15/10, B13/1-5 B12/7
1321	3	G→A	-	B12/5-6, B12/3-4 B15/5
1445	3	C→T	-	B9/4-5, B9/1-3, B15/4
1167	1	G→A	-	C12/1-4
1401	2	A→G	-	D1/2-5, B2/7-9
1181	1	T→A	-	B2/4-6
1282	1	G→A	-	B2/1-3
1381	1	C→T	-	A1/7-10

in 60 isolates, 27 strains (45%) showed variations in the gene sequence. The codon 315 was the most variable site showing point mutation in 14 strains. The amino acid substitutions at this codon were Ser → Thr (n=6), Ser → Asn (n=4), Ser → Gly (n=2), and Ser → Arg (n=2). Ten strains showed double point mutation, and three had triple point mutation.

A mutation in the *gyrA* gene related to ciprofloxacin resistance, was found in 28 mutant strains. Among these strains, the most common nucleotide substitution was a G→C mutation at codon 284 (n=11), followed by a G→C substitution at codon 61 (n=6).

Point mutations in the *rrs* gene, which cause kanamycin resistance, were observed in 25 strains (42%). Among them, a substitution mutation at codon 1260 (G→A) was the most common mutation (n=3), followed by A→T 1278, A→T 1279, C→T 1300, G→A 1321, and C→T 1445, each of which had a frequency of 3 out of 25.

MALDI-TOF MS Assay

Cell wall proteins of 23 *M. tuberculosis* strains resistant to the first and second treatment line drugs were compared with drug-susceptible and a standard strain by MALDI-TOF assay. Since genetic changes associated with drug resistance can cause changes in cell wall proteins of different bacterial strains, diverse MALDI-TOF patterns based on bacterial drug resistance patterns can be expected. The results showed different graphical algorithms and, thus, different peaks in different drug-resistant strains of *M. tuberculosis*. Figure 3 presents the MALDI-TOF spectrogram of the standard, and some isolated *M. tuberculosis* strains.

Discussion

The annual incidence of *M. tuberculosis* infection has been increasing in recent years, and immediate diagnosis and characterization of *M. tuberculosis* infection are urgently required. In addition, rapid drug-resistant *M. tuberculosis* testing could improve anti-tuberculosis approaches and provides shorter characterization and more efficient treatment strategies. In addition, timely drug resistance testing could reduce the risk of the spread of drug-resistant strains. Molecular methods are considered novel and rapid techniques for genotyping of drug resistance. However, their limitations include high costs and the need for advanced instruments that reduce their extensive application in biomedical laboratories (12). In contrast, MALDI-TOF MS is a novel and reliable tool for identifying bacterial pathogens, providing a bacterial diagnosis on time, especially for slow-growing microorganisms (10). Numerous studies have utilized the MALDI-TOF MS assay to detect *M. tuberculosis* strains from clinical samples (10,13). However, less research has been conducted on the rapid drug-resistance testing of *M. tuberculosis* strains. Therefore, *M. tuberculosis* strains were isolated from infected individuals and identified using

biochemical and molecular assays. Their drug resistance profile was characterized by culture-based antibiogram, sequencing of antibiotic target genes, and MALDI-TOF MS assay.

Antibiotic resistance testing by culture-based method showed that 20% (12/60) and 45% (27/60) of isolates were resistant to rifampin and, isoniazid, respectively. In addition, 11.6% (7/60) and 48% (29/60) of the strains were resistant to ethambutol and ciprofloxacin, respectively. Point mutations are considered the main cause of resistance to the mentioned antibiotics in *M. tuberculosis*. The genes encoding antibiotic target proteins were amplified and sequenced to identify the possible mechanism of drug resistance to rifampin, ethambutol, isoniazid, and ciprofloxacin. We found that a nucleotide substitution and, subsequently, amino acid substitution at codon 507 of the *rpoB* gene is the most prevalent mutation associated with resistance to Rifampin. Makadia et al (14) reported that mutations in the codon 507-533 region are associated with Rifampin resistance, which agrees with our results. However, they indicated that codon 531 was the highest variable site.

In 2016, Şamlı and İlki (15) studied MALDI-TOF MS' performance for identifying MTB and NTM mycobacteria compared with nucleic acid hybridization and MPT64 immunochromatographic test. Among the 69 isolates, MALDI-TOF MS identified 88% of the isolated mycobacteria. All strains of *M. tuberculosis* were correctly identified, but it was 38.5% compared to NTM. *Mycobacterium* identification using MALDI-TOF MS takes 45 minutes. The study involved the analysis of 20 selected samples of *Mycobacterium tuberculosis*. After confirming the mutations, the MALDI-TOF test was conducted. The results indicated that compared to the standard sample and various types of resistance, the MALDI-TOF detection was confirmed through graphical representation.

Sequencing of the *katG* gene among Isoniazid-resistant strains revealed that the point mutation in codon 314 is highly frequent in resistant strains. Similarly, Gupta et al (16) reported mutation at codon 314 of the *katG* in 82.95% of Isoniazid-resistant *M. tuberculosis* strains which agrees with our study. Also, mutation at codon 248 of the *gyrA* was highly associated with Ciprofloxacin resistance, while various point mutations in the *rrs* gene were related to Kanamycin resistance.

Characterizing drug-resistant strains by MALDI-TOF MS assay revealed different spectrograms for resistant *M. tuberculosis* strains. The findings indicate variations between the graphical algorithms and their associated peaks in clinical *M. tuberculosis* and the standard strain. Genetic changes cause the expression and production of new proteins, which will change the cell wall proteins of *M. tuberculosis*. This finding reveals the necessity and needs to prepare a protein library. Recent studies found that the MALDI-TOF method can distinguish non-tuberculous

mycobacteria from *M. tuberculosis* by providing a protein library. This technique has also the potential to distinguish drug-resistant and susceptible *M. tuberculosis* species based on developing a protein library.

We found that drug-resistant strains have different MALDI-TOF patterns, which could be mainly due to their cell wall lipid and glycoprotein content. Few MALDI-

TOF studies are available to characterize *M. tuberculosis* components and drug resistance.

Wu et al (17) performed a nucleotide MALDI-TOF study on *M. tuberculosis* strains to predict their drug resistance pattern and found the overall sensitivity and septicity of their assay were 92.2 and 100% for rifampin, 90.9% and 90.6% for isoniazid, 71.4 and 81.2% for

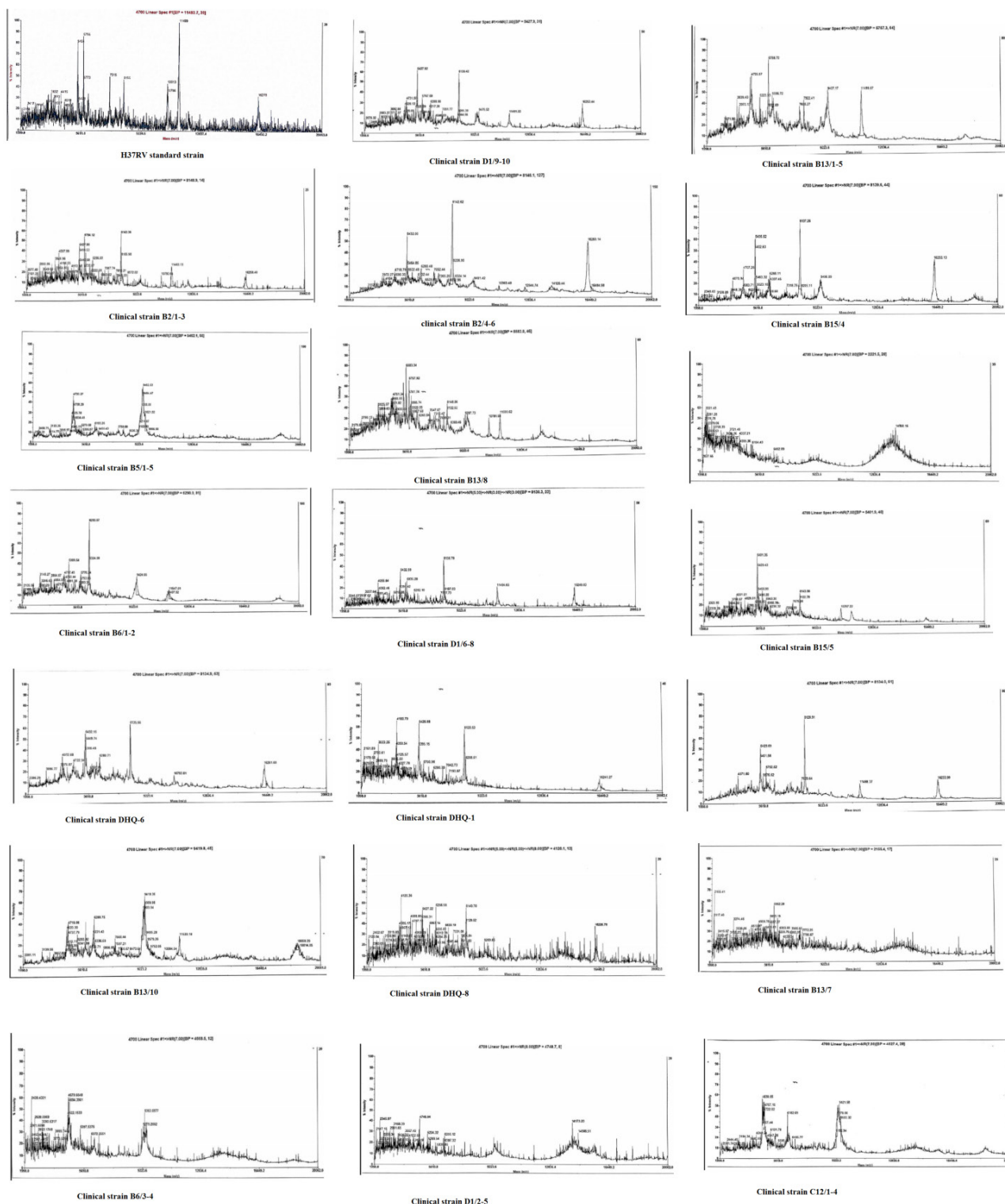


Figure 3. MALDI-TOF MS Spectrograms for the Standards and Some Clinical *M. tuberculosis* Strains. The results showed that there are different graphical algorithms and thus, different peaks in different drug-resistant strains of *M. tuberculosis* which are related to their different drug resistance pattern.

ethambutol, 77.8 and 99.3% for kanamycin, respectively. Their results showed that this assay is reliable for predicting antibiotic resistance in *M. tuberculosis* strains. In this work, we observed that the spectrograms of the MALDI-TOF assay in drug-susceptible strains were considerably different from those for antibiotic resistant ones. Although due to the lack of a proteomic database for *M. tuberculosis*, the association between the peaks and resistance to the studied antibiotics was not investigated. Our results showed that the characterization of bacterial protein could be considered a promising candidate to determine drug resistance.

The most mycobacterial MS peaks were observed at m/z values of less than 15 kDa. The differences between the observed peaks were mainly in their m/z values and not in the absence/presence of strain-specific peaks. Minor differences were observed for the peaks with the m/z range of less than 5 kDa, suggesting that these peaks are associated with the mycolic acid and conserved lipid components of the bacterial cell wall. However, the signals observed in the mass range of 5-10 kDa are associated with bacterial proteins, which showed a moderate variation between the strains in case of their intensity and possibly expression level. Furthermore, different MALDI-TOF MS spectrograms were observed for drug-resistant *M. tuberculosis* strains. In *M. tuberculosis*, resistance to different antibiotics occurs via different mechanisms, mainly reduced drug permeability, antibiotic efflux, and alteration of the antibiotic target site. Reduction of antibiotic permeability and drug efflux are directly associated with their cell wall and membrane lipid and protein content (18). In this regard, different lipid and protein content in drug-resistant and drug-susceptible strains is expected. Therefore, characterization of bacterial content, especially bacterial envelope, by MALDI-TOF assay could be a promising approach to predict drug susceptibility. However, no proteomics database is currently available for drug-resistant *M. tuberculosis* strains. Therefore, a homemade database based on reference and clinical isolates of *M. tuberculosis* could assist in predicting the antibiotic resistance profile of the strains and reduce diagnosis time.

Conclusions

In this work, we isolated clinical *M. tuberculosis* strains from infected patients whose identities were confirmed by biochemical and molecular assays. Furthermore, the strains' drug resistance profile was determined using classical antibiogram, target gene amplification and sequencing, and MALDI-TOF MS assay. This study revealed that, in accordance with the results from culture-based and molecular methods, the characterization of bacterial proteins by MALDI-TOF MS assay could be a valuable tool to predict bacterial drug resistance, which can reduce diagnostic and patient-care approaches.

Authors' Contribution

Conceptualization: Samer Montazeri.

Data curation: Samer Montazeri, Fariba Sharifinia, Alireza Ghasempour, Ahmad Majd, Abbas Akhavan Sepahi.

Formal analysis: Samer Montazeri.

Methodology: Samer Montazeri, Alireza Ghasempour, Shadi Faribania.

Validation: Fariba Sharifinia, Alireza Ghasempour, Ahmad Majd.

Resources: Samer Montazeri, Fariba Sharifinia.

Writing-original draft: Samer Montazeri.

Writing-review and editing: Samer Montazeri, Fariba Sharifinia.

Supervision: Fariba Sharifinia.

Project Management: Samer Montazeri.

Conflict of Interests

None.

Ethical Issues

All the experimental processes of this research were approved by the Ethical Committee of Islamic Azad University, Tehran, Iran (Ethics No. 1574800649173711399162358211).

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It was conducted at the researchers' own expense.

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