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CHARACTERIZATION OF *Mycobacterium tuberculosis* COMPLEX ISOLATED FROM IRANIAN AND AFGHANI PATIENTS BY SPOLIGOTYPING METHOD

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ABSTRACT

Designing newer drugs, vaccines, and diagnostic techniques is dependent on better understanding of *M. tuberculosis* virulence mechanism. In this study the prevalence of *pcaA* gene was determined in *M. tuberculosis* strains typed by spoligotyping. The associated risk factors among patients with different nationalities residing in Iran were also determined. The isolated *M. tuberculosis* strains have been characterized by performing susceptibility tests against four first-line antituberculosis drugs and were then subjected to spoligotyping characterization. PCR was used for detection of *pcaA* gene and its nucleotide sequence was also determined. Spoligotyping of *M. tuberculosis* strains resulted in 140 different patterns. One hundred twenty two (87.1%) of these spoligotype isolates were unique and reported for the first time. The remaining18 (12.8%) spoligotype patterns were previously reported from other geographical regions of the world. Haarlem family was most prevalent than other genotype. Antibiotic resistances were higher in those isolated from the Iranian patients. The *pcaA* gene was detected in *M. tuberculosis* clinical isolates but not in saprophyte strains such as *M. kansasi*. The results showed that, spread of *M. tuberculosis* strains belonging to the Beijing family among Iranian patients has to be considered seriously. This study confirmed the widespread existence of *pcaA* gene in almost all the clinical isolates. It is also important to undertake studies to identify which factors are the most significant to consider in tuberculosis control program.

Key words: tuberculosis; resistance; drugs; spoligotyping; *pcaA*

INTRODUCTION

Molecular technology applied to understand the basis of transmission patterns of TB in the world (38). The most extensively used molecular epidemiology technique is Restriction Fragment Length Polymorphism (RFLP) typing, which uses the insertion sequence IS6110 to differentiate clinical isolates (5,37). Polymerase Chain Reaction (PCR) is the most sensitive method in the diagnosis of clinically suspected tuberculosis (1,8,25). New typing methods based on the PCR, such as spoligotyping (18), and mycobacterial interspersed repetitive units (MIRU) typing have also been described (34).

Spoligotyping, developed by Kamerbeek *et al.*, in 1997 (18) can simultaneously detect and type *M. tuberculsis* strains. This method is based on DNA polymorphism within the direct repeat (DR) locus of *M. tubeculosis.* This locus contains multiple, well conserved 39-bp DRs interspersed with nonrepetitive spacer sequences which are 34 to 41 bp long. Strains vary in the number of DRs and in the presence or absence of particular spacers. This technique requires minimal quantities of DNA and has the potential to be used directly on clinical specimens without the need for prior culture.

Mycobacterium tuberculosis cells have a complex structure that contains many unique lipids and glycolipids (3,42). The bacterium synthesizes mycolic acids, very long chain α-alkyl,

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β-hydroxyl fatty acids, in three different classes that predominantly located in cell envelope. The three mycolic acids includ α-, methoxy-, and keto-mycolates (35,43). The resident cyclopropane rings and methyl branches can be modified through the combined action of a large family of *S*-adenosyl methionine (SAM)-dependent methyl transferases (2). The double bonds in the meromycolate chain are then modified resulting in the exhibition of exquisite substrate specificity (14).

Recently a mutant of *M. tuberculosis* have been created that failed to produce the rope-like (corded) colony structures characteristic of virulent strains and was unable to establish chronic infections in mice. The disrupted gene in the mutant strain coded for the enzyme, cyclopropane synthetase, which modifies mycolic acids moieties. The enzyme catalyzes the formation of a three-membered carbon ring structure on alpha mycolate. The gene was named *pcaA* for "proximal cyclopropanation of alpha mycolate" (15). *PcaA* was recently shown to be crucial to *M. tuberculosis* persistence and virulence *in vivo* (14). In this work, the frequency of *pcaA* gene occurrence was determined among the clinical *M. tuberculosis* isolates in Iran.

MATERIAL AND METHODS

This study involved a total of 523 patients that referred to the National Research Institute of Tuberculosis and Lung Disease (NRILTD), the referral tuberculosis center in Iran during March 21st-2004 to March 21st-2005. Laboratory procedures for determining drug resistance were performed by the indirect proportion method (30).

DNA extracts for spoligotyping were prepared by using the classical cetyltrimethylammonium bromide method (39). Spoligotyping by use of a set of 43 spacers was carried out as described by Kamerbeek *et al.* (18). Spoligotype patterns were designated with hexadecimal codes and/or arbitrary database numbers as described by Dale *et al.* (7). Typing results were analyzed and compared with world spoligotyping database as described by Sola *et al.* (4).

The *pcaA* gene was amplified by PCR, using two sets of primers; *pcaA*-1 and *pcaA*-2. To determine the sequence of *pcaA* (GenBank accession no. BX842573) from various strains of Mycobacteria isolated from humans, primer *pcaA* -1 (5'-ACGCCGCATTTTGGAAAC -3'), corresponding to bp 1-18 of the *M. tuberculosis pcaA* gene, and primer *pcaA*-2 (5'-TTTTCCAGTGTGAACTGGTCG -3'), corresponding to bp 824-845 of the same gene were used. PCR was performed using a reaction buffer composed of 10 mM Tris/HCl, pH 8.8; 50 mM KCl; 1.5 mM $MgCl_2$; 0.125 µM of the above-mentioned primers; 0.125 mM dNTPs; 1.25 units *Taq* DNA polymerase and 10-100 ng DNA. PCR amplification was performed in a DNA thermal cycler, set for 5 min at 94ºC denaturation step, followed by 30 cycles at 94ºC for 0.5 min, 58ºC for 0.5 min, and 72ºC for 1 min and followed by a 10-min extension at 72ºC. PCR products were electrophoretically fractionated in 0.8% agarose gel (1x Tris-borate-EDTA [pH 8.3]) and then visualized under UV after ethidium bromide staining. PCR products were purified with the PCR purification kit (Roch-Germany) and sequences of these gene products were determined by automated dideoxy sequencing method.

RESULTS

We enrolled 523 patients with TB, who presented to the NRITLD during the March 21st-2004 to March 21st-2005. Of the 523 patients, 338(65.6%) were Iranians and 185(35.4%) were Afghan patients. There was a striking difference in the prevalence of drug resistance in response to different drugs (Fig. 1). The rate of MDR strains was higher in the Iranian patients relative to those isolated from the Afghani patients, as shown in (Fig. 2). Microscopic smear for acid fast bacilli (AFB) were done on sputum and other specimens from 523 patients. Of these, 405(77.4%) had smear positive pulmonary tuberculosis (Table 1). One hundred forty distinct genotypes of *M. tuberculosis* were identified which belonged to three evolutionary groups (I, II, and III). There were 122 (87.1%) unique spoligotype patterns. The remaining isolates 18(12.8%) belonged to spoligotype clusters that shared with world spoligotyping database and were also reported from other geographical regions of the world. (Table 2) shows the number of patients in each cluster. Group III is more predominantly seen in Iranians but was less prevalent in Afghan patients (Table 1). Haarlem family was the most prevalent family in this study. T family and EAI family were in the second and third level (Table2). The Beijing strains were most common in the Beijing province

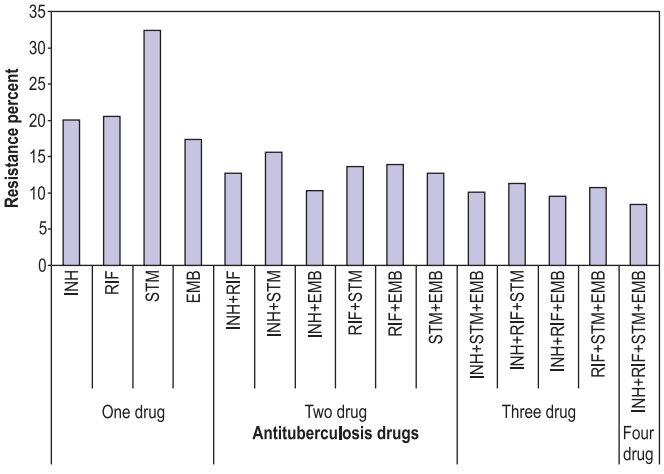

Figure 1. Resistance patterns to antituberculosis drugs among M. tuberculosis isolates from 523 TB patients (P<0.002).

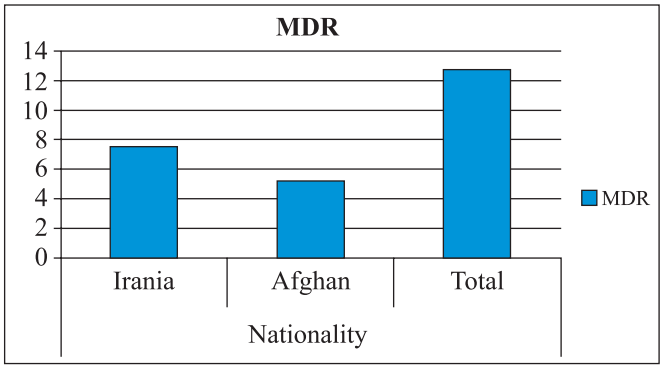

Figure 2. The distribution pattern of Multidrug-Resistance (MDR) strains in reference to patient's nationalities (P<0.005).

Table 1. Correlation between nationality and direct smear, sex, sample source, Beijing family and Major group

		Nationality			
		Iranian		Afghani	
		Count	%	Count	%
SMEAR	+	268	51.2	137	26.2
	-	70	13.4	48	9.2
Sample source	Sputum	285	54.5	173	33.1
	Bronchial	21	4	6	1.1
	Pleural fluid	1	0.2	2	0.4
	Urine	2	0.4	1	0.2
	Lymph node	2	0.4	1	0.2
	Biopsy	11	2.1	2	0.4
	Abscess	1	0.2	0	0
	G/W	8	1.5	0	0
	BAL	7	1.3	0	0
SEX	Male	216	41.3	128	24.5
	Female	122	23.3	57	10.9
Family	Non-Beijing	321	61.4	170	32.5
	Beijin	17	3.5	15	2.9
Major group	Group 1	181	34.6	150	28.7
	Group 2	104	19.9	29	5.5
	Group 3	53	10.1	6	1.1

of China, accounting for 92% of strains (28,40). These strains were also observed in some other Asian countries (16). Our study indicated that 6.4% of the strains belonged to the Beijing Family (Table 1). The *pcaA* gene was detected in all clinical *M. tuberculosis* isolates but it was not seen in any of the environmental Mycobacterial isolates such as in *M. kansas I* (Fig. 3). Also, the sequence of PCR fragment was determined and compared with Gene Data Bank (GenBank accession no. BX842573).

Table 2. Octal presentation of the clinical M. tuberculosis strains isolates Spoligotype.

Row Labels	No. of patients	octal code
AFRI	1	570071740030400
CAS1	18	703777740003771
EAI	31	777777775410771
Haarlem	75	777777775420771
LAM2	1	670000777760531
Manu	4	777777777423571
T	3	707757177760771
T1	34	777777777760771
T2	2	777777777760731
undefined	37	777777777777771
W-Beijing	14	000000000003771
Total	220	

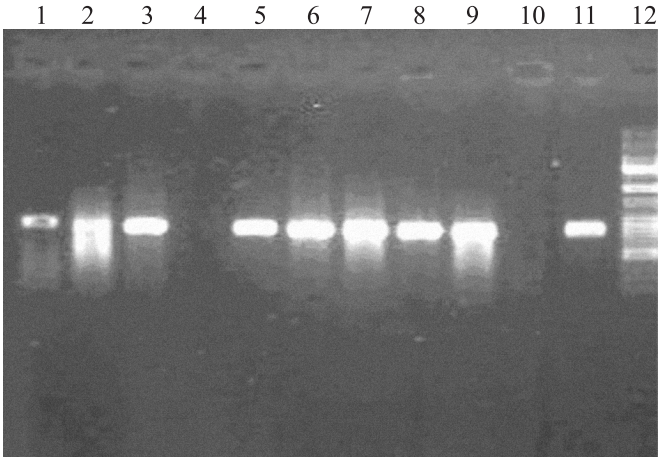

Figure 3. Gel electrophoresis of the PCR amplified *pcaA* gene products (M. tuberculosis lanes 1, 2, 3, 5, 6, 7, 8, 9, and 11; M. Kansasi lane 4; Negative control lane 10, lane 12 marker 50-1000bp).

DISCUSSION

Mycobacterium tuberculosis currently infects one-third of the world population, and cause 2.9 million deaths annually (11,24). It is reported that tuberculosis has an incidence of 8 million new cases annually in the world with >19,000 cases/year

in Iran (41). Therefore, development of new drug and vaccines is necessary for treatment and prevention of the disease. The primary aim of this study was: 1) determination of drug susceptibility in clinical isolates; 2) determination of spoligotyping pattern of the clinical strain isolated from Iranian and Afghani patients; 3) determination of the extent of Beijing strains prevalence among the clinical isolates; 4) detecting the prevalence of *pcaA* gene by PCR amplification with specific primers among the isolated strains.

The resistance rate for Isoniazid (INH), Rifampicin (RIF), Streptomycin (STM) and Ethambutol (EMB) were 20%, 20.5%, 32.4% and 17.4% respectively (Fig. 1). In a similar study conducted in Turkey, resistance rate were reported to be 16.2% for STM, and 11.6% for INH (19). In a Taiwanese study; 19.0% of the strains were resistant to INH, 6.1% to RIF, 15.7% to EMB, and 10.0% to STM (21). In a Brazilian study 13.8% of the strains were resistant to INH and 8.6% for STM (22).

We detected almost similar resistance rate to EMB, but much higher rate towards STM. The higher STM resistance rate may be due to the more widespread usage of these antituberculosis drugs in Iran. In comparison to the results of a similar study reported previously in Iran (23), we observed a much higher resistance rate to one drug as well as to a combination of two or more drugs.

Multidrug-resistance TB, defined as simultaneous resistance to the two most important drugs, INH and RIF, is a potential threat to tuberculosis control (29). Patients infected with strains resistant to multiple drugs are extremely difficult to cure, and the necessary treatment is much more toxic and expensive (27). Resistance to INH+RIF was 12.7% (Fig. 2). Other investigators reported INH+RIF resistance rates to be 11.4% (12), 8.9% (10) and 5.1% (21). Therefore, the MDR rate detected in this investigation was within the MDR isolation range observed elsewhere in the world.

Molecular epidemiology techniques can help to better trace infectious disease transmission, and monitor their mode of spread. We employed spoligotyping technique which is a rapid PCR-based identification system in order to differentiate between clinical *M. tuberculosis* isolates. Spoligotyping can be used to simultaneously detect and type *M. tuberculosis* present in clinical specimens, such as sputum, tissue, or bronchoalveolar lavage, a procedure that can be performed in less than 2 days. In addition, this method was used to type *M. tuberculosis* in sections of 40-years- old paraffin- embedded tissues and characterization of members of the *Mycobacterium tuberculosis complex* (MTB) in historic tissue samples (18,32,44). Evolutionary investigations indicated that, three genetic groups of *M. tuberculosis* are separable based on polymorphic nucleotides in katG codon 465 and gyrA codon 95 (33). Bacteria belonging to spoligotype major Group 2 and 3 failed to hybridize with spacer 33 to 36 (33). With respect to the world spoligotyping database and analysis of our spoligotyping patterns, 140 spoligotype patterns were observed which belonged to three major groups (Table 2). Additionally, 220 strains were subclassified into previously described families; 34% belong to the Haarlem family, 17.1% belong to the T family, 14% to the EAI familly, 8.6% to the CASI family, 6.3% to the W-Beijing family, and 1.3% to the Manu family (Table2). Although W-Beijing, Central Asian (CAS), and East-African-Indian (EAI) genotype families belong to principal genetic group 1; whereas, X (european-low banders), Latino-American and Mediterranean (LAM), Haarlem (H), and T families belong to principal genetic groups 2 and 3 (31). Ferdinand *et al.* (9) reported that 23.5% of isolates belonged to the EAI family, 9.1% to the CAS family, 4% to the Beijing family and 2.7% to the Manu family. In other study, 11.7% of the isolates belonged to T family, 7.3% to Haarlem family, and 3.2% to Beijing family (20). In the two mentioned investigations, most of the isolates were belong to EAI and T family whereas in this study, the majority belonged to the Haarlem family which indicates clonal spread of M. tuberculosis strains.

Beijing strains of *M. tuberculosis* are located in group 1 and are associated with MDR strains reported from most parts of the world (16). Strains of the Beijing genotype were first described in China and were already highly prevalent in 17 different areas around Beijing from 1956 to 1960 (40). These strains were also disseminated to the neighboring Asian countries, such as Mongolia, Thailand, South Korea, and Vietnam (40). Other far away places were later contaminated by these strains. Strains of the Beijing genotype have also been detected in South Africa, Colombia, and Gran Canaria Island (26,30,36). Spoligotyping seems to be both sensitive and specific for the Beijing family and is also easily comparable between different studies. IS6110 fingerprinting can also be used to detect this genotype family with results that correlate closely with the spoligotyping technique (6). In this study, 6.4% of the strains belonged to Beijing family (Table 1). Beijing family isolation rate in many Asian countries were reported to be >50% (32). This may be indicative of the fact that these Beijing strains are not local in origin and have entered Iran via Afghani immigrations from its Eastern border. Drug resistance is a growing problem in TB treatment and control. Furthermore, MDR isolates have become a major worldwide predicament (30). The TB strains isolated from the Iranian patients showed higher levels of drug resistance relative to these isolated from the Afghani group (Fig. 2). Thirty four percent of Iranian isolates belonged to Group 1; whereas, 28.7% of Afghani isolates were within this group (Table 1). As noted earlier, drug resistance in group 1 is higher than the other two groups. Group 1 is also mostly associated with MDR strains (26,36).

Designing newer drugs, vaccines, and diagnostic techniques is dependent on better understanding of *M. tuberculosis* virulence mechanism. Since it appears that TB virulence mechanism is multi-gene dependent, we sought to determine the prevalence of one of these virulence genes, namely *pcaA*, in clinical isolates. The cyclopropane moiety of mycolic acids is a

unique lipid structure that greatly affects the pathogenesis of *M. tuberculosis* (15). The bacterium uses a family of *S*-adenosylmethionine-dependent methyltransferases to modify the mycolic acids of its cell envelope with a variety of stereochemistries and positions for cyclopropyl groups (13). There are at least three mycolic acid cyclopropane synthases (*PcaA*, CmaA1, and CmaA2) that are responsible for these site-specific modifications of mycolic acids (17). *PcaA* act as a proximal *cis*-cyclopropane synthetase for -mycolate molecule that is essential for *M. tuberculosis* virulence (13,15). *PcaA* was identified as a gene necessary for the morphology of cording in *M. tuberculosis. PcaA* has clearly been shown to be essential for maintenance of a chronic infection (15). This suggests that the family of mycolic acid methyltransferases may have particular importance in virulence and persistence of *M. tuberculosis*. Since there is no known cyclopropanated lipid in mammals, it is conceivable that these structures together with biochemical assays will provide a foundation for the rational development of new anti-tuberculosis drugs. Moreover, the substrate similarity for the active sites of these enzymes points to the possibility of one potential inhibitor acting upon multiple targets. This reduces the potential for drug resistance which is a favorable feature for any new drug in the fight against tuberculosis. In conclusion, we confirmed that *pcaA* gene widespread existence in almost all the clinical isolates (Fig. 3) can be a potential target for drug, vaccine and diagnostic test designs. It is also important to undertake studies to identify which factors are the most significant to consider in tuberculosis control program.

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RESUMO

Caracterização do complexo *Mycobacterium tuberculosis* isolado de pacientes do Irã e Afeganistão pelo método de *spoligotyping*

O desenvolvimento de novas drogas, vacinas e técnicas de diagnóstico depende de uma melhor compreensão dos mecanismos de virulência de *Mycobacterium tuberculosis*. Neste estudo, a prevalência do gene *pcaA* em cepas de *M. tuberculosis* foi avaliada através de da técnica de *spoligotyping*. Os fatores de risco associados nos pacientes de diferentes nacionalidades vivendo no Irã foram também determinados. As cepas de *M. tuberculosis* isoladas foram submetidas a testes de sensibilidade a quatro drogas anti-tuberculose de primeira linha e à caracterização por *spoligotyping*. Empregou-se PCR para detectar o gene *pcaA*, determinado-se também a sequencia de nucleotidios. A espoligotipagem resultou em 140 grupos diferentes, sendo 120 (87,1%) reportados pela primeira vez. Os demais espoligotipos (12,8%) já foram descritos em outras regiões geográficas no mundo. A família Haarlem foi mais comum que os demais genótipos. A resistencia a antibióticos foi maior nas cepas isoladas dos pacientes iranianos. O gene *pcaA* foi detectado em isolados clínicos de *M. tuberculosis* mas não em cepas saprófitas, como *M. kansasi*. Os resultados indicaram a existência de *M. tuberculosis* pertencente à família Beijing nos pacientes iranianos. Este estudo confirmou a presença do gene *pcaA* em quase todos os isolados clínicos. Estudos que identifiquem os fatores mais significantes nos programas de controle da tuberculose são necessários.

Palavras-chave: tuberculose, resistência, drogas, *spoligotyping*, *pcaA*

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