

ORIGINAL ARTICLE

The most prevalent *Mycobacterium tuberculosis* superfamilies among Iranian and Afghan TB cases

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Abstract

This survey identified the spoligopatterns of *Mycobacterium tuberculosis* strains with an international designation responsible for transmission and prevalence of TB (2000 to 2005) among native and immigrant populations of Tehran. The spacer oligonucleotides typing was performed on 1742 *Mycobacterium tuberculosis* strains isolated from verified cases of TB. Clinical and demographic data of patients were collected using traditional methods. A total of 133 distinct spoligopatterns was observed. 1679 clinical isolates were clustered in 70 clusters (52.5%) and 63 isolates were defined as orphans pattern (47.3%). Based on an international spoligotype database, the east African-Indian (EAI, 24%), central Asian (CAS, 20.8%), T clade (20.7%), Haarlem I (4.4%), Beijing (3.2%) and shared type 253 (3.1%) were the major identified *M. tuberculosis* superfamilies. Our results showed that the intra-community TB transmission was 13.7%, whereas the inter-community transmission was 39.3% for Afghans and 20.3% for Iranians. The study highlighted the epidemic potential of specific genotypes (EAI, CAS, T clades) among tuberculosis cases in the Tehran territory. We also confirmed that the impact of transmission through immigration is low.

Introduction

In Iran, a country with 72 million inhabitants, the incidence rate of tuberculosis (TB) in 2004 was 28 per 100,000 [1]. In all parts of the country the prevalence of TB is variable, and it is higher in eastern and north-west regions (50–99 per 100,000) [1,2]. In these areas the high rate of immigration, HIV infection, poor access to health services and low income were correlated with high incidence of TB. In our previous investigations, we have demonstrated that 35% to 40% of all registered TB patients in Tehran (capital city of Iran) were Afghan immigrants [3,4]. With such a high number of TB-infected Afghans living in Tehran, the question was raised as to what extent the TB incidence is related to the immigrant population. In this context, the usual traditional methods are ineffective, and the impact of a TB control programme is often difficult to assess [3,5]. This difficulty could be overcome by

using an alternative approach in which molecular strain typing techniques are used [5,6]. Spacer oligonucleotide typing is 1 of the molecular typing methods that are used to differentiate *M. tuberculosis* complex [7,8]. The method is based on an analysis of polymorphism in the direct repeat (DR) chromosomal region consisting of identical 36-bp DRs alternating with 35 to 41 bp unique spacers. Its utility as an initial screening method is well documented. Recently, by systematically analysing spoligopatterns of *M. tuberculosis* strains essentially from Europe and the USA (<http://www.pasteur-gualdeloupe.fr/tb/spldb3>) the polymorphism database mining methods were constructed. A total of 9 potential superfamilies or clades of *M. tuberculosis* complex have been identified (*M. africanum*, Beijing, *M. bovis*, EAI, CAS, T group of families, Haarlem I, X family and LAM family). In this study we seek to determine the predominant spoligopattern among native and immigrant Afghan TB

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patients who developed the disease during the previous 10 y. In addition, we compared the genetic biodiversity of *M. tuberculosis* strains with international designation [9,10] that were responsible for transmission and prevalence of TB in Tehran.

Materials and methods

Data collection

The study was conducted from December 2000 to June 2005. Case data were collected by trained technicians using standard questionnaires. Information was obtained on gender, age, country of birth, close contacts (family member or sharing a night or day shelter), previous TB history, present address and associated medical data such as HIV infection, tuberculin skin test and chest radiography findings.

Characteristics of study population

From December to June 2005, 3812 patients with pulmonary TB were referred to the Iranian National Reference TB Laboratory (INRTL) Tehran, Iran (generally, all health facilities in Tehran refer their TB suspect to NRL for diagnosis and treatment). Of 3812 suspected TB patients, 1582 (41%) were excluded because they were either culture negative (916, 24%) or had smear examination only (667, 17.4%). In addition 394 patients (10.3%) were excluded because their cultures were contaminated. 93 patients (2.4%) were infected with diseases other than tuberculosis. The study therefore consisted of 1074 (28.1%) Iranian and 668 (17.5%) Afghan cases. Their demographic characteristics are shown in Table I. 935 (53.6%) were males and 807 (46.3%) were females. The mean age for Iranian cases was 46.1 ± 17.6 y (SD) and for Afghans was 39.8 ± 16.3 y (SD). HIV serology was performed in 200 cases (11.4%), of which only 12 were found to be positive (6%). HIV and intravenous drug abuse information was not available for Afghan cases. Reviewing the patients' questionnaires revealed that the majority of Afghan patients had either a previous history of TB (318, 47.6%) or had a close contact (240, 35.9%).

In comparison, 73.5% of Iranian patients ($n = 790$) were newly smear positive without any history of contact.

Bacterial strains

Primary isolation and culturing of *Mycobacterium* isolates was followed in accordance with the procedures manual [1]. All isolates were identified as *M. tuberculosis* complex by using biochemical tests, including production of niacin, catalase

Table I. Shows the detailed demographic data of the study population; groups I and II.

Total number of patients	Iranian	Afghani
1742	1074 (61.6%)	668 (38.3%)
Mean Age	46.1 ± 17.6	39.8 ± 16.3
Female	495 (46%)	312 (46.7%)
Male	579 (53.9%)	356 (53.2%)
Pulmonary	950 (88.7%)	613 (91.7%)
Extra-pulmonary	93 (8.6%)	34 (5.0%)
Both	31 (2.8%)	21 (3.1%)
HIV-positive	12/200*	NA
Intravenous drug abuse	118 (10.9%)	NA
Previous history of TB	117 (10.8%)	318 (47.6%)
History of family or contact tracing	167 (15.5%)	240 (35.9%)
New-smear positive	790 (73.5%)	110 (16.4%)
Crowded and poor living condition	863 (80.5%)	513 (76.7%)
Low salary	910 (84.8%)	604 (90.4%)
Poor access to health services	813 (75.8%)	417 (62.4%)
Unemployment	520 (48.5%)	348 (52.0%)

*Only 200 patients had a HIV test result, of which 12 were positive (6%).

NA: data not available.

activity, nitrate reduction, pigment production and growth rate. Drug susceptibility testing against isoniazid (INH), rifampicin (RIF), streptomycin (STM) and ethambutol (ETB) were performed by the proportional method on Lowenstein-Jensen media at a concentration of 0.2, 40, 4.0 and 2.0 µg/ml, respectively. Susceptibility to pyrazinamide (900 and 1200 µg/m) was tested using a 2-phase medium and if at the 21st d reading, the proportion of resistant colonies was higher than the critical proportion, the strain was reported as resistant to pyrazinamide. Multi-drug resistance was defined as resistance to at least isoniazid and rifampicin.

Spoligotyping

Spoligotyping was performed as previously described by Kamerbeek et al. [7] with a commercially available kit, according to the instructions supplied by the manufacturer (Isogen Bioscience BV, Maarsse, The Netherlands). In brief, DR region was amplified by PCR using primers [DRa (5'-CCG AGA GGG GAC GGAAAC-3') and [DRb (5'-GGT TTT GGG TCT GAC GAC-3')] derived from the DR sequence. The amplifications were carried out in a Master Cycler 5330 (Eppendorf, Germany) by using 53 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 55°C and extension for 30s at 72°C. The first denaturation and final extension steps were held for 10 min. The amplified DNA was hybridized to a set of 43 immobilized oligonucleotides derived from the spacer sequences

of *M. tuberculosis* H37Rv and *M. bovis* BCG P3 by reverse line blotting. Hybridized DNA was detected with ECL detection liquid (Amersham, Buckinghamshire, UK) and by exposing ECL-Hyper film (Amersham) to the membrane for 12 min [7].

Statistical analysis

The results obtained were entered in a binary format as Excel spreadsheets (Microsoft) and compared with published data [9,10,12,13]. Categorical data in each group (Iranian and Afghan TB cases) were compared by χ^2 test or Fisher’s exact test.

Results

Prevalence of drug resistance

The rate of resistance to each of the 5 first-line, anti-TB medications, as well as resistance to multiple drugs, is shown in Table II; 712 Iranian (66.2%) and 274 Afghani cases were susceptible to all 4 drugs tested. The results showed that 263 patients (15%) were multi-drug resistance cases. Notably, 175 of MDR-TB cases (66.5%) were isolated from Afghan immigrants. 80 (25.1%) had a previous history of TB, 96 (28.7%) were close contact and 26 (23.6%) were newly smear positive cases. As shown in Table II, 379 patients (21.7%) had mono-resistant strains (258 were SM, 52 were INH, 29 were RF, 30 were ETB and 10 were PZA) and 114 patients (6.5%) had a combined resistance. Overall, the resistant rate to any drug or drug combination including MDRTB was significantly higher in Afghan studied cases (58.5%) than in Iranian cases (33.9%).

Spoligotyping

A total of 133 distinct patterns was observed. 63 unique patterns (orphan patterns) were seen,

and the remaining 1679 strains were contained within 70 clusters. The diversity of observed clusters was 0.076 (diversity = the number of shared types divided by the total number of found isolates, i.e. 133 of 1742, 0.076). Among clustered isolates, 2 broad categories of clusters (shared types) could be identified; the clusters represented by more than 10 isolates, i.e. 11 clusters containing 1385 isolates, and those represented by fewer than 10 isolates, i.e. 59 clusters containing 294 isolates (12 shared types contained only 2 patients each, 9 shared types contained only 3 patients, 10 shared types contained 4 patients, 8 shared types contained 5 patients, 7 shared types contained 8 patients, 3 shared types contained 8 patients and 10 shared types contained 9 patients only).

Epidemiological analysis of patients in clusters

Afghan TB cases in clusters. Of 668 isolates, 640 (95.8%) were clustered in 32 clusters. Nine clusters contained 556 isolates (more than 10 isolates per cluster) and the remaining 84 isolates clustered in 23 clusters (fewer than 10 isolates per cluster) (Figure 1). A direct epidemiological link could be established for 252 Afghan patients (39.3%) in different clusters; 240 were family members. Among identified clusters, the Beijing family and ST253 had a unique appearance. In the Beijing family, 68.7% of patients were below the age of 30 y, 62.5% were family or close contact, and 58.7% were MDRTB. In comparison, 65.4% of patients in the cluster ST 253 were older than 45 y of age and 74.5% had a previous history of TB. The age differences with Beijing and ST253 family were statistically significant.

Iranian cases in clusters. These contained 829 isolates (more than 10 isolates per cluster) and 57 clusters contained 212 isolates (fewer than 10 isolates per

Table II. Rate of drug resistance according to nationality.

Variable	Iranian			Afghan-case			Total case
	n = 1742	Previous history of TB n = 117	Close contact n = 167	New smear positive n = 790	Previous history of TB n = 318	Close contact n = 240	New smear positive n = 110
Mono-resistant							
Isoniazid	52 (2.9%)	9 (7.6%)	10 (5.9%)	15 (1.8%)	10 (3.1%)	5 (2.0%)	3 (2.7%)
Rifampin	29 (1.6%)	6 (5.1%)	6 (3.5%)	5 (0.6%)	9 (2.5%)	2 (0.8%)	1 (0.9%)
Pyrazinamide	10 (0.5%)	3 (2.5%)	3 (1.7%)	2 (0.25%)	1 (0.3%)	–	1 (0.9%)
Ethambutol	30 (1.7%)	9 (7.6%)	5 (2.9%)	7 (6.8%)	4 (1.2%)	3 (1.2%)	2 (1.8%)
Streptomycin	258 (14.8%)	22 (18.8%)	34 (20.3%)	85 (6.7%)	59 (18.5%)	40 (16.6%)	18 (16.3%)
Two drugs	90 (5.1%)	8 (6.8%)	15 (8.9%)	18 (2.2%)	34 (10.6%)	9 (3.7%)	6 (5.4%)
>Two drugs	24 (1.3%)	4 (3.4%)	5 (2.9%)	3 (0.3%)	5 (1.5%)	5 (2.0%)	2 (1.8%)
MDRTB	263 (15.0%)	22 (18.8%)	19 (11.3%)	47 (5.9%)	80 (25%)	69 (28.7%)	26 (23.6%)
Sensitive	986 (56.6%)	34 (29%)	70 (41.9%)	608 (76.9%)	116 (36.4%)	107 (44.5%)	51 (46.3%)

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initial TB patients referred to our unit were from Afghan-born immigrants. Notably, the rate of resistance to any drug or drug combination (including MDRTB), was almost 2-fold that of Iranian cases. These findings highlight the need of screening immigrants from neighbouring countries, which is absent in the current system. In previous studies [1,2], the impact of the immigrant sub-population on the epidemiology of tuberculosis has been studied using classical methods. In the present study, intra-community TB transmission was 13.7%, whereas the inter-community transmission was 39.3% for Afghans and 20.3% for Iranians. Therefore, the impact of TB transmission through immigration was low and we suggest that most Iranians develop tuberculosis from reactivation and/or inter-nationality transmission. Recently, Sreevatsan et al. [14] assigned *M. tuberculosis* isolates into 3 distinct genetic groups. Group I are evolutionarily older and have further evolved into group II and III organisms. Later, Soini et al. demonstrated a relation between spoligotyping pattern and the major genetic groups of *M. tuberculosis* isolates [10]. *M. tuberculosis* isolates belonging to genetic groups II and III failed to hybridize with spacers 33 to 36, suggesting that these spacers and the DRs have become deleted from the genome of all these groups [10]. In the present investigation about half of *M. tuberculosis* strains that isolated from native cases belonged to the principal genetic group I and the remainder belonged to the genetic groups II and III. This feature was in sharp contrast to what is observed in Afghan TB cases, where 72% of TB bacilli belong to genetic group I organisms. Hence, the current epidemiological picture of TB in Iran is based on the persistence of ancestral clones of *M. tuberculosis* as well as those emerging recently (groups II and III). In comparison, the TB bacilli in Afghan immigrants were more likely to be of ancient origin. Our results showed that out of genetic group I organisms (Table II), the east African-Indian (EAI) and central Asia I (CAS) were the most frequent superfamilies in both communities. Until now, this family of strains has been reported in different countries of the Middle East (Iran, Pakistan, and India), Oceania (Australia), the United State and Europe. In Europe and Australia, these strains were regularly found to be linked with immigrants from central Asia and the Middle East [10,15,16]. However, in previous reports the number of obtained samples from these regions was very low ($n = 351$) and until today no single pattern could exceed $>5\%$. In this study EAI and CAS families reached 24.1% and 21.6%, respectively, of all clustered isolates. The other detected superfamilies

(T clade, Beijing family and shared type 253) belonged to the principal genetic group II and III organisms [15,16]. The T clade was dominant in Iranians, whereas the Beijing family and shared type 253 were dominant in Afghans. The T clade is characterized by the absence of spacers 33–36, and Duchene et al. [16] suggested the African origin of these alleles. The high prevalence of this family among Iranian TB cases (33.3%) might be of great interest for further study and genetic interpretation. The Beijing type of *M. tuberculosis* was originally described by van Soolingen et al. [17] in China, and are reported to be highly prevalent throughout Asia and in the countries of the former Soviet Union. The Beijing genotype is thought to possess selective advantages compared with strains of other *M. tuberculosis* genotypes and is sometimes associated with multi-drug resistance with specific pathogenic properties [18–20]. In our study, 55.4% of patients with Beijing strains were MDRTB. It has been claimed that alteration within DNA repair (mutator genes) might favour the emergence of MDR strains with an increased adaptability [19]. Association of Beijing strains with drug resistance pattern as demonstrated in this study might be related to accumulation of mutations within their putative mutator genes and that has to be further investigated. Doroudhchi et al. [4] have demonstrated that 40% of Iranian isolates in the Beijing family have come from Afghan refugees living in Iran. In the present data, 68.4% of Afghan patients with Beijing characteristic were below the age of 30 y, and had stayed in Iran for more than 10 y without any previous history of TB. We also understood that 68.7% of Afghan patients of Beijing family develop diseases because they are family or close contact. Therefore, the Beijing superfamily is 1 of the circulating MTB strains, and prevalence of these strains or other MDR strains in Afghans might be related to their low income, psychological stress, poor education and poor living conditions. We also found another conserved genotype of *M. tuberculosis* strain that was prevalent throughout the former Soviet Union. The presence of ST 253 in Afghans could be linked to the past former Soviet Union history in that region. However, further investigations are in progress to estimate the prevalence of this genotype in Afghanistan itself. Last but not least, the findings highlighted the need for developing new strategies to control TB especially in groups at high risk. Finally, to our knowledge this is the first introduction and report which specifically identified the major *M. tuberculosis* superfamilies in Tehran.

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