

# The Place and Importance of Pcr-Rflp Method in Determination of Mycobacteria Species in Routine Laboratory Practice



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## Abstract

**Background:** Rapid identification of mycobacterial species is the most important step for effective and true treatment. Various molecular methods have been used for rapid identification of mycobacterial species. In this study, we were aim to evaluate the place PCR-RFLP method in routine use and its application practice by the hsp65 gene PCR amplification from clinical isolates.

**Material and methods:** In our study, 80 mycobacterial isolates and 21 reference ATCC strains were evaluated to by in house PCR –RFLP of an amplified 439-bp segment of the gene encoding the 65-kDa heat shock protein. Restriction digests amplicons were separated by 8 % polyacrylamide and 4% Nusieve agarose gel electrophoresis (PAGE). By including a size standard in each sample, the restriction fragment profile was evaluated using algorithm.

**Results:** Of 80 mycobacteria strains, 70 (87.5%) were identified as *Mycobacterium tuberculosis* complex and 4 (5%) as *Mycobacterium scrofulaceum*, 3 (3.75%) as *Mycobacterium gordonae*. 3 (3.75%) strains displaying different restriction patterns could not be identified as they were found in the algorithm reported in the literature.

**Conclusion:** PCR-RFLP method is a rapid, practical and reliable method that can be used routinely in laboratories for typing mycobacterium strains isolated from clinical samples. Restriction enzymes and molecular weight standards play an important part in the efficacy of the test. It is suggested that quality control of materials used should be made comparatively with standards before the test and that more than one molecular weight standards at proper band size should be used in order to minimise mistakes at the stage of evaluation.

**Keywords:** Mycobacteria; PCR-RFLP; hsp65 gene

## Introduction

Tuberculosis (TB) is one of the most ancient infectious diseases of human beings, and it is still in a leading position among infectious diseases as a cause of morbidity and mortality. In recently years, the HIV epidemics and the use of drugs that depress the immun system have resulted in an increase in infections [1,2]. Mycobacteria other than *Mycobacterium tuberculosis* (MOTT) infections are getting increase in human [3]. Key factors in the control of tuberculosis are rapid detection, adequate therapy, and contact tracing to arrest further transmission [4,5]. Determination of the species of mycobacteria, that are active

agents in mycobacteria infections is important in developing appropriate treatment protocols and collecting epidemiological data. Conventional biochemical methods and phenotypic tests for species differentiation of Mycobacteria are laborious and time-consuming and frequently require specialized testing that is beyond the capacity of clinical laboratories. Various genotypic methods for the rapid identification of mycobacteria have been used in recent years [6-9]. With in the these methods, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was described by Plikaytis et al. for differentiating

among slowly growing *Mycobacterium* species by PCR and restriction fragment length polymorphism analysis in 1992 [10]. This method is an easy, rapid and inexpensive method of identifying mycobacteria species in a single experiment when compared with the other molecular typing methods. This technique has been applied to several genes, for example 16S ribosomal DNA (rDNA), hsp65, dnaJ, groES and rpoB genes. The fundamental mechanism of this method is based on evaluation of the gene coding for the 65-kDa heat shock protein by PCR and restriction enzyme analysis. The PCR-RFLP was routinely used for the taxonomic separation of rapidly growing mycobacteria, identification of mycobacteria strains [11-14].

The success of this method, that yields results quite rapidly, varies according to standardization of the method used of each step, activity of restriction enzymes employed, markers of molecular weight, algorithm and experience of the user. In all these studies the algorithm describing the mycobacteria species is based on the use of two restriction enzymes (*BstEII* and *HaeIII*) and separation of the restriction fragments on an agarose gel. PRA patterns are then interpreted by converting the running distance in electrophoresis to apparent molecular size. Difficulties in PCR-RFLP interpretation may stem from similarities in a number of band sizes that are critical for discrimination of species and are not sufficiently resolved by agarose-based gel electrophoresis [15-19]. In this study, we aim to evaluate the place PCR-RFLP method in routine use and its application practice by the hsp65 gene PCR amplification.

### Materials and Methods

#### Bacterial strains

80 mycobacterial isolates and 21 reference ATCC strains (*M. bovis* BCG ATCC 27291, *M. smegmatis* ATCC 14468, *M. kansasii* ATCC 12478, *M. fortuitum* ATCC 6841, *M. parafortuitum* ATCC 19686, *M. fortuitum* subs. *fortuitum* ATCC 6841, *M. abscessus* ATCC19977, *M. chelonae* subs. *chelonae* ATCC 14472, *M. scrofulaceum* ATCC 19981, *M. szulgai* ATCC 35799, *M. goodii* ATCC 14470, *M. intracellulare* ATCC 13950, H37Rv ATCC 25618, H37Ra ATCC 25177, *M. xenopi* ATCC 19250, *M. avium* ATCC 25291, *M. triviale* ATCC 23292, *M. diernhoferi* ATCC 19340, *M. marinum* ATCC 927, *M. terrae* ATCC 15755, *M. haemophilum* ATCC 29548) were evaluated to by in house PCR- RFLP. Twenty-one reference strains were obtained from Prof. Dr. Tanil Kocagöz, Acibadem University of Turkey. The mycobacterial isolates were obtained from the clinical samples submitted to National Tuberculosis Reference and Research Laboratory of Refik Saydam Hygiene Center, Ankara, Turkey.

#### Biochemical tests and growth characteristics

Bacteriologic determination of clinic isolates was based on growth rate, colony morphology, pigmentation, biochemical tests [2-20].

#### DNA extraction

A loopful of cells were suspended in 500µl of TE buffer (10mM Tris, 1mM EDTA, pH 8.0) in a 1.5ml screw- cap microcentrifuge tube. The suspension was centrifuged at 15,000xg for 10min. The supernatant was removed and then the pellet was washed twice with 500µl of TE buffer and then resuspended with 200µl of the same buffer. The samples were incubated in a boiling water bath for 20 min centrifuged, and supernatants containing DNA was transferred to clean microcentrifuge tubes and kept at - 20°C until used [13].

#### DNA Amplification

A total of 10µl of the DNA-containing supernatant was added to a reaction tube containing 50µl PCR mixture (1X PCR buffer, 1X PCR Enhancer, 2.5mM MgCl<sub>2</sub>, 200-µM (each) deoxyribonucleoside triphosphate, 50pM (each) primer and 1.25 U of Taq polymerase [Fermentase]). The reaction tube was first heated for 3min at 94 °C and then to 44 cycles of amplification (1min 94 °C, 1min at 58 °C, 1min 30s at 72 °C); this was followed by 4min of extension at 72 °C. Primers Tb11 (5-ACCAACGATGGTGTGCCAT) and Tb12 (5-CTTGTCGAACCGCA TACCCT) amplified a 441 bp fragment between positions 398 and 836 of the previously published sequence of hsp 65. The presence of amplified product was confirmed by agarose gel electrophoresis [11-13].

#### Restriction digestion and analysis

All steps of the test were carried out in accordance with the recommendations of the manufacturer (Salubris Inc., Turkey). For *BstEII* digestion, 20µl of PCR product was added directly to a mixture containing 0.5µl (~ 5U) of enzyme, 2.5µl of restriction buffer, and the mixture was incubated for 60min at 37°C. Similarly, 20µl of product was incubated for 18h at 37°C in a solution containing *HaeIII* enzyme, the corresponding buffer.

#### Evaluation of restriction patterns

After digestion, 4µl gel loading buffer (3% orange G, glycerol in water) was added, and 10µl of the mixture was loaded onto a NuSieve 3:1 agarose gel (FMC Bioproducts) and 8% polyacrylamide gel. Fragments were visualized by staining with ethidium bromide. Products were evaluated with a NuSieve 3:1 agarose gel and polyacrylamide gel electrophoresis (8%). In the stage of evaluation, Mayker B and Mayker H (Salubris Inc., Turkey) molecular weight standards specific to enzyme restriction regions *BstEII* and *HaeIII*, that contains certain band size were used. 21 standard strains of mycobacteria were also analysed with this method and *M. tuberculosis* H37Ra was used as a control in every run [13].

#### Results

In the beginning stage of our study, all steps in house PCR-RFLP method were evaluated comparatively with standardized Mycobacteria typing kit. In standardization procedure using reference strains, same PCR products (441bp) were obtained

with both methods (Figure 1) obtained amplification product was cut by two separate restriction enzymes in accordance with reference literature and protocols of manufacturer. Final product were evaluated with 8% polyacrilamid gel electrophoresis and 4% Nusieve Agarose (Figure 2). While Hae III enzyme yielded similar results with both methods , with Bst E II enzyme band patterns inconsistent with algorithm table and results of procedure same enzyme was evaluated in comparatively with new products and at different concentrations. In all studies ,similar results were obtained with the same enzyme. Therefore, study was continued with a different standardized enzyme.

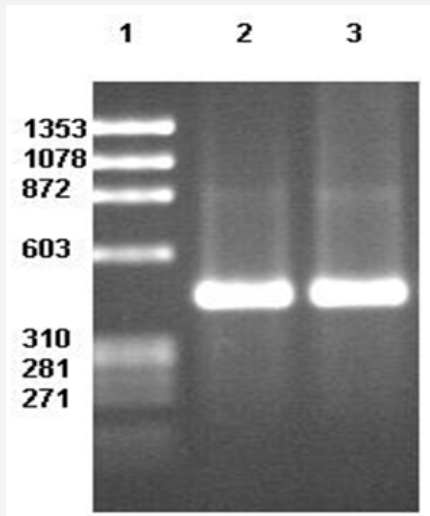


Figure 1: DNA amplification: M: marker ( $\Phi$  X174, HaeIII digest), 1-3 amplification product.

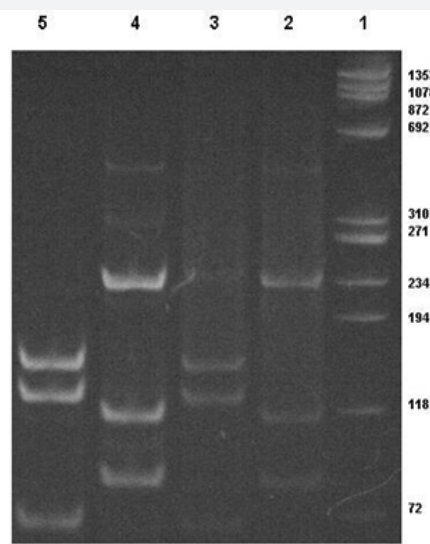


Figure 2: PCR-RFLP patterns of different BstE II in polyacrilamide gel electrophoresis. 1: MB: Myker B, 2-3: RFLP patterns of in house Bst E II enzyme, 4-5: RFLP patterns of Mycotype ALL Bst E II enzyme.

A total of 80 mycobacterial isolates were evaluated by using routine biochemical tests and PCR –RFLP. All the strains identified as *M.tuberculosis* by PCR-RFLP were identified as the same by biochemical tests and colony morphology. The 21 standard mycobacterial strains used as controls reproducibly produced RFLP patterns in accordance with the patterns previously reported in other studies. The number and rates of species isolated are shown in Table III. Of 80 mycobacteria strains, 70(87.5%) were identified as *Mycobacterium tuberculosis* complex and 4(5%) as *Mycobacterium scrofulaceum*, 3(3.75%) as *Mycobacterium gordonae*. 3(3.75%) strains displaying different restriction patterns could not be identified as they were found in the algorithm reported in the literature.

### Discussion

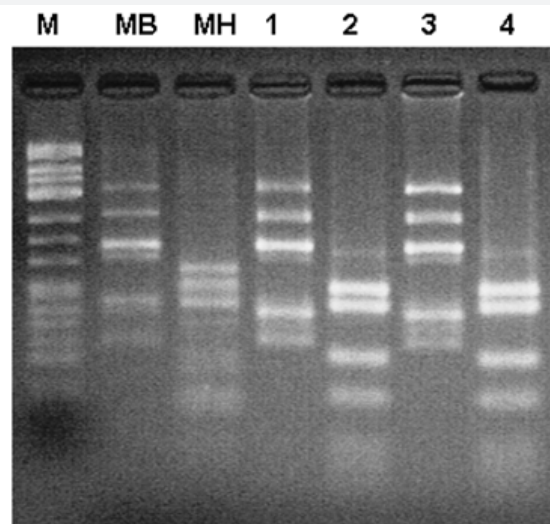


Figure 3: PCR-RFLP patterns of different BstE II in Nusive agarose: M: Myker HaeIII digest of  $\Phi$ X174 MB: Myker B, MH: Myker H, 1-2: RFLP patterns of in house Bst E II enzyme, 3-4: RFLP patterns of Mycotype ALL Bst E II enzyme.

The resurgence of tuberculosis and apperance of multidrug-resistant strains of *M.tuberculosis* have intensified the need for the increased use of rapid methods for isolation and identification of mycobacteria from patients. Mycobacterial species identification methods depending on colony morphology, pigment formation and biochemical analysis are very cumbersome and require several week [2,20,21]. Commercially available probe based methods are limited to identification of only limited number of species and expensive. Methods based on DNA sequencing are not practical enough to apply routinely and require expensive equipment. PCR-RFLP is simple to perform, easy to read, and reproducible and is used in several laboratories to identify MOTT. Mycobacterial identification by PCR-RFLP depends on amplification and restriction enzyme analysis of *hsp 65* gene ,which exist in all mycobacterial species but show

sequence differences from one to the other [10-15] (Figure 3). These differences are identified by restriction enzymes that recognize and cut specific DNA sequences. Obtained fragments are separated by electrophoresis and the size of fragments are compared to molecular weight marker. The most important stage in PCR-RFLP method is the enzyme cut of amplification product. In cases when enzymatic activity is low, this procedure cannot be performed in the expected period and misleading results may be obtained during evaluation. The activity of the enzymes used in the test varies with storage conditions employment period, heat during reaction and production characteristics. Therefore, in order to minimise any mistakes during evaluation, material to be used in all steps of the test should be controlled according to certain standards prior to the test. In the initial step of our study, MYCO TYPE ALL® (Salubris inc) kit was used as the standard for optimisation of the test and control of restriction enzymes [16]. Band patterns obtained with both tests were evaluated comparatively in algorithm [13,15,17]. While there was consistency between two methods in all steps of the test, in the evaluation made in in-house PCR-RFLP method after cut with BstE II enzyme, different band patterns were identified in algorithm. Hence, study was continued with another enzyme the activity of which was controlled with the same method. After the cut procedure with enzyme, gel with suitable characteristics and concentration for the separation of fragments should be used. Two methods used for this purpose are polyacrylamid gel (8-10%) and electrophoresis 4% NuSieve agarose. Both methods have certain advantages and disadvantages. The preparation of the former method is more practical and makes gel manipulation easier, but can not differentiate bands at similar magnitude. On the other hand, latter method enables detailed differentiation at suitable concentration and defines bands lower than 100bp clearly. It is disadvantage is that its preparation and gel manipulation is difficult.

Fragments in our study were evaluated with both methods. Although NuSieve agarose method was easier to employ, it was observed that it could not make differentiation as clear as polyacrylamide gel electrophoresis. In the evaluation stage of PCR-RFLP method, in order to differentiate species according to algorithm, the most important criterion is the selection of molecular weight markers containing suitable band size. The use of markers involving very far on very near band magnitude may cause incorrect evaluations. Therefore, the most commonly used molecular weight marker is  $\phi$ X174 Hae III DNA containing band sizes of 1353, 1078, 872, 603, 310, 281, 271, 234, 194 ve 118 with this marker, especially after cut with BstE II enzyme, band sizes can be discriminated easily. Yet, after cut with Hae III enzyme, misleading results may be obtained. Myker B and Myker H molecular weight markers just corresponding to the band sizes of at BstE II and Hae III enzyme cut regions are still at experimental stage. In our study, after amplification product were cut with BstE II and Hae III enzyme, we evaluated to band size by using three different marker ( $\phi$ X174 Hae III DNA, Myker B, Myker H). Band

sizes especially smaller than 100bp and close to each other were readily established.

In group and type differentiation, reference algorithm and Mycobacteria typing kit were evaluated comparatively with the modified algorithm. While similar results were obtained in group differentiation by both algorithms, in type differentiation algorithm produced by the manufacturer yielded more detailed information. Of 80 mycobacteria isolate investigated in our study, 70(87.5%) was identified as Mycobacterium complex, 4(5%) as Mycobacterium scrofulaceum and 3(3.75%) as Mycobacterium gordonae. 3 isolates with different band patterns could not be defined by either algorithm.

### Conclusion

In conclusion, PCR-RFLP method is a rapid, practical and reliable method that can be used routinely in laboratories for typing mycobacterium strains isolated from clinical samples. Restriction enzymes and molecular weight standards play an important part in the efficacy of the test. It is suggested that quality control of materials used should be made comparatively with standards before the test and that more than one molecular weight standards at proper band size should be used in order to minimise mistakes at the stage of evaluation.

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