IDENTIFICATION OF MYCOBACTERIAL SPECIES BY SEQUENCE SPECIFIC POLYMERASE CHAIN REACTION

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ABSTRACT

IDENTIFICATION OF MYCOBACTERIAL SPECIES BY SEQUENCE SPECIFIC POLYMERASE CHAIN REACTION

Tuberculosis continues to be one of the most important health problems in Turkey and around the world. *Mycobacterium tuberculosis* is the agent which is responsible for most of the mycobacterial infections in humans. On the other hand approximately 170 species of mycobacteria are identified.

Some of these may sometimes contaminate the cultures of the samples obtained from patients. When a type of *Mycobacterium* is isolated from a patient, its species should definitely be identified. Because, the antimycobacterial drugs used in the treatment of infections caused by *M. tuberculosis*, which is the most common cause of mycobacterial infections in humans, are not usually effective in the treatment of infection caused by other mycobacteria and different drugs are used for the treatment of these infections. Additionally, mycobacteria that contaminate culture media but which do not cause infection in humans are also identified by species identification and unnecessary treatment of the patients is thus eliminated. Classical identification of mycobacterial species depends on their rate of growth in culture media, their pigment formation and biochemical characteristics. In slow growing species like *M. tuberculosis*, classical species identification takes about two months and in many cases the results are controversial.

In recent years, several molecular methods were developed to speed up species identification and to increase the specificity. However the most developed and commercially available kits can only identify 16 mycobacterial species. In this project we have developed and evaluated a sequence specific polymerase chain reaction which is expected to be capable of differentiating all mycobacterial species easily, from organisms grown in culture.

In this method, which depends on obtaining or not an amplification product using sequence specific primers for the genes that encode 16S *ribosomal RNA*, *hsp65* and *gyrB*, the strategy is not to use primers specific for single species but primers that divide species into groups and identify species in an algorithmic way. With this new approach it is possible to identify all mycobacterial species, using fewer sequence specific primers with lower cost and effort compared to other methods.

ÖZET

DİZİYE ÖZGÜL POLİMERAZ ZİNCİRLEME TEPKİMESİ İLE MİKOBAKTERİ TÜRLERİNİN SAPTANMASI

Tüberküloz Türkiye'de ve dünyada en önemli sağlık sorunlarından biri olmaya devam etmektedir. İnsanda en sık enfeksiyona yol açan mikobakteri türü *Mycobacterium tuberculosis*'tir. Ancak yirmi kadar başka mikobakteri türünün daha insanda çeşitli enfeksiyonlara yol açtığı bilinmektedir.

Bugüne dek doğada 170 kadar mikobakteri türünün varlığı saptanmıştır. Bunların bir kısmı zaman zaman hastalardan alınan örneklerden yapılan kültürleri kontamine edebilmektedir. Hastalardan alınan örneklerden yapılan kültürlerde mikobakteri üretildiği zaman bunun mutlaka türünün saptanması gerekir. Çünkü insanda tüberküloza en sıklıkla yol açan *M. tuberculosis* enfeksiyonunun tedavisinde kullanılan ilaçlar diğer mikobakteri enfeksiyonlarında genelde etkili olmamakta, başka antimikobakteriyel ilaçlar tedavide kullanılmaktadır. Yine kültürleri kontamine eden ancak insanda enfeksiyona yol açmayan mikobakteriler, türlerinin belirlenmesi sayesinde saptanabilmekte ve hastaların gereksiz yere tedavi alması engellenmektedir.

Mikobakteriler için klasik tür saptama yöntemi kültürde üreme hızı, pigment oluşturma ve çeşitli biyokimyasal özelliklerine dayanmaktadır. Yavaş üreyen *M. tuberculosis* gibi mikobakterilerde bu yöntemle tür saptama 2 ay kadar sürmekte ve birçok kez kesin olmayan sonuçlar vermektedir. Bu süre içerisinde hastalara hatalı tedavi verilebilmektedir. Tür saptanmasını hızlandırabilmek, özgüllüğünü arttırabilmek için son yıllarda çeşitli moleküler yöntemler geliştirilmiştir. Bunlardan ticari kit haline gelen en gelişmişleri dahi sadece 16 kadar mikobakteri türünü saptayabilmektedir.

Bu projede diziye özgül polimeraz zincirleme tepkimesine dayanan, kültürde üretilmiş mikobakterilerin türlerinin tamamını kolayca ayırt etmesi beklenen bir yöntem geliştirdik. Mikobakterilerin 16S *ribozomal RNA*, *hsp65* ve *gyrB* gen dizilerine özgül

primerler ile çoğaltma ürünü elde edilip edilmemesine dayalı bu yöntemde, diğer yöntemlerde olduğu gibi tek türe özgül DNA dizileri yerine, türleri gruplara bölen aşamasal (algoritmik) ayırma sistemi kullandık. Böylece bu yeni yaklaşım ile az sayıda diziye özgü primer ile tüm mikobakteri türlerinin kolayca ve diğer yöntemlere kıyasla daha az harcama ile saptanması olanaklı kılan bir yöntem geliştirdik.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

bp	Base pair
C	Cytosine
C18	Carbon 18
C22	Carbon 22
C26	Carbon 26
C78	Carbon 78
C90	Carbon 90
G	Guanin
G	Centrifugal force
H	Hour
kDa	KiloDalton
mg	Miligram
ml	Mililitre
mm	Milimetre
mM	Milimolar
pH	Power of Hydrogen
pM	Pichomol
V	Volt
µg	Microgram
µl	Microlitre
µm	Micrometre
µM	Micromol
AIDS	Acquired Immune Deficiency Syndrome
DNA	Deoxyribonucleic Acid
dnaJ	Heat shock induced protein from the bacterium E. Coli
dNTP	Deoxyribonucleotide triphosphate definition
DSMZ	German Microbial Culture Collection

EDTA	Ethylenediaminetetraacetic acid
GLC	Gas-liquid chromatography
gyrB	DNA gyrase, subunit B
HIV	Human Immunodeficiency Virus
HPLC	High-performance liquid chromatography
Hsp65	Heat-shock protein 65
INH	Isonicotinic acid hydrazide
ITS	Internal transcribed spacer
LIPA	Line probe assay
L.J	Löwenstein jensen
MAC	Mycobacterium avium complex
MAIC	Mycobacterium avium intracellulare complex
MDR	Multidrug resistant
MOTT	Mycobacteria other than tuberculosis
MSPD	Matrix-solid phase dispersion
NTM	Non-tuberculosis mycobacteria
ORTE	Observable Real Time Electrophoresis
PCR	Polimerase Chain Reaction
PRA	PCR- Restriction Fragment Length Polymorphism Analysis
RD9	Region of Difference 9
rDNA	Recombinant Deoxyribonucleic Acid
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleci Acid
rRNA	Ribosomal Ribonucleci Acid
rpoB	RNA polymerase, subunit B
TB	Tuberculosis
T2H	Thiophene-2-carboxylic acid test hydrazide
TLC	Thin liquid chromatography
UV	Ultraviolet

1. INTRODUCTION

Today, as it has been for centuries, tuberculosis remains the leading cause of death in the world from infectious disease. Approximately a third of the world's population has been infected with *M. tuberculosis* and is at risk for developing disease. Globally, tuberculosis accounts for almost 3 million deaths annually and one-fifth of all deaths of adults in developing countries. Tuberculosis is a reemergent problem in many industrialized countries. In the modern world of global interdependency, rapid transportation, expanding trade, and changing social and cultural patterns, tuberculosis in any country is a threat to people in every country [1].

1.1. MYCOBACTERIA

The identification of mycobacteria began in the late 1800s with the discovery of the tubercile bacillus (originally named *Bacterium tuberculosis)* and the leprosy bacillus (originally named *Bacillus leprae*). The classification of mycobacteria started in 1896 when Lehmann and Neumann suggested the genus *Mycobacterium* to include these species (renamed *M. tuberculosis* and *M. leprae)* and placed this genus in the Mycobacteriaceae family, Actinomycetales order and Actinomycetes class. (*Mycobacterium* is now the only genus in the Mycobacteriaceae family). Classification is based on one or more characters or patterns of characters that all members of the group have and do not have. At the beginning of the twentieth century, the defining characteristics of the genus *Mycobacterium* were morphology (rod-shaped, non-motile) and acid-alcohol fastness (resistance to decolorization by acidified alcohol following staining with a basic fuchsin dye), which led to problems in distinguishing members of the genus *Mycobacterium* from others of the related genera *Nocardia, Rhodococcus* and *Corynebacterium.* Hence, more criteria were developed for differentiating these genera. Some of these differentiation criteria include gram staining, growth rate, penicillin resistance, mol %G + C, mycolic acid profiles and arylsulfatase production. The minimal criteria today for including a species in the genus *Mycobacterium* are acid-alcohol fastness, the presence of mycolic acids containing 60-90 carbons which are

cleaved to C22 to C26 fatty acid methyl esters by pyrolysis, a mol %G + C of 61-71% [2].

Mycobacteria are aerobic, acid fast bacilli (rods). They are stained poorly by the dyes used in gram stain because they are neither gram-positive nor gram negative bacteria. They are virtually the only bacteria that are acid-fast. The term acid-fast refers to an organism's avaliabilty to retain the carbol fuchsin stain despite subsequent treatment with an ethanol-hydrochloric acid mixture. Their cell wall makes them acid-fast because of the high lipid content [3].

1.2. GROUPING OF MYCOBACTERIA

In 1959, Runyon established a grouping for mycobacteria other than *M. tuberculosis* or *M. bovis* that occurred in clinical specimens. The groups were not species; rather, each group consisted of several species. Group I consisted of the photochromogenic mycobacteria, group II consisted of scotochromogenic species, group III contained the nonphotochromogenic and often, at least initially, nonchromogenic, slowly growing mycobacteria, and group IV consisted of the rapidly growing species that required less than 1 week of incubation at 25 or 37 °C. The Runyon grouping was based on pigmentation, colonial morphology, and growth rate. After several years, it became apparent that all species of mycobacteria did not fit within the Runyon grouping and that many newly described species could not be categorized. It is now recommended that all mycobacteria be identified to the species level and that the Runyon grouping scheme not be used by clinical laboratories. As other species of non-*M. tuberculosis* mycobacteria were described, they were placed in a general group of "atypical mycobacteria'', because the species were not typical of *M. tuberculosis*. Terms such as "mycobacteria other than tuberculosis (MOTT)'' or "non-tuberculosis mycobacteria (NTM)'' have been used, but they are also inadequate for describing the species of mycobacteria be reffered to by species name because of the therapeutic implications of infections caused by the various species of clinically important mycobacteria [4].

1.2.1. Slow Growing Pathogenic Mycobacteria

1.2.1.1. Mycobacterium tuberculosis

M. tuberculosis causes tuberculosis. Worldwide, *M. tuberculosis* causes more deaths than any other single microbial agent. Approximately one-third of the world's population is infected with this organism. Each year, it is estimated that 3 million people die of tuberculoisis and that 8 million new cases occur. *M. tuberculosis* grows slowly (it has a doubling time of 18 hours, in contrast to most bacteria, which can double in number in 1 hour or less). *M. tuberculosis* is an obligate aerob; this explains its predilection for causing disease in highly oxygenated tissues such as the upper lobe of the lung and the kidney [3].

Mycobacterial cell walls can induce delayed hypersensitivity and some resistance to infection and can replace whole mycobacterial cells. Mycobacterial cell contents only elicit delayed hypersensitivity reactions in previously sensitized animals. *M. tuberculosis* are rich in lipids. These include mycolic acids (long chain fatty acids C78-C90), waxes, and phosphatides. Lipids are to some extent responsible for acid fastness. Each type of *Mycobacterium* contains several proteins that elicit the tuberculin reaction. Proteins bound to a wax fraction can, upon injection, induce tuberculin sensitivity. They can also elicit the formation of a variety of antibodies. *M. tuberculosis* contains a variety of polysaccharides. Their role in the pathogenesis of disease in uncertain. They can induce the immediate type of hypersensitivity and can serve as antigens in reactions with sera of infected people [5].

Cord factor (trehalose dimycolate) is correlated with virulence of the organism. Virulent strains grow in a characteristic "serpentine'' cordlike pattern, whereas avirulent strains do not. Strains of *M. tuberculosis* is relatively resistant to acids and alkalis. Strains of *M. tuberculosis* resistant to the main antimycobacterial drug, isoniazid (isonicotinic acid hydrazide, INH), as well as strains resistant to multiple antibiotics (called multidrug resistant, or MDR strains), have become a worldwide problem. This resistance is attributed to one or more chromosomal mutations, because no plasmids have been found in this organism [3].

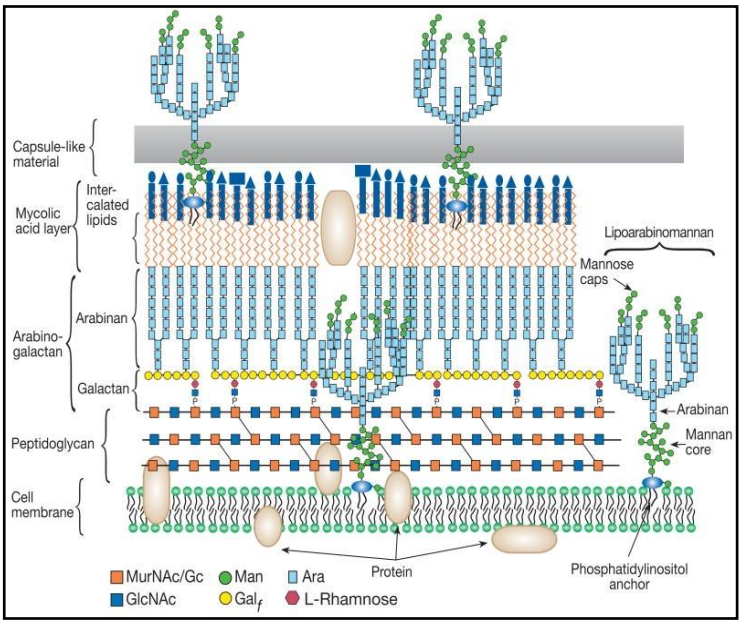

Figure 1.1. Mycobacterium Cell Wall [6]

Patients with active pulmonary tuberculosis shed large number of organisms by coughing, thus creating aerosol droplet nuclei. Because of resistance to dessication, the organisms can remain viable in the environment for a long time. The principal mode of contagion is person to person transmission by inhalation of the aerosol, and repeated or prolonged contact is usually required for transmission of infection. After being inhaled, mycobacteria reach the alveoli, where they multiply in the pulmonary epithelium or in macrophages. Within two to four weeks, many bacilli are destroyed by the immune system, but some survive, and are spread by the blood to extrapulmonary sites. The virulence of *M. tuberculosis* rests with its ability to survive and grow within host cells [7]. There are marked differences in the ability of different mycobacteria to cause lesions in various host species. Humans are highly susceptible to *M. tuberculosis* infection, whereas fowl and cattle are resistant [5].

Lesions are dependent on the presence of the organism and the host response. There are two types of lesions:

1- Exudative lesions, which consist of an acute inflammatory response and occur chiefly in the lungs at the initial site of infection.

2- Granulomatous lesions, which consist of a central area of giant cells containing tubercle bacilli surrounded by a zone of epithelioid cells [3].

In culture, colonies of *M. tuberculosis* are yellowish white and rough on solid media, although on moist media they may tend to be smoother [8].

1.2.1.2. Mycobacterium bovis

M. bovis causes tuberculosis in warm blooded animals but also in primates and humans. Human disease is very similar to that caused by *M. tuberculosis*. The organism grows poorly on Levenstein Jensen medium, but growth is stimulated if glycerol is replaced by pyruvate. *M. bovis* is able to grow in a reduced O_2 atmosphere. Colonies on egg based media are small and rounded, with irregular edges and a granular surface, and on agar media colonies are small and flat [8].

M. bovis is usually transmitted to humans via infected milk, although it can also spread via aerosol droplets [9].

1.2.1.3. Mycobacterium africanum

M. africanum is a cause of human tuberculosis in tropical Africa. Colonies of *M. africanum* resemble those of *M. tuberculosis*, and physiological and biochemical properties position the organism between *M. tuberculosis* and *M. bovis* [8]. *M. africanum* is a species of *Mycobacterium* that is most commonly found in West African countries. The symptoms of infection resemble those of *M. tuberculosis*. *M. africanum* is most commonly found in West African countries, causing up to a quarter of cases of tuberculosis in countries such as the Gambia. It is an infection of humans only and is spread by an airborne route from individuals with open cases of disease. It has a similar degree of infectivity to the regular *M. tuberculosis* organism but is less likely to progress to clinical disease in an immunocompetent individual. *M. africanum* is more likely to progress from infection to causing disease in an HIV positive patient, hence in countries where *M. africanum* is endemic, it represents an important opportunistic infection of the later stages of HIV disease. It is not fully understood how the genetic differences between *M. africanum* and *M. tuberculosis* give rise to the lower pathogenicity of the former. However, it is known that the Region of Difference 9

(RD9) is lacking in *M. africanum* but present in *M. tuberculosis*. *M. africanum* tuberculosis is treated with an identical regime to tuberculosis caused by *M. tuberculosis*. The overall rate of cure is similar, but as more *M. africanum* patients are likely to be HIV positive, they may have higher mortality from other HIV-related disease [4,8]

1.2.1.4. Mycobacterium avium

M. avium have been isolated from water, soil, plants, and other environmental sources. The clinical presentation is similar to that of tuberculosis. Patients usually present with persistent cough only. *M. avium* is well known for their heterogeneous colony morphology. *M. avium* colonies may often occur together with smaller, translucent colonies. A third, less frequent morphology resembles the dry and flat colonies of *M. tuberculosis* [8].

1.2.1.5. Mycobacterium asiaticum

M. asiaticum had previously isolated from monkeys. *M. asiaticum* is photochromogenic, dysgonic and yellow photochromogenic (pigment not produced in the dark) colonies. Slow growth on Löwenstein-Jensen medium at 37 °C after 15-21 days [8]. Biochemically M. asiaticum (photochromogenic) and *M. gordonae* (scotochromogenic) can only by the mode of pigmentation. Rarely causes human pulmonary disease [10].

1.2.1.6. Mycobacterium kansasii

M. kansasii is a photochromogenic species [8]. *M. kansasii* infection of the lung causes a pulmonary disease similar to tuberculosis. *M. kansasii* is not readily isolated from environmental sources. However, it has been isolated from a small percentage of specimens obtained from water supplies in areas with high endemicity. *M. kansasii* is acquired via either aspiration or local inoculation from the environment. Little evidence exists of person to person transmission. *M. kansasii* produces a yellow pigment when exposed to light [11].

1.2.1.7. Mycobacterium celatum

M. celatum is an uncommon cause of human infection, mainly occurring in patients with AIDS. Rarely, infections restricted to the lung and lymph nodes have been reported in immunocompetent hosts. *M. celatum* is indistinguishable from tuberculosis, especially in patients with a previous history of pulmonary tuberculosis [12]. *M. celatum* colonies are predominantly small, dome-shaped and unpigmented. They become pale yellow when cultures are old (8-12 weeks) [13].

1.2.1.8. Mycobacterium genavense

M. genavense causes in particular disseminated infections in patients with AIDS, and skin and genital infections have also been reported in these patients [14]. *M. genavense* has unusual fastidious growth requirements and shows poor and variable growth *in vitro*. Molecular biology techniques are necessary for accurate diagnosis of infection and have established *M. genavense* to be a definite cause of disseminated mycobacterial infection in immunosuppressed patients. *M. genavense* are similar to those of infection caused by *M. avium* complex (MAC) organisms [15].

1.2.1.9. Mycobaacterium haemophilum

M. haemophilum is fastidious, grows slowly, and requires a lower incubation temperature for growth than most other mycobacteria. Isolation of *M. haemophilum* also requires iron-supplemented medium. Because of these requirements, *M. haemophilum* would not be isolated using standart isolation techniques for tubercile bacilli [16]. *M. haemophilum* infections may be underrecognized because of the organism's predilection for a low incubation temperature (30 °C) and its uniqe requirement for ferric ammonium citrate or hemin for growth. If *M. haemophilum* is suspected in a clinical specimen but culture remains negative, the organism may be recovered by using a chocolate agar plate [8].

1.2.1.10. Mycobacterium interjectum

M. interjectum was recovered from children with chronic lymphadenitis and from an AIDS patient with diarrhea. Colonies are smooth and scotochromogenic. *M. interjectum* growth is produced within 21 to 28 days [8].

1.2.1.11. Mycobacterium intermedium

M. intermedium was repeatedly isolated from a patient with chronic pulmonary disease and can be easily confused with *M. asiaticum*, although differences exist in colony morphologies [8].

1.2.1.12. Mycobacterium intracellulare

M. intracellulare is gram-positive, nonmotile and acid-fast short to long rods, which usually make smooth, rarely rough and nonpigmented colonies. Aging colonies may become yellow. Growth on Löwenstein Jensen medium and Middlebrook 7H10 at 37 °C after 7 or more days. *M. intracellulare* and *M. avium* form the *M. avium intracellulare* complex, (MAIC) [17]. Most frequently encountered in pulmonary secretions from patients suffering from tuberculosis like disease and from surgical specimens from such patients. When isolated from human secretions, it is often the etiologic agent of pulmonary disease, although frequently isolated as apparent casual resident. First, it isolated from fatal systemic disease in a child and found in soil and water [18].

1.2.1.13. Mycobacterium leprae

M. leprae causes leprosy [3]. *M. leprae* is transmitted from human to human through prolonged contact, for example, between exudates of a leprosy patient's skin lesions, and the abraded skin of another individual. The infectivity of *M. leprae* is low, and the incubation period protracted, so that clinical disease may develop years or even decades after initial contact with the organism [7]. *M. leprae* has not been grown in the laboratory either on artificial media or in cell culture. It can be grown in the mouse footpad or in the armadillo. Humans are the natural hosts. The optimal temperature for growth (30 °C) is lower than body temperature; it grows pereferentially in the skin and superficial nerves. It grows very slowly with a doubling time of 14 days [3].

1.2.1.14. Mycobacterium malmoense

M. malmoense is gram-positive, nonmotile, acid-fast and coccoid to short rods. It has got smooth and nonpigmented colonies, growth below the surface of semisolid agar medium after deep inoculation (as seen with *M. bovis*), 0.9-1.7 mm in diameter. Growth on inspissated egg medium and oleic acid-albumin agar at a temperature range of 22-37 °C requires over 1 week. Usually infects young children with cervical lymphadenitis or

adults with chronic pulmonary disease, (mostly with previously documented pneumoconiosis). Rarely causes extrapulmonary diseases and disseminated infections [19].

1.2.1.15. Mycobacterium marinum

M. marinum is an atypical mycobacterium species found in cold or warm, fresh or salted water. *M. marinum* infection occurs following skin and soft tissue injuries that are exposed to an aquatic environment or marine animals. The infection usually presents as a localized granuloma but can envolve into an ascending lymphangitis that resembles sporotrichosis or can spread to deeper tissues. *M. marinum* grows best 32 °C; therefore, cooler extremities, particularly hands, are affected more often than central areas [11].

1.2.1.16. Mycobacterium scrofulaceum

M. scrofulaceum is found in water and as a saprophyte in adults with chronic lung disease. *M. scrofulaceum* causes chronic cervical lymphadenitis in children and, rarely, other granulomatous disease. Surgical excision of involved cervical lymph nodes may be curative, and resistance to antituberculosis drugs is common [5].

1.2.1.17. Mycobacterium shimoidei

M. shimoidei has been isolated only from the human respiratory tract and is often associated with tuberculosis like cavitary lesions. *M. shimoidei* causes pulmonary lesions in immunocompetent patients, particularly in those with preexisting lesions caused by tuberculosis, asthma, emphysema, carcinoma, or silicosis [20]. *M. shimoidei* is a thermophilic organism growing well at 45 °C [8].

1.2.1.18. Mycobacterium simiae

M. simiae is a species of nontuberculous *Mycobacterium* species commonly found in nature, but its role as a pathogen has been controversial. The slow-growing, photochromogenic *Mycobacterium* has been isolated from both surface and tap water and has been associated with a nosocomial pseudo-outbreak suspected to have originated from a contaminated hospital water supply. *M. simiae* rarely causes disease in immunocompetent patients; most infections are associated with AIDS patients [21].

1.2.1.19. Mycobacterium szulgai

M. szulgai is scotochromogenic at 37 °C and photochromogenic at 25 °C [8]. *M. szulgai* is an unusual pathogen that accounts for less than 1% of all cases of nontuberculosis mycobacterial infection. Infections with this organism usually involve the lung but may involve soft tissues. Although similar to tuberculosis in its clinical presentation, infection due to *M. szulgai* requires different management, and it is therefore important to distinguish disease caused by *M. szulgai* from that caused by *M. tuberculosis* [22].

1.2.1.20. Mycobacterium ulcerans

M. ulcerans infection is the third most frequent mycobacterial disease in humans after tuberculosis and leprosy. *M. ulcerans* produces a toxin that causes necrosis. Good recovery of *M. ulcerans* is obtained on Löwenstein Jensen medium with glycerol [8]. *M. ulcerans* causes an ulcerative skin disease, known as Bruli ulcer. The mode of transmission of the infection to humans is unknown [23].

1.2.1.21. Mycobacterium xenopi

M. xenopi is a slow growing scotochromogenic species. It was first reported by Schwabacher in 1959, having been isolated in lesions found on a *Xenopus laevis*, but the possibility of human infection was not confirmed until 1965 [24]. It has low pathogenicity in humans and where infections have been found they are closely associated with immunocompromised individuals [25].

1.2.2. Rapid Growing Pathogenic Mycobacterium

1.2.2.1. Mycobacterium abscessus

M. abscessus is a bacterium distantly related to the ones that cause tuberculosis and leprosy. *M. abscessus* is found in water, soil and dust. *M. abscessus* has been rarely known to cause lung infection in persons with various chronic lung diseases. *M. abscessus* can not be transmitted from person to person [26].

1.2.2.2. Mycobacterium chelonae

M. chelonae is a rare pathogen that causes infection among humans. *M. chelonae is* also ubiquitos in nature but have been found with increasing frequency in other

environments that include medical offices and surgical suites. The cultures of skin and soft tissue need to be plated at 28-30 °C, because *M. chelonae* will only grow on low temperatures on primary isolation [27].

1.2.2.3. Mycobacterium fortuitum

M. fortuitum has a worldwide distribution and can be found in natural and processed water, sewage, and dirt. It is uncommon for it to cause lung disease. *M. fortuitum* can cause local cutaneous disease, osteomyelitis (inflammation of the bone), joint infections, and occular disease after trauma. It is a rare cause of lymphadenitis. *M. fortuitum* can be a nosocomial (hospital acquired) disease. Surgical sites may become infected after the wound is exposed directly or indirectly to contaminated tap water. Other possible sources of *M. fortuitum* infection include implanted devices such as catheters, injection site abscesses, and contaminated endoscopes. *M. fortuitum* is gram-positive, nonmotile and acid-fast rods (1-3 μm x 0.2-0.4 μm). Sometimes long rods with occasional beaded or swollen cells having non-acid fast ovoid bodies at one end. Smooth hemispheric colonies, usually off-white or cream colored. May be butyrous, waxy, multilobate and even rosette clustered (dilute inocula). On Malachite green containing media, such as Löwenstein-Jensen media, colonies can absorb the green dye. Rapid growth on Löwenstein-Jensen media within 2–4 days. No growth at 45 °C, but grows on MacConkey agar. Differentiation from *M. fortuitum subsp. acetamidolyticum* by its ability to utilise *L-glutamate* and its inability to utilise acetamide as simultaneous nitrogen and carbon source. Both subspecies share an identical 5'-16S *rDNA* sequence [28]. However, the ITS sequences are different. Different types of sporadic infections: pulmonary disease, local abscesses. Postoperative sternal wound infections, endocarditis, meningitis, and osteomyelitis. Has produced postoperative infections after breast augmentation surgery. Biosafety level 2. It found world-wide in soil, dust, rivers, lakes and tap water. First, it isolated from a 25-year old patient (syringe abscess) in Rio de Janeiro. Also isolated from lymph glands of cattle and systemic or nodular infection of frogs [29].

1.2.2.4. Mycobacterium peregrinum

M. peregrinum is a rapidly growing *Mycobacterium* that is occasionally associated with disease at different locations. *M. peregrinum* is one of the taxa historically

included in the *M. fortuitum* group. A small number of cases of sporadic infection have been reported, including chronic lung disease, sterna wound infections, and cutaneous disease. Very recently several new cases included hard to treat infections, bacteremia, and fatal pneumonia. The treatment of rapidly growing mycobacteria may vary depending on the nature of the disease; single-drug therapy is often sufficient for minor disease, but disseminated cutaneous disease and pulmonary disease usually require multiple antibiotics. Knowledge of the susceptibility patterns of *M. peregrinum* to a large number of antibiotics, alone and in combination may be of great interest in view of the little information available on the treatment of this poorly known mycobacteria [30].

2. METHODS FOR SPECIES IDENTIFICATION OF MYCOBACTERIA

2.1. METHODS BASED ON CULTURE AND BIOCHEMICAL METHODS

Rounyon classification of mycobacteria requires culturing in the dark and exposed to light. The species of mycobacteria is determined according to pigment formation and growth rate. However, this is not sufficient to name all the species. Additional biochemical tests are also necessary to identify the species of mycobacteria. Biochemical methods used in species identification of mycobacteria.

2.1.1. Niacin Test

The niacin test is, perhaps, still the most widely used test for differential identification of mycobacteria. The nicacin test can be performed by two different methods as follows: 1- Modified Runyon Method: 0.25 ml of autoclaved extract is taken in a test tube and an equal amount of 4% aniline in ethanol and 10% aqueous cyanogen bromide is added to it. Positive results are indicated by the appearance of yellow colour and negative test by no colour. 2- Paper Strip Method: The niacin test strips are prepared with potassium thiocyanate, chloramine T, citric acid and sodium aminosalicylate. The test control is prepared with nicotinamide. The “disk”, when used according to the directions, yields a yellow solution, equivalent to approximately 5 mg niacin. The autoclaved extract 0.6 ml from the test strain is put in a special test tube with a stopper: the negative control is 0.6 ml, of distilled water and the positive control is the provided test control. The test strip is dropped in each tube with arrow downwards and stoppered immediately. The tubes are shaken gently but not tilted. After 12-15 minutes, but not later than 30 minutes, the colour of the extract is compared with the controls. A positive test is indicated by the appearance of yellow colour in the extract tube and positive control and no colour in the negative control [31].

2.1.2. MacConkey Agar

The differential inhibition of certain mycobacteria by MacConkey agar has been one method of value in the taxonomic separation of rapidly growing acid-fast bacilli. *M. fortuitum* and the two subspecies of *M. chelonei*, commonly regarded as the potentially pathogenic fast-growing mycobacteria, usually grow on MacConkey agar, whereas the normally saprophytic species are inhibited by this medium. An evaluation of clinical laboratories revealed that those laboratories that utilized MacConkey agar without crystal violet generally observed growth of the species *M. fortuitum*, whereas those laboratories employing the same medium with the dye often failed to grow this organism. MacConkey agar test for differential identification of certain rapidly growing mycobacteria [32].

2.1.3. Catalase Test

Essentially all mycobacteria are catalase positive. The only exceptions are some isoniazid-resistant mutants of *M. tuberculosis* and *M. gastri* and some nonpathogenic, isoniazid-resistant strains of *M. kansasii* [4].

2.1.3.1. Catalase drop method

The catalase drop test is very useful for the quick and easy determination of significant isoniazid resistance of *M. tuberculosis*, which ordinarily reflects prior contact with this drug [4].

2.1.3.2. Catalase after heating to 68 °C

The test for catalase after heating the cells to 68°C is valuable in conjunction with the niacin test for the recognition of tubercle bacilli. A positive test definitely indicates a species other than *M. bovis*, *M. tuberculosis*, *M. gastri*, or *M. haemophilum*, which are always negative [4].

2.1.3.3. Semiquantitative catalase test

The semiquantitative test for catalase has proved valuable in the separation of some species of mycobacteria. Two subgroups of *M. kansasii* have been recognized;

one produces < 45 mm of bubbles, whereas those strains more commonly associated with disease produce > 45 mm of bubbles. *M. tuberculosis, M. bovis*, *M. marinum*, the *M. avium* complex, *M. gastri*, *M. malmoense*, *M. xenopi*, and *M. haemophilum* produce a column of bubbles < 45mm high, but other species usually produce a higher column [4].

2.1.4. Pirazinamidase test

The deamidation of pyrazinamide to pyrazinoic acid in 4 days is useful for the differentiation of *M. marinum* (positive) from *M. kansasii* (negative) and of weakly niacin-positive *M. bovis* (negative) from *M. tuberculosis* (positive) and members of the *M. avium* complex (positive) [4].

2.1.5. Thiophene-2-carboxylic acid test hidrazid

The T2H test is used for distinguishing *M. bovis* from *M. tuberculosis* and other species. Only *M. bovis* is susceptible to low concentration of this compound. Some strains of *M. tuberculosis* are inhibited by 10 μg/ml, and some strains of *M. bovis* may show minimal growth at 1 μg/ml [4].

2.1.6. Arylsulfatase

The 3 day arylsulfatase test is used mainly to differentiate clinically significant rapid growers. With few exceptions, only *M. fortuitum* and *M. chelonae* split phenolphthalein from tripotassium phenolphthalein sulfate within 3 days. The 14 day test may be useful in the identification of slowly growing mycobacteria such as *M. marinum*, *M. szulgai*, *M. xenopi*, *M. triviale*, and *M. flavescens* [4].

2.1.7. Nitrate reduction

The nitrate reduction test is valuable fort he identification of *M. tuberculosis*, *M. kansasii*, *M. szulgai*, certain non-disease-associated strains of nonphotochromogens, and *M. fortuitum*, which are nitrate reductase positive. *M. bovis*, *M. marinum*, *M. simiae*, *M.*

avium complex, *M. xenopi, M. gastri*, *M. malmoense*, and *M. chelonae* are negative or only very weakly positive [4].

2.1.8. Tween 80 hydrolysis

The enzymatic hydrolysis of Tween 80 (a polyethylene derivative of sorbitan monooleate) releases complexed neutral red, resulting in a change in color of the test substrate. The change in color is not due to a pH shift from the formation of oleic acid but to the destruction (hydrolysis) of Tween 80. The test is helpful in the identification of scotochromogenic and nonphotochromogenic mycobacteria. Species of these two groups that hydrolyze Tween 80 readily are seldom of clinical significance. *M. scrofulaceum* strains are negative; *M. gordonae* and *M. malmoense* strains are positive. *M avium* complex, *M. xenopi*, and *M. haemophilum* are negative; other nonphotochromogens are positive [4].

2.1.9. Urease

The determination of the ability of an isolate to hydrolyze urea often helps in the characterization of mycobacterial strains aberrant in some other property. The urease test will help in the recognition of the occasionally encountered pigmented strain of *M. avium* complex [4].

2.1.10. Tellurite reduction

The reduction of colorless potassium tellurite to black metallic tellurium within 3 to 4 days is a distinctive property of *M. avium* complex strains [4].

2.1.11. Sodium chloride tolerance

The slowly growing mycobacteria, only *M. triviale* grows in the presence of 5% NaCl. Of the medically significant, rapidly growing mycobacteria, only *M. chelonae subsp. chelonae* fails to grow in the presence of 5% NaCl [4].

2.2. METHODS BASED ON CHROMATOGRAPHIC IDENTIFICATION OF MYCOLIC ACIDS

2.2.1. High-Performance Liquid Chromatography (HPLC)

The objective of this method is to identify mycobacteria by analysis of mycolic acids, using high performance liquid chromatography (HPLC). A suspension of acid-fast bacteria is saponified to cleave the mycolic acids bound to the cell wall. Mycolic acids are then separated by acidification and extraction into chloroform. After conversion to ultraviolet (UV)-absorbing *p-bromophenacyl* esters the mycolic acids are analyzed on a reverse-phase C18 column using HPLC. A gradient of methanol and dichloromethane (methylene chloride) generated by microprocessor-controlled pumps is used to separate the mycolic acid esters, which are detected with a UV spectrophotometer. Reproducible chromatographic patterns containing combinations of different diagnostic peaks are formed. Pattern recognition is by visual comparison of sample results with mycolic acid patterns from reference species of known mycobacteria. Correct pattern interpretation requires training. Computer-assisted pattern recognition technology and high-sensitivity fluorescence detection are being evaluated [33].

2.2.2. Gas Liquid Chromatography (GLC)

GLC methods have been developed in an attempt to differentiate mycobacteria by identifying their complex mycolic acids. GLC was used to examine mycolic acid methyl ester cleavage products of several *Mycobacterial* species and showed the potential of this system for differentiating mycobacteria; however, because of the limited number of species tested and the complexity of the methyl mycolates, the methodology had limited practical application. In recent years, GLC profiles of mycobacterial fatty acid methyl esters has appeared more useful for speciating mycobacteria. An impressive example of using GLC-mass spectrometry of fatty acid methyl esters for the identification of mycobacteria. Of 81 clinical isolates, 64% were identified to species level by chromatography alone and an additional 35% were differentiated to a group consisting of two or three organisms [34].

2.2.3. Thin Layer Chromatography (TLC) and Matrix Solid-Phase Dispersion (MSPD)

TLC is a relatively inexpensive technique which has been used to differentiate mycobacterial species. Limitations of standard TLC include its inability to clearly distinguish between serovars which have type-specific glycopeptidolipids with identical TLC mobilities because of similar molecular weights or polarities. This has resulted in overlapping and smearing of bands and, therefore, the inability to distinguish between many serovars in the *M. avium* complex and between other related species. In addition, extracting alkali-stable whole lipids from clinical isolates, in order to perform TLC for mycobacterial components, has historically been a tedious, time-consuming process. The great variability between TLC plates from different laboratories has made interpreting Rf values difficult, and the lack of standardization of the technique has further detracted from the procedure's practicality. For these reasons, TLC has rarely been utilized as the sole means for mycobacterial characterization. Matrix solid-phase dispersion (MSPD) has been recently demonstrated to be an effective technique for lysis and partial fractionation of mycobacterial components and a quick, relatively inexpensive method for obtaining samples of various mycobacterial lipids and glycolipids. In this study, the MSPD technique was combined with TLC to establish a procedure for identifying mycobacterial chemotypes that could be performed easily and cost effectively in clinical or research laboratories. The two-dimensional nature of the MSPD-TLC technique provided a means of overcoming the problem of overlapping bands that is associated with TLC alone. In addition, the time-consuming step of lipid extraction that is necessary with TLC of mycobacteria was circumvented with the combined MSPD-TLC technique, and comparison of relative retention (Rx) values provided a means of standardizing the technique between laboratories [35].

2.3. MOLECULAR DETECTION OF MYCOBACTERIA

Methods based on culture and biochemical methods for mycobacterial species identification require long time and extensive effort. To eliminate this problem researchers have been developing molecular methods that give results in a short period of time. In recent years, several successful methods for mycobacterial species identification are applied successfully. However, these methods are still cumbersome and require experienced personnel. Some of these methods are summarized below:

2.3.1. Species Identification by DNA probes

DNA probes (AccuProbe; Gen-Probe Inc.) have been available for some time for identification of clinically important mycobacterial species, including *M. tuberculosis* complex, *M. avium*, *M. intracellulare*, *M. avium* complex, *M. kansasii*, and *M. gordonae*. The tests are based on species-specific DNA probes that hybridize with rRNA released from bacteria. The probes are labeled with acridinium ester, and results are measured with a luminometer. For culture-positive specimens, the turn around time for the method is 2 h. The method is very easy to perform, and no special instrumentation is needed. However, probes are not available for all pathogenic mycobacterial species, and those isolates must be identified by other methods. In addition, the *M. tuberculosis* complex probe cannot differentiate between the members of this complex (*M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum*, and *M. microti*) [36].

The introduction and routine application of nucleic acid probes, such as the AccuProbe *M. tuberculosis* complex assay (TB AccuProbe; Gen-Probe Incorporated, San Diego, Calif.), has considerably shortened the time required for identification of the *M. tuberculosis* complex while providing high sensitivity and specificity. However, cross-reactivity was observed between *M. celatum* type 1, but not type 2, in the TB AccuProbe. DNA sequencing showed that *M. celatum* type 1 differs by a single nucleotide from the probe used in the assay, while type 2 differs by four nucleotides. *M. celatum*, type 3, that also showed cross-reactivity in the TB AccuProbe; however, the DNA sequence for the probe region was not reported. *M. terrae* with the TB

AccuProbe, it was found that changes in temperature influenced the specificity of the test results [37].

2.3.2. Reverse Hybridization Method

DNA strip technology, based on the reverse hybridization of PCR products to their complementary probes, has been applied to simultaneous detection and identification of mycobacteria. Currently, two DNA strip assays, INNOLiPA MYCOBACTERIA (Innogenetics N.V., Ghent, Belgium) (LiPA) and GenoType Mycobacteria (Hain Lifescience GmbH, Nehren, Germany) (GenoType), are commercially available. INNO-LiPA Mycobacteria assay targets the 16S-23S *rRNA* spacer region. Both assays provide probes for the *M. tuberculosis* complex, *M. avium*, *M. intracellulare*, *M. kansasii*, *M. chelonae*, *M. gordonae*, *M. xenopi*, and *M. scrofulaceum*. In addition, the LiPA strip can identify members of the *M. avium* complex and differentiate between the three *M. chelonae* and three *M. kansasii* subgroups. The GenoType strip has additional probes for *M. celatum*, *M. malmoense*, *M. peregrinum*, *M. phlei*, and two subgroups of *M. fortuitum* [38].

2.3.3. Restriction fragment length polymorphism (RFLP)

A restriction enzyme (or restriction endonuclease) is an enzyme that cuts double-stranded or single stranded DNA at specific recognition nucleotide sequences known as restriction sites [39-41].

Such enzymes, found in bacteria and archaea, are thought to have evolved to provide a defense mechanism against invading viruses [42, 43]. Inside a bacterial host, the restriction enzymes selectively cut up foreign DNA in a process called restriction; host DNA is methylated by a modification enzyme (a methylase) to protect it from the restriction enzyme's activity. Collectively, these two processes form the restriction modification system [44]. To cut the DNA, a restriction enzyme makes two incisions, once through each sugar-phosphate backbone (i.e. each strand) of the DNA double helix. Restriction enzymes recognize a specific sequence of nucleotides and produce a double-stranded cut in the DNA [40]. While recognition sequences vary between 4 and

8 nucleotides, many of them are palindromic, which correspond to nitrogenous base sequences that read the same backwards and forwards [45].

PCR-restriction fragment length polymorphism analysis (PRA) of the *hsp65* gene present in all mycobacteria offers an easy, rapid, and inexpensive procedure to identify several mycobacterial species in a single experiment. Other PCR restriction fragment length polymorphism methods based on the analysis of digestion products of specific genes, such as 16S *rRNA* or *dnaJ* genes, using three to five different restriction enzymes have also been reported but remain cumbersome compared to *hsp65* PRA, which uses only two restriction enzymes. *hsp65* PRA can identify almost all species of mycobacteria [46].

2.3.4. DNA Sequencing

PCR-based sequencing has become the gold standard for identification of mycobacterial species. The method consists of PCR amplification of mycobacterial DNA with genus-specific primers and sequencing of the amplicons. The organism is identified by comparison of the nucleotide sequence with reference sequences. As commonly practiced, only one sequencing reaction is needed for a definitive identification. This method also allows for direct detection of mycobacterial species that cannot be grown on conventional laboratory culture media, and several previously unrecognized species have been identified. The target most commonly used is the gene coding for the 16S *rRNA*. This gene is present in all bacterial species and contains both conserved and variable regions, making it an ideal target for taxonomic purposes. The 16S *rRNA* gene has been sequenced from a large number of mycobacterial species, and the identification method based on this gene has been evaluated extensively in diagnostic laboratories. Sequencing of two hypervariable regions of the 16S *rRNA* gene allows for identification of the majority of mycobacterial species. However, members of the *M. tuberculosis* complex cannot be distinguished. Similarly, an important pathogen, *M. kansasii*, has a sequence identical to that of a nonpathogenic species, *M. gastri*, and additional 16S *rRNA* gene regions need to be sequenced to differentiate *M. marinum* from *M. ulcerans.* Several other target genes have been characterized for this purpose. The genes coding for the 32-kDa protein, the 65-kDa heat shock protein, and

the 16S-23S *rRNA* internal transcribed spacer contain enough sequence diversity to distinguish all clinically important mycobacteria except for the members of the *M. tuberculosis* complex. These target genes also allow for differentiation of *M. kansasii* and *M. gastri*. In addition, because of the intraspecies variation observed in the 65-kDa protein gene, this target can also be used for distinguishing clones of certain mycobacterial species [36].

2.3.5. DNA Microarray

The DNA microarray or DNA chip generally comprises a glass surface on which multiple DNA probes with known identities are fixed for molecular hybridization with DNA samples, which allows the examination of parallel gene expression or genotyping. This method allows the simultaneous analysis of thousands of genes in a short assay time and so is useful for phylogenetic analysis and species identification. High-density oligonucleotide arrays (DNA microarrays) offer the possibility of rapid examination of large amounts of DNA sequences with a single hybridization step. This approach has recently been applied to simultaneous species identification and detection of mutations that confer rifampin resistance in mycobacteria. This technique is based on hybridization of fluorescently labeled PCR amplicons generated from bacterial colonies to a DNA array containing nucleotide probes. The bound amplicons emit a fluorescent signal that is detected with a scanner. The probes used in this array are based on 82 unique 16S *rRNA* sequences that allow for discrimination of 54 mycobacterial species and 51 sequences that contain unique *rpoB* gene mutations. The method correctly identified 67 of the 70 isolates representing 27 mycobacterial species. All three *M. szulgai* isolates were identified as *M. malmoense* because of an error in the probe sequence. The turnaround time for this method when performed on culture positive specimens was only 4 h. Molecular methods offer many advantages over conventional methods in the identification of mycobacterial species. The results are obtained rapidly, are reliable and reproducible, and even mixed or contaminated cultures can be analyzed. The probes are already widely used in clinical laboratories for the identification of the most common mycobacterial species. Because automatic DNA sequencers and the programs used for analyzing sequence data have become technically simpler, the PCR-based sequencing method is now being used in many mycobacterial reference

laboratories as the routine method for species assignment. The DNA microarray method holds great promise for the future because it is easy to perform, it can be readily automated, and it allows for identification of a large number of mycobacterial species in one reaction. DNA microarray technology described for mycobacterial species identification can also be used for rapid detection of mutations that are associated with resistance to TB drugs [36].

The main aim of this study, was to develop a PCR method that can identify single nucleotide differences between different species of mycobacteria. *rRNA* and *hsp65* gene sequences of mycobacteria have been targeted that are conserved in some regions and show heterogenity in other parts. However the sequences of these genes are exactly the same in *M. tuberculosis, M. bovis, M. africanum, M. microtii* which are all together called *M. tuberculosis* complex. Since it was not possible to differentiate *M. tuberculosis* complex bacteria from each other we have targeted *gyrB* (DNA gyrase, subunit B) which shows sequence differences within this group.

3. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Chemicals

Table 3.1. Manufacturers of some chemicals

Material Name	**Material Brand**
Agarose	Agarose Basica LE
NuSieve GTG Agarose	NuSieve GTG Agarose FMC Bioproduct, USA
Tris(hydroxymethyl)-aminomethane	MERCK, Germany
Acetic acid	MERCK, Germany
EDTA	MERCK, Germany
Sybr Gold Flourescense Dye	Invitrogen, USA
Sybr Green I Flourescense Dye	Invitrogen, USA
DNA Isolation Kit	Roche, Germany

3.1.2. Instruments

Table 3.2. Some instruments

Material Name	**Material Brand**
Techne TC-4000, Thermal Cycler	Techne, UK
Mycycler	Biorad, USA
Light Cycler 2.0	Roche Diagnostics, Germany
Techne TC-512, Thermal cycler	Techne, UK
Observable Real Time Electrophoresis	TİBO and Salubris Technica, Turkey
Electrophoresis Tank and Power Supply	Obitek EDU101, METU, Turkey
UV Transilluminator	BioRad, USA
Microwave oven	Boch Micro combi, Germany
Computer	Aidata
Pipets	Thermo/Finnpipette
Pipet Tips	Thermo/Finntip and Expell
Biosafety cabinet, Herasafe	Thermo
Biosafety cabinet Holten 2010	Thermo
Biosafety cabinet	Esco (TBC)
Biosafety cabinet	Metisafe

3.1.3. Culture Media

Table 3.3. Culture Media

Culture Media Name	Culture Media Brand
Löwenstein Jensen	Salubris, Turkey
TK Medium	Salubris, Turkey

3.1.4. Mycobacterial Species

Table 3.4. Mycobacterial species

Strain	Line#	CC#	Other CC#
M. kansasii	1	NT 701	ATCC 12478
M. simiae	2	NT 909	ATCC 25275
M. scrofulaceum	3	NT 1001	ATCC 19981
M. gordonae	4	NT 1101	ATCC 14470
M. szulgai	5	NT 1201	ATCC35799
M. gastri	6	NT 1301	ATCC 15754
M. nonchromogenicum	7	NT 1401	ATCC 19530
M. terrae	8	NT 1501	ATCC 15755
M. triviale	9	NT 1601	ATCC 23292
M. xenopi	10	NT 1901	ATCC 19250
M. vaccae	11	NT 2201	TMC 1526
M. diernhoferi	12	DSM43524	ATCC 19340, JCM 6371, 7H10, 37^{o}C
M. smegmatis	13	NT 2433	ATCC 14468
M. parafortuitum	14	DSM43528	ATCC 19686, CCUG 20999, CIP 106802
M. aurum	15	NT 4101	ATCC 23366
M. chitae	16	NT 4301	NCTC 10485
M. duvalii	17	NT 4601	NCTC 358
M. chubuense	18	NT 4701	ATCC 27278
M. aichiense	19	NT 4801	ATCC 27280
M. tokaiense	20	NT 4901	NCTC 10821
M. gadium	21	NT 5001	ATCC 27726

M. rhodesiae	22	NT 5201	NCTC 10779
M. austroafricanum	23	NT 5301	ATCC 33464
M. fallax	24	NT 5501	ATCC 35219
M. gallinarum	25	NT 5601	ATCC 19710
M. porcinum	26	NT 5801	ATCC 33775
M. sphagni	27	DSM44076	ATCC 33027
M. asiaticum	28	NT 6101	ATCC 25276
M. senegalense	29	NT 6801	TMC 806
M. pulveris	30	NT 7001	ATCC 35154
M. fortuitum	31	ATCC 6841	
M. chelonae	32	DSM43804	ATCC 35752, IMET 10609, NCTC 946, TMC 1544, 7H10,28^{o}C
M. bovis BCG	33	ATCC 27289	
M. kansasii	34	IMET 10659	
M. gordonae	35	SN 601	
M. intracellulare	36	ATCC 13950	TMC 1406
M. phlei	37	ATCC 11758	
M. terrae	38	ATCC 15755	
M. tuberculosis	39	14323/RIVM	
M. celatum	40	ATCC 51130	
M. celatum	41	DSM44243	ATCC 51131
M. heidelbergense	42	ATCC 51253	
M. branderi	43	DSM44624	ATCC 51789, CIP 104592, 7H10,37^{o}C
M. triplex	44	DSM 44626	
M. flavescens	45	DSM 43219	
M. peregrinum	46	DSM 43271	ATCC14467, 23023
M. confluentis	47	DSM 44017	ATCC 49920
M. intermedium	48	DSM 44049	ATCC 51848
M. interjectum	49	DSM 44064	ATCC 51457
M. mucogenicum	50	DSM 44124	ATCC
M. conspicuum	51	DSM 44136	
M. shimoidei	52	DSM 44152	ATCC 27962
M. malmoense	53	DSM 44163	ATCC
M. thermoresistibile	54	DSM 44167	ATCC 19527
M. abscessus	55	DSM 44196	ATCC 19977
M. novocastrense	56	DSM 44203	
M. marinum	57	DSM 44344	ATCC 927
M. alvei	58	DSM 44176	ATCC 51304
M. bohemicum	59	DSM 44277	

M. brumae	60	DSM 44177	ATCC 51384, CIP103465
M. farcinogenes	61	DSM 43637	ATCC 35753,CCM 6181,NCTC 10955
M. hassiacum	62	DSM 44199	
M. komossense	63	DSM 44078	ATCC 33013
M. lentiflavum	64	DSM 44418	ATCC 51985
M. moriokaense	65	DSM 44221	ATCC 43059, JCM 6375
M. murale	66	DSM 44340	HAMBI 2320
M. obuense	67	DSM 44075	ATCC 27023, JCM 6372, NCTC 10778
M. septicum	68	DSM 44393	ATCC 700731
M. tusciae	69	DSM 44338	
M. vaccae	70	ATCC 29678	
M. elephantis	71	DSM 44368	
M. hodleri	72	DSM 44183	CIP 104909T
M. heckeshornense	73	DSM 44428	
M. wolinskyi	74	DSM 44493	ATCC 700010, CIP 106348
M. hiberniae	75	DSM 44241	ATCC 49874
M. mageritense	76	DSM 44476	ATCC 700351, CIP 104973
M. cookii	77	DSM 43922	ATCC 49103
M. goodii	78	DSM 44492	ATCC 700504, CDC 860673, CIP 106349
M. tuberculosis$H_{37}R_V$	79		CDC
M. *agri*	80	DSM 44515	ATCC 27406
M. avium subsp silvaticum	81	DSM 44175	ATCC 49884
M. botniense	82	DSM 44537	ATCC 700702
M. *doricum*	83	DSM 44339	CIP106867
M. frederiksbergense	84	DSM 44346	NRRL B-24126
M. gilvum	85	DSM 44503	ATCC 43909, NCTC10742
M. neoaurum	86	DSM 44074	ATCC 25795
M. bönickei	87	DSM 44677	7H10
M. brisbanense	88	DSM 44680	7H10
M. canariasense	89	DSM 44828	7H10
M. cosmeticum	90	DSM 44829	7H10
M. florentinum	91	DSM 44852	LJ, 7H11
M. haemophilum	92	DSM 44634	7H10 w hemin, 30^{o}C
M. houstonense	93	DSM 44676	7H10
M. immunogenum	94	DSM 44764	7H10
M. kubicae	95	DSM 44627	7H10
M. lacus	96	DSM 44577	7H10

M. montefiorense	97	DSM 44602	7H10, 25°C
M. nebraskense	98	DSM 44803	7H10
M. neworleansense	99	DSM 44679	7H10
M. palustre	100	DSM 44572	7H10
M. parascrofulaceum	101	DSM 44648	7H10
M. parmense	102	DSM 44553	7H10
M. psychrotolerans	103	DSM 44697	7H10
M. pyrenivorans	104	DSM 44605	7H10,28°C
M. saskatchewanense	105	DSM 44616	7H10
M. vanbaalenii	106	DSM 07251	BHİA,30°C
M. angelicum	107	DSM45057	7H10,28°C
M. arupense	108	DSM44942	ATCC BAA-1242
M. barombii	109	DSM45059	7H10,28°C
M. chimaera	110	DSM44623	CIP 107892
M. conceptionense	111	DSM45102	CCUG 50187, CIP 108544
M. concordense	112	DSM44678	ATCC BAA-329
M. fürth	113	DSM44567	
M. flouranthenivorans	114	DSM44556	CIP 108203
M. hackensackense	115	DSM44833	ATCC BAA-823
M. holsaticum	116	DSM44478	CCUG 46266
M. kumamotonense	117	DSM45093	CCUG 51961, JCM 13453
M. manitobense	118	DSM44615	
M. massiliense	119	DSM45103	CCUG 48898, CIP 108297
M. monacense	120	DSM44395	CIP 109237
M. neglectum	121	DSM44756	20-31°C
M. paraseoulense	122	DSM45000	
M. petroleophilum	123	DSM44182	ATCC 21497, FERM P-385
M. phocaicum	124	DSM45104	CCUG 50185, CIP 108542
M. ratisbonense	125	DSM44364	
M. salmoniphilum	126	DSM43276	ATCC 13758
M. seoulense	127	DSM44998	CIP 109583, KCTC 19146
M. setense	128	DSM45070	CIP 109395
M.senuense	129	DSM44999	KCTC 19147
M. yunnanensis	130	DSM44838	KCTC 19022, YIM 70063, 7H10,28°C

There are 170 mycobacterial species but in this study the number of mycobacterial species found in our collection and used was 130. Since there is more than one from the same bacterial species in general mycobacterial collection.

3.2. METHODS

Most of the mycobacterial infections in humans is caused by *M. tuberculosis*. In Turkey, isolation rate of *M. tuberculosis* to MOTT ratio is usually over 95%. For this reason, when mycobacteria are isolated in culture, it would be wise to first differentiate if it is *M. tuberculosis* or MOTT with a PCR specific for only *M. tuberculosis.* This would identify most of the the clinical isolates as *M. tuberculosis* and only a few isolates need further evaluation.

Taking all these into account, a three step evaluation system have been designed with three panels of PCR called panel A, B and C.

Panel A was designed to differentiate if the isolate is *M. tuberculosis* or not. Since most of the clinical isolates are *M. tuberculosis* panel A is sufficient for species identification in most of the cases. If panel A determines that the isolate is not *M. tuberculosis* but some other mycobacteria then it belongs to either mycobacteria belonging to *M. tuıberculosis* complex other than *M. tuberculosis* or MOTT. Panel B was which consists of 5 reactions is designed to differentiate the members of *M. tuberculosis* complex group members from each other. Panel C consists of 13 different PCRs. It was designed to identify all species of MOTT. Today there are 170 different mycobacterial species recorded in German Microbial Culture Collection, DSMZ. The number of mycobacterial species found in our collection and used in this study was 130.

“Panel A” which consisted of a reaction, A1, that produces a PCR product if the studied isolate is any species of mycobacteria, a second reaction, A2, which produces a PCR product if the isolate is *M. tuberculosis* and finally a third reaction tube, A3, which contains exactly the same reagents as the first tube but no template DNA is included during the experiment, which is used as negative control to show that the reaction is completed without any contamination. A1 amplified *rRNA* gene common to all

mycobacteria with the same primer sequences. A2 amplified a region of gyrB and the primers only matched *M. tuberculosis* DNA sequences. The results of panel A was evaluated according to Table 3.5.

Table 3.5. The evaluation of panel A according to obtaining PCR products in three reaction tubes

A1	**A2**	**A3**	**Result**
+	+	+	Contamination
+	+	-	*M. tuberculosis*
+	-	-	MOTT or mycobacteria belonging to *M. tuberculosis* complex but not *M. tuberculosis*
-	-	-	PCR did not work or the isolate is not mycobacteria

Panel B consisted of five reactions (B1 to B5) targeting *gyrB* sequences that differentiated species belonging to *M. tuberculosis* complex. The results of panel B was evaluated according to Table 3.6.

Table 3.6. The evaluation of panel B according to obtaining PCR products in five reaction tubes

B1	**B2**	**B3**	**B4**	**B5**	**Result**
+	+	-	-	-	*M. tuberculosis*
+	-	+	-	-	*M. bovis*
+	-	-	+	-	*M. microtii*
+	-	-	-	-	*M. africanum*
-	-	-	-	-	PCR did not work

B1 is positive control for all *M. tuberculosis* complex species. B5 is the negative control. If positive, it will show contamination.

Panel C consisted of 13 reactions (C1 to C13) targeting 5 regions of *rRNA* and 8 regions of *hsp65* sequences, that differentiated MOTT species. The sequences of *rRNA* and *hsp65* is not available for all species of mycobacteria in the gene bank. The expected results of panel C, for mycobacteria with sequences available for *rRNA* and *hsp65*, is shown in Table 3.7.a and Table 3.7.b.

Table 3.7.a. Expected patterns for different species of mycobacteria in panel C

Reactions	**C1**	**C2**	**C3**	**C4**	**C5**	**C6**
Primers	**RR1F RR1R**	**RR2F RR1R**	**RR3F/ RR2R**	**RR4F RR3R**	**RR5F RR4R**	**HSP1F HSP1R**
Expected size of PCR Product / Mycobacteria	**294bp**	**160bp**	**181bp**	**190bp**	**268bp**	**360bp**
M.goodii	+	+	+	+	+	+
M.mageritense	+	+	+	+	+	+
M.smegmatis	+	+	+	+	+	+
M.thermoresistibile	+	+	+	+	+	—
M.wolinskyi	+	+	+	+	—	+
M.chitae	+	+	+	+	—	—
M.hassiacum	+	+	+	+	—	—
M.murale	+	+	+	—	+	+
M.tokaiense	+	+	+	—	+	+
M.neoaurum	+	+	+	—	+	—
M.lacticola	+	+	+	—	+	—
M.rhodesia	+	+	+	—	+	—
M.frederiksbergense	+	+	+	—	+	—
M.immunogenum	+	+	+	—	—	—
M.fallax	+	+	+	—	—	—
M.komossense	+	+	+	—	—	—
M.diernhoferi	+	+	—	—	+	+
M.abscessus	+	+	—	—	—	+
M.aichiense	+	+	—	—	+	+
M.flavescens	+	+	—	—	—	—
M.monacense	+	+	—	—	—	—
M.hodleri	+	+	—	—	—	—
M.chelonae	+	+	—	—	—	—
M.agri	+	—	+	+	+	+

M.doricum	+	—	+	+	—	—
M.alvei	+	—	+	—	+	+
M.mucogenicum	+	—	+	—	+	+
M.septicum	+	—	+	—	+	+
M.porcinum	+	—	+	—	+	+
M.parafortuitum	+	—	+	—	+	—
M.peregrinum	+	—	+	—	—	+
M.senegalense	+	—	+	—	—	—
M.farcinogenes	+	—	+	—	—	—
M.obuense	+	—	+	—	—	—
M.aurum	+	—	—	—	+	—
M.austroafricanum	+	—	—	—	+	—
M.vaccae	+	—	—	—	+	—
M.gilvium	+	—	—	—	+	—
M.asiaticum	+	—	—	—	—	+
M.malmoense	+	—	—	—	—	—
M.novocastrense	+	—	—	—	—	—
M.madagascariense	—	+	+	+	+	—
M.moriokaense	—	+	+	+	+	—
M.brumae	—	+	+	+	—	+
M.botniense	—	+	+	+	—	—
M.xenopi	—	+	+	+	—	—
M.pulveris	—	+	+	+	—	—
M.confluentis	—	+	+	+	—	—
M.terrae	—	+	+	+	—	—
M.phlei	—	+	+	+	—	—
M.petroleophilum	—	+	+	—	+	—
M.sphagni	—	+	+	—	+	—
M.nonchromogenicum	—	+	+	—	—	—
M.hiberniae	—	+	+	—	—	—
M.ulcerans	—	—	—	—	—	+
M.marinum	—	—	—	—	—	+
M.gadium	+	+	+	+	+	—
M.tusciae	+	+	+	+	—	—
M.heidelbergense	—	+	—	+	—	+
M.kubicae	—	+	—	+	—	+
M.chubuense	—	—	—	—	—	—
M.conspicuum	—	—	—	—	—	—
M.chlorophenolicum	—	—	—	—	+	—

M.gordonae	—	—	—	+	—	—
M.paraffinicum	—	—	—	+	—	—
M.microti	—	—	—	+	—	—
M.africanum	—	—	—	+	—	—
M.tuberculosis	—	—	—	+	—	—
M.bovis	—	—	—	+	—	—
M.lepraemurium	—	—	—	+	—	—
M.szulgai	—	—	—	+	—	—
M.intracellulare	—	—	—	+	—	+
M.leprae	—	—	—	+	—	+
M.avium	—	—	—	+	—	+
M.avium subs paratuberculosis	—	—	—	+	—	+
M.kansasii	—	—	—	+	—	+
M.scrofulaceum	—	—	—	+	—	+
M.bohemicum	—	—	—	+	—	+
M.haemophilum	—	—	—	+	—	+
M.gastri	—	—	—	+	—	+
M.cookii	—	—	+	+	—	—
M.branderi	—	—	+	+	—	—
M.heckeshornense	—	—	+	+	—	—
M.celatum	—	—	+	+	—	+
M.shimoidei	—	—	+	+	—	+
M.porifera	—	—	+	+	+	—
M.triviale	—	+	—	—	—	—
M.duvalii	—	+	—	—	—	—
M.genavense	—	+	—	+	—	—
M.triplex	—	+	—	+	—	—
M.interjectum	—	+	—	+	—	—
M.intermedium	—	+	—	+	—	+
M.simiae	—	+	—	+	—	+
M.lentiflavum	—	+	—	+	—	+

Table 3.7.b. Expected patterns for different species of mycobacteria in panel C

Reactions	**C7**	**C8**	**C9**	**C10**	**C11**	**C12**	**C13**
Primers	**HSP2F HSP2R**	**HSP1F HSP3R**	**HSP3F HSP2R**	**HSP4F HSP4R**	**HSP5F HSP6F HSP2R**	**HSP7F HSP5R**	**HSP1F HSP6R**
Expected size of PCR Product / Mycobacteria	**200bp**	**350bp**	**145bp**	**140bp**	**140-150bp**	**170bp**	**290bp**
M.goodii	+	+	+	+	+	+	+
M.mageritense	—	+	+	—	+	—	+
M.smegmatis	—	+	+	+	+	+	—/?NA
M.thermoresistibile	+	—	+	—	+	+	NA
M.wolinskyi	+	+	+	—	+	—	+
M.chitae	+	—	—	—	—	+	NA
M.hassiacum	+	—	+	—	+	+	+
M.murale	—	+	+	—	—	—	—/??+
M.tokaiense	—	+	+	—	—	—	+
M.neoaurum	+	+	—	+	+	—	NA
M.lacticola	+	+	—	+	—	—	+
M.rhodesia	—	—	—	—	+	—	NA
M.frederiksbergense	—	+	—	+	—	—	+
M.immunogenum	—	+	—	+	—	—	+
M.fallax	—	—	+	—	—	—	NA
M.komossense	—	—	—	—	—	—	+
M.diernhoferi	+	+	—	+	—	—	+
M.abscessus	—	—	+	+	+	—	NA
M.aichiense	—	—	—	—	+	—	NA
M.flavescens	+	—	—	—	+	—	+
M.monacense	—	+	+	+	+	—	NA
M.hodleri	—	+	+	+	—	+	+
M.chelonae	—	+	—	+	—	—	NA
M.agri	—	—	+	—	+	+	NA
M.doricum	—	+	+	—	+	—	+
M.alvei	—	+	+	—	+	+	NA
M.mucogenicum	—	+	—	—	—	—	NA
M.septicum	—	—	+	—	+	+	+

M.porcinum	—	—	+	—	+	—	NA
M.parafortuitum	—	—	—	—	—	—	+
M.peregrinum	—	—	+	—	+	+	NA
M.senegalense	—	+	+	—	+	—	—/NA
M.farcinogenes	—	+	+	—	+	—	—??+
M.obuense	—	—	—	—	+	+	NA
M.aurum	—	—	—	—	+?	—	+
M.austroafricanum	—	—	+	—	+	—	NA
M.vaccae	—	—	—	—	—	—	+
M.gilvium	—	—	—	+	—	—	NA
M.asiaticum	—	—	+	+	—	+?	NA
M.malmoense	+	—	+	—	+	+	NA
M.novocastrense	+	—	—	—	+	—	+
M.madagascariense	—	+	—	+	+	—	+
M.moriokaense	—	—	+	—	+	—	+
M.brumae	+	—	—	+	+	—	NA
M.botniense	—	+	—	+	—	—	+
M.xenopi	—	+	+	—	+	+	NA
M.pulveris	—	—	—	+	+	—	NA
M.confluentis	—	—	—	+	+	+	NA
M.terrae	—	—	+	+	—	+	NA
M.phlei	—	—	+	—	+	+	NA
M.petroleophilum	—	—	—	—	+	—	NA
M.sphagni	—	—	+	—	+	+	NA
M.nonchromogenicum	—	—	—	+	—	—	NA
M.hiberniae	—	—	+	+	—	—	+
M.ulcerans	—	—	+	+	+	+	+
M.marinum	—	—	+	+	+	—	+
M.gadium	—	—	—	—	—	+	NA
M.tusciae	—	—	—	+	+	+	NA
M.heidelbergense	+	—	+	+	+	—	+
M.kubicae	+	—	+	—	+	—	+
M.chubuense	—	—	+	—	+	—	+
M.conspicuum	+	—	+	—	+	+	+
M.chlorophenolicum	—	—	+	—	+	—	+
M.gordonae	—	+	+	+	—	+	NA
M.paraffinicum	+	—	+	—	+	+	NA
M.microti	+	+	+	—	+	+	+
M.africanum	+	+	+	—	+	+	+

M.tuberculosis	+	+	+	—	+	+	NA
M.bovis	+	+	+	—	+	+	+
M.lepraemurium	+	—	—	—	+	—	NA
M.szulgai	+	—	—	+	+	+	NA
M.intracellulare	—	—	+	+	+	+	NA
M.leprae	—	+	—	—	—	—	NA
M.avium	+	—	+	+	+	+??	NA
M.avium subs paratuberculosis	+	—	+	+	+	+	+
M.kansasii	+	—	+	+	+	+	NA
M.scrofulaceum	+	—	+	—	+	+??	—?NA
M.bohemicum	+	—	+	—	+	+??	+
M.haemophilum	+	+	—	+	+	—	+
M.gastri	+	+	+	+	+	—	NA
M.cookii	—	—	—	+	+	—	+
M.branderi	—	—	+	+	+	—	NA
M.heckeshornense	—	—	+	—	+	+	+
M.celatum	—	—	+	—	+	—	NA
M.shimoidei	+	+	+	+	+	—	NA
M.porifera	—	—	+	+	+	—	NA
M.triviale	—	—	—	—	—	+	NA
M.duvalii	—	—	+	—	—	—	NA
M.genavense	+	—	—	—	+	—	NA
M.triplex	+	—	—	+	+	+	NA
M.interjectum	+	—	+	—	+	+	NA
M.intermedium	—	—	+	+	+	+	+
M.simiae	—	+	+	—	+	—	+
M.lentiflavum	+	—	—	+	+	+	+

In this study different types of primers were designed and used in Table 3.8 and Table 3.9.

Table 3.8. Panel A *rRNA* and *gyrB* primers

Panel A *rRNA* and *gyrB* Primers	
Primers Name	**Primers Sequence**
RRFC	5' GGCGTGCTTAACACATGCAAGTCG 3'
RR1R	5'CCTACGAGCTCTTTACGCCCAGTA 3'
MtbgyrBF	5’ GCC GAA GTC GGC AAG TGA ACG C 3’
MtbgyrBR1	5’ AGC CTT GTT CAC AAC GAC TTT CGC 3’

Table 3.9. Panel B *gyrB* primers

Panel B *gyrB* Primers	
Primers Name	**Primers Sequence**
MtbgyrBF	5’ GCC GAA GTC GGC AAG TGA ACG C 3’
MtbgryBR1	5’ AGC CTT GTT CAC AAC GAC TTT CGC 3’
NTbgyrBR	5’ AGC CTT GTT CAC AAC GAC TTT CGA 3’
MbovgryBF	5’CCA AGA AGA CGG GGT CAA CGG TA 3’
MmicgyrBF	5’TCG AGA TCA AGC GCG ACG GGT AT’3
MtbgyrBR2	5’TGG ATT CGG CTG CGC GTT CAC TT’3

Some of them designed by adding inosine as shown as below in Table 3.10 and Table 3.11.

Table 3.10. Panel C *rRNA* primers with inosine

Panel C *rRNA* Primers with Inosine	
Primers Name	**Primers Sequence**
RR1FI7U6	5'- CGA CGA CGG GTA GCC GGI III IIAGGG TGA-'3
RR1R7U6	5'- AAA CCA CCT ACG AGC TCI III IIC CCA GTA-'3
RR2FI7U6	5'- GAC GGC CTT CGG GTT GTI III IIC TTT CAG-'3
RR2RI7U6	5'- GGC TAC CCG TCG TCG CCI III IIG GCC ATT-'3
RR4F7U6	5'- GGG TAC TCG AGT GGC GAI III IIG AGT AAC-'3
RR3RI7U6	5'-GGC TAC CCG TCG TCG CCI III IIG GCC ATC-'3
RR5FI7U6	5'- ATA AGC ITG GGA AAC TGI III IIA TAC CGA-'3
RR4RI7U6	5'- GIG GTT TAC AAC CCG AAI III IIC ATC CCT-'3

Table 3.11. Panel C *hsp65* primers with inosine

Panel C *hsp65* Primers with Inosine	
Primers Name	**Primers Sequence**
HSP1FI7U6	5'-GCC CCC ACG ATC ACC AAI III IIT GTG TCC- '3
HSP2FI7U6	5'-TTG GTC AAA GAG GGC CTI III IIC GTC GCG-'3
HSP3F7U6	5'-CGC IGG CAT CGA GAA GG I III IIA GAA GGT- '3
HSP4FI7U6	5'-CAA GGA IGT CGA GAC CAA III III GAT CGC T-'3
HSP5FI7U6	5'-ATC GAI AAG GCC GTC GAG AII III ICC CAG AC-'3
HSP6FI7U6	5'-GAA GCG CGG CAT CGA GAA III III CGA GGC C-'3
HSP7FI7U6	5'-GAI AAG GCI GTC GAG AAG GTI III IIG ACCCTG-'3
HSP1RI7U6	5' -CIA CIT TGT CCA TCG CCT CII III ICA GGT CG-'3
HSP2RI7U6	5' – CGT TGC CIA CCT TGA CCA III III CGG CGA T – 3'
HSP3RI7U6	5' – CTC GGC GAT CAG GTC GCC III III CTG GTC A – 3'
HSP4RI7U6	5' – TGA GCT CIA GCT GCA GGC CII III IGT TGG AC – 3'
HSP5RI7U6	5' – CCI AAG GTG TTG GAC TCC TCI III IIG ATG ACA -3'
HSP6RI7U6	5' - CIG TGG CIG CGA TCT GG(C) T CI III IIT CGA CGT – 3'

3.2.1. Culture and Isolation of DNA

Mycobacterial stocks stored in 85 °C, were thawed and cultures were made using Löwenstein Jensen media. Several colonies from fresh cultures were suspended in 750 µl of TE buffer (10 mM Tris pH 8.0, 1 mM EDTA) in 1.5 ml microcentrifuge tubes. The tubes were centrifuged at 12.000 g for 2 minutes. The supernatant was discarded and the sediment was resuspended in 750 µl of TE buffer. After spinning the tubes the sediment was suspended in 200 µl of TE buffer and the tubes were incubated on boiling water for 20 minutes to lyse the cells and release their DNA. The tubes were spinned again at 12.000 g for 2 minutes and the supernatant which contained mycobacterial DNA was transferred into a clean tube. The DNA samples were kept at -20 °C until used for PCR.

3.2.2. PCR Amplifications

For PCR, generally 25 µl of PCR mixes consisted of 1x PCR buffer (diluted from 10X), dNTP (0.2 mM each), Mg^{++}, primers (20 pMoles each), heat stable DNA polymerase and 3 µl of template DNA, prepared as described above. To obtain specificity to differentiate a single nucleotide difference, which was the main and most critical aim of this study, several different polymerases, Mg^{++} concentrations, primers and primer concentrations were assayed. These were reported in detail in results and discussion sections of this report. Different temperature cycles were tried for PCR amplification but highest specificity was obtained with 2 minutes at 95 °C followed by 45 cycles of 30 seconds at 95 °C and 10 seconds at 64 °C.

3.2.3. Multiplex PCR for Minimizing the Number of PCR Mixes

Panel C consisted of 13 different PCR mixes which produced PCR products of different sizes. It has been realized that these products could be obtained in 4 multiplex PCR and the sizes of the products could be adjusted in a way that when analyzed by electrophoresis, all the PCR products could be separated by electrophoresis and visualized distinctly. It was possible to prepare 4 multiplex reactions which provided a distinct pattern of PCR products for individual species of mycobacteria.

3.2.4. Evaluation of PCR products

PCR products were evaluated by agarose gel electrophoresis in Observable Real Time Electrophoresis (ORTE) which is a newly developed system that integrates electrophoresis and gel documentation. For the analysis, PCR products were labeled by SYBR Gold dye, by adding 1/10 volume of 10x Tibo Gold dye which contains this fluorescent dye. The PCR products were separated using 2% agarose gel. The electrophoresis was run approximately 30 to 45 minutes at 100 V and the bands were continuously monitored and photographed to obtain the optimum separation.

3.2.5. Evaluation of PCR product patterns

Panel A and B produced patterns for each reaction as presence and absence of PCR products. The combination of these results enabled us to determine if the isolate was *M. tuberculosis* or belonged to an other species belonging to *M. tuberculosis* complex group. The panel C patterns were evaluated to identify the species of all mycobacteria.

3.2.6. Drying Primers in Tubes

When applying the tests, it took very long to prepare different combination of primers for panel tubes. In an effort to simplify the application, the primer combinations were dried in tubes. For this purpose primer mixtures prepared in sterile deionized water were put into 0.2 ml PCR tubes and were incubated at room temperature with their lids open in the biosafety cabinet for one day. These ready to use reaction tubes were kept at room temperature until being used for PCR. When needed PCR mixes and DNAs are added into these tubes and then PCR was performed.

4. RESULTS

4.1. IDENTIFICATION OF *M. tuberculosis* FROM OTHER MYCOBACTERIA SPECIES

M. tuberculosis is the causative agent in most of the cases of tuberculosis infection. For this reason, when a species of mycobacteria is isolated from a clinical sample it is primarily important to identify if it is *M. tuberculosis* or not. This is important in guiding the therapy, and eliminating further need for species identification. For this purpose a triplet PCR, which can detect if the isolate is first of all mycobacteria or not, and if it is, it can identify if it is *M. tuberculosis* or not, was prepared. Negative control have been also included in this triplet PCR, to make sure the PCR amplification is completed without any contamination. This panel was named as panel A. Experiments showed that panel A can detect the isolate as mycobacteria and also differentiate if it is *M.tuberculosis* from other species as shown in Figure 4.1.

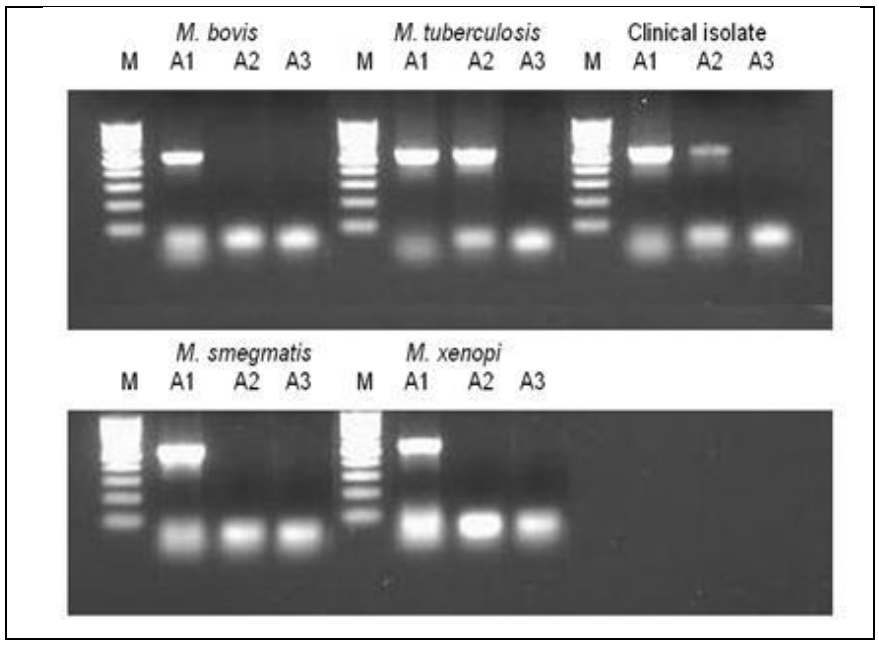

Figure 4.1. The patterns obtained in gel electrophoresis of PCR products obtained by panel A from different species of mycobacteria and a clinical isolate

M is Molecular weight marker, 100bp ladder; *A1* is Amplification with mycobacterial general primers *A2* is Amplification with primers specific to *M. tuberculosis A3* is Negative control, without DNA.

4.2. PROBLEMS OF SPECIFICITY IN PANELS B AND C AND OPTIMIZATION OF PCR CONDITIONS

PCR reactions were designed to differentiate single nucleotide differences. The most important part of PCR primers that determines specificity is 3' end where polymerase binds and starts polymerization. Primers were designed so that their 3' ends matches the nucleotides that show differences from one species to the other. If there is a mismatch in 3' end of the primer the classical knowledge tells that the polymerization can not start and a PCR product is not produced. However, PCR experiments showed that in several reactions included in panels B and C produced PCR products, there were mismatches in 3'end of the primers. To eliminate the non-specific amplifications became the main challenge of this study. Several approaches described below were tried and finally a combination of these enabled us to eliminate non-specific amplification and make reactions specific.

4.3. PRIMER DESIGN AND OPTIMIZATION

Primers were redesigned to eliminate non-specific priming. Inosine nucleotides were exchanged by sequence specific nucleotides at positions close to 3' end of the primers. Inosine is a normal nucleotide but it does not have side groups to make hydrogen bonds. The aim of inosine addition was to weaken the 3' end of primers so that if there is a mismatch at the 3' end this would inhibit the binding and polymerization. To determine the optimum number of inosines and their optimum location in the primer that eliminate non-specific amplification several models of a single primer were designed and tested their efficiencies. The original forward primer in panel C, 3rd reaction, RR3F: 5'- AACGGGTGAGTAACACGTGGGTG 3', was redesigned as follows:

1- Primer with 5 inosines, begining 5 nucleotides from 3' end:

RR3FIN6: 5’- AAC GGG TGA GTA III IIT GGG TG -3’

2- Primer with the same features but with a longer part at 5’ end:

RR3FINU: 5’- A GTG GCG AAC GGG TGA GTA AII III GGG TG -3’

3- Primer with 5 inosines, begining 6 nucleotides from 3’ end:

RR3FIN6U: 5’- A GTG GCG AAC GGG TGA GTA III IIT GGG TG -3’

4- Primer with 6 inosines, begining 6 nucleotides from 3’ end:

RR3FIN6U6: 5’- A GTG GCG AAC GGG TGA GTI III IIT GGG TG -3’

5- Primer with 6 inosines, begining 7 nucleotides from 3’ end:

RR3FIN7U6: 5’- A GTG GCG AAC GGG TGA GII III IGT GGG TG -3’

6- Primer with an additional mismatch at the 3rd nucleotide from 3’ end:

RR3FM3: 5'- AACGGGTGAGTAACACGTGGCTG 3'

7- Primer with an additional mismatch at the 3rd nucleotide from 3’ end and with inosines, begining 6 nucleotides from 3’ end:

RR3FIN6U: 5’- A GTG GCG AAC GGG TGA GTA III IIT GGC TG -3’

4.3.1. Experiment One

Table 4.1. PCR conditions 1

PCR conditions 1	
Number of cycles	40
$MgCl_2$	3.6 μM
Primer Annealing	67 °C

4.3.1.1. Template DNA

Exactly matching with the primers: Mycobacteria number 7 (*M. nonchromogenicum*) and 66 (*M. murale*). Single nucleotide mismatch: Mycobacteria number 19 (*M. aichiense*) and 49 (*M. interjectum*). Double nucleotide mismatch: Mycobacteria number 6 (*M. gastri*) and 59 (*M. bohemicum*).

4.3.1.2. Used Primers

1- RR3F (5' AACGGGTGAGTAACACGTGGGTG 3') - RR2R

2- RR3FIN (5' AAC GGG TGA GTA AII III GGG TG 3') - RR2R

3- RR3FIN6 (5’ AAC GGG TGA GTA III IIT GGG TG 3’) - RR2R

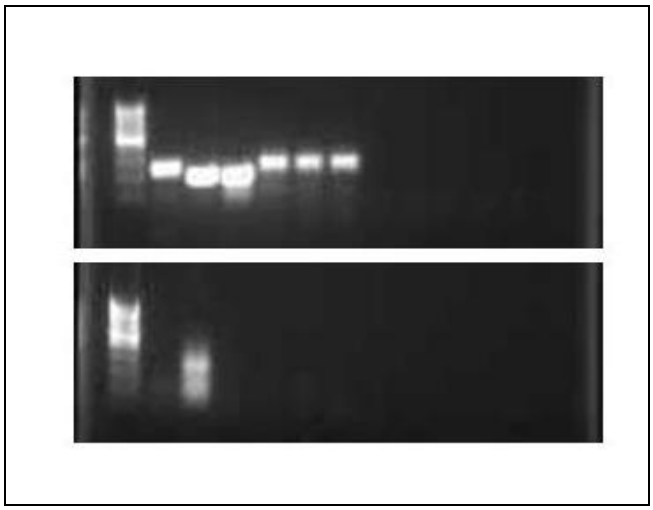

Figure 4.2. Primer Experiment

Samples with order: Molecular Standard, 1-7 (*M. nonchromogenicum*), 1-66 (*M. murale*), 1-19 (*M. aichiense*), 1-49 (*M. interjectum*), 1-6 (*M. gastri*), 1-59 (*M. bohemicum*), 2-7 (*M. nonchromogenicum*), 2-66 (*M. murale*), 2-19 (*M. aichiense*), 2-49 (*M. interjectum*), 2-6 (*M. gastri*), 2-59 (*M. bohemicum*) Molecular Standard, 3-7 (*M. nonchromogenicum*), 3-66 (*M. murale*), 3-19 (*M. aichiense*), 3-49 (*M. interjectum*), 3-6 (*M. gastri*), 3-59 (*M. bohemicum*)

Table 4.2. Patterns of different Mycobacteria with 1., 2., 3., multiplex PCR

Primer Mycobacteria	**17**	**166**	**119**	**149**	**16**	**159**	**27**	**266**	**219**	**249**	**26**	**259**	**37**	**366**	**319**	**349**		**359**
Expected Result	+	+	-	-	-	-	+	+	-	-	-	-	+	+	-	-		-
Result	+	+	+	+	+	+	-	-	-	-	-	-	-	+	-	-		-

When primers without inosine were used, nonspecific amplifications occurred with both single and double mismatch sequences. When binding of 3' end of the primers was weakened by inosine there were no amplification if there was 6 nucleotides after inosines at 3' end. So the specificity was increased but binding was not sufficient even for completely matching sequences. When nucleotide number after inosines was increased to seven there was amplification from one strain with complete match while there were no amplification from single or double mismatches.

4.3.2. Experiment Two

Table 4.3. PCR conditions 2

PCR conditions 2	
Number of cycles	40
$MgCl_2$	3.6 μM
Primer Annealing	67 °C

4.3.2.1. Template DNA

Exactly matching with the primers: Mycobacteria with number 7 (*M. nonchromogenicum*) and 66 (*M. murale*). Single nucleotide mismatch: Mycobacteria with number 19 (*M. aichiense*) and 49 (*M. interjectum*). Double nucleotide mismatch: Mycobacteria with number 6 (*M. gastri*) and 59 (*M. bohemicum*).

4.3.2.2. Used Primers

RR3FIN6 (5'- AAC GGG TGA GTA III IIT GGG TG -3') - RR2R

Table 4.4. Comparison of PCR done by new and old isolated DNA

Mycobacteria	7	66	19	49	6	59
Expected result	+	+	-	-	-	-
Result	-	+	-	-	-	-

There was amplification from DNAs with complete match and no amplification with single or double nuclotide mismatch with inosine primers. There was no amplification from mycobacteria with number 7 (*M. nonchromogenicum*). It was thought that there could be a problem with template DNA (DNA isolation was repeated from fresh culture).

4.3.3. Experiment Three

Table 4.5. PCR conditions 3

PCR conditions 3	
Number of cycles	45
$MgCl_2$	3.6 μM
Primer Annealing	72 °C

4.3.3.1. Template DNA

Exactly matching with the primers: Mycobacteria with number 7N (*M. nonchromogenicum*) (Newly isolated DNA), 7 (*M. nonchromogenicum*) (Old DNA), 26 (*M. porcinum*) and 68 (*M. septicum*). Single nucleotide mismatch: Mycobacteria with number 5 (*M. szulgai*) and 28 (*M. asiaticum*). Double nucleotide mismatch: Mycobacteria with number 1 (*M. kansasii*) and 36 (M. intracellulare).

4.3.3.2. Used Forward Primers

1- RR3FIN6: 5'- AAC GGG TGA GTA III IIT GGG TG -3'

2- RR3FINU: 5'- A GTG GCG AAC GGG TGA GTA AII III GGG TG -3'

3- RR3FIN6U: 5'- A GTG GCG AAC GGG TGA GTA III IIT GGG TG -3'

4- RR3FIN6U6: 5'- A GTG GCG AAC GGG TGA GTI III IIT GGG TG -3'

5- RR3FIN7U6: 5'- A GTG GCG AAC GGG TGA GII III IGT GGG TG -3'

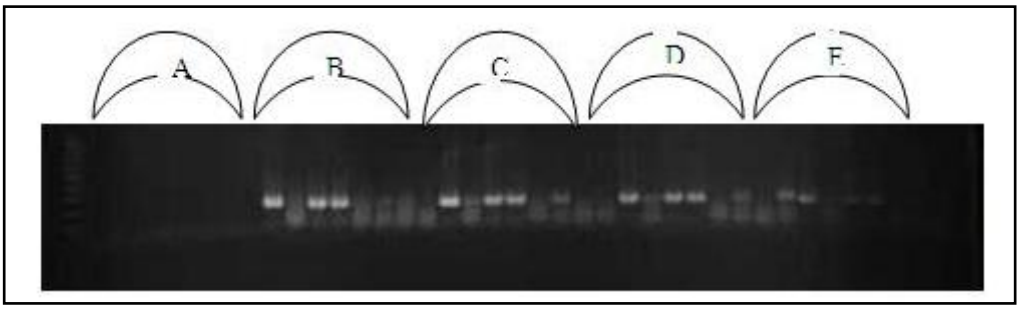

Figure 4.3. Experiment with different primers and mycobacteria

4.3.3.3. Samples with order

A7N (*M. nonchromogenicum*)- A7 (*M. nonchromogenicum*)- A26 (*M. porcinum*)- A68 (*M. septicum*)- A5 (*M. szulgai*)- A28 (*M. asiaticum*)- A1 (*M. kansasii*)- A36 (*M. intracellulare*), B7N (*M. nonchromogenicum*)- B7 (*M. nonchromogenicum*)- B26 (*M. porcinum*)- B68 (*M. septicum*) - B5 (*M. szulgai*)- B28 (*M. asiaticum*)- B1 (*M. kansasii*)-

B36 (*M. intracellulare*), C7N (*M. nonchromogenicum*)- C7 (*M. nonchromogenicum*)- C26 (*M. porcinum*)- C68 (*M. septicum*) - C5 (*M. szulgai*)- C28 (*M. asiaticum*)- C1 (*M. kansasii*)- C36 (*M. intracellulare*), D7N (*M. nonchromogenicum*)- D7 (*M. nonchromogenicum*)- D26 (*M. porcinum*)- D68 (*M. septicum*) - D5 (*M. szulgai*)- D28 (*M. asiaticum*)- D1 (*M. kansasii*)- D36 (*M. intracellulare*), E7N (*M. nonchromogenicum*)- E7 (*M. nonchromogenicum*)- E26 (*M. porcinum*)- E68 (*M. septicum*) - E5 (*M. szulgai*)- E28 (*M. asiaticum*)- E1 (*M. kansasii*)

1- RR3FIN6 (5'- AAC GGG TGA GTA III IIT GGG TG -3') (5'end short primer) RR2R

Table 4.6. Results of PCR done by new designed 5'end short primer

Primer **Mycobacteria**	A 7 Y	A 7	A 26	A 68	A 5	A 28	A 1	A 36
Expected Result	+	+	+	+	-	-	-	-
Result	-	-	-	-	-	-	-	-

It was confirmed that the primer could not bind at 72 °C, because its 5' end was short.

2- RR3FINU (5'- A GTG GCG AAC GGG TGA GTA AII III GGG TG -3') RR2R

Table 4.7. Results of PCR done by new designed 5'end long primer

Primer **Mycobacteria**	B 7N	B 7	B 26	B 68	B 5	B 28	B 1	B 36
Expected Result	+	+	+	+	-	-	-	-
Result	+	-	+	+	-	Faint +	Faint +	-

Extending the 5' end of the primer enabled binding and amplification. DNA prepared from fresh culture of mycobacteria number 7 (*M. nonchromogenicum*) enabled DNA amplification confirming the problem with template DNA in previous experiment.

This primer did not completely eliminate non-specific amplification as seen in mycobacteria with number 28 (*M. asiaticum*) and 1 (*M. kansasii*) which had single and double nucleotide mismatch.

3- RR3FIN6U (5'- A GTG GCG AAC GGG TGA GTA III IIT GGG TG -3') - RR2R

Table 4.8. Results of PCR done by new designed primer as increased the number of inosines from 5 to 6 on 3' end of the primer

Primer **Mycobacteria**	C 7 N	C 7	C 26	C 68	C 5	C 28	C 1	C 36
Expected result	+	+	+	+	-	-	-	-
Result	+	-	+	+	-	Faint +	-	-

Increasing the number of inosines from 5 to 6 on 3' end of the primer increased specificity. Amplification from mycobacteria with number 28 (*M. asiaticum*) which had a sequence with single nucleotide mismatch was not eliminated completely with this primer.

4- RR3FIN6U6 (5'- A GTG GCG AAC GGG TGA GTI III IIT GGG TG -3') - RR2R

Table 4.9. Results of PCR done by new designed primer as increased the number of inosines from 5 to 6 decreased the specificity

Primer **Mycobacteria**	D 7N	D 7	D 26	D 68	D 5	D 28	D 1	D 36
Expected result	+	+	+	+	-	-	-	-
Result	+	Faint +	+	+	-	Faint +	-	+

Increasing the number of inosines from 5 to 6 decreased the specificity. Amplification products are obtained from mycobacteria number 28 (*M. asiaticum*) with single mismatch and number 36 (*M. intracellulare*) with double mismatch.

5- RR3FIN7U6 (5'- A GTG GCG AAC GGG TGA GII III IGT GGG TG -3') - RR2R

Table 4.10. The results of newly designed primer as inosine number kept at 6 but the number of specific nucleotides at 3'end of the primer was increased from 6 to 7

Primer	E	E	E	E	E	E	E	E
Mycobacteria	7N	7	26	68	5	28	1	36
Expected result	+	+	+	+	-	-	-	-
Result	+	-	+	+	-	-	-	-

When inosine number kept at 6 but the number of specific nucleotides at 3'end of the primer was increased from 6 to 7, the specificty was increased and expected results were obtained. Amplification was obtained only with sequences of complete match. Even a single nucleotide mismatch eliminate amplification.

Results of all these experiments, showed that 7 specific nucleotide on 3' end of primer, and 6 inosines adjacent to them, 20-25 nucletides on 5' end adjacent to inosines provides best specificty for priming. Having concluded on primer features the other optimization experiments were continued using this type of primers. Since these new primers did not solve completely non-specific amplification, other parameters for PCR were tested to increase specificity.

4.4. OPTIMIZATION OF MAGNESIUM CONCENTRATION FOR PCR

Magnesium optimization is very important for PCR. Primer binding to template DNA becomes easier with higher Mg^{++} concentration. Mg^{++}, is also a cofactor of DNA polymerase enzyme. Mg^{++} concentration has to be at optimum concentration for the best enzymatic activity. If Mg^{++} concentration is not optimized either PCR amplification will not occur or primer will bind to nonspecific regions leading to non-specific amplification. To prevent nonspecific amplificatons in our experiments magnesium optimizations were studied in Figure 4.4.

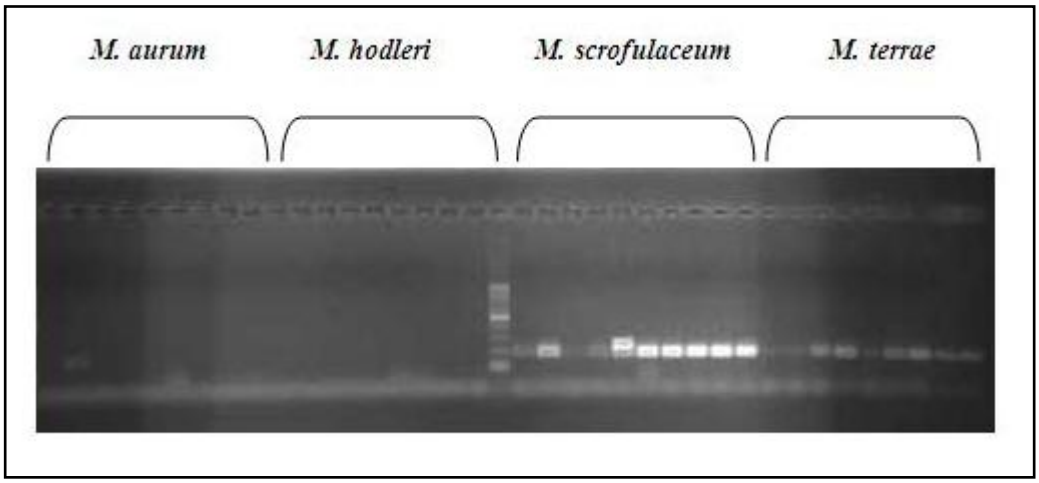

Figure 4.4. Magnesium concentrations of different mycobacterial species

PCR amplification at different magnesium concentrations from DNAs of different mycobacterial species with complete match with primers and mismatches: Mg^{++} concentration from left to right: 2.4, 2.6, 2.8, 3.0, 3.2, 3.4, 3.6, 3.8, 4.0 µM. Amplification was not expected from *M. aurum* and *M. hodleri*; approximately 200 bp amplicon was expected from *M. scrofulaceum* and *M. terrae*. According to these results 3.6 mM Mg^{++} was determined as the optimum concentration.

4.5. REDUCTION OF PRIMER CONCENTRATION

Primer concentration may affect DNA amplification. It may be expected that by decreasing the primer concentration it may be possible to increase the specificity. Different concentration of primers were tested to evaluate the effect of Mg^{++} concentration on amplification by PCR in Figure 4.5.

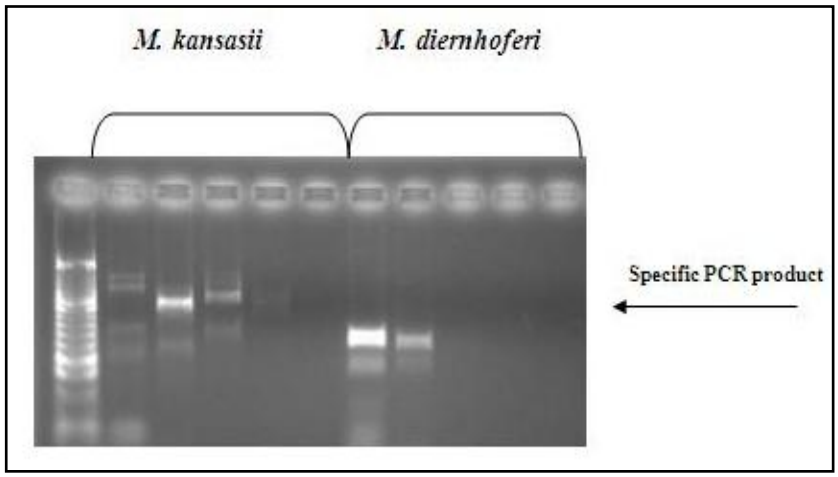

Figure 4.5. Amplified of the same primers at different concentrations

DNAs amplified from *M. kansasii* and *M. diernhoferi* with the same primers at different concentrations: Primer concentration from left to right 20; 10; 5; 2,5 ve 1,25 pM . There were unexpected amplification from *M. kansasii* DNA; there were amplification from *M. diernhoferi* DNA as expected at normal primer concentrations, but when primer concentration is decreased the amplicon quantity also decreased. It was concluded that decreasing primer concentration did not eliminate non-specific products.

4.6. DIFFERENT TAQ POLYMERASES

Taq polymerases are heat resistant enzymes used in PCR. At the beginning of our study, regular Taq DNA polymerase enzyme was used for PCR. However, different kinds of taq polymerases obtained by *in vitro* mutagenesis on their amino acid sequences are available. Some of these bind to template DNA tightly and successful to amplifiy long PCR products, some bind to template DNA loosely and are succesfully to do more specific DNA amplification. These are called high fidelity enzymes. In our study, a high Fidelty Taq polmerase enzyme which is called “Dream Taq” was tested. It was observed that it prevented the nonspecific amplifications in Figure 4.6.

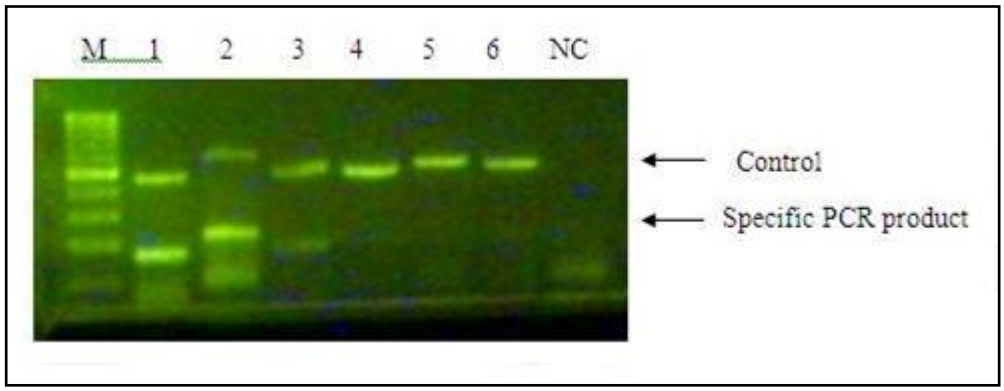

Figure 4.6. PCR products obtained with high fidelity Taq polymerase "Dream Taq"

M is Molecular weight standard (100bp ladder); 1,2 and 3 specific amplification product as expected; 4, 5 and 6 absence of specific PCR products and presence of internal control as expected. *NC* is Negative control.

4.7. TEMPERATURE CYCLES

Classically, PCR cycles consist of three different temperatures: 95 °C to denaturate the template DNA, 48-72 °C for primer annealing and 72 °C for taq polymerase activity. Although Taq polymerase works fastest at 72 °C, high speed may bring alone non-specific amplification. In this study, it was discovered that decreasing the time spent at 72 °C increased the specificity of PCR. The most specific amplifications was obtained when it the 72 °C step was completely eliminated. After this observation all the PCR amplification were did by two step cycles consisting of 30 seconds at 95 °C and 10 seconds at 64 °C.

4.8. ADDITION OfF AMPLIFICATION CONTROL TO REACTION

When there is no specific template primers in reaction tubes, primers tend to bind to the best possible binding sequences although there may not be a complete match and non-specific amplification products are produced. Adding an amplification control to each reaction uses up any dNTPs available in the mixture and Taq polymerase molecules are busy amplifiying the internal control. Since, this may help to eliminate non-specific PCR products, an internal control has been added into our reaction tubes.

If *rRNA* primers were used to identify species, primers specific for *hsp65* gene region were used as amplification control and if *hsp65* primers were used to identify species, primers specific for *rRNA* gene region were used as amplification control in Figure 4.7. Adding internal control helped to eliminate non-specific amplification products.

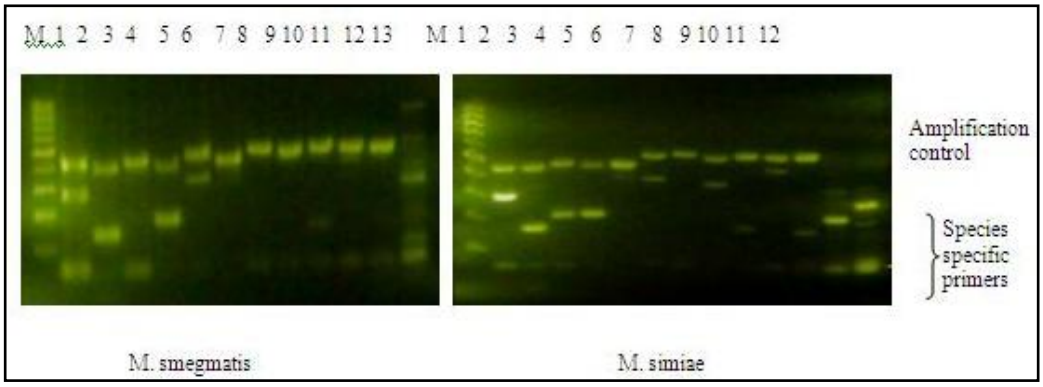

Figure 4.7. PCR products obtained by panel C with internal control from *M. smegmatis* and *M. simiae*

As expected *M. smegmatis* amplified in all reaction except 7th reaction, *M. simiae* only amplified 1, 2, 4, 5, 10 and 12th reactions.

4.9. MULTIPLEX PCR METHOD

Panel C consisted of 13 different PCR mixes which was produced PCR products of different sizes. To minimize the number of reaction tubes 13 pairs of primers were divided into 4 reaction tubes (Table 4.19, Table 4.20, Table 4.21 and Table 4.22). When PCR products were analyzed by electrophoresis, they can be separated by electrophoresis and visualized distinctly. 4 multiplex reactions provided a distinct pattern of PCR products for individual species of mycobacteria in Figure 4.8.

Table 4.11. The multiplex PCR combinations of Panel C's 1.reaction

. Reaction: Primer Combinations and the amount of addittion	
RRFC	0,4 µl
Hsp3FI7U6	0,4 µl
Hsp2RI7U6	0,4 µl
RR4FI7U6	0,4 µl
RR3RI7U6	0,4 µl
RR1FI7U6	0,4 µl
RR1RI7U6	0,4 µl
Hsp1FI7U6	0,4 µl
Hsp4RI7U6	0,4 µl
Total	3,6 µl (The amount distribute each tube)

Table 4.12. The multiplex PCR combinations of Panel C's 2.reaction

2. Reaction: Primer Combinations and The amount of addittion	
RRFC	0,4 µl
RR1RI7U6	0,4 µl
Hsp5FI7U6	0,4 µl
Hsp2RI7U6	0,4 µl
Hsp2RI7U6	0,4 µl
Hsp2FI7U6	0,4 µl
Hsp1FI7U6	0,4 µl
Hsp3RI7U6	0,4 µl
Hsp6FI7U6	0,4 µl
Total	3,6 µl (The amount distribute each tube)

Table 4.13. The multiplex PCR combinations of Panel C's 3.reaction

Reaction: Primer Combinations and The amount of addittion	
RRFC	0,4 µl
RR1RI7U6	0,4 µl
RR3FI7U6	0,4 µl
RR2RI7U6	0,4 µl
RR5FI7U6	0,4 µl
RR4RI7U6	0,4 µl
Hsp1FI7U6	0,4 µl
Hsp1RI7U6	0,4 µl
Total	3,2 µl (The amount distribute each tube)

Table 4.14. The multiplex PCR combinations of Panel C's 1.reaction

Reaction: Primer Combinations and The amount of addittion	
RRFC	0,4 µl
Hsp7FI7U6	0,4 µl
Hsp5RI7U6	0,4 µl
RR2FI7U6	0,4 µl
RR1RI7U6	0,4 µl
Hsp1FI7U6	0,4 µl
Hsp6RI7U6	0,4 µl
Total	2,8 µl (The amount distribute each tube)

In reactions primers with 6 inosine was used.

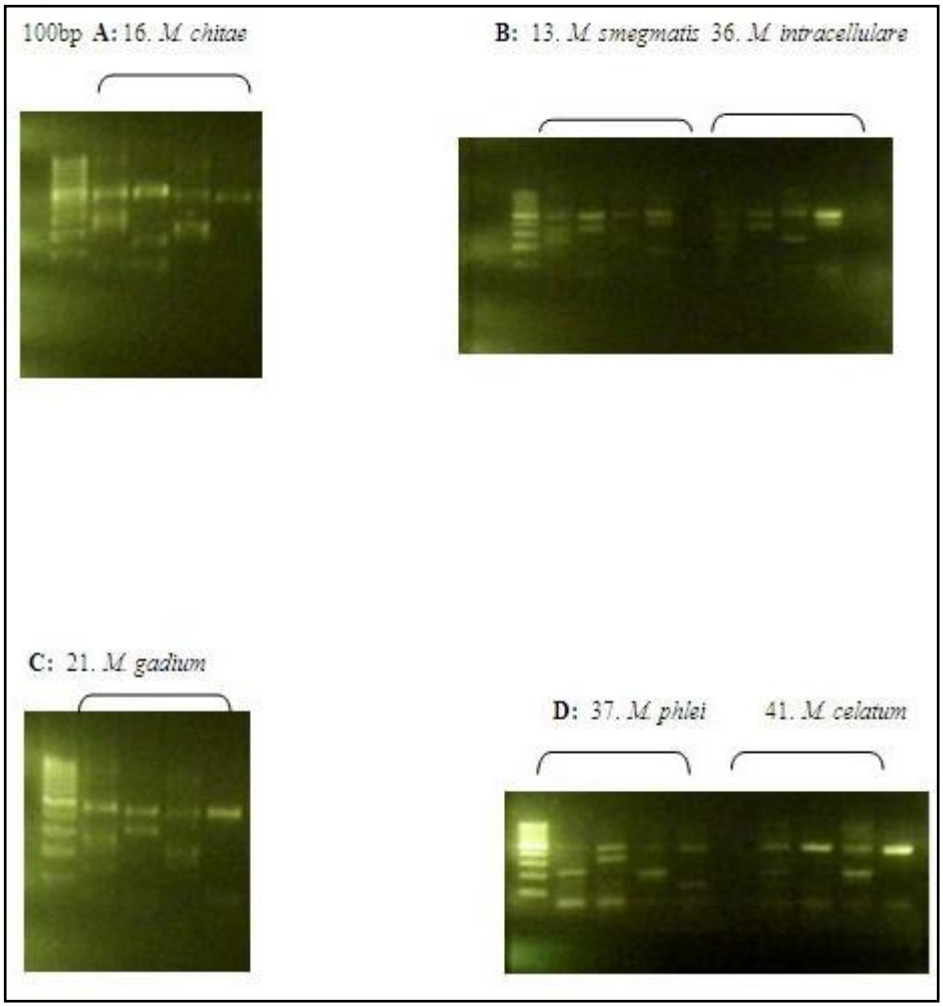

Figure 4.8.Multiplex PCR results of different mycobacteria results

Multiplex PCR results of different mycobacteria results: 100 bp, primer combination 1 is primer combination 2 is primer combination 3 and primer combination 4. *A* is 16. *M. chitae, B* is 13. *M. smegmatis* and 36. *M. intracellulare, C* is 21. *M. gadium, D* is 37. *M. phlei* and 41. *M. celatum*.

As seen on Figure 3.10 with multiplex PCR which concicts of 4 reaction mycobacteria can separate from each other.

4.10. PCR EXPERIMENT WİTH PRIMERS DRIED IN TUBES

In this project to identify the mycobacteria species different kinds of primers and PCR mix were used. The primers used in PCR was dried ın tubes for ready to use because it was thought that the enhanced method could be made a kit to identify mycobacteria species. It was seen that effective results were taken from experiments when only PCR mixture added on dried primers. Primers were dried two years before also worked effectively. That showed the long life usage of dried primers.

5. DISCUSSION

M. tuberculosis is responsible for the majority of mycobacterial infections and it is the leading pathogen microorganism which is responsible for the highest number of patients and death around the world as a single infectious agent. Therefore, early detection of *M. tuberculosis* in clinical samples and application of proper treatment regimen is very important for the patient and also for breaking the chain of infection [3, 5].

When mycobacteria are isolated from clinical samples, it is critical to identify the species. The standard treatment regimen used for infections caused by *M. tuberculosis* is different than infections caused by other mycobacterial species [3, 5].

The number of mycobacterial species detected so far in nature is reaching 170 and increasing with identification of new species every year. At least twenty of these can cause infections in humans. In recent years, increase in the number of immune suppressive patients because of the HIV virus infection and chemotherapy of cancer patients leads to the increase in number infections cuased by MOTT [3, 5, 8].

Classical way to identify the species of mycobacteria, as suggested by Rounyon, depends on growth time on culture, pigment formation of colonies in dark or exposed to light, and different biochemical properties. However, this type of identification requires long time in slow growing mycobacteria and is very are hard to apply [4]. For that reason, phenotypic methods depending on chromatic separation of mycolic acids by HPLC, GLC or TLC have been developed. Although, these methods speed up the species identification they suffer from being cumbersome and inefficiency of differentiating all mycobacterial species from each other [33-35]. In recent years, several molecular methods for species identification of mycobacteria have been developed. All of these methods depend on sequence differences in certain genes like *rRNA* and *hsp65*. The most specific way is to sequence these genes. However, DNA sequencing require expensive equipment. Manual methods like SSCP or PCR restriction enzyme analysis (PRA) are hard to apply and require experienced personnel.

The aim of this project, was to develop a PCR method that can identify single nucleotide differences. By showing the single nuclotide differences it was possible to differentiate mycobacterial species by only PCR and electrophoresis. Using this method, any laboratory that can perform PCR and agarose gel electrophoresis can determine mycobacterial species easily and quickly.

Firstly, standard primers were used for identification of single nucleotide sequence differences. The compatibility of 3' end nucleotides of these primers to nucleotides on binding sites of DNA templates were investigated by obtaining or not a PCR product which depends on specific priming and initiation of DNA polymerization. Although, several PCR conditions were tried, it was not possible to obtain specifity with standard primers. In many cases, a PCR product was obtained even the 3' end of the primer did not match exactly to template DNA. It was possible to obtain specificity by a combination of several changes in the application.

Our first approach was to redesign the primers. It was aimed to release the primers 3’ end when there was a mismatch so that it can not start polymerization. To weaken the three prime end binding, the original nucleotides of the primer close to the 3' end were exchanged, by inosines which do not make hydrogen bonds with the template. Different primers were designed by adding 5 to 8 inosines to 3’ end with a distance from 5 to 8 nucleotides away. The best results were obtained with primers having 7 nucleotides at 3’end and 6 inosines adjacent to these. Having less than 7 specific nucleotide at 3’ end may lead to non-specific binding to several regions in the genome sequences. 7 nucleotide sequence that match the primers’ 3’ end are much less frequent in the genome than 6 nucleotide sequences. When the number of nucleotides are increased to 8, this may be decreasing specificity by binding to tight to the template DNA. When inosines were included in primers. It was also observed that at least another 18 nucleotides adjacent to inosines were required at 5’ end of the primer for proper binding.

Germer et. al, used a method which they called *T*m-shift genotyping, combined allele-specific PCR with the discrimination between amplification products by their melting temperatures (*T*m). Two distinct forward primers, each of which contains a 38-

terminal base that corresponds to one of the two SNP allelic variants, were combined with a common reverse primer in a single-tube reaction. A GC-tail was attached to one of the forward allele-specific primers to increase the *T*m of the amplification product from the corresponding allele. PCR amplification, *T*m analysis, and allele determination of genomic template DNA were carried out on a fluorescence-detecting thermocycler with a dye that fluoresces when bound to dsDNA. A truncated form of *Taq* DNA polymerase, Stoffel DNA polymerase was used here. Stoffel fragment has been shown to enhance discrimination of 38 primer–template mismatches *Taq* polymerase does not discriminate well mismatches of T with G, C, or T [47].

Chun et. al, used a method which they reported as a novel dual priming oligonucleotide (DPO) which contains two separate priming regions joined by a polydeoxyinosine linker. The linker assumes a bubble-like structure which itself is not involved in priming, but rather delineates the boundary between the two parts of the primer. This structure results in two primer segments with distinct annealing properties: a longer 50-segment that initiates stable priming, and a short 30-segment that determines targetspecific extension. This DPO-based system is a fundamental tool for blocking extension of non-specifically primed templates, and thereby generates consistently high PCR specificity even under less than optimal PCR conditions. They evaluated the DPO-based system in a multiplex PCR application for the detection of five different human respiratory viruses. In their study, long conventional primers generated many non-specific bands, most likely due to non-specific annealing or primer competition. In contrast, the DPO primer generated target-specific viral fragments, and no false positives [48].

Jiang et. al, achieved type determination of *Pneumocystis carinii* isolates by type-spesific PCR assays. Type-spesific PCR primers were made so that they differ at their 3’ ends by the two nucleotides which distinguish type A from type B of ITS1 plus an additional ‘’A’’ residue at the extreme 3’ ends of the primers. These two primers were paired seperately with a general primer which anneals to a region downstream from ITS2 to specifically amplify Ax or Bx. The amplified products were then reacted seperately with ITS2 specific probes 2-a, 2-b, and 2-c to identify their types. Also in this study, a negative type-specific PCR generated no products, so no bands were seen when

the reaction mixtures were run on the gel. However, almost all positive type-specific PCRs produced multiple bands in addition to the specific band. Although numerous PCR conditions were tried, a set of conditions which would result in production of only the specific band remained to be discovered. However the presence of multiple bands in positive type-specific PCR reactions did not affect the subsequent typing [49].

Magnesium ion concentration is important for the binding of primer and DNA polymerase activity. Excess or inadequate amount of Mg^{++} can lead to non-specific or lack of amplification. During this study, it was determined that Taq polymerase showed its activity best at 3.6 mM of Mg^{++} and high fidelity Taq polymerase (Dream Taq) showed its best activity at 2.0 mM Mg^{++} concentration. These results exclaim that it is needed to apply different Mg^{++} concentrations for different Taq polymerases and optimize Mg^{++} concentration for all newly designed PCRs.

Another possible way to eliminate non-specific PCR products was to lower the primer concentration. But our experiments show that when primer concentration is lowered, the amount of amplification product decreases accordingly and there is no increase in the specifity of reaction.

Another approach that we tried for increasing specifity was to assay different taq polymerase enzymes which have different features like proof-reading. “Dream taq” which is a high fidelity enzyme is a taq polymerase with proof-reading activity which is not present in standard taq polymerase. Dream taq polymerase increased the specifity of our PCR and prevented in many cases the nonspecific amplification. This enzyme may be an important tool when it is needed to differentiat single nucleotide differences.

Taq polymerases show highest speed in polymerization in 72°C. In this study, decreasing the polymerization temperature to 64°C in PCR cycles became an important application for increasing PCR specificity. Decreasing the polimerization rate by decreasing the heat may be an important factor for preventing the false polymerization by unmatched nucleotides.

If primers can not find an appropriate template, they tend to bind the best possible binding sequences and non-specific amplification products are obtained as long as dNTP's and Taq polymerase are available in the reaction mixtures. Adding an amplification control to each reaction uses up any dNTPs available in the mixture and keep Taq polymerase busy, probably preventing its non-specific binding and polymerization. Adding internal control increased specificity of our PCR especially when a specific product of target sequence was not expected.

The approaches that can be applied for designing PCR methods that can differentiate single nucleotide differences, can be summarized as follows:

1- The nucleotide that is aimed to be differentiated by PCR should be placed at 3' end of the primer.
2- Primers containing inosines should be used. These should have 7 nucleotides matching to template DNA at their 3' end, 6 inosines adjacent to this sequence which control specific binding, and 18 to 25 nucleotides adjacent to inosines at 5' end.
3- The optimum magnesium ion concentration should be determined by experiments.
4- High fidelity taq polymerases shoud be used.
5- Polimerization temperature has to be lower than 72°C, the most effective and proper temperature should be identified by experiment. (64 °C was identified for our reactions as best for amplification and specificity.)
6- Amplification control must always be included in the reaction. This verifies that the amplification was achieved and also increases specificity.

6. CONCLUSION

A PCR method, which can detect mycobacteria species easily and quickly due to the single nucleotide DNA sequence differencess, was developed. The method has important advantages over the classical ones. By the methodology developed during this Project will help to identify mycobacteria species easily and fastly. Also, it can be applied to detect genetic disease due to single nucleotide differences and multiple drug resistance mutation. It is easy and cheaper than the other methods to apply for the DNA sequence analysis, mutation detection, and enzyme analysis. By drying the primers in tubes and long life usage of them is elicited the method to make an available kit.

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