Eimani and Seyyedi, J Obes Weight Loss Ther 2015, 5:6
DOI: 10.4172/2165-7904.1000289

Journal of
Obesity & Weight Loss Therapy

Research Article Open Access

Genomic Analysis of *Mycobacterium tuberculosis* in Respiratory Positive-Smear Patients Using PGRS-RFLP in Northwest and West Provinces of Iran

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Abstract

Determining and clustering of Mycobacterium tuberculosis strains is of great application in control programs of tuberculosis. Identification of transmission type and tracking the infection source is also highly necessary. The present study was performed aiming to track and determine the type of *Mycobacterium tuberculosis* infection, as well as its relationship with demographic factors, using PGRS-RFLP.

Materials and methods: In this cross-sectional study, 84 smear-positive patients from 5 frontier provinces (East Azerbaijan, West Azerbaijan, Ardebil, Kurdistan, and Kermanshah) were investigated according to PGRS-RFLP. Demographic data were collected using a questionnaire. The results were analyzed by SPSS-18 and G-Box.

Findings: Based on clustering, recent transmission was 66%. Most clusters were obtained from Kurdistan and Kermanshah. Vaccination record (p=0.49) and treatment group (without previous treatment) (p=0.004) had a significant relationship with clustering. Other demographic factors including age, gender, religion, drug abuse, smoking, history of migration, and marital status did not show a significant relationship with clustering.

Conclusion: Genetic variation of *Mycobacterium tuberculosis* is high in this region. The rate of recent transmission based on clustering was unexpected (global average is 30-40%). Recent transmission was more dynamic in the west than the northwest Iran. The strong relationship between the treatment group 1 (without previous treatment) and clustering based on PGRS-RFLP can demonstrate the high correlation between molecular and classic information. In addition, the significant relationship between vaccination record and clustering highlights the necessity to conduct more extensive studies.

Keywords: *Mycobacterium tuberculosis*; PGRS-RFLP; Northwest; West; Demographic

Introduction

About one-third of the world population is affected with Mycobacterium tuberculosis (MTB) and a new infection occurs every second on a global scale. TB is the second leading cause of death from infectious diseases [1] and, if untreated, the infection can kill more than 50% of the affected people. Global prevalence of Mycobacterium tuberculosis in 2012 is estimated to be about 0.0018 which results in an annual death of 15 per 100,000 patients with TB. In its latest report, the World Health Organization has stated that 19 cases per 100,000 Iranians are infected with tuberculosis [2]. Tuberculosis shows a sudden rise after 40 years of steady decline, leading to a TB pandemic. According to the researchers estimate, about 30-40% of TB cases occurs due to recent transmission of the disease [3]. It is noteworthy that estimation of recent transmission and recurrence rate of TB is of great application in control programs of the infection [4]. According to WHO, nearly one billion people will be infected worldwide between 2000 and 2020 in case of failure to control the disease, which may result in 200 million cases and 35 million deaths [4].

Vicinity of Iran at west and northwest to Iraq and Azerbaijan with high burden prevalence of tuberculosis and drug resistance, and relatively easy cross-border traffic, as well as tracking of the infection source and transmission dynamics of the disease all highlight the need to focus more on tuberculosis. So it seems necessary to track the infection in Iran, especially in the frontier provinces of northwest and west [5]. Clustering of bacteria is a new option for identifying the source of an infection and tracking a disease. Genotyping of *Mycobacterium tuberculosis* is commonly used in epidemiologic surveys. RFLP-based genetic analysis of IS6110 is a powerful epidemiological tool for isolation of different strains of *Mycobacterium tuberculosis* [6]. This method is of the highest value in terms of relative stability over time, variable copy number, and location of genes [7]; however, its low resolution for isolates with fewer than five copies of IS6110 is a major limitation. Hence, a secondary typing method is performed using GC-rich sequence, in general, to increase the power of detection of IS6110 isolates with equal to or less than 5 copy numbers. More bands with different tonality are produced with PGRS compared to IS6110, making difficult its reading and analysis [7].

GC content is an important parameter for classification of bacteria and an appropriate indicator for many of the physical properties of bacterial genome. Different mycobacteria have various GC contents which seems normal according to the genome size of each bacterium [8].

In the present study, we used GC-Rich profile of *Mycobacterium tuberculosis* through the genomic RFLP method to cluster the bacteria

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Received November 18, 2015; **Accepted** December 17, 2015; **Published** December 21, 2015

Citation: Eimani BG, Seyyedi M (2015) Genomic Analysis of *Mycobacterium tuberculosis* in Respiratory Positive- Smear Patients Using PGRS-RFLP in Northwest and West Provinces of Iran. J Obes Weight Loss Ther 5: 289. doi:10.4172/2165-7904.1000289

and to track the infection. The objective of this study was to identify the genetic diversity of *Mycobacterium tuberculosis* strains using PGRS-RFLP based on GC-rich sequences in five frontier provinces at northwest and west of Iran and to evaluate the demographic and clinical factors associated with different transmissions of the disease [9].

Materials and Methods

Sampling and bacteria culture

Ninety sputum samples were collected from smear-positive patients with pulmonary TB from 2011 to 2012 in health centers in East Azerbaijan (23 samples), West Azerbaijan (13 samples), Ardebil (11 samples), Kurdistan (26 samples), and Kermanshah (17 samples). After decontamination with N-acetyl L-cysteine, the samples were cultured in glycerin-contained Löwenstein-Jensen gradient media to reproduce the bacteria and study their growth rate. Finally, 84 samples grew in the media, of which 24.8%, 14.1%, 12.9%, 20.2%, and 28% pertained to East Azerbaijan, West Azerbaijan, Ardebil, Kermanshah, and Kurdistan, respectively. Biochemical tests including production of niacin and catalytic activity at 22 and 68 °C were run to differentiate the species.

DNA extraction

Extraction of genomic DNA from bacteria was carried out through the standard method of van Solingen in which unbroken genomic DNA is completely extracted and used for RFLP. The quality and quantity of the extracted DNA were evaluated using short electrophoresis and nanodrop.

PGRS-RFLP

Enzymatic digestion was performed by ALU1 (Fermentas) restriction enzyme using the minimum acceptable levels of bacterial DNA. The method of Kepler was used for Southern blot on a positively charged nylon membrane (Roche). Hybridization and detection was performed after cross-linking of the membrane by UV radiation. To this end, the membrane yielded from Southern blot using pre-hybridization solution (Roche) was placed in a hybridization oven at 65 °C. PGRS probes (Technology, Denmark) labeled with digoxigenin (Roche) were then added to the membrane and incubated for 24 h at 65 °C. Nonspecific probe connections were eliminated through washing and anti-digoxigenin (Roche) antibody conjugated with alkaline phosphatase (Merck) were added to the nylon membrane. The probe-detected bands were finally visualized by NBT/BCLP substrates (Roche).

Computer analysis

In addition to visual observation, the images resulted from hybridization of the probes with DNA fragments were evaluated with G-Box. Association of clinical and demographic factors with bacterial strains was evaluated with X^2 and Fisher's exact tests using SPSS-18.

Results

Of the 84 isolates examined in this study, 52% (n=43) were from patients in the northwest provinces and 48% (n=41) from patients in the west provinces; of them 59% (n=50) were Shiites and the remaining were Sunnis; 42.9% (n=36) were in the age group of 40-60 years; 51.2% (n=43) were female; 50% (n=42) were married; 58% (n=49) were new patients (no previous treatment); 59% (n=51) had a history of smoking; 63.9% (n=53) had no record of vaccination with BCG; and 69% (n=58) were illiterate or had an elementary literacy.

Of the samples genomically analyzed and classified in this study, 33.3% (n=28) were designated in the single-member clusters and 66.7% (n=56) in the multi-member clusters. Thus 42 genetic types were emerged of which 28 types (33.3%) were unique and 16 types were distributed in four 2-member clusters (9.51%), two 3-member clusters (7.14%), three 4-member clusters (14.28%), two 5-member clusters (11.9%), two 6-member clusters (14.28%), and one 8-member cluster (9.52%) (Table 1). The largest one obtained in this study was an 8-member cluster which demonstrated a strong correlation between the classical epidemiological data and molecular results, so that all 8 members of the cluster pertained to the west provinces' patients; actually, 80% and 20% of the isolates were from Kermanshah and Kurdistan, respectively. The frequency of clustering distribution is separately depicted for the studied provinces in Table 1.

Comparative analysis of the northwest and west provinces based on the clustering probability showed that the northwest provinces tend to form clusters more than the west provinces; actually, clusters with larger populations were seen in isolates from Kurdistan and Kermanshah.

Our study revealed a strong correlation between clustering and the treatment group 1 (patients without previous treatment); so that, of patients without previous treatment, 75.5% were in similar clusters and 24.5% were from unique strains (p=0.004). A significant relationship was also found between vaccination and the formed clusters (p=0.049), so that 58.5% of the patients with vaccination record were included in the multi-member clusters.

It should be noted that no significant relationship existed between gender (p=0.24), religion (p=0.33), Addiction history (p=0.47), smoking (p=0.79), history of migration from rural the city (p=0.47), geographic region (p=0.25), age, education, and marital status (p>0.05) with unique groups and clustering (Table 2).

Discussion

It is now clear that the bacillus *Mycobacterium tuberculosis* is able to spread the infection immediately after affection of an individual or show the disease symptoms years after, which may be due to reactivation of a previous infection or a reinfection [10]. As we know, the incidence of TB depends on factors such as the prevalence of TB in a region, duration of infection, and exposure to sick people [5]. On the other hand, the lack of powerful markers to identify different species of *Mycobacterium tuberculosis* hampered epidemiological studies. However, the advent of molecular techniques in the epidemiology of *Mycobacterium tuberculosis* has created an appropriate opportunity to use effective biomarkers for tracking the disease transmission in the human environment and for identifying the phylogenetic features of *Mycobacterium tuberculosis* strains [7].

In the present study, carried out on indigenous people of East Azerbaijan, West Azerbaijan, Ardebil, Kermanshah, and Kurdistan, 42 different genetic types were obtained from 84 subspecies pertained to the patients. Based on research, it is believed that *Mycobacterium tuberculosis* has a high genetic diversity worldwide. For example, Rafee Mozaffari Farnia [6], and Chavez [4] isolated 45, about 50, 60, and 35 different genetic types from this bacterium, respectively. Our results are consistent with the mentioned studies and confirm the high levels of *Mycobacterium tuberculosis* genetic diversity in the region.

As an objective, the source of infection was evaluated and tracked geographically in this study; thus the religion was considered as a confounding variable and no significant correlation was found between the religion and clustering in statistical analyses (p=0.33).

Another demographic factor which was assumed to be examined in

8 -members Cluster n(%)	6 -members Cluster n(%)	5-members Cluster n(%)	4 -members Cluster n(%)	3-members Cluster n(%)	2 -members Cluster n(%)	Unique n(%)	clustering / Provinces
8(19.52)	5(12.2)	5(12.2)	8(19.52)	2(4.88)	2(4.88)	11(26.8)	**west**
0(0.0)	7(16.52)	5(11.6)	4(9.28)	4(9.28)	6(13.92)	17(39.5)	**Northwest**

Table 1: Frequency distribution of *Mycobacterium tuberculosis* strains clustering according to geographical regions.

P-value	^2X	unique	cluster	factor	Variable
-	-	2(25.0)	6(75.0)	<20	age
		1(16.7)	5(83.3)	20-40	
		15(41.7)	21(58.3)	40-60	
		10(29.4)	24(70.6)	>60	
0.024	1	14(32.6)	29(67.4)	female	gender
		14(34.1)	27(65.9)	men	
0.33	1.05	15(30.0)	35(70.0)	Shiite	religion
		12(41.4)	17(58.6)	Sunni	
0.25	1.52	17(39.5)	26(60.5)	Northwest	Provinces
		11(26.8)	30(73.2)	west	
0.049	4.08	3(15.8)	16(84.2)	yes	Vaccination history
		22(41.5)	31(58.5)	N0	
0.47	0.58	1(50.5)	1(50.5)	yes	Addiction history
		10(25.6)	29(74.4)	No	
0.79	0.16	16(31.4)	35(68.6)	yes	smoking
		9(36.0)	16(64.0)	No	
0.47	0.73	2(22.2)	7(77.8)	yes	history of migration
		21(36.8)	36(63.2)	No	
-	-	2(18.2)	9(81.8)	Single	marital status
		17(40.5)	25(59.5)	Married	
		4(23.5)	13(76.5)	Widow/Divorced	
0.004	9	12(24.5)	37(75.5)	new patients	treatment group
		26(60.9)	9(39.1)	With previous treatment	

Table 2: Relationship between demographic factors and clustering.

this study was the addiction to drugs; however, low number of addicted patients (n=2) has limited the statistical analysis. This limitation can be addressed through carrying out studies with larger sample sizes, provided that the subjects answer sincerely.

Vaccination record had a significant association with clustering in this study, so that people with no history of vaccination had a higher index of disease transmission and contamination. According to some studies, vaccination can prevent the disease from developing more severe in children [11]. It also exerts a strong protective effect against extra-pulmonary TB [12].

In terms of smoking, although 68.6% of smokers were classified in similar clusters, no significant relationship was found between smoking and clustering in the statistical analysis (p=0.79).

In general, the relative frequency of clustering groups was estimated as 66.7%, which was higher than another study (45%) performed in Markazi Province through PGRS-RFLP [4]; the frequency of clustering was higher in our study. In general, PGRS patterns have often low variability in areas with a lower incidence of TB in African and Asian countries; *i.e.*, the frequency and the size of clusters are higher than the unique isolates and this issue has been similarly stated for IS6110 patterns [13].

The comparative analysis of patients in the two treatment groups (new and previously treated patients) based on clustering showed a strong association between treatment group 1 (no treatment) and clustering; so that 75.5% of these patients were classified in similar clusters. This fact confirms also the recent transmission among patients with no previous treatment; unfortunately, unavailability of relevant articles performed through RFLP-GRSP made impossible comparing our results with other studies.

A strong relationship existed in this study between geographical region and clustering and hence the possibility of a new transmission; so that, the majority of patients in the west provinces (Kurdistan and Kermanshah) were affected *via* recent transmission. Clusters larger than 4, which included a significant part of our research, can be a serious warning of outbreaks in a particular area. These outbursts can arise from the high-burden countries neighboring with these areas. According to the results of various studies, the probability of clustering is low in countries with lower incidence of the disease. Comparing the incidence of TB in the northwest and west regions of Iran [14] also confirms this fact that clustering of the northwest provinces is higher than the west provinces.

Gender and age of the patients were other variables investigated in this study. While an association was reported between male gender and clustering in a study in French [15], no significant correlation was found in the present study between gender and age and clustering of tuberculosis isolates (p>0.05); it seems that recent transmission of the disease has no difference between men and women. The results reported in the majority of studies conducted on gender and age, were similar to ours.

The other demographic factor examined in the present study was the history of migration from rural to urban areas. Although reports

of relationship has been found between migration and clustering [16], since the present study investigated merely the history of migration from rural to urban, the evaluated patients were necessarily natives of their own areas, making impossible the comparison with other studies.

In general, we concluded that the genetic variation of *Mycobacterium tuberculosis* is high in this region. The rate of recent transmission based on clustering was unexpected (global average is 30-40%). Recent transmission was more dynamic in the west than the northwest Iran. The strong relationship between the treatment group 1 (without previous treatment [17] and clustering based on PGRS-RFLP can demonstrate the high correlation between molecular and classic information. In addition, the significant relationship between vaccination record and clustering highlights the necessity to conduct more extensive studies.

The limitations of this study include the difficulty of sampling from different provinces and unavailability of articles performed through the same molecular method. In addition, we lost a number of data due to large distance between the provinces and unavailability of the patients.

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