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Original Research

Identification of the Mycobacterial Strains Isolated From Clinical Specimens Using hsp65 PCR-RFLP Method

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Abstract

Objectives: It is important to identify mycobacteria at the species level, to distinguish pathogen from non-pathogenic species, to choose the appropriate treatment regimen and to collect epidemiological data. For the identification of mycobacteria, which are time-consuming and laborious with traditional methods, faster, more sensitive and reliable methods are needed. This study aims to investigate the suitability of the hsp65 Polymerase chain reaction-restriction fragment polymorphism (PCR-RFLP) method for routine laboratory use.

Methods: In this study, 141 mycobacterial isolates were obtained from 1632 samples, which were sent to the Medical Microbiology Laboratory.

Results: In the culture, mycobacteria were identified as 138 M. tuberculosis complex (*MTBC*) and three non-tuberculosis mycobacteria (NTM) by conventional methods. Using the hsp65 PCR-RFLP method, 137 isolates were identified as MTBC, four isolates as NTM. An isolate that was evaluated as MTBC because it was PNB sensitive by the conventional method was determined as NTM with the hsp65 method. In the identification of non-tuberculosis mycobacteria with the hsp65 PCR-RFLP method, one isolate was identified as M. abcessus and three isolates were identified as M. avium complex.

Conclusion: In our study, it was concluded that the hsp65 PCR-RFLP method, which allows identification of mycobacteria, including NTMs, is a method that is cheap, easy and suitable for routine use to provide rapid information to the clinic. The scope of the agar and database used in the method is effective in the definition of the correct species.

Keywords: *hsp65; M. tuberculosis complex*; non-tuberculous Mycobacteria; PCR-RFLP.

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The most frequent disease -causing Mycobacterium species is M. *tuberculosis*, which is the cause of tuberculosis. Today, tuberculosis remains a major health problem worldwide. It was reported that 10 million new cases of tuberculosis were seen worldwide in 2017, and 1.3 million patients with tuberculosis and 300 000 HIV (+) patients died due to tuberculosis.[1] Atypical or non-tuberculosis mycobacteria (NTM) have more than 150 species and are found in environmental resources and water distribution systems, such as soil and water, and cause opportunistic infections in humans.[2] Outbreaks that arise from contaminated medical equipment have also been reported in hospital settings.[3]

It is generally taken in by inhalation in people with predisposing factors and causes lung infections.[4] In recent years, it has been reported that NTM infections tend to increase [2, 5] and are often associated with lung, lymphatic system, skin or bone involvement.[4, 6]

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Identification of mycobacteria at the species level; is important for the selection of the appropriate treatment regimen and the collection of epidemiological data. Since the biochemical tests used in classical identification take time and are labor-intensive, faster, sensitive and reliable methods are required for the identification of mycobacteria.

While priorly biochemical properties, analysis of mycolic acids in the cell wall were used in the identification of mycobacteria, many molecular methods, hybridization -based commercial probes and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry methods have been developed recently.[7]

Polymerase Chain Reaction Restriction-Fragment Length Polymorphism (PCR-RFLP) method is one of the inexpensive and easy to apply molecular methods. Different gene regions (16S-23S rRNA, hsp65, rpoB) can be used with this method.[8–11] AccuProbe system (Hologic Gen-Probe, San Diego, CA, USA), INNOLiPA Mycobacteria system (Fujirebio Europe, Ghent, Belgium), GenoType Mycobacterium system (Hain Lifescience, Nehren, Germany) are commonly used systems. While the identification time is shortened with these systems, identification of limited number of species and cross-reaction between species with some probes can be disadvantages.[12] In the hsp65 PCR-RFLP method, a certain region of the hsp65 gene of mycobacteria is reproduced and cut with restriction enzymes, and the obtained band patterns are compared with the reference species in species detection algorithms. This study aims to determine the suitability of hsp65 PCR-RFLP method for routine laboratory use in identifying mycobacteria at the species level.

Methods

In this study, 141 consecutive Mycobacterium isolates among clinical samples from 1632 patients, each belonging to a single patient , were included. Sterile samples were directly inoculated into the Mycobacterial-Growth Indicator Tubes (MGIT) and Lowenstein-Jensen (LJ) media for culture, while non-sterile samples were inoculated after decontamination-homogenization-concentration processes applied using NaOH-Nalc method. Erlich -Ziehl Neelsen staining was used for microscopic examination.

Differentiation between MTBC and NTM isolates with growth and acid-resistant staining characteristics was performed by applying niacin test strip (BD BBL Taxo TB Niacin Test Strips) and MGIT-PNB (p-nitro benzoic acid) susceptibility tests and evaluating the results in combination.

by applying niacin test strip (BD BBL Taxo TB Niacin Test Strips) and MGIT-PNB (p-nitrobenzoic acid) susceptibility tests and evaluating the results in combination. In addition to differentiation between MTBC-NTM using the PCR RFLP method NTM species were identified. H37Rv (ATCC 27294) strain of *M. tuberculosis* was used for quality control.

Cost analysis of test reagents and materials was performed, excluding device setup and consumables of tests.

Niacin Test

The niacin test was performed according to the manufacturer's recommendations. In this study, 1.5 ml sterile distilled water was transferred to the LJ medium in the active growth phase (three to four weeks old). To obtain niacin released from the colonies, the colonies were gently scraped with a loop from the medium and kept in an inclined position for 20-30 minutes. At the end of the period, 600 µl of this solution was taken and placed in the tubes marked as control and test tubes. The niacin strip (Becton Dickinson BBL Taxo TB Niacin Test Strips) was placed in the test tube with the arrow pointing down, then the tubes were closed and then shaken gently. They were gently shaken again after 5-10 minutes and the test result was evaluated after 15 minutes. Colorless appearance was evaluated as niacin negative and yellow color as niacin positive.

MGIT/PNB (p-Nitrobenzoic acid) Inhibition Assay

One ml of liquid was taken from the positive MGIT tube and transferred onto a 4 ml sterile saline to make a 1: 5 dilution. Two MGIT tube media were taken and the first tube was marked as a control tube and the other tube as a test tube for the PNB test. Under aseptic conditions, 500 µl of MGIT OADC solution was added to both tubes, and 100 µl of 4% PNB solution was added to the test tube only. A 500 µl of the diluted bacterial suspension was added to each tube and agitated. At the same time, the passage was performed on the blood agar medium to control contamination from the bacterial suspension.

Tubes were incubated at 37 °C. The control tube was evaluated daily on the MGIT device and the test tube was evaluated from the first day when it was positively read. At the end of the third day, if the fluorescence was detected in the tube containing PNB, Mycobacterium was evaluated as NTM, and if no fluorescence was detected, it was evaluated as an *M. tuberculosis complex*.[13]

DNA Extraction

The MGIT tube where bacterial growth was detected was vortexed for one minute. Then, 500 µl of this solution was taken with a sterile Pasteur pipette and placed in a 1.5 ml Eppendorf tube. Bacteria were inactivated in a dry heat block at 80 °C for 10 minutes. The bacteria were precipitated by centrifugation at 14000 rpm for five minutes, and the supernatant was discarded. Before each use 10% Chelex 100 mixture, vortexed and 250 µl of the solution was trans-

ferred into an Eppendorf tube. The solution was vortexed again for 10-15 seconds, then incubated for 10 minutes at 60°C in a dry heat block, and left to cool down at room temperature. It was vortexed for 10-15 seconds again, and incubated at 100 °C for 15 minutes in a dry heat block and allowed to cool down at room temperature.

The solution was centrifuged at 14000 rpm for three minutes. The supernatant containing Mycobacterium DNA was transferred into another Eppendorf tube and kept at -70 °C until used for PCR processing.[14]

DNA Amplification and Cutting with Restriction Enzyme

For the identification of mycobacteria using PCR-RFLP method, and selection of primers, and restriction enzymes appropriate for the target region, the study carried out by Telenti et al.[8] was taken as a reference. To reproduce the Hsp65 gene, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 200 M (each) deoxynucleoside triphosphate (dNTP) (Promega), 50 pmol from each primer (Tb11 = 5-ACC AAC GAT GGT GTG TCC AT-3] and Tb12 = CTT GTC GAA CCG CAT ACC CT-3]), 1 U Taq DNA polymerase (Promega) were added into the PCR mixture, and 45 µl was distributed into PCR tubes. A final concentration of 50 µl was obtained by adding 5 µl of the resulting bacterial DNA.

For PCR process, 30 thermal cycles set at 94 °C for 5 min; at 94 °C for 1 min, at 60 °C for 1 min, and at 72 °C for 10 min on the (Bio-Rad, USA) thermal cycler.

Samples demonstrating the bands (440 base pairs) of the appropriate target DNA region were completed to 20 µl with 2 µl 10X RE buffer, 0.2 µl acetylated BSA, 1 µl restriction enzyme, 10 µl PCR product and distilled water, and incubated for 2.5 hours at 60 °C for BstEII enzyme (Promega), and for 2.5 hrs at 37 °C for HaeIII enzyme (Promega) in dry heat block or thermal cycler.

To display the products obtained after cutting with restriction enzymes, a 2% agarose gel (AppliChem, Germany) was prepared and *M. tuberculosis complex* strains were identified. A 3% NuSieve GTG Agarose (Cambrex, USA) gel was used to identify non-tuberculosis mycobacteria. The resulting band sizes were determined by comparing with ϕX174 DNA/Hinf I DNA (Fermentas) and DNA 100bp ladder molecular weight standards.

Species were identified by evaluating the band patterns of the isolates and the algorithms in the studies in combination.[8, 9, 15]

Statistical Analysis

SPSS 15.0 for Windows program was used for the statistical analysis. Descriptive statistics were given as numbers and percentages for categorical variables. Comparison of rates in dependent groups was made using Mc Nemar Analysis. The consistency of the results was analyzed with Cohen's Kappa compliance test. The level of alpha significance level was set at $p<0.05$.

Results

The age range of 141 patients in whose culture mycobacterial growth was demonstrated ranged between 0-81 years, while 33% of these patients were in the age groups of 0-18, 35% were in 19-49 years. Mycobacterial isolates were grown in samples of sputum (n=4), gastric fasting fluid (n=32), abscess (n=24), biopsy material (n=14), cerebrospinal fluid (n=10), peritoneal fluid (n=7), pleural fluid (n=4), urine (n=4) and bronchoalveolar lavage fluid (n=1). Niacin test-positivity was detected in 137, and negativities in four isolates. PNB (p-Nitrobenzoic acid) could inhibit the growth of 138 isolates of mycobacteria, while three isolates were resistant to PNB.

The 441 base pair (bp) long DNA segment obtained by replication of the hsp65 gene region was cut with BstEII enzyme and HaeIII enzyme. As a result, 137 isolates of *M. tuberculosis complex* were identified, which formed DNA fragments with a length of 231-116-79 bp and 152-127-69 bp, respectively (Figs. 1, 2). The strains identified as M.

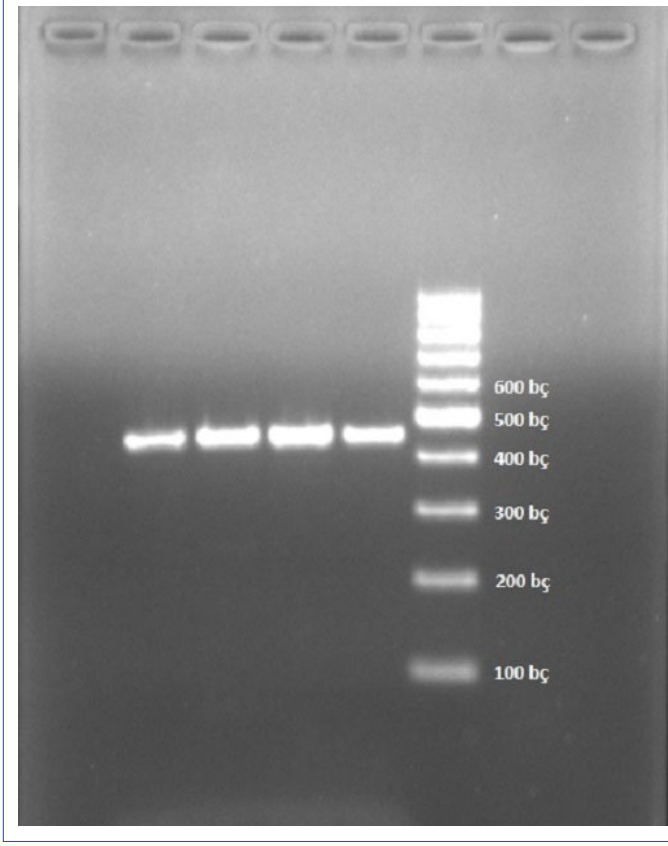

Figure 1. *hsp65* gene (440 bp).

Figure 2. Cutting of hsp65 gene with BstEII and HaeIII restriction enzymes.

tuberculosis complex demonstrated niacin test-positivity and PNB test-sensitivity. The results obtained with the niacin test and PCR RFLP method were 100% compatible in the identification of the isolates (Kappa=1.000 p=1.000).

An isolate evaluated as MTBC based on its sensitivity to PNB was identified as NTM by the PCR-RFLP method (Kappa= 0.855 p=1.000). The bands formed as a result of cutting the isolates determined as NTM with PCR RFLP method with BstEII and HaeIII restriction enzymes were evaluated. Subsequently, three isolates were identified as *M. avium-intracellulare* (MAC) and one isolate as *M. abscessus*. The properties of NTM isolates are shown in Table 1. The approximate costs of the tests were calculated as follows: Niacin test $3, PNB test $5 and PCR-RFLP method $4.

Discussion

Tuberculosis is one of the oldest diseases in human history. Although its cause is known and its treatment is possible, it is still a widespread contagious disease with high mortality.[16] An increase in non-tuberculous mycobacterial infections has been observed in recent years.[5, 17] Especially in individuals with risk factors, the mortality is high and the distribution of mycobacterial species varies geographically.[18] NTM types are resistant to many antibiotics and disinfectants.[2]

Accurate discrimination between MTBC/NTM and the definition of species is important in developing the appropriate treatment strategy. In four patients with a lung infection, treatment failure was reported with the 12-month use of clarithromycin, which was the first choice in treatment, as a result of misidentification of the agent as *M. abscessus* instead of macrolide- resistant *M. bolletii*.[19]

In the identification of mycobacteria by conventional methods, growth rate, colony structure, the formation of pigmentation and biochemical methods (such as niacin production, nitrate reduction tween hydrolysis, urease and arylsulfatase) are being used.[7] Niacin test can give positive results in MTBC isolates (except *M. bovis* and *M.bovis BCG*), and also in *M. simiae* species. Therefore, it should be evaluated together with other methods when used in the preliminary diagnosis of MTBC.[20]

One of the biochemical methods used is the observation of reproduction inhibition in the presence of inhibitory substrates added to suitable media. While NTMs can grow (resistant) in 500µg / ml concentration of p-nitrobenzoic acid (PNB), one of the substrates used in this method, MTBC is inhibited (sensitive). However, as is the case with an isolate in our study, though in a scarce number of isolated, PNB sensitivity[13] or PNB resistance in MTBC isolates has been reported in some of the NTMs.[21, 22] Differentiation between MTBC and NTM using the PNB test could be achieved with similar accuracy rates (Sharma et al.[23], 99.05%; Giampaglia

Table 1. Characteristics of the NTM produced in culture

NTM species	Sample	EZN	PNB Test	BstEII Pattern	HaeIII Pattern	Clinics
M. abcessus	Sputum	Positive	D	231-210 bp	145-69-58-52-48 bp	Chest diseases
MAC	Sputum	Negative	D	440 bp	145-127-42-40 bp	Chest diseases
MAC	Sputum	Negative	H	440 bp	145-127-42-40 bp	Pediatrics
MAC	BOS	Negative	D	440 bp	145-127-42-40 bp	Infection Diseases

NTM: Non-tuberculous mycobacteria; MAC: M.avium complex; CSF: cerebrospinal fluid; EZN: Ehrlich Ziehl Neelsen staining; PNB: para nitrobenzoic acid sensitivity test; bp: base pair.

et al.[13] 99.4%, and in our study, 99.2%.

It takes an average of 4-11 days to report the test results.[23] Because the biochemical tests used in the identification of mycobacteria require extensive labor, test results can be obtained in a few weeks (4-8 weeks) in addition to their low reproducibility, researchers have turned to molecular identification methods that are easier, faster and more reliable.[24]

Various identification methods have been developed based on the detection of specific DNA patterns by hybridization with commercially available probes, or species or genus-specific PCR that can be produced commercially or in-house. However, these methods can only distinguish a certain number of mycobacteria and are costly for routine use.[25]

PCR RFLP method was first used in 1992 to identify slow-growing mycobacteria[26], and it was stated in many subsequent studies that it is a fast, easy and cost-effective method for identifying Mycobacterium species.[8, 9, 27–29] In a study investigating the compatibility of the PCR-RFLP method with biochemical tests, 100% compliance was found for *M. tuberculosis*, 83.3% for slow-growing mycobacteria and 98.8% for fast-growing mycobacteria.[27] Chimara et al.[28] compared the hsp65 PCR-RFLP method with biochemical experiments in the species diagnosis of mycobacteria and defined 321 of 434 strains as compatible with both methods.

A total of 113 incompatible strains were identified by the hsp65 gene sequence analysis and 71 were found to be compatible with hsp65 PCR-RFLP results, and 12 strains were misidentified with the PCR-RFLP method. In the other 30 isolates, 13 new PCR-RFLP patterns that were not previously identified were identified. In a similar study, biochemical tests and hsp65 PCR-RFLP method gave compatible results in 43 of 50 isolates. In the confirmation of seven incompatible isolates by 16S rRNA sequence analysis, the two isolates yielded the same results as PCR-RFLP.[29]

In a study in which identification was made using sequence analysis and PCR RFLP methods, the results were consistent with two methods in common species (such as *M. fortuitum*, *M. avium-M.intracellulare*) in which only 30% of isolates can be identified with the PCR-RFLP method. Identification could not be made due to the presence of different species with the same restriction pattern or the presence of new species whose restriction pattern is unknown.[19] In our study, both methods yielded concordant results in 140 isolates (99.2%) in the MTBC-NTM distinction, and only MTBC-NTM distinction could be made with the biochemical tests (niacin and PNB tests) we used. Many additional biochemical tests are required for the identification of the NTM species.

An important difficulty with the Hsp65 PCR-RFLP method is that when evaluating with agarose gel electrophoresis, the distinction between the bands of nearly equal size is quite difficult to make and the bands smaller than 60 base pairs (bp) cannot be used in the distinction between mycobacteria.[30] In a study, 43 reference species and 65 clinical isolates were identified by hsp65 PCR-RFLP method, and it has been emphasized that NuSieve agar is very useful in the visualization of small DNA fragments and applicable in comparison to polyacrylamide gel.[9] With the preliminary experiments we conducted in our study, there was no difficulty in evaluating the *M. tuberculosis complex* using 2% agarose gel culture media. Therefore, it was concluded that it would be beneficial to use 2% agarose gel in the differentiation of MTBC because it is more cost-effective and most of ourisolated constitute MTBC strains; however, it will be more beneficial to prefer NuSieve agar where small DNA bands can be better observed.

The majority of mycobacteria produced in clinical mycobacteriology laboratories are composed of *M. tuberculosis complex*. However, there is an increase in the frequency of non-tuberculosis mycobacteria.

NTMs are held responsible for 0.5-30% of all mycobacterial infections.[5, 18, 31] In our country, MTBC[31–34] is the most common mycobacterial agent (83-87.5%) found in studies where the hsp65 PCR-RFLP method is used to identify mycobacteria. There are differences in the distribution of NTM species. In the study conducted by Tarhan et al.,[32] *M. scrofulaceum* (5%), *M. gordonae* (3.75%), in the study by Agacayak,[33] *M. scrofulaceum* (8%), *M. avium* (4%) and *M. intracellulare* (2%); in the study by Çiftçi[34] *M.intracellulare* (4.9%), and in the study by Bayram and Emekdaş.[35] *M. fortiutum* (16%) were the most frequently detected species.

In a study on pulmonary samples collected from 30 different countries and 62 centers outside of our country, and in another current study on clinical samples in which 10 member countries of the European Union were included, the *M. avium complex* (MAC) was isolated most frequently among the NTM species followed by *M. gordonae* and *M xenopi*.[6, 18] In our study, 97% of our mycobacterial strains were *M. tuberculosis complex*, while 3% of them were identified as NTM. Among the NTM isolates, *M. avium-intracellulare* group (MAC) was isolated most frequently in accordance with the data of the world and our country.

In a study where the cost analysis of the methods used for the identification of mycobacteria was made at the species level, the hsp65 PCR-RFLP method was found to be a cost-effective method in terms of both devices in the installation phase and methods such as GLC, HPLC, AccuProbe per patient. When consumables and test reagents were calculated, a cost of $1.2 was reported for identifica-

tion of species by PCR RFLP method and $0.9 for distinction between MTBC, and NTM with the use of niacin test and PNB test in combination.[36] In another cost analysis study, using the sputum sample directly, distinctioning between MTBC, and NTM , and identification of their species using the PCR RFLP costed nearly $6 per sample.[37]

In our study, the only distinction between MTBC-NTM was made with biochemical tests and niacin, and PNB tests cost approximately $3 and $5, respectively. With the PCR -RFLP method, in addition to the distinction between MTBC and NTM, species of NTM have been identified and its cost has been calculated as approximately $4.

When these methods are examined concerning time spent per test, the most advantageous method has been specified as the hsp65 PCR-RFLP method.[36]

In the MTBC –NTM distinction, results can be obtained on the same day in the niacin test, 4-11 days later with PNB test,[23] and within 24-48 hours with PCR-RFLP method.[36] To identify the species of NTM isolates, it is necessary to use many additional biochemical tests together, intense workload and an average of 4-8 weeks.[24, 36] The advantage of the PCR-RFLP method is that identification up to species level in two days in addition to the distinction between MTBC and NTM[36] One of the limitations of our study is that it is not possible to verify NTM isolates by sequence analysis.

Another limitation is that identification of NTM species cannot be achieved with the biochemical tests used. Since the identification of NTM species can be made in a limited number of tuberculosis laboratories in our country, studies with a high number of isolates are needed in comparisons between epidemiological data and methods used.

Thanks to its advanced technology, it is beneficial to use the user-friendly, and cost-effective *hsp65 PCR-RFLP* method in mycobacteriology laboratories, which is faster and more accurate than biochemical tests.

Disclosures

Ethics Committee Approval: This study was approved by University of Health Sciences, Sisli Hamidiye Etfal Training and Research Hospital Ethics Committee, Decision number: 2332.

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Conflict of Interest: There is no conflict of interest declared.

Authorship Contributions: Concept – A.B., B.B.; Design – A.B., B.B.; Supervision – B.B.; Materials – A.B.; Data collection &/or processing – A.B.; Analysis and/or interpretation – A.B.; Literature search – A.B.; Writing – A.B.; Critical review – B.B.

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