Molecular characterization of multidrug and extensive drug-resistant *Mycobacterium tuberculosis* isolates from Iran

Hossien Kazemian[1], Jalil Kardan-Yamchi[2], Nader Mosavari[3], Mohammad Mehdi Feizabadi[1,4]

[1]Department of Microbiology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran;
[2]Division of Microbiology, Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran;
[3]Reference Bovine Laboratory, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran;
[4]Thoracic Research Center, Tehran University of Medical Sciences, Tehran, Iran

SUMMARY

Tuberculosis (TB) is one of the main causes of death among curable infectious diseases and one of the top 10 causes of death worldwide. Hence, molecular typing of MTB strains is necessary for epidemiological studies and helps to identify risk factors for TB transmission. Therefore, the present study was conducted to determine molecular typing of drug-resistant *M. tuberculosis* strains isolated from Iran using the RFLP-PGRS method. Thirty-two MDR strains and one XDR strain were isolated from TB patients in four major cities of Iran. MTB isolates were subjected to drug susceptibility testing. Whole genomic DNA from mycobacterial colonies were extracted and hybridized with PGRS probe in RFLP analysis. All fingerprinted MDR and XDR isolates were grouped into 13 clusters. The largest cluster (cluster 3) contained 48.4% (n = 16) of all isolates. Clusters 1, 4, and 6 included 2, 4, and 2 isolates, respectively. Two isolates were in cluster 7, one was H37Rv standard strain, which was used as a control strain in this study, and eight isolates were placed in single clusters. This study provides information about molecular epidemiology of MDR-TB in Iran. The alarming increase in the incidence of MDR isolates, especially Beijing strains, raises concerns for TB control programs in Iran.

Keywords: Tuberculosis, *M. tuberculosis,* MDR, XDR, genotyping

INTRODUCTION

Tuberculosis (TB) is one of the main causes of death among curable infectious diseases, and according to global tuberculosis report by WHO, TB is one of the top 10 killers among infectious diseases worldwide. In 2018, almost 1.4 million of 10.4 million new TB cases died due to tuberculosis. Africa and Southeast Asia had the most TB deaths among HIV negative people and the combined total TB deaths in HIV-negative and HIV-positive people in 2018 [1].

Molecular typing of strains for epidemiological studies can help to identify risk factors for TB transmission and to respond to important epidemiological questions, such as the origin of infection in a family or community [2-4]. Transmission of *Mycobacterium tuberculosis* can be studied using standard molecular fingerprinting techniques that are used to compare strains between laboratories, regions, and countries. Also, molecular genotyping methods can elucidate the evolutionary origins, investigate outbreaks, identify laboratory cross-contamination, facilitate the estimation of the extent of recent transmission, and distin-

Corresponding author
Mohammad Mehdi Feizabadi
E-mail: mfeizabadi@tums.ac.ir

guish between reactivation and re-infection [2, 3, 5]. Among molecular typing methods, restriction fragment length polymorphism (RFLP) with *IS6110* probe is the gold standard method for genotyping of MTB strains. However, it is unreliable for analyzing strains with a low copy number of *IS6110,* such as W/Beijing family members [6, 7]. Hence, polymorphic GC-rich repetitive sequence (PGRS) is a good target for typing strains with both high and low *IS6110* copy number. PGRS elements are more stable than *IS6110* elements, and RFLP-PGRS based typing has a higher discriminatory power compared to RFLP-*IS6110* technique [3, 8-12]. Spoligotyping is a rapid polymerase chain reaction (PCR)-based method that provides the possibility of MTB complex differentiation along with identification of Beijing strains of MTB simultaneously [1, 13]. It is also useful to investigate the evolutionary genetic events. It has been reported that spoligotyping together with RFLP genotyping can provide an accurate genotyping system for MTB strains typing [1, 13]. Therefore, the aim of the present study was to determine the molecular typing of drug resistant *M. tuberculosis* isolated from Iran using the RFLP-PGRS method.

METHODS

Bacterial strains

Thirty-two MDR strains and 1 XDR strain isolated from sputum of TB patients in 4 major cities of Iran (Tehran, Mashhad, Kermanshah, and Zahedan) during 2012 and 2015 were included in this study. All isolates were supplied by the Regional TB reference laboratory. The samples were decontaminated by N-acetyl L-cysteine method, and all isolates were identified as MTB by standard biochemical tests, including niacin accumulation test, nitrate reduction test, and heat-labile catalase test. For MDR confirmation, MTB isolates were subjected to drug susceptibility testing by the proportion method on Lowenstein-Jensen media containing isoniazid (0.2 µg/mL) and rifampin (40 µg/mL).

Whole DNA extraction from mycobacterial colonies

Mycobacterial colonies were transferred into a microcentrifuge tube containing 400 µL of 1×Tris–EDTA (TE buffer) and inactivated by heating in water bath at 80°C for 30 minutes. Next, 50 microliters of 10 mg/mL lysozyme were added and the mixture was incubated overnight at 37°C. Sodium dodecyl sulfate (SDS)/proteinase K (70 µL of 10% [w/v] SDS and 5 µL of 10 mg/mL of proteinase K) were added and incubated at 65°C for 10 minutes. Then, 100 microliters of 5M NaCl and N-cetyl-N,N,N trimethylammonium bromide (CTAB)-NaCl solution (4.1g of NaCl and 10 g of CTAB per 100 mL) was added and incubated at 65°C for 10 minutes. DNA was then extracted by the standard phenol-chloroform extraction method [10].

RFLP

RFLP was performed using the protocol as described previously by van Embden et al. [14]. Briefly, chromosomal DNA (2-3.5 µg) was restricted by *PvuII* at 37°C overnight. Restricted fragments were separated overnight in 1% (w/v) agarose gel by electrophoresis. The fragments were transferred onto positively charged nylon membranes by Southern blotting and hybridized with DIG labeled PGRS probe (5′ -CGG CCG TTG CCG CCG TTG CCG CCG TTG CCG CCG-3′ [15]. After hybridization, the hybridized probe was visualized colorimetrically using the DIG DNA Labeling and Detection Kit (Roche).The PGRS fingerprint patterns were analyzed using GelCompar II software version 6.5 (Applied Maths, Sint- Martens-Latem, Belgium) and compared with spoligotyping data from our previous study performed on the same samples [16]. MTB H37Rv was used as standard strain.

RESULTS

According to band-based similarity coefficient of 85% in RFLP-PGRS method, all fingerprinted MDR and XDR isolates were grouped into 13 clusters. The largest cluster (cluster 3) contained 48.4% (n = 16) of all isolates. Clusters 1, 4, and 6 included 2, 4, and 2 isolates, respectively. Two isolates were in cluster 7, one of which was H37Rv standard strain and used as a control strain, and 8 isolates were placed in unique clusters (No. 2, 5, 8, 9, 10, 11, 12, and 13) (Figures 1 and 2). According to our previous study, spoligotyping classified 33 isolates into 8 clusters, including Beijing (n = 13; 39.39%), Ural (former Haarlem 4) (n = 6; 18.18%), Central Asian strain (CAS) (n = 4; 12.12%), Latin American-Mediterranean (LAM) (n = 2; 6.01%),

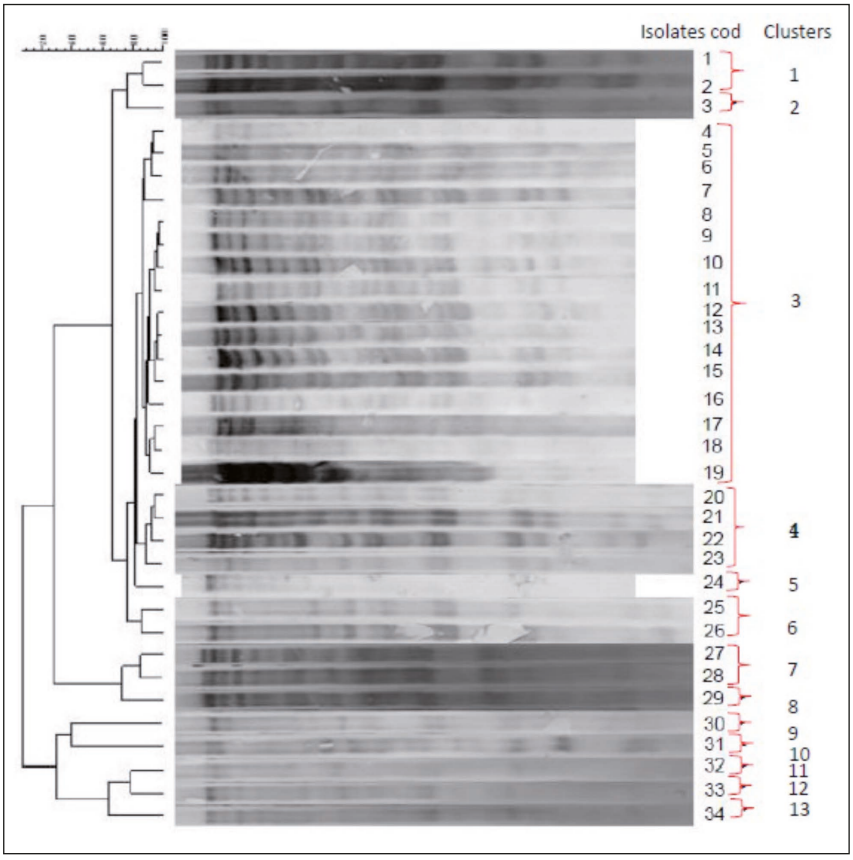

Figure 1 - Dendogram of different clusters of MDRTB isolates by RFLP-PGRS analysis.

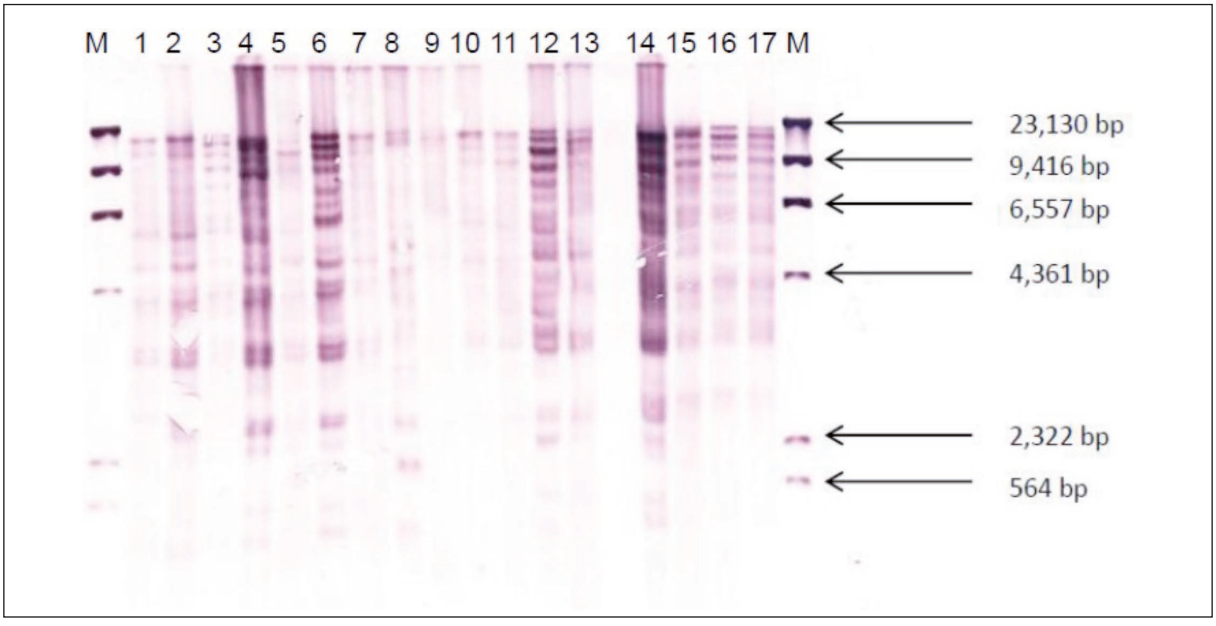

Figure 2 - RFLP-PGRS binding pattern of strains (M; Marker).

Table 1 - Spoligotype pattern of MDR and XDR MTB isolates.

Spoligotypes	*Code of isolates in dendrogram*
Beijing	4,5,7,8,9,10,11,13,24,29,30,33,34
H4	3,6,12,15,19,31
T	1,2
LAM	16,22
EAI	14
CAS1	17,18,25,26
MANU2	32
H37Rv	20,23, 27
New	21

H37Rv (n = 3; 9.07%), T (n = 2; 6.06%), Manu 2 (n = 1; 3.03%), and East African-Indian (EAI) (n = 1; 3.03%). One isolate was characterized by novel genotype not previously described in the SITVIT2 database; 61.5% of Beijing genotypes were placed in cluster 3 and other Beijing types were placed in unique clusters.

DISCUSSION

The combined data from different probes commonly increase the differentiation accuracy of *M. tuberculosis* strains, but it is limited to strains with more than 6 copies of *IS6110*. Therefore, typing results using different probes may not be consistent in strains without or with few copies of *IS6110*. The differential power of each molecular typing method depends on the evolutionary rate of its target sequence. Thus, spoligotyping is an appropriate method that shows the long-term genetic changes, but RFLP can demonstrate recent genetic events [11]. It has been reported that Beijing strains may appear unrelated with the *IS6110* marker, but they share a characteristic of PGRS pattern, which may indicate that they belong to a common lineage [11]. It has also been shown that different samples taken from the same patient over time or MTB isolates obtained from different body sites of the same patient can show different patterns of RFLP-*IS6110,* and this is not surprising because of rapid changes in *IS6110* [17]. Flores et al. indicated that RFLP, using PGRS marker for isolates of *M. tuberculosis* that have 5 or fewer *IS6110* hybridizing bands, improves the discriminatory power of RFLP-*IS6110* [10]. Since most of our isolates were classified in Beijing family (strains commonly with low copy number of *IS6110*), the PGRS probe was preferred to *IS6110* probe. In this study, genetic relatedness between 33 MDR and XDR MTB isolates were analyzed using RFLP-PGRS method and compared to our previously published spoligotyping results performed on the same samples. All 33 isolates were classified into 13 different clusters, showing the high genetic diversity of MDRTB isolates in Iran. Cluster 3, with 48.3% (16 isolates) of all studied isolates, was the largest cluster that included 8 of all 13 Beijing strains. The 5 remaining Beijing strains were in 5 unique clusters (clusters 5, 8, 9, 12, and 13). MDR isolates in cluster 3 belonged to Mashhad (North-east), Kermanshah (Southwest), and Zahedan (Southeast), demonstrating that MDR isolates were circulated among different regions of Iran. In addition, similarities were found between other MDRTB strains isolated from Kermanshah, Mashhad, and Tehran using RFLP-PGRS method. The single strain in cluster 12 was XDRTB, which was isolated from a patient in Mashhad and was correlated with Afghani immigrants.

Many studies were conducted for molecular epidemiology of tuberculosis in different areas of Iran, such as Tehran, East Azerbaijan, Khorasan, and Markazi provinces [18-26]. According to these studies, it is confirmed that a considerably high genetic diversity is seen in the population of *M. tuberculosis* strains in Iran. This genetic diversity is due to the transmission of tuberculosis to Iran through neighboring countries with high burden of tuberculosis, such as Iraq, Azerbaijan, Afghanistan, and Pakistan. It seems that tuberculosis control program was neglected in these countries due to bad conditions, such as war. Feyisa et al. reported that a higher prevalence of Ural family MTB isolates among Afghani patients than among Iranian patients suggests the possible transmission of this lineage following the immigration of Afghans to Iran [27].

MDR-TB outbreaks have been reported to be associated with Beijing family genotype [28]. Factors responsible for the selection and dissemination of these hypervirulent strains are not known. Beijing strains are more common in areas where BCG vaccination coverage is extensive, including most countries in Southeast Asia that have used

BCG vaccination for the past 2 to 6 decades. It has been suggested that BCG vaccination may have favored the selection of *M. tuberculosis* strains that resist BCG-induced immunity [29]. BCG vaccination is used in Iran as TB control program, but according to past studies, the prevalence of Beijing strain was very low [1, 27, 30]. Indeed, about 40% of our MDR-MTB isolates was recognized as Beijing type, which is considerably high.

In conclusion, this study provides information about the molecular epidemiology of MDR-TB in Iran. The alarming increase in the incidence of MDR isolates, especially Beijing strains, raises concern for TB control programs in Iran. This warrants high priority for rapid diagnosis of patients, identification of persons latently infected with MDR/XDR strains, and comprehensive screening of close contacts with MDR/XDR-TB patients to reduce the reactivation of latent drug resistance TB and disease transmission within the community.

ACKNOWLEDGMENT

This project was supported by Tehran University of Medical Sciences (Tehran, Iran) [project no. 25108].

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