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 HaeIII & BstEII 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.

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 in-
fected 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 differentia-
tion of NTM,⁶⁻¹⁰ investigation of genetic variation within M. scrofulaceum,¹¹ and identifying
the subspecies of M. kansasii.¹²
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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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 MgCl\textsubscript{2}, 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 an 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’-CTTGTCGAACCCGCATAACCT.

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.\textsuperscript{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.\textsuperscript{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 \textit{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
previously published patterns for MTB as 160/140/70 bp fragments for \textit{Hae} III and 245/125/80 for \textit{Bst} EII digests.\textsuperscript{5} The diverse restriction patterns were observed for five clinical isolates in \textit{Hae} III digest only, while their \textit{Bst} EII digestion patterns showed no variation and were similar to other isolates. Two strains were showed different \textit{Hae} III patterns as 180 / 100 / 80bp and 194/ 72bp (Figure II). The third different \textit{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.\textsuperscript{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.\textsuperscript{5,5} 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,\textsuperscript{9} that the DNA sequence selected, was identical for species studied.

We noticed a possible intra-species variation, which resulted in three patterns among \textit{Hae} III digests of PCR-amplified segments of the HSP gene sequence from clinical isolates of MTB. For the strains with different \textit{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.\textsuperscript{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 \textit{M. tuberculosis}
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. 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