Mycobacterium tuberculosis Complex Drug Resistance in a High Tuberculosis Incidence Area from the WHO Eastern Mediterranean Region

Noormohamad Mansoori[1], Masoumeh Douraghi[1, 2], Ali Akbar Rajabloo[3], Masoomeh Taziki[3], Mehdi Yaseri[4], Farzam Vaziri[5, 6]

[1]Division of Microbiology, Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran; [2]Food Microbiology Research Center, Tehran University of Medical Sciences, Tehran, Iran; [3]Tuberculosis Reference Laboratory of Golestan Province, Golestan University of Medical Sciences, Gorgan, Iran; [4]Department of Epidemiology and Biostatistics, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran; [5]Department of Mycobacteriology and Pulmonary Research Pasteur Institute of Iran, Tehran, Iran; [6]Microbiology Research Center (MRC), Pasteur Institute of Iran, Tehran, Iran.

ABSTRACT - Purpose. The incidence of tuberculosis (TB) in Golestan province of Iran has been ranked 10th among countries of World Health Organization (WHO) Eastern Mediterranean Region. The province is residence of ethnically heterogeneous groups. However, there are limited data on *Mycobacterium tuberculosis* drug resistance in this province. The main aim of this study was to determine the resistance profile of *M. tuberculosis* complex (MTBC) isolates to first-line anti-TB drugs. **Methods:** The clinical specimens were collected from 11807 cases diagnosed during this study. MTBC isolates were tested for susceptibility to first-line anti-TB drugs. **Results:** A total of 176 new cases were diagnosed as culture positive for MTBC. There was one case that had multidrug-resistant (MDR) isolate and 18 (10.2%) had isolates that were resistant to at least one drug (any drug resistant). Resistance to streptomycin and isoniazid was noted in 15 (8.5%) and 5 isolates (2.8%), respectively. Also, a statistically significant association was observed between age groups and any drug resistance pattern ($p = 0.022$): 1-24 years *vs*. 25-45 years ($p = 0.033$), 25-45 years *vs*. >65 years ($p = 0.010$), 46-65 years *vs*. >65 years ($p = 0.050$). One third of any drug resistant isolates were obtained from TB patients of Persian ethnic group. **Conclusion:** Despite the high incidence of TB, the rate of MDR-TB in Golestan province was similar to those reported by WHO for Iranian new cases from other regions. One-tenth of the studied isolates showed any drug resistance pattern. This rate of any drug resistance implies the possibility of initial resistance of MTBC isolates circulating in this region.

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INTRODUCTION

Mycobacterium tuberculosis (MTB) and *Mycobacterium bovis* are important members of *Mycobacterium tuberculosis* complex (MTBC). Although MTB is frequently identified as a main causative agent of human tuberculosis (TB), the implication of *M. bovis*, as a relatively common cause of TB should not be neglected (1). TB is one of the most common infectious diseases worldwide and still remains as a hygiene problem particularly in developing countries. Numerous studies have reported the age-specific trend of TB and have also indicated that the rate of TB varies significantly by ethnic groups, immigration, and other risk determinants (2, 3). Armed with the capacity to emerge drug-resistant MTB, the treatment failure of patients harboring such isolates is a common issue (4). There is mounting evidence that 3.5% of new TB cases harbor multidrug-resistant TB (MDR-TB) worldwide (5). The widespread distribution of MDR-TB has been also highlighted in World Health Organization (WHO) reports that pointed to MDR-TB as a public health crisis (4).

Correspondence Author: Masoumeh Douraghi, Ph.D., Division of Microbiology, Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, PO Box: 14155-6446, Tehran, Iran. Tel: +98 21 42933152, Email: mdouraghi@tums.ac.ir.

Nine countries in the WHO's Eastern Mediterranean Region including Pakistan, Afghanistan, Sudan, Morocco, Somalia, Iraq, Egypt, Iran and Yemen are known as the TB burden countries contributing to 95% of TB burden in this region (6). Iran is one of the middle-income countries with intermediate TB burden and an estimated incidence rate of 22 per 100000 population (5). According to a 2016 report from the Ministry of Health and Medical Education of Iran, the incidence of TB varies in different provinces of Iran and the Golestan province has a high incidence of TB (38.2 per 100000 people) (7). Golestan province is situated in north of the country in the vicinity of the Caspian Sea. This province houses several ethnic groups such as Baluch, Cossack, Kurd, Persian, Sistani, Turk, Turkmens and other non-Iranian immigrants from Afghanistan and Turkmenistan (8, 9).

In addition, Golestan is bordered by Turkmenistan where the rate of MDR-TB reaches 14% among new TB cases (10). Despite the high incidence of TB, there are scarce data about drug resistance in large numbers of TB patients in this province which is a residence of ethnically heterogeneous groups. As this province is unique in terms of the TB incidence and ethnicity groups, therefore, we aimed to determine the resistance profile of MTBC isolates to first-line anti-TB drugs and also assess the distribution of these isolates in different ethnic groups.

MATERIALS AND METHODS

Setting
The study proposal was reviewed and approved by the ethics committee of Tehran University of Medical Sciences. This cross-sectional study was conducted in Golestan province, north of Iran, during a period from July 2014 to July 2015. This province is surrounded by the other provinces of Iran in all directions except in northern direction bordering Turkmenistan country. In addition to housing population of various ethnicities of Iran (including Baluch, Kurd, Persian, Sistani, Turk and Turkmens), Golestan is a residence or destination of foreign nationals. As this province was identified previously as a high-incidence area of TB, the study included cases who were referred from 47 urban and 743 rural health care units to the Tuberculosis Reference Laboratory of Golestan, Iran. Demographic characteristics were gathered from the information recorded in medical forms of Iranian TB registry including age, sex, ethnicity and nationality. Afterward, the culture positive TB patients were divided into four age groups including: 1-24, 25-45, 46-65 and >65 years (Table 1).

Table 1. Comparison of patients with pan-susceptible *Mycobacterium tuberculosis* complex isolates and patients harboured the isolates with any drug resistance profile

Susceptibility profile	Pan-susceptible	Any drug resistance	*p*
	n (%, 95% CI)		
Sex			0.459
Female	67 (42.4, 34.9-50.2)	6 (33.3, 15.3-56.3)	
Male	91 (57.6, 49.8-65.1)	12 (66.7, 43.7-84.7)	
Age (Year)			0.022*
1-24	18 (11.4, 7.1-17)	0 (0)	
25-45	49 (31, 24.2-38.5)	10 (55.6, 33.2-76.3)	
46-65	44 (27.8, 21.3- 35.2)	7 (38.9, 19.4-61.7)	
>65	47 (29.7, 23- 37.2)	1 (5, 0.6-23.2)	
Ethnicity			0.051*
Sistani	64 (40.5, 33.1-48.3)	2 (11.1, 2.4-31.1)	
Turkmen	38 (24.1, 17.9-31.1)	4 (22.2, 8-44.6)	
Persian	32 (20.3, 14.6-27)	6 (33.3, 15.3-56.3)	
Baluch	11 (7, 3.8-11.7)	4 (22.2,8-44.6)	
Turk	4 (2.5, 0.9-5.9)	1 (5.6, 0.6-23.2)	
Kurd	2 (1.3, 0.3-4)	0 (0)	
Non-Iranian	7 (4.4, 2-8.5)	1 (5.6, 0.6-23.2)	

* Significant based on Fisher exact test.

Specimen Collection and Isolates

The clinical specimens were collected from 11807 cases diagnosed during this study. All specimens were subjected to smear microscopy by Ziehl-Neelsen staining and cultured into Lowenstein-Jensen (LJ) culture media after decontamination by Petroff's method. The colonies were identified as MTBC using conventional biochemical tests including morphology of colony, growth rate, pigment production, niacin production, catalase activity and nitrate reduction (11, 12).

Drug Susceptibility Testing (DST)

Isolates were tested for susceptibility to first-line anti-TB drugs. DST against rifampicin (RMP, 40 μg/mL), isoniazid (INH, 0.2 μg/mL), ethambutol (EMB, 0.2 μg/mL), and streptomycin (STM, 4.0 μg/mL) (Sigma-Aldrich) was performed using the proportion method on LJ media. Briefly, bacterial suspensions were prepared and added to LJ media containing the critical concentration of the drugs and drug free LJ media, as bacterial growth control. Resistance was defined as 1% or more bacterial growth on the media containing the critical concentrations of the drugs. The drug susceptible *M. tuberculosis* H37Rv strain was used for quality control (12, 13).

Definitions

New case of tuberculosis is a patient with TB who had never been previously treated for TB or treated for less than one month (13). Any drug resistance was defined as strains that were resistant to one or more first-line drugs. Mono-resistance was defined as resistance to only one of four first-line drugs. MDR-TB was defined as strains showing resistance to at least RMP and INH. Pan-susceptible is defined as MTBC isolates that are susceptible to all first-line anti-TB drugs (13).

Statistical Analysis

Data analysis was performed using SPSS ver. 18.0 (SPSS Inc., Chicago, IL, USA). Descriptive analysis of the data was conducted using frequencies (counts) with 95% confidence intervals and mean± SD. For comparison of the categorical variables, the Fisher's exact test was used at *p* <0.05. To compare the any drug resistance rate among age groups, we used generalized linear model and to consider the pairwise comparison in this analysis, we used Least Significant Difference (LSD) test.

RESULTS

A total of 11807 cases were studied and 176 were diagnosed as culture positive for MTBC - including 174 MTB and 2 *M. bovis* isolates - and correspondingly were considered as TB patients These patients had no history of TB treatment or treated for less than one month according to patient's information, therefore, were considered as new cases. The majority of isolates (n=160, 90.9%) were recovered from sputum while 15 (8.5%) were from bronchoalveolar lavage and only one MTB isolate was obtained from gastric juice. In terms of ethnicity, more than one third (n=66, 37.5%) of MTB isolates belonged to patients of Sistani, followed by Turkmen (n=42, 23.9%), Persian (n=38, 21.6%), Baluch (n=15, 8.5%), Turk (n=5, 2.8%), and Kurd (n=2, 1.1%) ethnic groups. The remaining were isolated from non-Iranian (n=8, 4.6%).

Demographic Information

The age of patients ranged from 16 to 90 years and the mean age ±SD was 50.38 ± 19.51 years. The male to female ratio was 1.41 as 103 (58.5%) of patients were male. One hundred sixty-eight patients (95.4%) were native Iranians while the remaining eight patients were non-Iranian: seven (4%) were immigrant patients from Afghanistan and one (0.6%) from Turkmenistan.

Drug Susceptibility Patterns

Among 176 isolates included in this study, a set of 158 (89.8%) isolates were pan-susceptible. Although all the isolates were susceptible to EMB (Table 2), resistance to STM was noted in 15 isolates (8.5%, 95% CI = 4.5%-12.5%). In regard to other first-line anti-TB drugs, 5 isolates showed resistance toward INH (2.8%, 95% CI = 0.6%-5.7%) and only one isolate (0.6%, 95% CI = 0.0%-1.7%) was resistant to RMP. The latter isolate was identified as MDR showing resistance to INH and RMP. This isolate was recovered from an Iranian 62-year-old female living in a village. As depicted in Figure 1, the resistance profile of MTB isolates was different among the ethnic groups. Only two STM-resistant isolates were found in TB patients of Sistani ethnicity, despite that these patients harbored higher proportion of MTB isolates

Table 2. First line anti-tuberculosis drugs resistance in *Mycobacterium tuberculosis* complex isolates

Resistance profile	No.	%
Pan-susceptible	15	89.8
Any drug resistance	18	10.2
Mono-resistance	16	9.1
Any drug resistance to STM	15	8.5
Any drug resistance to INH	5	2.8
Any drug resistance to EMB	0	0.0
Any drug resistance to RMP	1	0.6

Keys: STM, Streptomycin; INH, Isoniazid; EMB, Ethambutol; RMP, Rifampicin
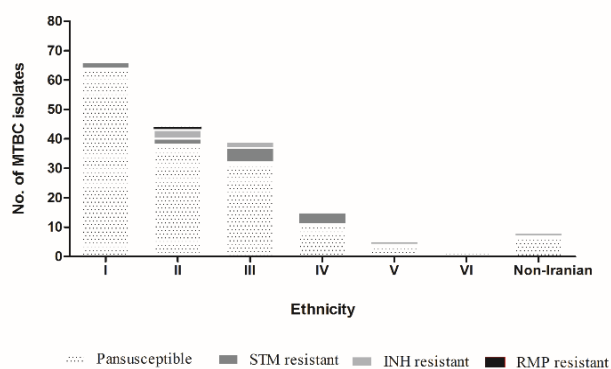
Figure 1. The distribution of *Mycobacterium tuberculosis* complex (MTBC) isolates in relation to resistance profile in different ethnic groups. Non-Iranians comprised of seven patients from Afghanistan and one from Turkmenistan. Keys: I, Sistani; II, Turkmen; III, Persian; IV, Baluch; V, Turk; STM Streptomycin; INH, Isoniazid; EMB, Ethambutol; RMP, Rifampicin

compared with other ethnicities. Even though mono-resistance to RMP was not found in our study, a MDR isolate showing resistance to RMP and INH was obtained from a patient of Turkmen ethnic group. In addition, INH mono-resistant (n=2) and STM mono-resistant isolates (n=2) were identified in Turkmen ethnic group. In comparison to other groups, number of STM resistant isolates was higher in those patients of Persian (n=5) and Baluch (n=4) ethnic group. Overall, 18 (10.2%, 95% CI = 5.7%-15.3%) isolates were resistant to at least one drug and categorized as any drug resistant: one (12.5%, 95% CI = 0.0%-37.5%) was observed among non-Iranian immigrants and 17 (10.1%, 95%

CI = 5.4%-14.3%) in Iranian patients. As indicated in Table 1, higher any drug resistance rate was observed in MTB isolates from males than females, but this difference did not reach statistical significance (*p*= 0.459). In addition, more than half of isolates (10 out of 18, 55.6%) with any drug resistance profile were found in age range of 24-45 years and this difference was statistically significant (*p* =0.022). Pairwise comparisons (adjusted by LSD test) were showed the statistically significant difference between following age groups: 1-24 years *vs.* 25-45 years (*p* = 0.033), 25-45 years *vs.* >65 years (*p* = 0.010), 46-65 years *vs.* >65 years (*p* = 0.050), but other pairwise comparisons were not statistically significant (all were *p* >0.05). A notable point is that one third of any drug resistant isolates were obtained from TB patients of Persian ethnic group (Table 1).

DISCUSSION

Mismanagement in TB treatment stems, in part, from a lack of information on drug susceptibility patterns of MTBC isolates. Although the dug susceptibility testing has been incorporated into TB control programs, there is a limited data regarding anti-TB drug patterns in Golestan. Here we found that the percentage of MDR-TB among new TB cases in this high-incidence area was less than one percent; which is lower than the global estimated rate of 3.5%. This figure in Iran's neighboring countries, i.e., Turkey, Afghanistan, Pakistan, Armenia, Azerbaijan and Turkmenistan is estimated as 2.5%, 3.2%, 3.7%, 9.4%, 13% and 14% respectively (5, 10). In addition, a recent study in Turkmenistan (14) has reported a 13.9% MDR-TB, an incident closely in agreement with that reported by WHO. The limited number of studies conducted previously on the drug resistance pattern of MTBC isolates in Golestan province, targeted the same area as we did, had different sample size and study period. In addition, they did not study the drug resistance to EMB and STM. Javid *et al.* (15) used the molecular methods for detection of mutations in the genes corresponding to resistance toward RMP and INH for 87 MTBC isolates recovered from new TB cases in Golestan province and showed that the percentage of MDR-TB, RMP and INH resistance were 2.3%, 4.6% and 6.9%, respectively. However, the sample size used in the mentioned study was rather small to represent the entire province. In

another study performed in this region, Livani *et al*. (16) studied drug susceptibility test on 148 MTB isolates using the mycobacteria growth indicator tube system and showed that 3.4% were MDR, 3.4% resistant to RMP and 17.6% resistant to INH. The drug resistance percentages reported in the latter study are higher than our findings; however, it is important to note that they included some isolates recovered from non-new TB cases as some isolates were recovered from patients that treated for TB for more than one month.

The National Research Institute of Tuberculosis and Lung Disease of Iran conducted a retrospective analysis for drug resistance patterns among 47 MTB isolates from Golestan province using Multiplex PCR to detect mutations in the genes responsible for RMP and INH resistance from 2010 to 2011 and showed that 4.2% of isolates were MDR (17). This percentage seems to be overestimated because they included TB patients who remained sputum smear positive after two to nine months of standard therapy.

Another finding of the present study is that one-tenth of isolates were recognized with any drug resistance profile. Studies conducted in Pakistan, Iraq and Turkey indicated that 10.5%, 21.3% and 27.9% of isolates had any drug resistance pattern, respectively (18-20). In addition, we found a statistically significant association between any drug resistance and some age groups including, 1-24 years *vs*. 25-45 years, 25-45 years *vs*. >65 years and 46-65 years *vs*. >65 years. Several reports have demonstrated that drug resistant TB is predominantly associated with patient's age, for instance, Garcia *et al*. (21) showed significant association between drug resistance and patients aged older than 40 years. In contrast, Faustini *et al*. (22) divided the TB patients in two age groups (age < 65 and age > 65 years) and reported that MDR-TB was more common in patients under 65 years age. Also Lukoye *et al*. (23) revealed that among new cases, those aged older than 35 years were more likely to have MDR-TB, compared with the patients less than 35 years. In present study, 94.4% of the drug resistant isolates were found to be from patients in age groups of 24-45 and 46-65. The high frequency of drug resistance among young age group may indicate the occurrence of recent transmission, whereas this status in the older age group may suggest the distant past acquisition of infection.

Although we did not find RMP mono-resistant isolate, 8.5% of isolates were STM-resistant, followed by 2.8% resistance toward INH. These findings are consistent with lower values of previous reports from Iran ranging from 7.8-27% for STM and 1.9-23% for INH (24-27). There are reports from Pakistan, Turkey and Iraq indicating 0-16.1% resistance to STM and 8.9-18.9% to INH (18-20). The main cause of STM resistance in Iranian new TB cases has remained unknown. It might be due to the extensive use of STM for treatment of different infectious diseases, or may be partially attributed to cross-resistance which occurred due to imprudent use of other aminoglycosides (27, 28). This high rate of STM resistance has considerably significant impact on TB control strategies as it is often used as second-line drugs for TB treatment in Iran. As a consequence, the treatment regimens might be less effective due to the effectiveness of STM (25, 29, 30). Salek *et al*. (31) studied the incidence of pulmonary TB in different ethnic groups residing in Golestan province from 1999-2003 and reported that 45% of TB patients belonged to Sistani, followed by Persian (22%), Turkmen (19%) and Baluch (9%) ethnicity. Also the Kurd and Baluch groups collectively comprised 2% of TB patients and the remaining was from immigrant patients (2%). These percentages are in close agreement with our results and highlight that the distribution of TB has not changed in different ethnic groups in Golestan province during recent years. Another finding of the present study is that one-third of all drug resistant isolates were obtained from patients of Persian ethnic group, followed by Turkmen and Baluch ethnic groups. These results are not unexpected as 45.5% of TB cases are related to these three ethnic groups. Additionally, Turkmen and Persian ethnic groups are native residents of Golestan while Baluch ethnic group is originally from the highest TB incidence area of Iran; Sistan and Baluchestan. This province is bordering the high TB burden countries, Pakistan and Afghanistan. Also, this finding was supported by a study conducted in Sistan and Baluchestan province that 17% of isolates were designated as any drug resistant and 5% as MDR (27). The traffic between this province and other areas may contribute to the circulation and spread of resistant MTB. Despite the differences in the incidence of TB in different provinces bordering Golestan, drug resistance

patterns were approximately similar (24, 27). The rest of provinces had the proportions of drug resistance and MDR ranging from14.5-39% and 0.95-7%, respectively (26, 32-34). Given the geographic, social and economic circumstances, Golestan province has been received a large number of Iranian immigrants by inter-province movement from Sistan and Baluchestan. Besides, we found non-Iranian new TB cases including one from Turkmenistan and seven Afghan patients. It seems that traveling between these countries and Golestan province might partly affect the high TB incidence in this province, but this finding needs to be proven by further studies.

CONCLUSION

The TB burden in Golestan province is similar to those of 0.3-1.4% reported by WHO for the MDR-TB for Iran. The MDR-TB was found in a new case; therefore, it is not unlikely that this case was infected with an already circulating MDR isolate. The highest mono-resistance was observed against STM, indicating a high risk for treatment failure in such population. Moreover, the rate of 18% for any drug resistance implies the possibility of initial resistance of MTB complex isolates recovered from new cases. On the other hand, the data point toward the success of TB control programs during recent years, despite the high incidence of TB in Golestan province. However, it should be taken into consideration that these programs are threatened by the possible transmission of MDR-TB from neighbor countries. Consequently, there is an increasing demand for monitoring the antibiotic susceptibility profile in order to interrupt the transmission of resistant isolates. This study provided information to understand the TB resistance trends and also highlighted the effectiveness of TB control measures.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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