Research Article

DNA Fingerprinting of Resistant Mycobacterium tuberculosis Isolates in Iran by IS6110-RFLP Method

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Abstract

The objective of the research was to identify resistant Mycobacterium tuberculosis strains and recognize their molecular epidemiology and how they are disseminated in order to determine the cause and the effect of drug resistance and the process of its development.

Materials and methods. Genomic deoxyribonucleic acid obtained from 37 drug-resistant Mycobacterium tuberculosis isolates were examined and fingerprinted by the IS6110- restriction fragment length polymorphism method. The data obtained were then analyzed using SPSS statistics software.

Results. The mean patients’ age was 51 ± 15.5 years. There were 46% of male patients, 67.6% of the patients from urban areas, 86.5% of Iranians, 21.6% of relapsed cases, 8.1% of human immunodeficiency virus-positive patients, and 10.8% of the patients with a history of contact with tuberculosis patient. Based on IS6110 - restriction fragment length polymorphism, 30 different genetic types were observed which indicated a significant variation of this pathogen in Markazi province, Iran. The number of cluster genotypes was determined by 6 clusters; the number of unique types was 24. There were no relationships between age, gender, nationality, residence, close contact with tuberculosis patients, recurrence of tuberculosis, positive human immunodeficiency virus status and clustered or non-clustered strain genotype.

Conclusions. Considering the high genetic diversity in Mycobacterium tuberculosis strains, it can be concluded that, based on IS6110 - restriction fragment length polymorphism, about 65% of cases occurred due to reactivation, and 35% of the cases were due to recent transmission. The information obtained through the molecular typing method can be very effective in future planning for tuberculosis control in Iran.

Keywords

drug resistance; genetic fingerprinting; pulmonary tuberculosis

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Problem statement and analysis of the latest research

Tuberculosis is an infectious disease, and about 1.7 billion people, 23% of the world’s population, are estimated to have latent tuberculosis (TB) infection and are thus at risk of developing active TB disease during their lifetime. In 2017, there were an estimated 10 million cases of TB globally; about 1.3 million deaths among human immunodeficiency virus (HIV) - negative people were caused by TB; there were additional 300, 000 deaths from TB
among HIV-positive people. Drug-resistant TB continues to be an important public health problem. According to the World Health Organization (WHO), about 558,000 people worldwide developed TB that was resistant to rifampicin in 2017; 82% of these cases were multidrug-resistant TB (MDR-TB). Globally, in 3.5% of new TB cases and 18% of previously treated cases, MDR TB was observed. Among cases of MDR-TB in 2017, 8.5% were estimated to have extensively drug-resistant TB (XDR-TB). Due to the emergence and spread of drug resistance, the WHO ranked the disease above acquired immunodeficiency syndrome (AIDS) and hepatitis. TB remains one of the 10 most communicable diseases [1]. About 95% of all TB cases and 98% of deaths from TB occur in developing countries. One of the main factors of TB control is the identification of infected people and a rapid detection of the origin of infection. Therefore, the diagnosis of the patients with active TB plays an important role in controlling TB and its effective management. The disease may result from the reactivation of latent infection, or from recent infection and active transmission. Therefore, the distinction between recent infection and reactivation of infection in TB control programs is of particular importance [2].

According to the WHO, the incidence of TB in Iran in 2017 was reported to be about 14 per 100,000 people, and the incidence of MDR-TB was 0.24 per 100,000 people [1]. Racial and geographical diversity and socioeconomic differences of the population affected the prevalence of TB in different geographical regions of Iran. The WHO has developed a standard short-course therapy for TB. This therapy includes the treatment of new cases using isoniazid, rifampin, pyrazinamide, and ethambutol for two months, followed by two-drug treatment with rifampin and isoniazid [3]. Drug-resistant TB was first discovered in 1943 and was followed by the extensive and unpredictable use of rifampin. MDR-TB was observed in 1990 being a leading cause of morbidity worldwide. In 1993, the WHO declared TB a “global emergency” 1993 [2]. Multidrug resistance is defined as resistance to isoniazid and rifampin. The problem of drug resistance is a major threat to TB control and a major concern for global health [4, 5].

Drug-resistant TB occurs when a genetic mutation occurs in mycobacterium. In addition, defective treatment results in the degradation of susceptible bacilli and, resistant strains survive, multiply, and become dominant strains in the body. It should be noted that in Iran, drug-resistant TB is an important health problem as well [6]. The analysis of TB transmission and the tracing of infectious resources requires the ability to differentiate between Mycobacterium tuberculosis species. Some methods which, until now, have been based on the study of phenotypic traits such as serotypes, bio-types, or antimicrobial susceptibility, were used to classify these species [7]. Molecular epidemiology is used to better understand the status, spread, and pathogenicity of diseases. Techniques such as restriction fragment length polymorphism (RFLP) are suggested to differentiate mycobacteria and determine the characteristics of isolated species, and in addition, this technique has been used successfully to prove laboratory cross-contamination and is applicable to detect the occurrence of TB [8].

The objective of the research was to determine different drug-resistant strains of Mycobacterium tuberculosis, which are likely to be responsible for infections through recent person-to-person transmissions or to identify strains indicating the activation of latent infection in a person with TB using IS6110-RFLP fingerprinting techniques.

### 1. Materials and Methods

This is a descriptive cross-sectional study that was conducted on the patients over 3 years in Markazi province in Iran. All the patients with smear positive pulmonary TB diagnosed by health centers and candidates for anti-TB treatment were included in the study.

The sample size was calculated based on statistical estimation with a 95% confidence interval and previous study [9] taking into account:

\[
p = 0.23 \\
q = 0.77 \\
d = 0.135 \\
n = Z_{(1-\alpha/2)}^2 \times p \times q \div d^2
\]

\[
n = 37
\]
Thus, a total of 37 patients with drug resistance were studied. This study was approved by the Arak Medical University’s Ethics Council (code number: 93-110-72).

1.1 Sampling and culture
Sampling was carried out by a simple random method and gradually completed over a 36-month period. After receiving the patients’ consent, a questionnaire on demographic information and the results of sputum examination was completed by the health experts. Then, sputum samples were taken using the Kubica’s method by means of decontamination and condensation. Concentrated suspensions were cultured on the Lowenstein-Jensen media containing glycerin and pyruvate and stored at 37°C for 6-8 weeks. Then, biochemical tests (such as Niacin test, Nitrate Reduction test, Heat Resistant Catalase test, etc.) were used to diagnose Mycobacterium tuberculosis.

All these strains were cultured on the Lowenstein-Jensen medium containing antibiotics and that without antibiotics (as control). Also, the Mycobacterium tuberculosis strain H37Rv, which is susceptible to all drugs, was used as a qualitative control. To determine the susceptibility of isolated strains, the proportional method was used according to the WHO guidelines and the Clinical and Laboratory Standards Institute (CLSI) [10, 11]. Drug susceptibility was tested on the Lowenstein-Jensen medium containing isoniazid – 0.2 mcg/ml, rifampicin – 40 mcg/ml, ethambutol – 2 mcg/ml, streptomycin – 4 mcg/ml. Pyrazinamide susceptibility testing was not performed.

Thirty-seven isolates were found to be resistant to at least one of the first-line drugs. Subsequently, deoxyribonucleic acid (DNA) extracted from 37 isolates for genomic fingerprinting and polymorphism of resistant Mycobacterium tuberculosis was studied.

1.2 IS6110-RFLP
To obtain the IS6110-RFLP pattern, DNA isolated from bacterial culture was purified. Then, DNA was digested with the Proteus vulgaris (Pvu) II enzymes (0.9 to 10.9 kb) and the fragments of DNA were separated on the agarose gel. To view the components containing the IS6110, a peroxidase-labeled probe containing complementary DNA of IS6110 was added to DNA hybridization buffer that was subsequently poured on a nitrocellulose membrane. Compounds produced by the Pvu II enzyme which were hybridized with the IS6110 probe were detected by a chemiluminescence reaction; the RFLP pattern was detected on a light-sensitive film. Gel-campair software was used to compare the fingerprints.

2. Results
The mean patients’ age was 51 ± 15.5 years. There were 46% of male patients, 67.6% of the patients from urban areas, 86.5% of Iranians, 21.6% of relapsed cases, 8.1% of HIV-positive patients, and 10.8% of the patients with a history of contact with TB patients.

The number of copies of IS6110 in each of the isolates was determined by the number of bands hybridizing with the probe, and the number of copies varied from zero to seventeen; therefore, the number of copies of isolates was divided into four groups: no copies; low copy number (1-2 copies); moderate copy number (3-5 copies); high copy number (6-17 copies). The average number of IS6110 copies was 9; 5 samples had low copy number; 7 samples had moderate copy number; 23 samples had high copy number; 2 samples had no copies. Twenty-four strains (64.9%) showed a unique pattern; six other patterns (35.1%) were related to 13 patients.

Among the species studied, three MDR Mycobacterium tuberculosis isolates were involved in 3 separate isolates in recent infections. The similarity between strains was very low; and one MDR Mycobacterium tuberculosis isolate and one non-MDR Mycobacterium tuberculosis isolate belonging to two Afghan refugee patients were similar to Iranian patients, and the remaining isolates of Afghan patients had no similarity to Iranian patients. There were found no strains belonging to the Beijing family of strains. There were no relationships between age, gender, nationality, residence, close contact with TB patient, recurrence of TB, positive HIV
Table 1. Risk factors associated with clustered and non-clustered cases among 37 patients with resistant Mycobacterium tuberculosis strains in Iran (IS6110-RFLP)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Clustered strain genotype</th>
<th>Non-clustered strain genotype</th>
<th>Adjusted OR (CI 95%)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;45 years old</td>
<td>8 (21.6%)</td>
<td>12 (32.4%)</td>
<td>0.63 (0.16-2.47)</td>
<td>0.7307</td>
</tr>
<tr>
<td>≥ 45 years old</td>
<td>5 (13.5%)</td>
<td>12 (32.4%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>6 (16.2%)</td>
<td>11 (29.7%)</td>
<td>0.98 (0.26-3.82)</td>
<td>0.6267</td>
</tr>
<tr>
<td>Females</td>
<td>7 (18.9%)</td>
<td>13 (35.1%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type of patient</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>New case</td>
<td>12 (32.4%)</td>
<td>17 (45.9%)</td>
<td>0.2 (0.02-1.87)</td>
<td>0.2164</td>
</tr>
<tr>
<td>Recurrence</td>
<td>1 (2.7%)</td>
<td>7 (18.9%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nationality</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iranian</td>
<td>11 (29.7%)</td>
<td>21 (56.8%)</td>
<td>1.27 (0.18-8.78)</td>
<td>0.5856</td>
</tr>
<tr>
<td>Afghan</td>
<td>2 (5.4%)</td>
<td>3 (8.1%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Residence</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urban</td>
<td>8 (21.6%)</td>
<td>17 (45.9%)</td>
<td>1.52 (0.37-6.30)</td>
<td>0.7161</td>
</tr>
<tr>
<td>Rural</td>
<td>5 (13.5%)</td>
<td>7 (18.9%)</td>
<td></td>
<td></td>
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<tr>
<td>Close contact</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>with TB patient</td>
<td>Yes</td>
<td>3 (8.1%)</td>
<td>1 (2.7%)</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>10 (27%)</td>
<td>23 (62.2%)</td>
<td>(0.01-1.57)</td>
</tr>
<tr>
<td>HIV infection</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>1 (2.7%)</td>
<td>2 (5.4%)</td>
<td>1.09 (0.09-13.31)</td>
<td>0.7223</td>
</tr>
<tr>
<td>No</td>
<td>12 (32.4%)</td>
<td>22 (59.5%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

status and clustered or non-clustered strain genotype (Table 1).

3. Discussion

Thirty genetic variants were detected by the IS6110-RFLP method. There was observed a significant variety of this pathogen in Markazi province. Consequently, in this study, similar to other studies, activated TB with more drug resistance occurred due to reactivation of infection, and recent transmission played a less important role. However, there were observed some recent transmissions that should be considered as a danger to the health system, especially the system dealing with TB.

The similarity of the genetic variants of Mycobacterium tuberculosis isolated from Iranian and Afghan patients in 2 cases and the similarity of 3 isolates indicated that the prolonged presence of non-Iranian minorities in the country plays a certain role in the transmission of strains with resistance to Iranian citizens. In this group, there was found one of the MDR isolates.

In other studies, conducted in Iran, there was a wide genetic variation in Mycobacterium tuberculosis strains, which suggested that TB affected people of different origins. Therefore, reactivation of infection seems to play a greater role in the spread of TB. In a study carried out in Tehran, among 292 strains of Mycobacterium tuberculosis, 232 strains showed a unique pattern indicating re-activation of infection and 60 strains showed the same pattern indicating the transfer and activation of these strains. Similarly, in another study conducted in West Azerbaijan Province and East Azerbaijan Province, among 154 strains isolated from the patients, 69.5% of them had a unique RFLP pattern. Therefore, reactivation of infection was found to play a greater role in the epidemiology of TB in these areas. It is estimated that 90 % of TB cases in developed countries are due to reactivation of the disease at an advanced age and transmission plays a less important role in infection [12, 13, 14].

In another study, the prevalence of TB among Afghan refugees living in Tehran was determined by genotyping IS6110-RFLP. Among 60 immigrant patients, only 2 Afghan refugees were diagnosed with the disease in Tehran; therefore, the transmission of
the disease from Afghan refugees to Iranian people was unusual [15]. While in our study, there was a certain similarity between two Afghan isolates and Iranian isolates, which could be attributed to the transmission of the disease and the presence of epidemic isolates. This study did not fully investigate the social relationships between these patients, and it was impossible to determine the index case. The molecular technique used in this study was selected according to the WHO method and provided the highest distinction for *Mycobacterium tuberculosis* polymorphism [16]. High patients’ age and the presence of a wide range of genetic diversity, despite the relatively limited number of isolates, could confirm the role of reactivation of old infections at the onset of the disease as compared to transmission of infection between the patients.

### 4. Conclusions

In our study, there was observed a transmission of resistant *Mycobacterium tuberculosis* strains in the study population, indicating the lack of timely identification of the patients and their role in transmission of the disease in the community. Considering the high genetic diversity in *Mycobacterium tuberculosis* strains, it can be concluded that based on IS6110-RFLP about 65% of cases occurred due to reactivation, and 35% of the cases were due to recent transmission. A similar pattern of antibiogram in the patients with similar strains suggested extensive transmission of a single strain in the community. It can be assumed that the strain was initially susceptible, and as a result of transfer from one host to another host (for example due to failure of treatment in the patients), it turned into a non-MDR strain and eventually became MDR strain. The molecular typing method of *Mycobacterium tuberculosis* strains can be very effective in future planning for TB control in Iran. Considering the importance of drug resistance in TB, the necessity of creating and expanding the ability to detect resistance, including the use of rapid methods such as polymerase chain reaction, is inevitable and necessary in all provinces of the country.

### 5. Prospects of Further Researches

This study was carried out with a relatively small sample of the patients. To confirm the obtained findings, it is necessary to study a larger sample of the patients.

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### Conflict of interest

The authors declare no competing interests.

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### Authorship

All the authors contributed to the content of this manuscript and participated in the editing of the final manuscript and approved the final version.

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