Antimicrobial resistance profile and prevalence of *Mycobacterium tuberculosis* complex in Western Iran using spoligotyping method

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ABSTRACT

Tuberculosis (TB) is a chronic infectious disease with multiple manifestations and gradual progression that remains a major health problem and a leading cause of death worldwide. In recent years, a number of DNA fingerprinting techniques have been developed to identify strains of the *Mycobacterium tuberculosis* (MTB) complex. Spoligotyping is one of the first PCR-based genotyping methods. Information on the number and identification of common strains among MTB complex samples in clinical samples from Kermanshah city is needed to develop more effective therapeutic strategies.

This is a descriptive cross-sectional study of 41 sample patients with TB referred to Kermanshah Tuberculosis Centre between December 2021 and June 2022, including sputum, aspiration, urine, etc. First, the susceptibility of the developed bacteria to culture media was compared with that of isoniazid using the proportional method, and rifampin was determined according to the standard protocol. Demographic data of patients referred to the Centre for the Control of Lung Diseases were also recorded.

In the next step, spoligotyping was carried out using the standard method and each strain pattern was recorded as an OCTAL code and compared with the information available at the World Bank on spoligotyping and its strains. Forty-one patients with pulmonary TB were tested using spoligotyping. Four MTB strains were identified, including H4, CAS, T1 and H1.

The H4 strain also had the highest frequency with 16 samples (39%) among the MTB complex strains isolated using spoligotyping.

The highest frequency of strains isolated using spoligotyping was associated with the H4 strain. It can be concluded that spoligotyping is very cost effective, simple, repeatable and highly sensitive.

1. Introduction

It is estimated that 9.9 million people get ill from tuberculosis (TB) in 2020. TB remains one of the world's top infectious killers. 1.3 million people died from TB, including 214,000 people with HIV. According to the report of the World Health Organization (WHO), the rate of TB in Iran is 16 per 100,000, which indicates the low TB incidence in this country [1–3]. In recent years, the emergence and spread of multidrug-resistant (MDR) strains of MTB poses a critical public health problem and make TB more difficult to control and treat [4,5].

The published articles indicate that Kermanshah province is one of the endemic centers of TB in the west of Iran. One of the reasons for this high prevalence can be due to the number of immigrants from neighboring countries such as Iraq, which can increase the spread of the disease and increase the cases of MDR TB [6]. The high mortality rate due to TB worldwide, spread of a rapid and accurate diagnostic methods

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could significantly reduce the related mortality [7]. See (Table 1).

Therefore, molecular typing of MTB complex isolates can be helpful in the control of TB by identifying the dynamics of transmission and the source of infection, which can infect a person endogenously (recurrence of a previous infection) or exogenous (new transmission of the disease) [8,9]. The existence of unique genetic markers in the conserved genome of MTB can explain the difference in typing technique, such as Insertion Sequence *IS6110* [10], Mycobacterial Interspersed Repetitive Units-Variable Number of Tandem Repeats (MIRU-VNTR) [11], and Large Sequence Polymorphism (LSP) [12]. Restriction Fragment Length Polymorphism (RFLP) and reverse line dot blot spoligotyping [13].

There is a specific region within the MTB complex genome that is composed of a number of repeats of the DR locus polymorphism of 36 bp, that are connected to one or two non-repetitive 43 bp spacer sequences [14]. The spacer sequences are unique and can be hybridized with the spacer oligonucleotide. The hybridization plot shows which spacer oligonucleotide is present in which strain. This method is called spoligotyping (spacer).

In examining the DR locus of several strains, it has been observed that the order of the spacer sequences was almost the same, but deletion and addition of the spacer and DR sequences may also occur. This is the reason for the identification of a different species of MTB complex [11].

Among the advantages of this method is its ease and repeatability, which requires two days to perform. This method is very sensitive and can detect even two to three bacteria in a patient sample without culture. It is also useful for samples with a small number of *IS6110*. PCR-based methods (such as spoligotyping) are generally faster than RLFP, but have a lower resolution limit. Disadvantages of this method is the lower resolution of spoligotyping than RFLP because it targets only one percent of the genome. Also, this method does not have the ability to separate samples with multi-strain contamination. This method is not effective for non-tuberculosis mycobacteria [15].

Genotyping of MTB complex isolates using the spoligotyping technique was carried out for the first time in Kermanshah province as a regional reference in the west of the country. This technique provides useful information to compare the genetic diversity of the same strains with other techniques such as MIRU-VNTR and PFGE. In the past, the only indicators available to study TB epidemiology were drug susceptibility profiles and phage typing, which limited the use of both methods. In recent years, many DNA fingerprinting methods have been proposed to determine the species of the Mycobacterium tuberculosis complex. In order to control the spread of tuberculosis, it is necessary to carry out investigations to identify the strains and types of the organism. Spoligotyping is one of the methods used to achieve this. So, our aim in this study was to determine antimicrobial resistance profile and prevalence of MTB complex in Western Iran using the spoligotyping method. By recognizing the types of strains as well as identifying the most common strains, the strains can be investigated more and more accurately from a genetic point of view, and by identifying the drug resistance of the strains, it is possible to develop effective preventive and therapeutic policies.

2. Materials and methods

2.1. Sample collection

Among the patients who referred to the laboratory of Kermanshah Lung Disease Center, 90 cases of sputum smear positive were reported, of which 41 cases were culture positive. Positive culture cases were defined as cases in which at least one colony had grown on Lowenstein-Jensen (LJ) medium. Then, biochemical characteristics such as niacin accumulation, nitrate reduction, and catalase test were recorded for definitive diagnosis of TB.

2.2. Antibiotic sensitivity test

The sensitivity test for isoniazid (INH), ethambutol (EMB), and rifampin (RIF) was performed using the proportional method, and the amount of resistance to these drugs and the frequency of MDR isolates among the investigated isolates were determined.

2.3. DNA extraction

G spin kit was used for DNA extraction according to the manufacturer's instructions.

2.4. Polymerase chain reaction (PCR)

After DNA extraction, PCR using primers DRa (CCG AGA GGG GAC GGA AAC) and DRb (GGT TTT GGG TCT GAC GAC) where DRb is labeled at the 5′ end using biotin and optimized concentrations of components. The reaction was carried out according to the kit instructions. The temperature program of the thermocycler device is 96 °C, 3 min; (96 °C, 60 s; 55 °C, 60 s; 72 °C, 30 s) × 30; 72 °C, 5 min; 4 °C forever. The reaction was carried out [16].

2.5. Spoligotyping

Spoligotyping was performed using the available spoligotyping kit according to the manufacturer's protocol. Then the amplified products were hybridized on a membrane precoated with spacer oligos. After incubation with streptavidin–peroxidase and enhanced chemiluminescence detection, the presence of spacers was imaged on X-ray films as black squares [17]. *M. tuberculosis* H37Rv and BCG strain were used as the positive control (spoligotyping kit for detecting TB; Mapmygenome, Hyderabad, Andhra Pradesh, India).

2.6. Statistical analysis

Demographic and clinical characteristics were collected and included gender, age, and place of residence. The Fisher's exact test was used to examine the relationship between the frequency of isolates and gender. Using Fisher's test, there was no significant relationship between the frequency of isolates and gender (P value 0.751). The relationship between resistance to isoniazid, rifampin, and ethambutol antibiotics and gender was also checked by the Fisher's *t* test. Using Fisher's test, there was no significant relationship between resistance to antibiotics and gender (P value > 0.546). Statistical analysis using the Chi-Square test showed a significant relationship between the frequency of isolates and MDR samples (P-value = 0.006). All statistical analyses were performed using SPSS version 20.0 (SPSS Inc., Chicago, USA).

3. Results

In a study that was conducted, out of 90 sputum samples that were investigated, 41 isolates of MTB were identified and isolated after performing confirmatory tests such as colony morphology on LJ medium, Zil-Nelson staining, niacin production test, catalase test, and nitrate reduction test. Among the 41 isolates in the examined patients, 29 were

Table 1
The frequency of each cluster.

Cluster	Frequency (%)
H4	16 (39 %)
CAS1-Delhi	9 (22 %)
T1	7 (17.1 %)
H1	1 (2.4 %)
Unknown	8 (19.5 %)
Total	41

Table 2
Result of proportional method.

Lineage	Rif		INH		EMB		MDR	
	R	S	R	S	R	S	Yes	No
H4	2	0	2	0	1	0	1	0
CAS-Delhi	0	0	0	0	0	0	0	0
T1	2	0	2	0	1	0	1	0
H1	2	0	1	0	1	0	1	0
Unknown	0	0	0	0	0	0	0	0
Total	14.6 %) 6)		12.2 %) 5)		7.3 %)3)		7.3 %)3)	

male (70.7 %) and 12 were female (29.3 %).

Since the pulmonary TB laboratory of Kermanshah province is considered a reference center in the west of Iran, many samples from other centers in neighboring provinces are sent to this center for identification. The sample sent using the province is as follows:

Twenty-one samples (51.21 %) were collected from Kermanshah province, 3 samples (7.3 %) from Hamadan province, 11 samples (26.8 %) from Lorestan province, and 6 samples (14.63 %) from Kurdistan province.

Analysis of pattern spacer sequences obtained from spoligotyping of species in the SITVIT database (https://www.pasteur-guadeloupe.fr:8081/SITVIT2/) showed that the strains are in 4 clusters that include CAS1-Delhi and H4, and H1 and T1 were classified. Also, samples whose OCTAL code was not defined for any strain were defined as Unknown. The frequency of each cluster was as follows:

3.1. Drug susceptibility testing (DST)

DST was performed for all culture positive specimens (n = 41) using the proportional method, for all first-line anti-TB drugs like rifampicin (RIF), isoniazid (INH), and ethambutol (EMB) [18]. use LJ medium supplemented with INH at 0.2 µg/mL, RIF at 40 µg/mL, EMB at 2 µg/mL. After performing the proportional method on INH, RIF, and EMB, the resistance to these drugs among the investigated isolates was determined to be 5 isolates (12.2 %), 6 isolates (14.6 %), and 3 isolates (7.3 %), respectively. Also, the number of MDR isolates is 3 (7.3 %). Out of 5 samples resistant to INH, 2 samples are related to type T1, 2 samples are related to type H4, and 1 sample is related to type H1 (Table 2).

4. Discussion

Despite all the plans made at the global level, the eradication of TB is still considered one of the main challenges of global treatment. One of the main reasons for this is the high genotypic diversity of TB strains, which can affect virulence, transmissibility, host response and the emergence of drug resistance. [19].

The best strategy to control TB is to identify the disease in its early stages and treat it in time using selected drugs (including the first and second lines of tuberculosis treatment) [20].

In recent years, a large number of DNA fingerprinting methods have been proposed to determine the species of MTB. Methods that produce fingerprint profiles, such as Restriction Fragment Length Polymorphism (RFLP), and methods based on PCR, such as spoligotyping [10–21]. The study results indicate that the H4 clusters had the highest frequency among our isolates, accounting for 39 %. This finding is consistent with previous studies [22].

However, it should be noted that the frequency of H4 clusters in our study differs from that of other studies. This difference may be due to the limited number of MTB isolates screened in our study [23–26].

In the second resistance, the most frequent isolates are related to the CAS family, including CAS, CAS2, CAS1-Delhi, and CAS1-Kili, with a frequency of 22 %. This indicates a high prevalence of the CAS family in Iran (25 %) [27,28], Sudan (53.9 %) [29], Iraq (41.8 %) [30], Syria (10.4 %) [23], and Pakistan (CAS 39 %) [31].

Considering that in many Asian countries, TB has a higher rate than the prevalence of this disease in Iran, therefore, the migration pattern can be considered in increasing the incidence rate and genetic diversity of this bacterial strains in Iran [32,33]. The frequency of MDR-TB isolates in East Africa, Latin America and India as endemic Center of tuberculosis were 4 %, 7 %, and 5.4 %, respectively [34–36].

Taken in Asian countries, this indicates the high frequency of CAS strain. It seems that this strain is native to West Asian regions, and therefore, it makes possible the transfer of this genotype through migration [37–40]. The rate of frequency of MDR strains in our study was 7.3 %. The reported prevalence of MDR strains in the west of Iran in a previous study, it was higher than in our study [41].

TB control was successful in the west of Iran. Notably, the Beijing genotype was not detected in our study. The prevalence of resistance in Iran is lower than in other parts of the world.

5. Conclusion

In this study, population structure analysis using spoligotyping verified that H4 and CAS-Delhi lineages were the most frequent lineages of MTB clinical isolates in Kermanshah (western border province). This provides baseline information on the genetic diversity of MTB in this region. To our knowledge, the rate of MDR was low. A good control program needs to understand the dynamic transmission of local isolates; Therefore, we conducted this study to identify the circulating isolates in the western region of Iran.

Ethical statement

This study was approved by the Ethics Committee of Kermanshah University of Medical Sciences (Ref. ID: IR.KUMS.REC.1396.65).

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CRediT authorship contribution statement

Soroush Borji: Writing – review & editing, Writing – original draft, Visualization, Data curation. **Sara Kooti:** Writing – original draft, Data curation. **Rashid Ramazanzadeh:** Methodology, Conceptualization. **Sepide Kadivarian:** Methodology, Data curation. **Sara Atashi:** Data curation. **Parviz Mohajeri:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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