

APPLICATION OF RESTRICTION ENZYME ANALYSIS TECHNIQUE BASED ON 65KDA HEAT SHOCK PROTEIN GENE FOR FINGERPRINTING AND DIFFERENTIATION OF MYCOBACTERIUM TUBERCULOSIS CLINICAL STRAINS ISOLATED FROM TUBERCULOSIS PATIENTS IN AHWAZ, IRAN

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ABSTRACT

Objective: Application of identification methodology of restriction enzyme analysis (REA) for fingerprinting of the expanded population of *Mycobacterium tuberculosis* (MTB) isolates.

Methodology: A total of 150 clinical isolates from patients referred to TB reference laboratory, Public Health Centre, Ahwaz, Iran, were identified as MTB by using conventional culture and biochemical tests from January to December 2004. The PCR-REA method uses a PCR step based on amplification of a 439 bp fragment of hsp65 gene involving genus specific primers and restriction enzyme analysis by digestion of products with *Hae*III & *Bst*EII enzymes were employed.

Results: The identical restriction patterns similar to MTB reference strains equal to 160 / 145 / 72bp fragments for *Hae* III and 250 /120/82bp fragments for *Bst* EII digests were seen in 145 isolates (96.6%). The diverse patterns were observed for five isolates in *Hae* III digest as 180 / 100 / 80 bp, 194/ 72 bp and 160/ 145 bp representing the possible intra-species variation within studied MTB strains , while their *Bst* EII digestion patterns showed no variation.

Conclusions: The PCR-REA technique revealed three different new patterns for *Hae* III digest. However to verify that they are indeed MTB isolates, a sequence-based analysis of the exceptional isolates should be performed.

KEY WORDS: *M. tuberculosis*, Restriction enzyme, 65 heat shock protein gene, PCR-REA.

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INTRODUCTION

Diseases caused by pathogenic mycobacteria remain a major cause of human morbidity and mortality. According to WHO data, One-third of the world's population is currently infected with *Mycobacterium tuberculosis* (MTB), and three million human deaths annually are attributed to the organism.¹ The 65kDa heat shock protein (*hsp65*) is one of the major

immuno-reactive proteins of the mycobacteria, which the gene encoding that in MTB, and its nucleotide sequence, was reported previously.² In 1993, a method for differentiating among slow growing mycobacterium species by *hsp65* gene- based polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP)analysis was developed.³ The technique was later conducted to identify non-tuberculous mycobacteria (NTM) on a genetic basis.⁴ A similar approach was used for rapid identification of mycobacteria to species level based on evaluation of the gene coding for the *hsp65* by PCR and restriction endonuclease analysis, REA.⁵ Subsequently, this approach was used for the taxonomic separation and differentiation of NTM,⁶⁻¹⁰ investigation of genetic variation within *M. scrofulaceum*,¹¹ and identifying the subspecies of *M. kansasii*.¹²

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While detailed differentiation of NTM has been described by this technique, only a small number of MTB strains have been studied, which in these, one REA pattern for MTB strains was reported.⁵⁻¹³ Since the technique has proven to be useful for the species differentiation and investigation of genetic diversity, we conducted the present study to apply this identification methodology for differentiation of an expanded population of MTB isolates and to investigate the possible variation among the strains in the region of study. For this purpose, the PCR-REA method employed uses a simple DNA extraction followed by a PCR step based on amplification of a 439 bp fragment of *hsp65* gene involving genus specific primers.⁵

PATIENTS AND METHODS

It was a prospective cross-sectional study conducted in TB reference laboratory, Public Health Centre, Ahwaz, Iran, over a period of one year (January to December 2004).

a. sampling: A total of 150 clinical isolates of MTB were collected from referred tuberculosis patients. Despite that the sampling was a part of patients' diagnosis protocol, permission was obtained from human ethics committee at the university and the relevant authorities during the approval of the proposal. The patients were 93 men and 57 women and their age ranged from 23 to 69 years with a mean of 45.6.

b. MTB identification procedure: Acid fast staining was performed for the isolates and they were identified as MTB by using conventional culture and biochemical tests.¹⁴

c. DNA extraction: Chromosomal DNA was extracted from growth harvested from surface of Lowenstein Jensen (LJ) medium by simple boiling method. In short, few colonies were removed and suspended in 500µl of sterile double distilled water and was boiled for 10 min. After centrifugation at 12000g for 3 min, 5µl of supernatant was used for the PCR.

d. PCR: The reaction volumes (25µl) composed of 50 mmol KCl, 10mmol Tris-HCl (pH 8.3), 1.5 mmol MgCl₂, 0.2 mmol of each deoxynucleotide triphosphate, 0.5 µmol of each primer, 1.25 units of Taq polymerase, 14 µl of

sterile distilled water and 5µl of DNA template. The reaction conditions were as follows:

An initial denaturation at 95°C for 60s; 45 cycles of 94°C for 60s, 60°C for 60s and 72°C for 60s; and a final extension at 72°C for 10 min. The PCR products were loaded on a 1% (w/vol) agarose gel with 0.5 mg/ml of ethidium bromide and were analyzed by gel electrophoresis. The genus specific primers which were used were Tb11: 5'-ACCAACGATGGTGTGTCCAT and Tb12: 5'-CTTGTCGAACCGCATACCCT.

e. Restriction enzyme analysis: *Bst* EII and *Hae* III restriction enzymes were used in this study. Briefly, 10µl of amplified sample was added to the *Bst* EII enzyme mix (6µl of sterile distilled water, 2µl of restriction enzyme and 2µl of corresponding buffer) and incubated for 2-3 hrs in a 60°C water bath. In addition, 10µl of product was added to *Hae* III enzyme mix (6µl of sterile distilled water, 2µl of restriction enzyme and 2µl of corresponding buffer) and incubated for 1-2 hrs in a 37°C water bath.⁵ The results were analyzed on a 2% (w/vol) agarose gel with 0.5mg/ml of ethidium bromide. Gels were photographed and the digestion bands were measured. The base pair size of each DNA fragment was determined by comparison of migration distances of different strains with the molecular markers visually. Appropriate positive and negative controls were included in every gel as recommended by other investigators.⁵ The two reference strains of MTB H37Rv (Pasteur Institute, Tehran, Iran) and MTB BKH37 (Razi Institute, Karaj, Iran) were used as positive controls. PCR reagents and restriction enzymes were purchased from Cinnagen Co. (Tehran, Iran).

RESULTS

Based on the obtained results, 145 clinical isolates (96.6%) showed the identical restriction patterns similar to *M. tuberculosis* reference strains of MTB H37Rv and MTB BKH37 (Razi Institute, Karaj, Iran), equal to 160 / 145 / 72 bp fragments for *Hae* III and 250 / 120 / 82 bp fragments for *Bst* EII digests (Table-I and Figure-I). The patterns were also similar to

Table-I: Restriction enzyme analysis: patterns obtained in MTB standard strains and clinical isolates

MTB strains	<i>Bst</i> EII digest (bp)	<i>Hae</i> III digest (bp)
H37Rv & BKH 37	250/120/82	160/145/72
Clinical isolates (No.145)	"	"
Strains I,II,III	"	160/145
Strain IV	"	194/72
Strain V	"	180/100/80

previously published patterns for MTB as 160/140/70 bp fragments for *Hae* III and 245/125/80 for *Bst* EII digests.⁵ The diverse restriction patterns were observed for five clinical isolates in *Hae* III digest only, while their *Bst* EII digestion patterns showed no variation and were similar to other isolates. Two strains were showed different *Hae* III patterns as 180 / 100 / 80bp and 194/ 72bp (Figure II). The third different *Hae* III digest pattern were seen in three strains as 160/ 145bp (Figure III). The latter strains were found to be originated from a common source, and were isolated from patients in the same prison.

DISCUSSION

The differentiation of species and subgroups of the genus mycobacterium has traditionally entailed evaluation of growth characteristics and biochemical testing and more recent rapid methodologies including high-performance liquid chromatography (HPLC), DNA probes and rRNA sequencing, which are hampered by the limited number of available commercial probes, extensive standardization, high initial equipment expense, and/or intensive labor requirements and are not readily adaptable to routine use in the clinical laboratory.⁶

In 1993, a rapid, sensitive method for differentiation of mycobacterial species and subgroups, through PCR amplification of HSP gene sequences coupled with REA was described.³⁻⁵ However, Despite that large numbers of NTM were screened, only a few number of MTB species were included in their studies. In another study, employing the same PCR-based methodology, 15 strains of MTB were included,⁹ that the DNA sequence selected, was identical for species studied.

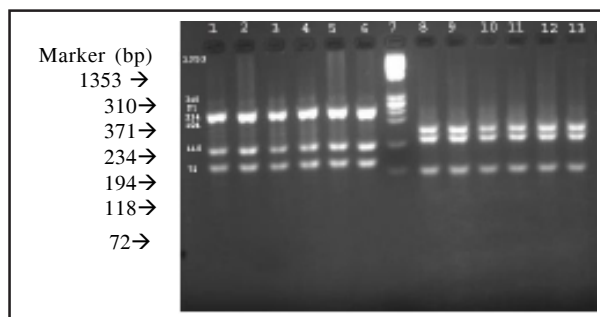


Figure-1: REA profiles for MTB isolates representing identical patterns in standard and clinical strains after digestion with *Hae* III and *Bst* EII restriction enzymes. lanes 1-6: *Bst* EII digestion; lane 7: DNA size marker; lanes 8-13: *Hae* III digestion Marker positions are indicated on the left (base pairs)

We noticed a possible intra-species variation, which resulted in three patterns among *Hae* III digests of PCR-amplified segments of the HSP gene sequence from clinical isolates of MTB. For the strains with different *Hae* III patterns, triple REA testing was performed and the patterns remained constant in all tests. Besides the new patterns were compared to the REA patterns of few closely related mycobacteria and no similarity was seen between those. The band sizes determined in this study for 145 clinical isolates, showed a small differences in comparison to those reported.⁵ This difference is most likely the result of different gel matrix, and direct band size measurement in present study.

The three subgroups identified, are estimated <3.5% of clinical respiratory isolates. However, to verify that they are indeed *M. tuberculosis*

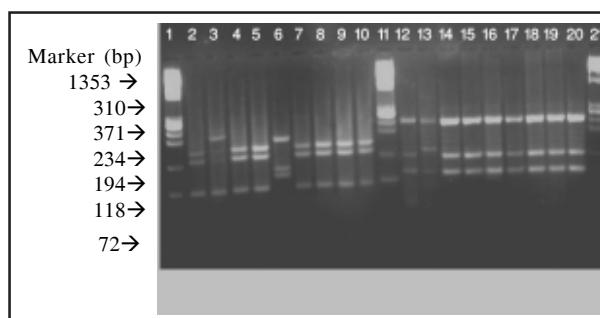


Figure-2: Diverse patterns obtained in few MTB clinical isolates after digestion with *Hae* III restriction enzyme. Lanes 1, 11, 21: DNA size marker; lanes 2-10: *Hae* III digestion (note to lanes 3 and 6 with a diverse patterns); lanes 12-20: identical patterns resulted from *Bst* EII digestion. Marker positions are indicated on the left (base pairs)

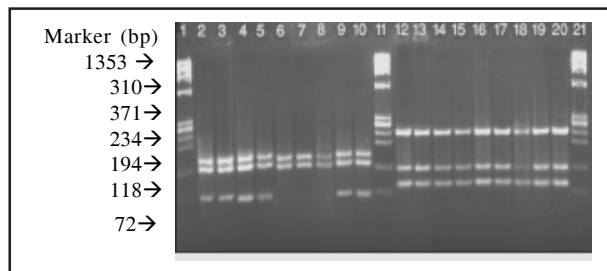


Figure-3: Diverse patterns obtained in few MTB clinical isolates after digestion with *Hae* III restriction enzyme lanes 1, 11, 21:DNA size marker; lanes 2-10: *Hae* III digestion (note to lanes 6-8 with a 72 bp fragment missing); lanes 12-20; identical patterns resulted from *Bst* EII digestion. Marker positions are indicated on the left (base pairs)

isolates a sequence-based analysis of the exceptional isolates should be performed additionally. This could be done with sequence analysis of the 16s rRNA gene. Although, based on the results, it seems that improvement are still required to differentiate all the subgroups of MTB with testing more strains, but since the majority of the strains showed identical REA patterns, so this PCR based technology, provides a rapid, sensitive, time- and labor- efficient method for identification and separation of the species and subgroups of mycobacteria.⁶ Such a system should not be difficult to implement in reference laboratories, which would then be enabled to provide species identification of MTB and other NTM in as few as 1 or 2 working days.

CONCLUSIONS

The technique revealed three different new patterns for *Hae* III digest in MTB isolates, representing the possible intra-species variation within the MTB strains in the region of study. However, ignoring this minority which should be verified by a sequence-based analysis, the results from present study represented PCR-REA as a rapid, accurate and reliable system for the identification and fingerprinting of MTB isolates which should be particularly useful for reference laboratories.

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REFERENCES

1. World Health Organization: Tuberculosis. Fact Sheet No. 104, Revised 2004. WHO. Geneva, Switzerland.
2. Shinnick TM. The 65-kilodalton antigen of *Mycobacterium tuberculosis*. *J Bacteriol* 1987;169:1080-88.
3. Plikaytis BB, Crawford JT, Woodley CL, Butler WR, Eisenach KD, Cave MD, et al. Rapid, amplification-based fingerprinting of *Mycobacterium tuberculosis*. *J Gen Microbiol* 1993;139:1537-42.
4. Hafner B, Haag H, Geiss HK, Nolte O. Different molecular methods for the identification of rarely isolated non-tuberculous mycobacteria and description of new *hsp65* restriction fragment length polymorphism patterns. *Molecular and Cellular Probes* 2004;18:59-65.
5. Telenti A, Marchesi F, Balz M, Bally F, Bottger EC, 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.
6. Steingrube VA, Gibson JJ, Brown BA, Zhang Y, Wilson RW, Rajagopalan M, et al. PCR amplification and restriction endonuclease analysis of a 65-kilodalton heat shock protein gene sequence for taxonomic separation of rapidly growing mycobacteria. *J Clin Microbiol* 1995;33:149-53.
7. Devallois A, Goh KS, Rastogi N. Rapid identification of mycobacteria to species level by PCR-restriction fragment length polymorphism analysis of the *hsp65* gene & proposition of an algorithm to differentiate 34 mycobacterial species. *J Clin Microbiol* 1997;35:2969-73.
8. Bahrmand AR, Bakayeva TG, Bakayeva VV. Use of restriction enzyme analysis of amplified DNA coding for the *hsp65* gene and polymerase chain reaction with universal primer for rapid differentiation of mycobacterial species in the clinical laboratory. *Scand J Infect Dis* 1998;30:477-80.
9. da Silva Rocha A, da Costa Leite C, Torres HM, de Miranda AB, Pires Lopes MQ, Degraive WM, et al. Use of PCR-restriction fragment length polymorphism analysis of the *hsp65* gene for rapid identification of mycobacteria in Brazil. *J Microbiol Methods* 1999;37:223-9.
10. Ringuet H, Akoua-Koffi C, Honore S, Varnerot A, Vincent V, Berche P, et al. *hsp65* sequencing for identification of rapidly growing mycobacteria. *J Clin Microbiol* 1999;37:852-7.
11. Khosravi AD, Stanford JL, Donoghue HD and Rook GA. Variation within *Mycobacterium scrofulaceum*. *J App Microbiol* 1997;83:596-602.
12. Richter E, Niemann S, Rusch-Gerdes S, Hoffner S. Identification of *Mycobacterium kansasii* by using a DNA probe and molecular techniques. *J Clin Microbiol* 1999;37:964-70.
13. Wong DA, Yip PC, Cheung DT, Kam KM. Simple and rational approach to the identification of *Mycobacterium tuberculosis*, *Mycobacterium avium* complex species and other commonly isolated mycobacteria. *J Clin Microbiol* 2001;39:3768-71.
14. Forbes BA, Sahn DF, Weissfeld AS. Baily & Scott's Diagnostic Microbiology. 11th edn. St. Louis: C.V. Mosby Inc. 2002;pp.546-61.