Molecular Diversity of Mycobacterium tuberculosis Strains in Northwestern Iran

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Abstract

Background: Years after the development of antituberculosis (TB) drugs, many people continue to suffer from this disease. To control the spread of TB, strains of the Mycobacterium tuberculosis complex need to be determined, and sources of infection must be identified. Such steps should help to prevent transmission of the infection.

Objectives: The aim of this study was to perform molecular genotyping of isolates of the M. tuberculosis complex obtained from patients in northwestern Iran.

Methods: One hundred ninety-four culture-positive M. tuberculosis isolates obtained from patients in northwestern Iran were analyzed using the mycobacterial interspersed repetitive unit-exact tandem repeats (MIRU-ETR) method.

Results: The MIRU-ETR method distinguished 162 different patterns in the 194 isolates, comprising 23 clusters and 139 unique patterns. Its discriminatory power according to the Hunter-Gaston discriminatory index (HGDI) was 0.9978. The largest cluster contained six isolates.

Conclusions: This research indicated that various strains of M. tuberculosis were responsible for TB and that the majority of cases were due to reactivation.

Keywords: Tuberculosis, Molecular Diversity, Iran, ETR

1. Background

Years after the development of antituberculosis (anti-TB) drugs, deployment of a variety of diagnostic methods, and global attempts to control the disease, the number of people with TB remains high. According to WHO reports, there were 9.6-million TB cases in the world in 2014, with the rate of mortality as high as 1.5 million. In 2014, the prevalence rate of TB in Iran was reported to be 22 per 100,000 population (1). Increasing numbers of drug-resistant isolates pose a threat to human health. Thus, using methods to identify TB, especially drug-resistant isolates, can be effective in controlling the disease (2). TB-control plans depend on the identification of sources of infection (3), transmission routes (4), and reasons for treatment failure. Preventing the transmission of infection will limit the spread of the disease (5). Molecular genotyping of Mycobacterium tuberculosis isolates obtained from patients can detect the prevalence of new epidemics and distinguish TB due to reactivation from TB due to recent transmission (6). It can also identify risk factors for transmission (7, 8). Thus, the results of molecular genotyping can aid disease control.

Multiple molecular typing methods have been used to differentiate strains in molecular epidemiological studies. One such method is based on repeated sequences of variable number tandem repeats (VNTRs) (9). The exact tandem repeats (ETR)-VNTR method, which is also based on tandem repeats (i.e., A, B, and C loci), is of greater value in typing of strains (10). Another type of VNTR method utilizes mycobacterial interspersed repetitive units (MIRUs). Normally, loci 2, 4, 10, 16, 20, 23, 24, 26, 27, 37, 39, and 40 are used to determine the genotype (11). Different strains of M. tuberculosis possess different numbers of repeats. The re-
peats can be identified through PCR-based methods, and the number of repeats can be calculated by the size of the amplified products (12). Both VNTR- and PCR-based methods are suitable for assessing a variety of genetic strains.

2. Objectives

The aim of the present study was to perform molecular genotyping of isolates of the M. tuberculosis complex to obtain a better understanding of the disease and aid appropriate disease-prevention decision making in northwestern Iran, thereby reducing the burden of TB.

3. Methods

3.1. Mycobacterial Isolates

Isolates of the M. tuberculosis complex were obtained from patients who were referred to central TB laboratories in eastern and western Azerbaijan provinces in northwestern Iran from March 2004 to March 2005. The study population consisted of patients from whom at least one sample was culture positive for the M. tuberculosis complex. One hundred ninety-four isolates of the M. tuberculosis complex were collected. The isolates was identified as M. tuberculosis complex by Ziehl-Neelsen staining and standard microbiological tests, including the production of niacin, catalase activity, nitrate reduction, pigment production, and growth rate on Lowenstein-Jensen medium (13).

3.2. MIRU Typing

DNA was extracted from the mycobacterial isolates using lysozyme, SDS, proteinase K, and CTAB (14). PCR was carried out in a volume of 20 µL containing 10-100 ng of DNA; 0.5 µM of specific primers (3) (Metabion, Germany); 1.5 mM of MgCl₂; 100 µM of dATP, dCTP, dGTP, and dTTP; 50 mM of KCl; 20 mM of Tris-Cl (pH 8.4); and 1.25 U of DNA polymerase (Cinnagen, Iran). The PCR was performed in a volume of 20 µL containing 10-100 ng of DNA; 0.5 µM of specific primers (15) (Metabion, Germany); 1.5 mM of MgCl₂; 100 µM of dATP, dCTP, dGTP, and dTTP; 50 mM of KCl; 20 mM of Tris-Cl (pH 8.4); and 1.25 U of DNA polymerase (Cinnagen, Iran). The PCR product was visualized under UV light. The size of the fragments was determined using a 100-bp DNA ladder and a size marker (Fermentase, Lithuania).

3.3. ETR Typing

The PCR was performed in a volume of 20 µL containing 10-100 ng of DNA; 0.5 µM of specific primers (15) (Metabion, Germany); 1.5 mM of MgCl₂; 100 µM of dATP, dCTP, dGTP, and dTTP; 50 mM of KCl; 20 mM of Tris-Cl (pH 8.4); and 1.25 U of DNA polymerase (Cinnagen, Iran). The PCR was done with an initial 7-minute denaturation step at 94°C and a final extension step at 72°C. The temperature cycles for the different types of PCRs were as follows: 35 cycles of 45 seconds at 94°C, annealing temperatures for 50 seconds, and 72°C for 65 seconds. The annealing temperatures used for ETR-A, ETR-B, and ETR-C were 66, 68, and 69°C, respectively.

3.4. Statistical Analysis

All the isolates in this study were classified into two groups: clustered and nonclustered. Clustered were defined as two or more isolates with identical MIRU-ETR types. Isolates with unmatched genetic patterns were considered nonclustered. Clustered isolates were assumed to have arisen from recent transmission. The MIRU-ETR allelic diversity (h) at a locus was calculated as follows:

\[ h = 1 - \sum x_i^2/n \times (n - 1) \]

where xi was the frequency of each allele at the locus, and n was the total number of strains in the typing scheme. The Hunter-Gaston discriminatory index (HGDI) was used as a numerical index of the discriminatory power of the MIRU-ETR method (16). The minimum estimate of the proportion of TB cases related to recent transmission was calculated (the number of clustered isolates - the number of clusters)/total number of isolates (17). Categorical data were compared by a chi-square test or Fisher’s exact test. A p value below 0.05 was considered significant.

4. Results

One hundred ninety-four positive cultures of the M. tuberculosis complex were evaluated using the MIRU-ETR and MIRU-VNTR methods. Sixty-six isolates were from patients in western Azerbaijan, and 128 isolates were from patients in eastern Azerbaijan. The ages ranged from 40-day-old infants to elderly patients (88 years). In total, 162 different patterns were detected: 23 clusters and 139 unique patterns (71.65%), as shown in Table 1. The range of clustering was 28.35% and 32.03% in eastern Azerbaijan and 21.21% in western Azerbaijan. The largest cluster had six members (five females and one male), four of whom were from western Azerbaijan and two of whom were from eastern Azerbaijan. The isolates in some of the clusters showed no epidemiological correlation. Two clusters, one with four
members and three with three members, were detected in eastern Azerbaijan (Table 2). In the 23 clusters, 14 isolates were obtained exclusively from patients in eastern Azerbaijan, two isolates were obtained from patients only in western Azerbaijan, and seven isolates were obtained from patients in both provinces. Table 3 presents the allelic diversity. Loci 10, 26, 40, and A had the highest allelic diversity, and locus 26 had the highest allelic diversity using the MIRU method. The MIRU-ETR method provided appropriate discriminatory power (HGDI = 0.9978).

In this study, 17% of TB cases were due to recent transmission ([55 - 23]/194). To distinguish the risk factors for recent transmission, 55 patients inside the cluster and 139 patients outside the cluster were compared (Table 4). The following factors did not show a significant relationship with the risk of recent transmission: age, sex, hospitalization during the previous year, a family member with TB, contact with a TB patient, smoking habit, diabetes, asthma, and imprisonment (P > 0.05). Individuals with extrapulmonary TB and patients with a history of anti-TB treatment were less common in the clusters (P < 0.05).

5. Discussion

In this study, the MIRU-ETR method was used for genotyping isolates of the M. tuberculosis complex in northwestern Iran. The rate of clustering among Mycobacterium strains was 28.35%, indicating that the majority of TB cases in this region of Iran were due to reactivation rather than recent transmission. Urban development has increased in northwestern Iran, especially in large cities (e.g., Tabriz and Urmia) and in towns, such as Maragheh, Mianeh, and Khoi, inhibiting the rate of Afghan migration to this territory. The numbers of TB cases were lower than in other parts of the country. Thus, the rate of clustering was also lower. The majority of the strains that were detected had unique patterns. In the present study, only 17% of TB cases were due to recent transmission in comparison to 37% in Casablanca, Morocco (18). Therefore, TB appears to be under control in northwestern Iran. However, many people with TB in the republic of Azerbaijan come to Iran for diagnosis and/or treatment, as TB treatment is free in Iran (19, 20). Thus, Iran will likely face increasing numbers of patients with TB, especially resistant TB, unless appropriate interventions are taken.

As shown by the results of the MIRU-VNTR analysis, allelic diversity varied throughout the different geographical regions. Regarding MIRU loci, locus 26 (h = 0.74) showed the highest variation. Among the ETR loci, the highest variation was observed at locus A (h = 0.65). Among MIRU loci in hospitals of Paris, France, locus 40 (h = 0.74) showed the highest variation (9). In three provinces in Iran, locus 16 (h = 0.64) exhibited the greatest variation (21), whereas in the province of Khuzestan in Iran, locus 31 (h = 0.73) displayed the highest variation (22). In a genotyping study of M. tuberculosis in China, locus C (h = 0.54) had the highest variation among ETR loci (23). In a similar survey in Taiwan, locus 26 (h = 0.77) had the highest variation (24).

The level of allelic variation is due to the presence of different isolates in various countries. In common with other research, in this study, the allelic diversity of some loci was lower than 0.6. Omitting these loci in future studies, and adding loci, such as VNTR3820 (23), QUB11a, QUB11b, and QUB3232 (15), which show greater allelic diversity in different populations, would likely make it easier to differentiate between strains.

The discriminatory power of MIRU-ETR in this study was 0.9978 for 194 cases, and 162 patterns were observed. In a study of TB cases in Singapore, Sun et al. (25) reported a discriminatory power of 0.994 for 68 cases. Studies of TB cases performed in Khoozestan province in Iran, Taiwan, and Samara in Russia reported a discriminatory power of 0.991 for 61 cases, 0.972 for 502 cases, and 0.625 for 129 cases, respectively (22, 24, 26). As the study in Singapore was based on a small number of isolates, its discriminatory power was slightly lower than in the present study. In the Russian study, all the isolates were from the Beijing family, and 43.6% of strains were from the Beijing family in the Taiwanese study. Therefore, the HGDI was low in those studies. In the present study in northwestern Iran, the high number of different patterns was due to the older age of the study population, increase in the percentage of reactivation of latent infection, and control of infection among young people. Therefore, the MIRU-VNTR method, together with the ETR method, may be appropriate for assessing recent cases of TB transmission in Iran. If further discriminatory power is required, Queen’s university of Belfast could be used.

In this study, the number of clusters identified by MIRU typing was lower than that detected by ETR typing. Based on MIRU typing, there were 30 clusters containing 86 isolates, with 2 - 12 patients in each cluster. Based on ETR typing, there were 164 isolates in each cluster. When ETR typing was added, the rate of recent transmission dropped from 29% ([86 - 30]/194) to 17% ([55 - 23]/194), which led to the omission of false clustering. The largest cluster contained six members, and the mean number of cases in each cluster was 2.39 (55/23). In studies conducted in Hong Kong (27), Samara in Russia (21), Anambra in Nigeria (28), three provinces in Iran (21), and Stockholm in Sweden (29), the
Table 1. Discriminatory Power of MIRU and ETR Typing Alone and Together*  

| Method       | Distinct Pattern | Unique Pattern | No. of Clusters | Isolates Included in Clusters | HGDI  |
|--------------|------------------|----------------|-----------------|-------------------------------|-------|
| MIRU         | 138              | 108            | 30              | 86                            | 0.9930|
| ETR-VNTR     | 55               | 30             | 25              | 164                           | 0.9322|
| MIRU-ETR     | 162              | 139            | 23              | 55                            | 0.9978|

*A score of 1 on the HGDR denoted the highest discriminatory power.

Table 2. MIRU-ETR Clusters of Isolates

| MIRU-ETR Pattern* | No. of Isolates in the Cluster |
|-------------------|--------------------------------|
| 2331 - 2325 - 2322 - 425 | 6                              |
| 2341 - 2525 - 3223 - 323 | 4                              |
| 2331 - 1624 - 3222 - 224 | 3                              |
| 2331 - 2325 - 2322 - 422 | 3                              |
| 2231 - 2525 - 3222 - 556 | 3                              |
| 2323 - 1325 - 3222 - 314 | 2                              |
| 2331 - 1515 - 3423 - 314 | 2                              |
| 2331 - 1515 - 3222 - 314 | 2                              |
| 2321 - 2524 - 3223 - 312 | 2                              |
| 2341 - 2521 - 3222 - 324 | 2                              |
| 2323 - 1525 - 3221 - 216 | 2                              |
| 2323 - 2513 - 3223 - 314 | 2                              |
| 2221 - 2525 - 3223 - 314 | 2                              |
| 2323 - 1512 - 3223 - 314 | 2                              |
| 2331 - 2323 - 3222 - 424 | 2                              |
| 1324 - 1515 - 3224 - 424 | 2                              |
| 2332 - 1524 - 3224 - 324 | 2                              |
| 2363 - 2523 - 3221 - 322 | 2                              |
| 2241 - 2325 - 3222 - 425 | 2                              |
| 2342 - 2524 - 3223 - 314 | 2                              |
| 2332 - 2615 - 3222 - 224 | 2                              |
| 2331 - 2516 - 3221 - 314 | 2                              |
| 2323 - 2526 - 3313 - 314 | 2                              |

*Order of MIRU and ETR loci 2, 4, 10, 16, 20, 23, 24, 26, 27, 31, 39, 40, A, B, and C.

In addition, clustering is time consuming and not accurate (30). In this study, seven of the 23 clusters were common in the two provinces, indicating that transmission occurred between the provinces, possibly due to them being adjacent. However, the presence of a common cluster does not necessarily signify an epidemiological correlation. It is possible that two patients who live in different regions and have not had any contact with each other have bacteria with the same pattern and are therefore located in one cluster (31). In such cases, isolates with high prevalence might lead to erroneous epidemiological correlations.

In Iran, large numbers of patients from towns in western provinces attend treatment enters in eastern provinces (Tabriz city). They are then admitted to hospitals in the city. They may transmit or contract the disease during brief interactions with others in various places, such as restaurants, cafes, offices, and stadiums, or by using public transportation, such as buses. Golub et al. (32) reported that a TB patient can transmit the disease through random and short contact with susceptible individuals. In this study, age was not a significant risk factor for bacterial transmission (P > 0.05). The majority of individuals in the clusters were older than 60 years (43.64%), with the remainder young (25.45%) and medium aged (30.90%) (Table 4). Although younger age is considered a risk factor for the transmission of new strains of TB, the disease is more common among the elderly (33).

With aging, the rate of entrance into the clusters declines. In a study in Tehran city, 85.7% of patients in the cluster were younger than 35 years. The increase in the percentage of elderly in the clusters in the present study may be attributed to unemployment, a poor diet lacking in nutrients, a low level of life study, a low prevalence of HIV, and even successfully controlling TB infection among young people in northwestern Iran. In the current study, gender was also not a risk factor (P > 0.05), with males and females accounting for 50.9% and 49.1% of those in the clusters. In a previous study of TB transmission in Tehran city, being male was a risk factor for inclusion in clusters (8). It seems that the difference was because of under-employment, low literacy, and high poverty among females in the male-dominated, patriarchal region of northwestern Iran. Due to
Table 3. Allelic Diversity of Each MIRU and ETR Locus

| Locus | Number of Isolates with the Specified MIRU and ETR Copy Number | Allelic Diversity (h) |
|-------|---------------------------------------------------------------|-----------------------|
| 2     | 2 1 177 5                                                   | 0.46                  |
| 4     | 4 1 46 145 2                                               | 0.39                  |
| 10    | 1 60 56 32 7 4 2 1                                         | 0.04                  |
| 16    | 1 11 11 11 11                                               | 0.35                  |
| 20    | 13 130 2 1                                                   | 0.41                  |
| 23    | 1 1 2 37 5 127 19 1 1                                       | 0.56                  |
| 24    | 14 10 2                                                     | 0.44                  |
| 25    | 27 27 75 3 2                                               | 0.74                  |
| 27    | 8 40 60 140 2                                              | 0.39                  |
| 36    | 17 320 18 1                                               | 0.32                  |
| 39    | 5 183 2                                                     | 0.10                  |
| 40    | 5 67 81 32 7 2                                           | 0.67                  |
| A     | 1 1 48 16 50 4 1                                           | 0.45                  |
| B     | 54 120 2 5 5                                             | 0.45                  |
| C     | 22 15 124 23 3 2                                         | 0.55                  |

to the low prevalence of HIV in the region, the ratio was higher in men than women.

In this study, previous TB treatment was considered a risk factor. Thus, patients who had received treated were not included in the clusters (P < 0.05). According to previous research, TB transmission can be prevented by identifying people with active TB, treating the disease, and educating patients about noncontact with others (5). In this study, previous hospitalization was also considered a risk factor, but the results showed that this factor was not statistically significant (P > 0.05). However, among 55 individuals in clusters, 30 (54.55%) had been hospitalized dur-

Table 4. Risk Factors for Clustering in Northwestern Iran

| Risk Factor                                  | No. (%) of Patients | No. (%) of Clustered Patients | No. (%) of Non Clustered Patients | P Value |
|---------------------------------------------|---------------------|--------------------------------|----------------------------------|---------|
| Age, y                                       |                     |                                |                                  | 0.825   |
| ≤ 40                                        | 55 (28.35)          | 14 (25.45)                     | 41 (29.50)                       |         |
| 41 - 59                                      | 60 (30.93)          | 17 (30.91)                     | 43 (30.93)                       |         |
| ≥ 60                                        | 79 (40.72)          | 24 (43.64)                     | 55 (39.57)                       |         |
| Sex                                         |                     |                                |                                  | 0.945   |
| Male                                        | 98 (50.53)          | 28 (50.9)                      | 70 (50.36)                       |         |
| Female                                      | 96 (49.49)          | 27 (49.1)                      | 69 (49.64)                       |         |
| Site of TB                                   |                     |                                |                                  | 0.454   |
| Pulmonary                                   | 171 (88.14)         | 50 (90.9)                      | 121 (87.05)                      |         |
| Extrapulmonary                              | 23 (11.86)          | 5 (9.1)                        | 18 (12.95)                       |         |
| Previous TB Treatment                       | 16 (8.25)           | 0 (0)                          | 16 (11.51)                       | 0.007   |
| Previous hospitalization, during the last year | 78 (40.21)        | 30 (54.55)                     | 48 (34.53)                       | 0.01    |
| Family history of TB                        | 34 (17.53)          | 11 (20)                        | 23 (16.55)                       | 0.569   |
| Smoking                                     | 64 (32.99)          | 19 (34.55)                     | 45 (32.37)                       | 0.772   |
| History of contact with a TB patient        | 33 (17.01)          | 11 (20)                        | 22 (15.83)                       | 0.486   |
| Diabetes                                    | 26 (13.40)          | 9 (16.36)                      | 17 (12.23)                       | 0.446   |
| Imprisonment                                | 7 (3.61)            | 3 (5.46)                       | 4 (2.88)                         | 0.407   |
| Asthma                                      | 6 (3.09)            | 2 (3.64)                       | 4 (2.88)                         | >0.999  |

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ing the last year, suggesting that those who were admitted to hospitals had low immunity. The isolation of these individuals, some of whom tested positive for the M. tuberculosis complex following hospitalization, is not appropriate. TB can spread in the hospital during intubation and tests, such as bronchoscopy, radiology, scans, and sonography, due to connective air conditioning.

We conclude that various strains of Mycobacteria are responsible for the spread of TB in northwestern Iran. Most of the TB cases were due to reactivation. Given the discriminatory power of the MIRU-VNTR method (0.9978), it is suitable for detecting the disease since sufficient numbers of cases are available for evaluation. Thus, it can be employed as a first-line method in genotyping of TB strains in Iran.

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Footnotes

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