INVESTIGATION OF GENETIC HETEROGENEITY IN *MYCOBACTERIUM TUBERCULOSIS* ISOLATES FROM TUBERCULOSIS PATIENTS USING DNA FINGERPRINTING

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ABSTRACT

BACKGROUND: DNA fingerprinting of Mycobacterium tuberculosis (MTB) based on IS6110 has been shown to be a powerful epidemiologic tool. Restriction enzyme analysis (REA) is a fingerprinting technique, which is used for differentiation and investigation of genetic diversity among mycobacterial species. AIMS: To investigating the genetic heterogeneity in MTB isolates in Ahvaz, Iran. SETTINGS AND DESIGN: It was a cross-sectional study conducted in Ahvaz, Iran. METHODS AND MATERIAL: One hundred and eighty clinical isolates of MTB were collected from TB reference unit, PHLS, Ahvaz, Iran. The PCR-REA employed uses a simple DNA extraction followed by a PCR step involving a single primer based on the insertion sequence IS6110. Restriction enzyme analysis was performed on the amplification products using HaeIII enzyme. STATISTICAL ANALYSIS: Data was analyzed using SPSS software and chi-square test/ Fishers' exact test was applied wherever applicable. RESULTS: The isolates were divided into four clusters based on their REA patterns. Cluster I contained 71.1% of strains with two fragments of 72 and 118. Cluster II with three fragments of 72, 118, and 194; cluster III with three fragments of 118, 194, and 234; and cluster IV with four fragments of 72, 118, 194, and 234 base pairs. As many as 73.8% of the identical fingerprint patterns were seen in male patients. Accounting the men as the major population in the study, there was no significant difference between REA patterns and sex; similarly, with age, patients' occupation and degree of smear positivity. However, we found significant correlation between REA patterns and patients' origin. As many as 61.6% of identical patterns were found in the patients who were lived in the same suburb. CONCLUSIONS: By PCR-based REA typing, the isolates studied were grouped into four clusters each containing between two and four fragments. However, in order to ascertain the level of heterogeneity of MTB isolates in their sample, further testing with a more discriminatory method is needed.

KEY WORDS: Fingerprinting, Mycobacterium tuberculosis, restriction enzyme

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INTRODUCTION

Tuberculosis (TB) is still a major health hazard in both developed and developing countries. According to the latest WHO data (2004), one third of the world's population is currently infected by *Mycobacterium tuberculosis* (MTB), and approximately 10 million people become sick with TB each year.[1],[2]

The genome of MTB, the causative agent of human TB, has been shown to contain several polymorphic repetitive DNA elements that can be used to discriminate between isolates.[3] One of these which has been used in molecular typing studies is IS*6110* which belongs to IS*3* family of enterobacterial insertion sequence (IS) elements.[4],[5]

DNA fingerprinting of MTB based on IS*6110* has been shown to be a powerful epidemiologic tool.[6]–[8] However, this method as a 'gold standard' typing technique, is labor intensive, requires a large amount of DNA, does not include an amplification step and takes a number of weeks to obtain results.[9]

In 1993 Telenti et al. introduced a polymerase chain reaction (PCR)-based restriction enzyme analysis (REA) for differentiation of mycobacteria to species level.[10] The later works on the method revealed that it could be successfully applied for investigation of genetic variation among different nontuberculosis mycobacteria.[11],[12] Recently a modified method based on IS*6110* of MTB was evaluated as an epidemiological typing method for investigating MTB outbreaks in UK.[9] Since the technique was rapid and easy to apply, we have decided to investigate genetic diversity within MTB species and studying this heterogeneity in relation to different criteria such as age, sex and living conditions in the patients, that may have some epidemiological value.

MATERIALS AND METHODS

It was a prospective cross-sectional study conducted in TB reference center, PHLS, Ahvaz, Iran, over a period of 9 months (March–November 2002). A total of 180 isolates derived from all new-diagnosed TB patients referred to the center, were analyzed. Despite that the sampling was a part of patients' diagnosis protocol, informed consent was taken in the presence of the research assistants. Besides, permission was obtained from human ethics committee at the university and the relevant authorities during the approval of the proposal. The patients were 133 men and 47 women and their age ranged from 20 to 60 years with a mean of 34.6. The isolates were identified as MTB by acid fast staining of direct smears prepared from sputum samples, culture in Lowenstein Jensen (LJ) medium and subsequently tested biochemically, using the niacin accumulation test, the nitrate reduction test, and heat-labile catalase test.[13]

Chromosomal DNA was extracted from growth harvested from the surface of LJ medium by the simple boiling method. In short, few colonies were removed and suspended in 500 ml of sterile double distilled water in a microfuge tube and was boiled for 10 min. After centrifugation at 12 000 **g** for 3 min in a microcentrifuge, 10 ml of supernatant was used for the PCR.[12]

An IS*6110*-based PCR approach was performed with a single primer, 5¢-GAGTCTCCGGACTCACCGG-3¢, targeted at the inverted repeat sequence of the IS*6110* insertion.[14] Reaction volumes were 25 ml and contained 1 ´ PCR buffer, 1.5 mmol MgCl$_2$, 0.2

mmol of each deoxynucleotide triphosphate, 25 *p*mol of primer, 1 unit of Taq polymerase and 5 ml of DNA template primer. The reaction conditions were as follows.

An initial denaturation at 95°C for 120 s; 1 cycle of 95°C for 20 s, 45°C for 360 s and 72°C for 120 s; 30 cycles of 95°C for 20 s, 62°C for 30 s and 72°C for 180 s; and a final extension at 72°C for 10 min.[14] The PCR products were loaded on an 1.5% (w/vol.) agarose gel with 0.5 mg/ml of ethidium bromide and were analyzed by gel electrophoresis.

Hae*III* restriction enzyme was used in this study and the experiment was carried out as described by other investigators.[15] In brief, 10 ml of PCR product was added to 6 ml of sterile distilled water, 2 ml of restriction enzyme Hae*III* (Cinnagen Co., Tehran, Iran), and 2 ml of corresponding buffer. The mixture was incubated for 1–2 h at 37°C in a water bath. The results were analyzed on a 2% (w/vol.) agarose gel with 0.5 mg/ml of ethidium bromide. Gels were photographed and the digestion bands were measured. The base pair size of each DNA fragment was determined by comparing the migration distance of the bands with the molecular markers visually.

Statistical analysis
SPSS software was used for data analysis.

The data was analyzed using SPSS software, chi-square test and Fishers' exact test was applied wherever applicable.

RESULTS

Based on the REA data, four clusters were identified. One cluster (I) contained 71.1% of the strains (128 isolates) with two fragments of 72 and 118 base pair (bp). Cluster II contained three fragments of 72, 118, and 194 bp (11 isolates); cluster III contained three fragments of 118, 194, and 234 bp (eight isolates); and cluster IV contained four fragments of 72, 118, 194, and 234 bp (33 isolates) [Figure 1].

Clustering was observed more in males than females [Table 1]. We did not find any significant correlation between diversity of the REA patterns and sex of the patients. Similarly neither correlation was seen between patients with different age groups and REA patterns nor any relevance was seen between the patterns and patients' occupation according to data extracted from questionnaires.

In order to investigate whether direct smear results could be related to heterogeneity in fingerprint patterns, patients were divided into four groups of 3.9% of negative; 44.4% of 1+; 33.3% of 2+ and 18.4% of 3+, according to their direct smear results. No significant
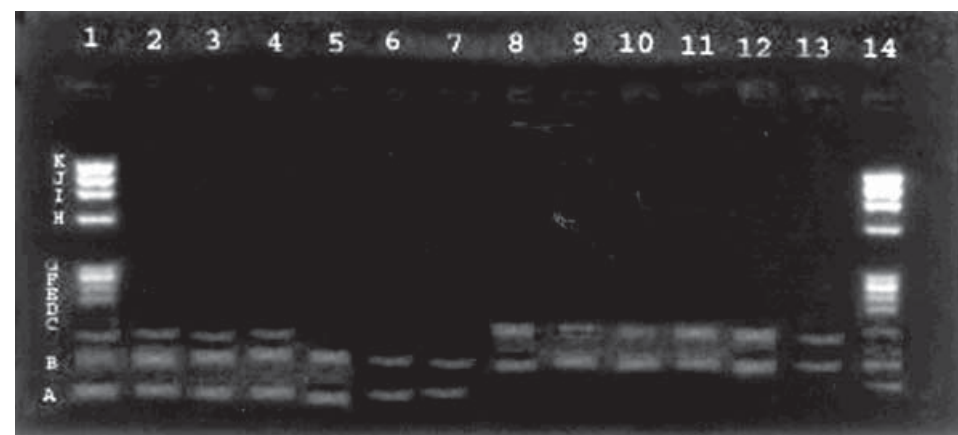
Figure 1: REA patterns showing restriction digestion of IS*6110* element using Hae*III* enzyme. Lanes 1 and 14, DNA size marker; lanes 2 to 4 (cluster I), giving identical 2-fragment patterns; lanes 5–7 (cluster II) and 8–10 (cluster III) each showing two different 3-fragment patterns; and lanes 11–13 (cluster IV) giving identical 4-fragment patterns. The base pair sizes of DNA ladder: K = 1353; J = 1078; I = 872; H = 603; G = 310; F = 281; E = 271; D = 234; C = 194; B = 118; A = 72

correlation was seen between the diversity of REA patterns and the smear results or degree of smear positivity.

Based on a survey which was undertaken during the study, probable correlation was seen between patients' residential area and living conditions to diversity of patterns. According to [Table 2], the results indicated that most of the identical REA patterns were found in patients who were lived in certain overcrowded suburb with low quality sanitation (data from questionnaire), which the correlation was statistically significant ($P = 0.049$, d.f. = 2).

DISCUSSION

Mycobacterial identification to the species level is not only of academic interest but also is important because by use of fingerprinting techniques, that provides a great deal of useful information on the epidemiology and pathogenesis of the organisms, suggesting potential intervention strategies including successful treatment of patients.[15]

In present study, we investigated whether the PCR-based REA can be apply as a suitable fingerprinting technique for studying genetic variation among MTB isolates in the region of

Table 1: Restriction endonuclease analysis fragments of isolates in relation to sex of the patients

Cluster	Fragment	Total No. (%)	Male No. (%)	Female No. (%)
I	72/118	128 (71.1%)	86 (67.2%)	42 (32.8%)
II	72/118/194	11 (6.1%)	10 (91%)	1 (9%)
III	118/194/234	8 (4.5%)	8 (100%)	0
IV	72/118/194/234	33(18.3%)	29 (87.8%)	4 (12.2%)

Table 2: Distribution of clusters of REA fragments in relation to residential area of the patients

Cluster	Suburb No. (%)	Central Ahvaz No. (%)	Province No. (%)
I	76 (59.3)	23 (18)	29 (22.6)
II	7	0	4
III	2	6	0
IV	26 (78.7)	4 (12.1)	3 (9)

study, which is one of the endemic foci of TB in Iran.

Based on the results, the prevalence of IS*6110*-REA patterns with two fragments in the area of survey were high which suggests a high genetic similarity among the isolates.
We were not able to find any evidence for transmission or possible laboratory contamination events under these circumstances. All the strains under investigation underwent duel REA testing and the patterns remained constant in both tests. It is unclear whether grouping of IS*6110*-REA is of significance but it seems that a secondary typing method is required in such a large-scale surveillance study.

The possible relevance between diversity of the REA patterns and residential area (three categories of Ahvaz suburb, central, and province) of the patients were investigated. Despite that collected data suggested possible relationship and infection from the same source in each category; we still need to have a precise interpretation for such data by using a more discriminatory method, as.[16],[17]

In conclusion, by PCR-based REA typing, the isolates studied were grouped into four clusters each containing between two and four fragments. However, in order to ascertain the level of heterogeneity of MTB isolates in their sample, further testing with a more discriminatory method is needed.

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REFERENCES

1. Grange JM. Mycobacteria and human disease. 2nd edn., London: Edward Arnold Publishers Ltd., 1996.pp.

2. World Health Organization: Tuberculosis. Fact Sheet No. 104, Revised 2004. WHO. Geneva, Switzerland.

3. Barlow RE, Gascoyne-Binzi DM, Gillespie SH, Dickens A, Qamer S, Hawkey PM. Comparison of variable number tandem repeat and IS6110-restriction fragment length polymorphism analyses for discrimination of high- and low-copy-number IS6110 Mycobacterium tuberculosis isolates. J Clin Microbiol 2001; 39:2453-7.

4. Thierry D, Brisson-Noel A, Vincent-Levy-Frebault V, Nguyen S, Guesdon JL, Gicquel B. Characterization of a Mycobacterium tuberculosis insertion sequence, IS6110, and its application in diagnosis. J Clin Microbiol 1990; 28:2668-73.

5. McAdam RA, Hermans PW, van Soolingen D, Zainuddin ZF, Catty D, van Embden JD, Dale JW. Characterization of a Mycobacterium tuberculosis insertion sequence belonging to the IS3 family. Mol Microbiol 1990 ;4:1607-13.

6. Yang Z, Barnes PF, Chaves F, Eisenach KD, Weis SE, Bates JH, Cave MD. Diversity of DNA fingerprints of Mycobacterium tuberculosis isolates in the United States. J Clin Microbiol 1998 ;36:1003-7.

7. Edlin BR, Tokars J I, and Grieco M H.: An outbreak of multidrug-resistant tuberculosis among hospitalized patients with the acquired immunodeficiency syndrome. N Engl J Med 1992; 326: 1514-21.

8. Frieden TR, Woodley CL, Crawford JT, Lew D, Dooley SM. The molecular epidemiology of tuberculosis in New York City: the importance of nosocomial transmission and laboratory error. Tuber Lung Dis 1996 ;77:407-13.

9. Yates MD, Drobniewski FA, Wilson SM. Evaluation of a rapid PCR-based epidemiological typing method for routine studies of Mycobacterium tuberculosis. J Clin Microbiol 2002 ;40:712-4.

10. Telenti A, Marchesi F, Balz M, Bally F, Bottger E C, Bodmer T. Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. J Clin Microbiol 1993 ; 31:7175-8.

11. Plikaytis BB, Crawford JT, Woodley CL, Butler WR, Eisenach KD, Cave MD, Shinnick TM. Rapid, amplification-based fingerprinting of Mycobacterium tuberculosis. J Gen Microbiol 1993 ;139:1537-42.

12. Khosravi A D, Stanford J L, Donoghue H D and Rook G A.: Variation within Mycobacterium scrofulaceum. J App Microbiol 1997; 83: 596-602.

13. Forbes BA, Sahm D F, Weissfeld AS. Baily & Scott's Diagnostic Microbiology. 11th edn. St. Louis: C.V. Mosby Inc. 2002. pp. 546-61.

14. van Embden JD, Cave MD, Crawford JT, Dale JW, Eisenach KD, Gicquel B, Hermans P, Martin C, McAdam R, Shinnick TM, et al. Strain identification of Mycobacterium tuberculosis by DNA fingerprinting: recommendations for a standardized methodology. J Clin Microbiol 1993 ;31:406-9.

15. Diel R, Schneider S, Meywald-Walter K, Ruf CM, Rusch-Gerdes S, Niemann S. Epidemiology of tuberculosis in Hamburg, Germany: long-term population-based analysis applying classical and molecular epidemiological techniques. J Clin Microbiol 2002 ;40:532-9.

16. Montoro E, Valdivia J, Leao SC. Molecular fingerprinting of mycobacterium tuberculosis isolates obtained in havana, cuba, by IS*6110* restriction fragment length polymorphism analysis and by the double-repetitive-element PCR method. J Clin Microbiol 1998 ;36:3099-102.

17. Park YK, Bai GH, Kim SJ. Restriction fragment length polymorphism analysis of Mycobacterium tuberculosis isolated from countries in the western pacific region. J Clin Microbiol 2000 ;38:191-7.

18. de Boer AS, Kremer K, Borgdorff MW, de Haas PE, Heersma HF, van Soolingen D. Genetic heterogeneity in Mycobacterium tuberculosis isolates reflected in IS6110 restriction fragment length polymorphism patterns as low-intensity bands. J Clin Microbiol 2000 ;38:4478-84.