ÇUKUROVA UNIVERSITY
INSTITUTE OF NATURAL AND APPLIED SCIENCES

MSc THESIS

Mahmoud Khaleel Mohammad SHOMAN

COMPARISON BETWEEN MULTIPLEX PCR OF THE REGIONS OF DIFFERENCE (RD) AND SPOLIGOTYPING FOR IDENTIFICATION OF *MYCOBACTERIUM BOVIS* AND *MYCOBACTERIUM BOVIS* BCG STRAINS.

DEPARTMENT OF BIOTECHNOLOGY

ADANA-2019

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We certify that the thesis titled above was reviewed and approved for the award of the degree of the Master of Science by the board of jury on 04/09/2019.

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This study supported by Çukurova University Scientific Research Projects Unit.
Project No: FYL-2017-8056

ABSTRACT

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Supervisor : Prof. Dr. Fatih KÖKSAL
Year: 2019, Pages: 117
Jury : Prof. Dr. Fatih KÖKSAL
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Identification of Mycobacterium isolates is important for appropriate treatment of tuberculosis. BCG is difficult to distinguish from other strains of *M. bovis* and other members of the *M. tuberculosis* complex by conventional methods. The molecular methods that demonstrate the presence of RD regions, which also play a significant role in the evolutionary process, are very important in rapid diagnosis. We aimed to make this distinction by performing multiplex PCR with 7 RD regions taking into account the evolutionary tree of the *Mycobacterium tuberculosis* complex. The strains used for this study were randomly selected among the Mycobacterium tuberculosis complex, which includes *M. tuberculosis*, *M. bovis* and *M. bovis* BCG and other strains; isolated during 2007-2017 in the Regional Tuberculosis Laboratory, Çukurova University. These strains were already identified using the biochemical and growth tests. Genomic DNA was obtained by the Mickle apparatus and all strains tested by RDs-based Multiplex PCR based on the presence or absence of the seven RD regions. Spoligotyping was performed to confirm sublineages. The molecular identification by RDs-based Multiplex PCR has high sensitivity and specificity in differentiation between the MTBC subspecies. We conclude that this molecular method allows accurate and rapid identification of the MTBC members including *M. bovis* and BCG strains at low cost.

Keywords: *M. tuberculosis*, *M. bovis*, BCG, Multiplex PCR, RD regions.

ÖZ

YÜKSEK LİSANS TEZİ

MYCOBACTERIUM BOVIS'IN VE _MYCOBACTERIUM BOVIS_ BCG SUŞLARININ TANIMLANMASI IÇIN RD BÖLGELERININ MULTIPLEX PCR'I VE SPOLIGOTYPING KARŞILAŞTIRMASI.

Mahmoud Khaleel Mohammad SHOMAN

ÇUKUROVA ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ
BİYOTEKNOLOJİ ANABİLİM DALI

Danışman : Prof. Dr. Fatih KÖKSAL
Yıl: 2019, Sayfa: 117
Jüri : Prof. Dr. Fatih KÖKSAL
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: Prof. Dr. Gönül ASLAN

Mycobacterium izolatlarının tanımlanması, tüberkülozun uygun tedavisi için önemlidir. *M. bovis* BCG'nin, *M. bovis*'in diğer suşlarından ve *M. tuberculosis* Kompleksinin diğer üyelerinden geleneksel yöntemlerle ayırt edilmesi zordur. Evrim sürecinde de önemli rol oynayan RD bölgelerinin varlığını gösteren moleküler yöntemler hızlı tanıda çok önemlidir. *Mycobacterium tuberculosis* kompleksinin evrim ağacını dikkate alarak 7 RD bölgesinin multipleks PCR'ı ile *M. bovis* ve *M. bovis* BCG ayrımını amaçladık. Çalışmamızdaki kullanılan *M. tuberculosis*, *M. bovis* ve *M. bovis* BCG ve diğer suşları içeren *Mycobacterium tuberculosis* suşları 2007-2017 yılları arasında Çukurova Üniversitesi, Adana Bölge Tüberküloz Laboratuvarı'nda izole edilen suşların arasında rastgele seçilmiştir. Genomik DNA, Mickle cihazı kullanılarak ekstrakte edildi ve bütün suşlar, yedi RD bölgesinin varlığını veya yokluğunu anlamak amacı ile multiplex PCR yönetmi ile amplifiye edildi. Doğrulama testi için spoligotipleme yapıldı. RDs tabanlı Multiplex PCR ile moleküler tanımlama, MTBC alt tipleri arasındaki farklılaşmada yüksek duyarlılığa ve özgüllüğe sahiptir. Bu moleküler yöntemin, *M. bovis* ve BCG suşları dahil olmak üzere MTBC elemanlarının düşük maliyetle doğru ve hızlı tanımlanmasına olanak sağladığı sonucuna vardık.

Anahtar Kelimeler: *M. tuberculosis*, *M. bovis*, BCG, Multiplex PCR, RD bölgeleri

EXPANDED ABSTRACT

The most widely used vaccine in the world is the attenuated strain of Mycobacterium bovis bacille Calmette – Guérin (BCG). The inoculation of live BCG vaccine is safe in most children, though it often results in benign regional adenitis (Lotte et al. 1984). In rare cases, however, vaccination causes disseminated BCG infection, which may be lethal. Impaired immunity of the host is generally thought to be the pathogenic mechanism. Disseminated BCG infection has been reported in children with inherited immune disorders. It is very important to identify *Mycobacterium bovis* among clinical instances, particularly for the appropriate management of patients or for an epidemiological purpose. *Mycobacterium bovis* is naturally resistant to the anti-TB drug pyrazinamide (PZA), a first-line anti-tuberculosis agent (Scorpio et al. 1997) while most of the other MTBC members are susceptible to this antimicrobial agent. Definitive distinguishing between *M. tuberculosis* and *M. bovis* enables adequate medication and decreases the development of drug-resistant strains.

Attenuated Bacillus Calmette-Guérin (BCG) has been derived from the virulent *M. bovis* and used as a live attenuated vaccine to control the threat of tuberculosis. The particular strain of *M. bovis*; *M. bovis* BCG 1173P2, has been passaged 1173 times at the Pasteur Institute. For over fifty years BCG has been used in TB immunization programmes for immunizing more than three billion people. Although, its protection against TB varies significantly, the average BCG vaccine has decreased the risk of the disease by 50% and the severe types of TB by 70 to 80% according to the latest meta-analysis estimates (Colditz et al. 1994). BCG has been recently proposed and produced as a live recombinant vehicle for new multivalent reliable vaccines against other diseases with effective non-specific immunostimulatory features (Stover et al. 1991, Stover et al. 1993). It is widely given to children as part of the WHO Expanded Programme on Immunization (EPI) in countries where TB is endemic.BCG is comparatively safe, but it can also

cause diseases such as Sepsis, Osteitis, and Meningitis occasionally (Lamm 1992).

Recent comparative genomic analysis has given useful data on the region of difference (RD) in the MTBC chromosome in order to show that the detection of these regions can achieve the specific identification of MTBC (Brosch et al. 2002). RDs-based Multiplex PCR techniques can be easily performed in different clinical laboratories with low cost (Huard et al. 2003). Spoligotyping and RDs Multiplex PCR based on the regions of difference (RDs) are two useful molecular methods for differentiating MTBC members. Spoligotyping (spacer oligonucleotide typing) is a rapid polymerase chain reaction (PCR)-based method for genotyping strains of the MTBC. It is depends on the detection of 43 different spacers between direct repeats locus (DR). The molecular methods that demonstrate the presence or absence of the RD regions, which also play a significant role in the evolutionary process, are very important in the rapid diagnosis (Kamerbeek et al. 1997). Comparative analysis of genomes have detected at least 20 variable regions among the MTBC genomes resulting from insertion-deletion events. For examples, in compression to *M. tuberculosis* H37Rv, there are 14 regions of difference (RD1-RD14), ranging in size from 2 to 12.7 kb, are absent in the genome of *M. bovis* BCG-Pasteur (Ru et al. 2017).

ACKNOWLEDGEMENT

First and foremost, I thank the Almighty ALLAH for preserving me and for the abundant grace He continues to shower on me. I have always been interested in higher education; and combining research, teaching and outreach activities a goal I value greatly.

I would like to thank the Turkish Government and people for their hospitality, financial supports offered during this study program.

I owe an enormous debt of gratitude to my supervisor Prof. Dr. Fatih KÖKSAL for giving me the opportunity to work on this exciting project and for the encouragement along the way. His continuous support throughout the study period and discussions were always rich in advice, suggestions and thought-provoking ideas.

I am sincerely grateful to the staff of the Tropical Diseases and Research Center, particularly to Dr. Begüm KAYAR for her endless time and patience in guiding me through my study, her expertise has been invaluable. Thanks to the staff at Biotechnology Research Center for their assistance and providing the necessary facility for my laboratory work.

I would like also to acknowledge the research council of Çukurova University for their financial supporting of this research project. I would like to thank my family for supporting me spiritually throughout writing this thesis and my life in general.

Last but not least, I thank my colleagues at Biotechnology department for their supporting and the exciting times we had together during the period of my study.

LIST OF CONTENTS **PAGE**

LIST OF TABLES **PAGE**

LIST OF FIGURES **PAGE**

SYMBOLS AND ABBREVIATONS

AFB	: Acid-Fast Bacilli
BCG	:Bacillus Calmette guérin
BTB	:Bovine Tuberculosis
DNA	:Deoxyribonucleic Acid
DR	:Direct Repeat
ECL	:Enhanced Chemiluminescence
EPI	:Expanded Programme On Immunization
GC	:Guanine And Cytosine
HIV	:Human-Immunodeficiency Virus
IFN-γ	:Gamma Interferon
INH	:Isonicotinicacid Hydrazide
IR	:Intergenic Region
I	:Insertion Sequences
LSP	: Large Sequence Polymorphisms
L.J. Medium	:Löwenstein–Jensen Medium
LOSs	:Lipooligosaccharides
LTBI	:Latent Tuberculosis Infection
MAP	:*M. avium* subspecies *paratuberculosis*
MGIT	:Mycobacterial Growth Indicator Tube
MIRU	:Mycobacterial Interspersed Repetitive Units
MOTT	:Mycobacteria Other Than Tuberculosis
MTBC	:Mycobacterium Tuberculosis Complex
NTM	:Non-Tuberculosis Mycobacteria
PCR	:Polymerase Chain Reaction
PGRS	:Polymorphic GC-Rich Repetitive Sequence
PPD	:Purified Protein Derivative
PZA	:Pyrazinamide

RD	:Region Of Difference
RFLP	:Restriction Fragment Length Polymorphism
SPOLIGOTYPING	:Spacer Oligonucleotide Typing
STB	:Smooth Tubercle Bacillus
TB	:Tuberculosis
TbD1	:Tuberculosis-Specific Deletion 1
TBM	:Tuberculous Meningitis
TCH	:Thiophen-2-Carboxylic Acid
TST	:Tuberculin Skin Test
VNTR	:Variable Number Tandem Repeat
WHO	:World Health Organization
ZN STAIN	:Ziehl-Neelsen Stain

1. INTRODUCTION

Tuberculosis (TB) is one of the most common deadliest contagious infectious diseases worldwide with distinctive clinical and pathological features and characterized by progressive development of granulomatous lesions or tubercles in the lung tissue, lymph nodes, or other organs. Tuberculosis occurs in humans and many animal species including cattle.

Tuberculosis (TB) has a long history and is present even before recording history begins. In 1882, Robert Koch discovered that bacteria called *Mycobacterium tuberculosis* is responsible for TB disease. This disease left detrimental impacts on all aspects of life grasping international attention with an increase of new cases every year. It is estimated that one-third of the world's population is infected with *M. tuberculosis* (~2 billion people) and that each year three million people die due to this disease.

WHO reported that over 10 million new cases and nearly 1.6 million deaths recorded every year with an estimated 1 million children became ill with TB and 230 thousand children died of TB (Organization 2018). The geographic incidence of infection and therefore the burden of disease differs widely.

Most of the estimated number of cases in 2017 occurred in South-East Asia Region (44%), African Region (25%) and Western Pacific Region (18%); smaller proportions of cases occurred in the Eastern Mediterranean Region (7.7%), the Region of the Americas (2.8%) and the European Region (2.7%) (Organization 2018). Widely varying incidence rates are demonstrated in Figure 1.1.

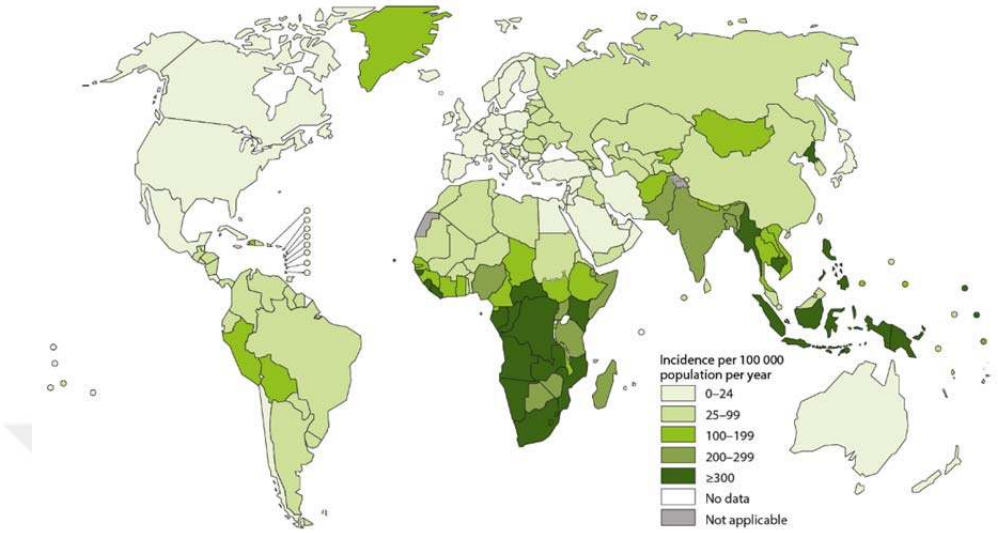

Figure 1.1. Estimated TB incidence rates, 2017 (WHO 2018)

It is thought that two billion individuals carry TB bacilli as a latent tuberculosis infection (LTBI) and that 10% of them will become ill with active TB during their lives. *M. tuberculosis* is the most prolific and poorly understood pathogen of humans. The main human tuberculosis-related microorganism is *M. tuberculosis*.

Pulmonary disease is the classic characteristic, and if untreated, the disease may eventually advance to death the host. However, when the appropriate mixture of medicine is made available and taken by the patient, TB could be cured significantly; and in some particular populations, vaccination can reduce the manifestations of the disease and even prevented by chemotherapy (Murray 2004). The catastrophic impacts of the disease have varied extensively over time and in different areas. However, human-made factors such as urban crowding and poverty have greatly affected its occurrence (Fätkenheuer et al. 1999).

Transmission of TB from person to person happens when an infected person spreads TB bacilli into the air during coughing, talking, sneezing and singing (Beresford and Sadoff 2010). *M. tuberculosis* causes the majority of human

TB and a small proportion of TB disease caused by *M. bovis* and *M. africanum.*

M. bovis is a member of the *Mycobacterium tuberculosis* (MTBC) complex which also comprising *M. caprae*, *M. microti*, *M. africanum*, *M. canetti*, *M. pinnipedii*, and *M. Bovis* Bacillus Calmette Guérin (BCG) (Cousins et al. 2003).

Mycobacterium bovis is the tuberculosis causative agent in livestock and infect both animals and humans. Zoonotic tuberculosis which also called Bovine tuberculosis (bTB) caused by *Mycobacterium bovis* is a chronic infectious disease with major health impacts and major economic burden for the global agricultural industries. Since the 19th century, bTB was recognized as a major zoonosis and a cause of human tuberculosis. bTB is widely distributed throughout the world and affects mammals including humans. It can also impact the national and international economy, affects the ecosystem via transmission to wild-life. It has been estimated that over fifty million cattle are infected with *M. bovis* globally resulting in economic losses of approximately 3 billion dolar per year (Ashford et al. 2001). In addition, it has been estimated that 5–10% of the global TB burden may be due to *M. bovis* (Dankner and Davis 2000). Bovine tuberculosis remains to be a big concern due to the susceptibility of humans to the disease and there is increasing evidence that *M. bovis* infections may be much more significant than generally considered.

Mycobacterium bovis infection in humans can occur through the consumption of contaminated raw or undercooked dairy and/or meat products. Meanwhile, the occupational infection may occur due to exposure through airborne infection among farmers, veterinarians, and slaughterhouse workers. Testing and slaughter of positive livestock is one of the primary methods for controlling bTB, but the case detection rates remain low in the study regions. Therefore, it requires intervention to detect the disease and to identify it with some control measures. The conditions that promote the transmission of tuberculosis between different animal species and between humans remain vague.

Bovine tuberculosis also has zoonotic potential (Grange 2001), so cattle can contribute to the human burden of TB primarily through the consumption of unpasteurized milk and dairy products. Early understanding of cross-species infection or zoonotic nature of these agents starts at the time that humans domesticated and lived closely with animals (Biet et al. 2005). Estimates of the incidence of zoonotic TB are shown in **Table 1.1.**

Table 1.1. Estimated incidence and mortality of tuberculosis caused by *M. bovis*. Best estimates (absolute numbers) are followed by the lower and upper bounds of the 95% uncertainty interval (WHO, 2018)

WHO REGION	INCIDENT CASES		DEATHS	
	Best Estimate	Uncertainty Interval	Best Estimate	Uncertainty Interval
Africa	70 000	18 800–154 000	9 270	2 450–20 500
The Americas	821	222–1 800	45	12–98
Eastern Mediterranean	7 660	1 980–17 100	733	194–1 620
Europe	1 150	308–2 550	87	24–191
South-East Asia	44 900	11 500–100 000	2 090	568–4 590
Western Pacific	18 000	4 740–40 000	309	84–678
GLOBAL	142 000	70 600–239 000	12 500	4 910–23 700

1.1. Study Objectives

The aim of this study is to evaluate the differentiate between infection with *M. tuberculosis* or *M.bovis* and *M. bovis* BCG strains using Multiplex PCR of the 7 Regions of Difference (RD14, RD13, RD12, RD9, RD7, RD4, RD1) and

Spoligotyping method, in order to assist the diagnosis of early infection as well as enhance the use of tuberculosis vaccines on a wider scale.

1.1.1. Specific Objectives:

The corresponding five specific objectives were:

- To review the knowledge about *Mycobacterium bovis* infection as a source of tuberculosis and to identify the needs of the community.
- To make a comparison between different two methods, Multiplex PCR of the Regions of Difference (RDs) and Spoligotyping, for identifying tuberculosis associated with *Mycobacterium bovis* infection.
- To introduce a new kit from different genes of the RD regions to facilitate identification of *Mycobacterium tuberculosis* complex (MTBC) subspecies. (RDs-kit).
- To develop a software tool for rapid identifying *Mycobacterium tuberculosis* complex (MTBC) subspecies and associated lineages. (RDs-tool).
- To study the other different species of *Mycobacterium tuberculosis* complex found in the samples obtained from Çukurova region.

2. LITERATURE REVIEW

2.1. Mycobacteria

Mycobacteria are a type of bacteria and have a waxy thick cell walls. They are classified in the suprageneric class of actinomycetes, which generally have a large DNA content of guanine and cytosine (G-C) and a high content of lipids in the wall, which is probably the highest among of all bacteria (Palomino, Leão and Ritacco 2007). *Mycobacterium* is a genus of Actinobacteria, given its own family, the Mycobacteriaceae.

This genus involves over 120 species and includes mammals pathogens such as (*Mycobacterium tuberculosis*) as well as non-pathogenic species (Tortoli 2003). It is acid fast and cannot be stained by the Gram stain procedure and have a special staining characteristics facilitated by mycolic acid layer in the cell wall. Infections with mycobacteria are extremely difficult to treat and some are becoming worldwide pandemics. The Mycobacteria includes over 80 species in the related complex and they are poorly studied organisms (Rainey et al. 1995).

The genus expresses genetic similarity and phenotypic characteristics, which includes: Nocardia, Corynebacterium and Actinomycetes. Mycobacteria display noticeable tropism in the lungs, probably due to the abundance of oxygen there (Wayne and Kubica 1986, Van Soolingen 2001).

The bacillus of *M. tuberculosis* also has enzymes used in the transfer of aerobic, microaerophilic and anoxic electrons and is able to survive in a number of different environments, such as the oxygen-rich lung, macrophage and caceous granuloma center (Cole et al. 1998). Mycobacteria are classified as acid fast because of their reaction to a type of dye called carbolfuchsin. The dye is taken up by the special cell wall of mycobacteria using heat, a decolorizer is then added to strip the stain from any other bacteria that do not have the special cell wall. If they are acid-fast, the outcome is red stained bacteria (Harada, Gidoh and Tsutsumi 1976). The cell wall of mycobacteria is a distinctive structure consisting mainly of

mycolic acid and peptidoglycan, which gives it a fast characteristic of its acid, enabling it to be visualized microscopically using Ziehl-Nielsen stained sputum smears in which the bacteria are seen as red rods integrated in a blue background of the counter stain (Che-Engku-Chik et al. 2016) .

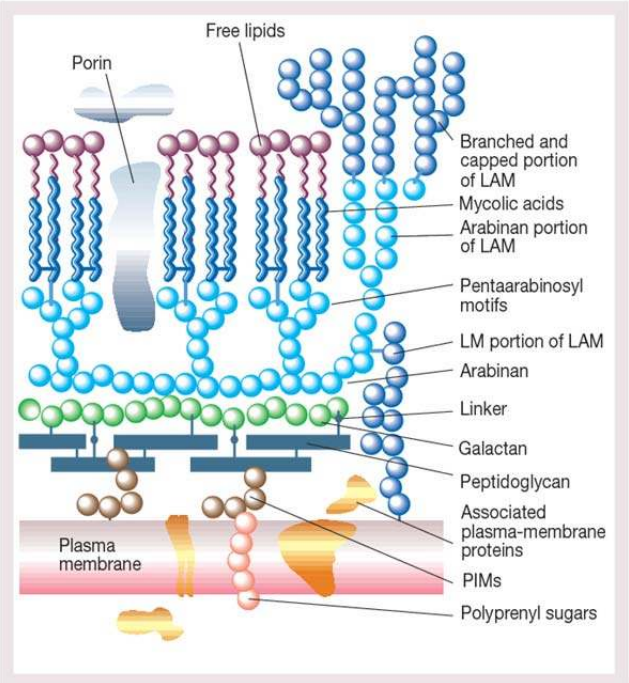

Figure 2.1. Mycobacterial cell envelope (Park and Bendelac 2000)

The majority of mycobacteria present as saprophytes except for a small group which is pathogenic which commonly found in the environment with relatively few causing diseases in animals or immunosuppressed individuals (Pfyffer 2015). Mycobacteria can be divided into three groups: *Mycobacterium tuberculosis* complex (causative pathogen of tuberculosis), Non-tuberculous mycobacteria (NTM) and *Mycobacterium leprae* (causative pathogen of leprosy). *M. avium* subspecies *paratuberculosis* (MAP) is one instance of the NTMs, which is the causative agent of Johne's disease in cattle characterized by a chronic

wasting in cattle (Sweeney et al. 2012). In addition, potentially pathogenic species of the NTMs are include *M. kansasii*, *M. marinum* and *M. avium* complex species (Grange 1996). The species of the *M. tuberculosis* copmplex are require a host to multiply and can be transmitted directly through aerosols, sputum, feces, milk, and body tissues and indirectly through exposure to contaminated feed bunks, water, equipment, and other items shared by cattle (Good and Duignan 2011). *Mycobacterium* genus consists of 71 species that are divided into two main groups on the basis of growth rate; the fast-grow mycobacteria and the slow-grow mycobacteria (Godreuil et al. 2007). The fast-grow mycobacteria produce obvious colonies within seven days under adequate conditions. They include many species such as *M. fortuitum* and *M. abscessus* that have restricted pathogenic impacts and generate atypical clinical symptoms in humans and livestock. The slow-grow species, which include MTBC, *M. avium*, *M. leprae* and *M. kansasii* trigger more pathogenicity in human and livestock and cause chronic diseases (Godreuil et al. 2007).

2.2. Mycobacterium Tuberculosis Complex (MTBC):

Mycobacterium tuberculosis Complex (MTBC) includes genetically closely related organisms which are: *M. tuberculosis*, *M. africunum*, *M. bovis, M. bovis* BCG vaccines strains, *M. caprae*, *M. pinnipedii* and two rarely seen members, *M. cannetii* and *M. microti* (Parsons et al. 2002). The primary causative agents of tuberculosis are mainly: *M. tuberculosis*, cause vast majority of tuberculosis in human cases; *M. africanum*, an agent of human tuberculosis in west Africa; *M. microti*, the agent of tuberculosis in voles and infect humans in rare cases; *M. bovis*, which infects a vast diversity of mammals comprising humans and the attenuated variant of *M. bovis* BCG; and *M. canettii*, variant that produces smooth and glossy colonies that is very rarely encountered but causes disease in human (Sreevatsan et al. 1997). Mycobacteria are widespread in soil and water, but *M. tuberculosis* is mainly identified as a pathogen lives in the host.

2.2.1. Mycobacterium tuberculosis:

Mycobacterium tuberculosis is a species of pathogenic bacteria from Mycobacteriaceae family and it is the causative agent of TB disease (Gordon and Parish 2018). *M. Tuberculosis* has an uncommon waxy layer, mainly due to the existence of mycolic acid on its cell surface. This layer makes the cells impermeable to Gram staining so *M. tuberculosis* can appear as Gram-negative or Gram-positive (Fu and Fu-Liu 2002). Under light microscope, *M. tuberculosis* has appear as a shape from coccobacilli to long rods with a width ranging from 0.2 - 0.6 μm and a length ranging from 1-10 μm (Cardona 2012). *M. tuberculosis* has the capability to synthesize most of the essential nutrients for its survival, including amino acids, vitamins and cofactors of enzymes (Godreuil et al. 2007). *M. tuberculosis* has circular chromosomes with a long of approximately 4,200,000 nucleotides and the G-C content is approximately 65% (Ouellet et al. 2010), a feature more frequently has been associated with aerobic prokaryotes (Naya et al. 2002). It has been reported that *M. tuberculosis*has a large percentage of genes are involved in lipogenesis and lipolysis encoding enzymes.

2.2.2. Mycobacterium africanum:

Mycobacterium africanum comprises of two different phylogenetic lineages within the complex of *M. tuberculosis* and mainly found in the countries of West African, causing up to a third of cases of tuberculosis in countries such as in Gambia (De Jong, Antonio and Gagneux 2010). It was first described as a distinct subspecies within the *M. tuberculosis* complex by Castets and his group in 1968 and it has been found that *M. africanum* is heterogeneous and has characteristics that emerge to lie in between *M. bovis* and *M. tuberculosis* (Castets et al. 1968).

M.africanum comprises of two phylogenetically distinct lineages; *M. africanum* subtype I which are closer to *M. bovis, and M. africanum* subtype II which are closer to *M. tuberculosis* (Niemann et al. 2002). The genetic differences

between *M. africanum* and *M. tuberculosis* is not completely understood give rise to the lower pathogenicity of the MTBC. However, the Region of Difference number 9 (RD9) is known to be absent in *M. africanum* but present in *M. tuberculosis* (Brosch et al. 2002).

2.2.3. Mycobacterium microti:

Mycobacterium microti commonly causes disease voles, wood mice and shrews. In 1946, Wells identified a sort of tuberculosis discovered in more than 20% of 4,309 voles (*Microtus agrestis*) obtained from many locations distributed throughout Great Britain between 1936 and 1942 (Wells and Robb-Smith 1946).

This bacterium has rarely been isolated from other animals, including: cats, pigs, llama, a rock hyrax, and a ferret (Pattyn, Portaels and Spanoghe 1970, Huitema and Jaartsveld 1967, van Soolingen et al. 1998). In addition, both attenuated and non-attenuated vole bacillus vaccines have been shown to be safe and effective vaccination with *M. microti* was found to be no more efficient than the typically used attenuated BCG of *M. bovis*. However, *M. microti* infections were considered as causing pathology in both immunocompetent and immunocompromised humans from The Netherlands (van Soolingen et al. 1998).

More recently, specific deletions of chromosomal DNA have been identified this group by the lacking of chromosomal region $RD1^{mic}$ (Cole et al. 2014) .

2.2.4. Mycobacterium caprae:

Mycobacterium caprae is a pathogen that can infect humans and animals but the incidence of humans is very limited globally. There are some characteristics differentiate *M. caprae* from its epidemiological twin, *M.bovis*; *M. caprae* is evolutionarily older, accounts for a smaller burden of zoonotic tuberculosis and is not distributed worldwide and it has been identified mainly in Central Europe, where it has occasionally been isolated from tuberculous lesions from cattle, pigs,

red deer (*Cervuselaphus*), and wild boars (*Susscrofa*) (Pavlik et al. 2002, Duarte et al. 2008, Prodinger et al. 2002, Erler et al. 2004). *M. caprae* occurs only in a small proportion of human tuberculosis instances and this small proportion may even reduce, if progress continues to eradicate livestock tuberculosis (Prodinger et al. 2002). Strains of this member of the MTBC indicated a special combination of pncA, oxyR, katG and gyrA gene polymorphisms. Although, sequence analysis of the gyrB gene for these strains revealed particular nucleotide substitutions not found in the other MTBC members which could be used to distinguish *M.caprae* from *M. bovis* and other members of the MTBC (Aranaz et al. 2003).

2.2.5. Mycobacterium pinnippedii:

Mycobacterium pinnipedii is a slowly growing member of the MTBC. This species is named after the Pinnipeds; the organisms from which *M. pinnipedii* was first isolated (Cousins et al. 2003). *M.pinnipedii* primarily infects pinniped species such as seals and sea lions (*Otariaflavescens*). Howevere, transmission to cattle and humans has been recorded. The prospective hosts of *M. pinnipedii* tends to be wide and multiple non-marine mammal species infections including humans also have been recorded (Thompson et al. 1993, Jurczynski et al. 2011, Loeffler et al. 2014). A genetic study has reported that Peruvian human skeleton dating back to 1000 CE had been infected with a type of TB most closely related to *M. pinnipedii*, indicating that seals had served as a transmitting vector of TB from the ancient time to the current (Bos et al. 2014) .

The first study about tuberculosis in seals assumed that the disease caused by *M. bovis*; the prevalent cause of tuberculosis in cattle (Cousins et al. 1990). Lately, this strain has been proved and distinguished from *M. bovis* and was identified as *M. pinnipedii* based on spoligotyping and other molecular techniques (Cousins et al. 2003).

2.2.6. "Mycobacterium canetti":

"*Mycobacterium canetti*", a novel pathogenic taxon of the *M. tuberculosis* complex and was first isolated in 1969 by Georges Canetti at the Institute Pasteur, Paris, France. "*Mycobacterium canetti*" is in quotation marks as it is not yet listed within the approval list of Bacterial Names (http://www.bacterio.cict.fr).

"*M. canettii*" formed smooth and glossy colony morphotype, which is highly exceptional for the *M. tuberculosis* complex. These smooth "*M. canettii*" strains differentiate from the classical rough *M. tuberculosis* strains by the existence of high amounts of a characteristic lipooligosaccharides (LOSs) (Daffe, McNeil and Brennan 1991). "*M. canettii*" associated tuberculosis seems to be an emerging disease in the Horn of Africa (Fabre et al. 2010, Koeck et al. 2011).

The prevalence of "*M. canettii*" taxon isolates may be underestimated in the normal microbiology laboratory because of their close similarity to *M. tuberculosis*, especially as the smooth colony type easily returns to give a stable rough colony morphotype in "*M. canettii*". The taxon of "*M. canettii*" may be further distinguished by a particular spoligotyping pattern, as recently shown by the publication of 26 new "*M. canettii*"-specific spacers (Van Embden et al. 2000).

2.2.7. Mycobacterium bovis:

Mycobacterium bovis, is a member of the *Mycobacterium tuberculosis* complex (MTBC) and it is a slow-growing acid-fast bacillus. *Mycobacterium bovis* was first observed in 1898 by isolation of tubercle bacilli from bovine sputum, which was inoculated into rabbits. Therefore, depending on the pathological results, it was found to be more virulent than human tubercle bacilli in rabbits (Smith 1898). *M. bovis* is the cause of tuberculosis in food-producing livestock and accounts for a comparatively tiny percentage of human instances. Infection with these bacteria is chronic and the infected human host may remain completely asymptomatic or may have mild to moderate illness that does not come to medical attention for long periods. The infection can eventually advance to serious systemic

disease in a percentage of human or animal hosts infected with these microorganisms.

Robert Koch was the first who observed and cultivated the tubercle bacillus in 1882 (Daniel 2006). Koch thought at first that there was only one type of tubercle bacillus that influenced both humans and animals, small but distinct differences between human and bovine strains were then discovered. Bovine strains were shorter bacilli and human strains tending to be longer.

2.2.8. Mycobacterium bovis BCG:

BCG (BacilleCalmette-Guérin) is derived fromthe closely related virulent tubercle bacillus, *M. bovis*, by prolonged serial passage that culminated in its attenuation and is currently the available vaccine against childhood and disseminated tuberculosis. BCG vaccine is injected intradermally into the upper arm (deltoid muscle) as a single injection, and the injection is preceded by a skin test of tuberculin, conducted by a qualified nurse and without a false positive result. BCG vaccine is routinely given to infants in settings in areas where tuberculosis is endemic. However, its effectiveness in the protection of adult and aging populations against pulmonary tuberculosis is extremely variable. More than 3 billion people have been immunized with BCG without significant side effects (Bloom and Murray 1992). BCG stimulates the immune system against *M. tuberculosis* (MTB) and tuberculosis infection to produce antibodies. The original BCG Pasteur strain has been created through 230 serial passages in liquid culture and was never shown to revert to virulence in animals, indicating that BCG's attenuating mutation(s) are stable deletions and/or multiple mutations that do not revert easily. BCG also has some efficacy against infection with Buruli ulcer and other non-tuberculous mycobacterial diseases (mondiale de la Santé and Organization 2018). Furthermore, it is sometimes used in bladder cancer therapy (Fuge et al. 2015).

2.3. Evolutionary History and Clonal Development of *M. tuberculosis* Complex (MTBC):

Phylogenetic analyzes have shown that *Mycobacterium tuberculosis* complex is the clonal progeny of an ancient strain of *M.canettii*. The MTBC was assumed to emerge as a clonal development from a progenitor group of a smooth tubercle bacillus (STB) (Gutierrez et al. 2005, Galagan 2014). The animal-adapted ecotype branch of *M. bovis* from a suspected human-adapted ancestry of *M. africanum* currently restricted to West Africa region. The Human-adapted *M. tuberculosis* strains are divided into seven main ancestries, each is mainly related to a different geographical distribution. The dates of branching events are gross estimates only (Galagan 2014) **Figure 2.2.** TbD1 indicates the deletion events specific for *M. tuberculosis* ancestries 2, 3 and 4. There is no scale shown for the evolutionary distances.

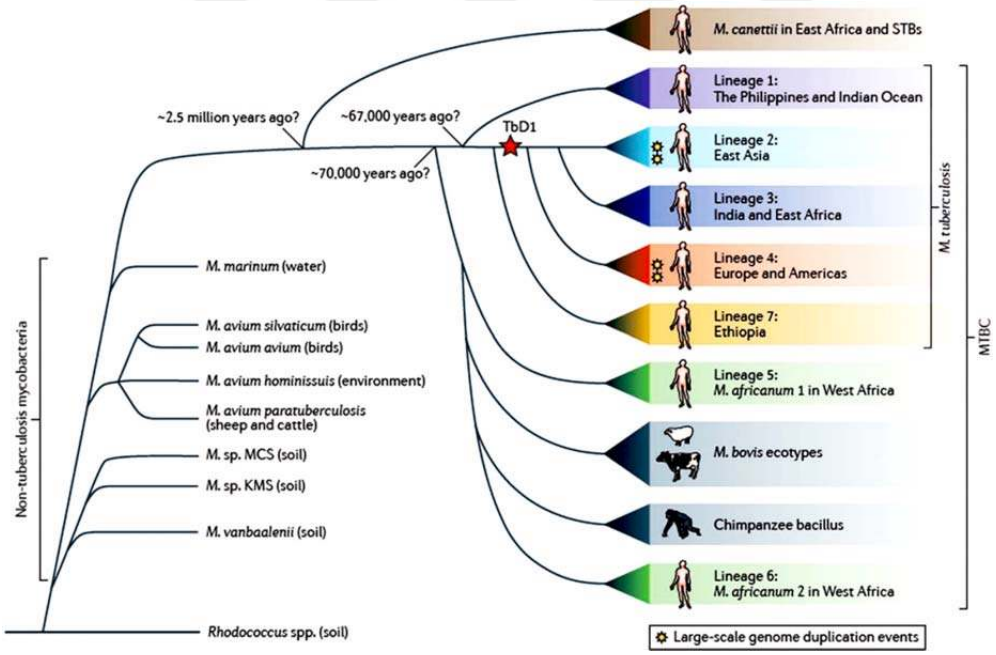

Figure 2.2. Evolutionary relationships with members of the Mycobacterium tuberculosis complex and selected mycobacteria (Galagan 2014)

The research of Comas et al. gives details in the context that the disease was originated long before. This research analyzed and compared the whole genome of 259 strains of *M. tuberculosis* complex to see their evolutionary history and showed that, over 70,000 years ago, going with the modern humans out of Africa and expanding to the world as a result of the increase in population density during the Neolithic period (Comas et al. 2013) **Figure 2.4.** In this study, 34,167 SNPs polymorphic sites within the 4.4 Mb genome of this species have been identified (i.e.: DNA sites that have some distinct nucleotides depending on strains and have been able to reconstruct this phylogeny of the bacterium). This research thus confirmed seven lineages that had been proposed by other methods. In addition, This phylogeny of genomes of *M. tuberculosis* is extremely similar to the phylogeny of the human mitochondrial genome as indicated in **Figure 2.3.** Therefore, this indicates that the evolution of the tuberculosis bacteria is related to that of modern humans.

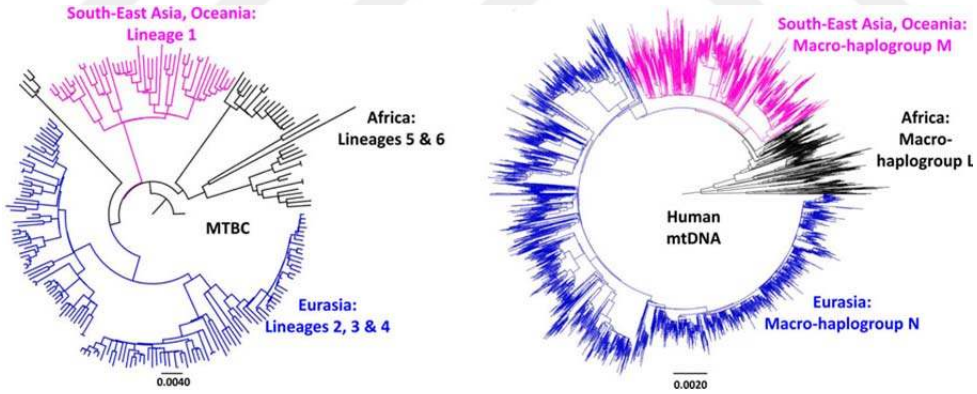

Figure 2.3. The genomic phylogeny of 220 of *M. tuberculosis* complex with different lineages (left) is comparable to that of 4995 human mitochondrial genomes (right) with their primary haplo groups (Comas et al. 2013)

Depending on these phylogenies and mutation frequencies found in *M. tuberculosis* genomes, the origin of the different lineages of the MTBC has been

established. Currently, it is evident that MTBC has developed alongside humans for a long time, thus influencing their evolution reciprocally (Banuls et al. 2015). Among these lineages, the ancestral lineage 1 coincided with the original migration of humans from Africa to the Indian Ocean as early as 67,000 years ago. The modern lineages 2, 3 and 4 might have emerged approximately 46,000 years ago, correlating with the second phase of human migration to the Middle East, Europe and East Asia (Comas et al. 2013). The lineage 7 is still exclusively related with the Horn of the African and might emerged 64,000 years ago. Lastly, lineages 5 and 6 tend to have emerged before other lineages and lineage 7 have remained in Africa (Comas et al. 2013, Firdessa et al. 2013). **Figure 2.4.**

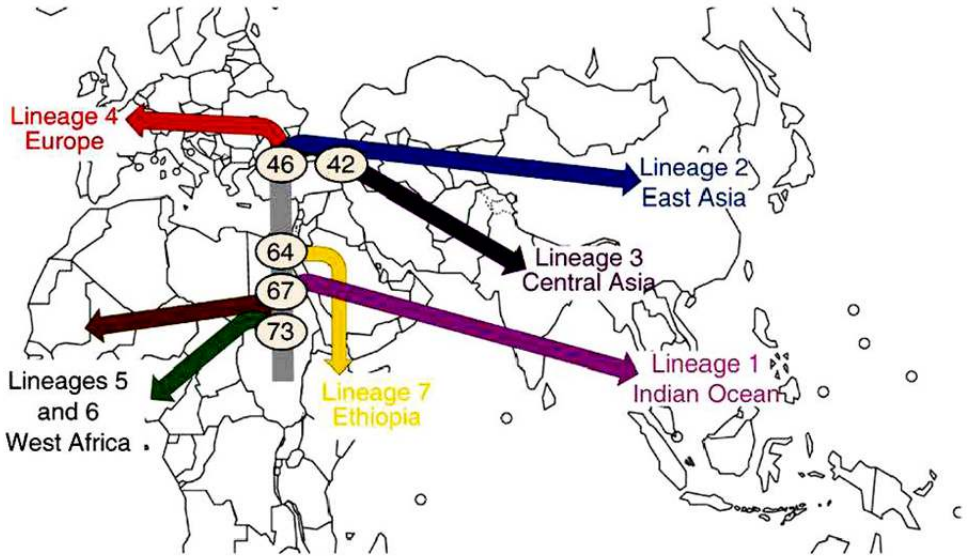

Figure 2.4. Neolithic expansion of M. tuberculosis complex going out of Africa, with the origin of different lineages and the estimated data of the evolutionary branches (thousands of years) (Comas et al. 2013)

2.4. *M. tuberculosis* Complex Genomic Diversity

The *Mycobacterium tuberculosis* complex (MTBC) includes a group of highly related subspecies which have similarity at the nucleotide level and shares 99.9% of identical genome sequences; identical 16s rRNA sequences. However,

they varies widely in terms of host tropisms and pathogenicity (Ru et al. 2017). These comprise the human-adapted *M. tuberculosis*, *Mycobacterium canettii*, and *Mycobacterium africanum* and the animal-adapted *M. bovis*, *Mycobacterium caprae*, *Mycobacterium microti*, *Mycobacterium pinnipedii*, *Mycobacterium orygis*, and *Mycobacterium mungi* (Brosch et al. 2002).

After sequencing, the genotypical classification of MTBC found that only two loci were present at high frequency; KatG codon 463 CTG (Leu) and gyrA codon 95A ACC (Thr). Therefore, depending on the polymorphism combination located at these regions ; all isolates of *M. africanum*, *M. bovis* and *M. canetti* had group 1 (KatG463 CTG (Leu) and gyrA95 ACC (Thr) characteristics. Although, *M. tuberculosis*, in addition to group 1 arisen in groups 2 and 3; KatG 463 CGG (Arg) and gyrA95 ACC (Thr) and KatG463 CGG (Arg) and gyrA95 AGC (Ser), respectively (Sreevatsan et al. 1997).

Human strains have been able to grow in vitro for longer periods, which was not the case with bovine strains (Thoen, Steele and Gilsdorf 2008). In 2002, *M. bovis* genome sequence has been completed by Sanger Institute (Sanger.ac.uk, 2002). The genome is an approximately 4.4 million base pairs (bp) long double-stranded circular deoxyribonucleic acid (DNA) molecule with a guanine and cytosine (GC) content of approximately 65% (Durr, Clifton-Hadley and Hewinson 2000a).

2.5. Genetic relatedness among strains of the *M. tuberculosis* complex

Molecular epidemiology studies use DNA genotyping depending on *M. tuberculosis* genome polymorphisms to characterize the bacteria into different strain groups (Mathema et al. 2006). Molecular typing of *M. tuberculosis* has been used for various epidemiological purposes as well as for clinical management. There are many methods for *M. Tuberculosis* type available at the present and it is essential to choose the most suitable method in accordance with the laboratory conditions and the specific features of the geographic area. The genotyping techniques allow a high level of differentiation among different *M. tuberculosis* strains (Ei et al. 2016). There are several genotyping methods have been developed

for discrimination between different *M. tuberculosis* strains, including: IS*6110* restriction fragment length polymorphism (IS*6110* RFLP) (Goyal et al. 1997, Das et al. 1995), Spoligotyping (Kamerbeek et al. 1997), Mycobacterial interspersed repetitive units typing variable-number tandem repeat typing (MIRU-VNTR) (Supply et al. 2001)and polymorphic GC-rich repetitive sequence typing (PGRS) (Poulet and Cole 1995). The main genotyping methods of *M. tuberculosis* are summarized in **Figure 2.5.**

Currently, IS*6110*-RFLP was regarded as the gold standard for molecular epidemiological studies because of its discriminatory strength; the ability to distinguish between two distinct species, and because it has been commonly used since the early 1990s (Houben and Glynn 2009). Spoligotyping and MIRU-VNTR typing assays are less discriminatory than IS6*110* RFLP. PGRS-RFLP Polymorphic GC-rich repetitive sequence RFLP has been used as a secondary typing method in *M. tuberculosis* isolates with 5 or less copies of IS*6110*(Rhee et al. 2000).

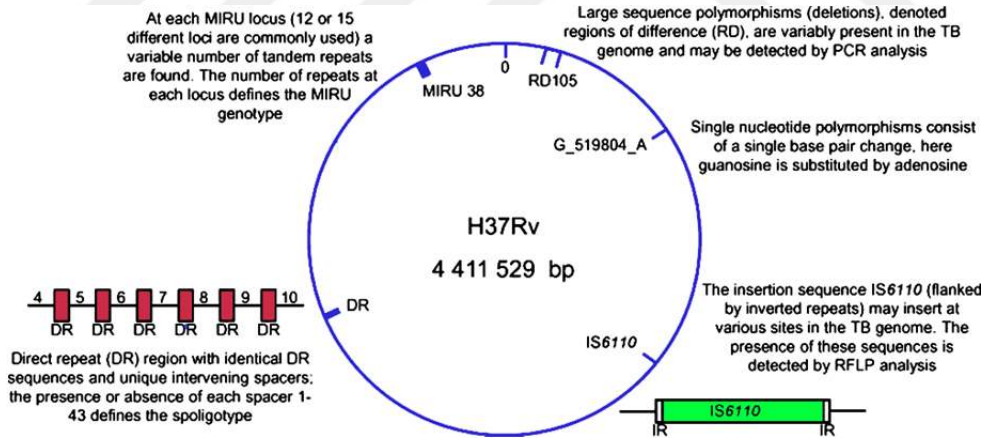

Figure 2.5. Schematic depiction of the *M. tuberculosis* genome representing the genetic basis of the main genotyping methods. Examples of main genetic elements used in strain genotyping are shown in the circular chromosome of the reference strain H37Rv (Nicol and Wilkinson 2008)

2.6. Background to tuberculosis disease in human and animals:

Tuberculosis (TB) disease in human and animal is caused by the tubercle bacilli of the *M. tuberculosis* complex (MTBC) and other environmental non-tuberculous or atypical mycobacteria (Cosivi et al. 1998, Une and Mori 2007). TB is characterized by progressive development of granulomatous lesions or tubercles in the lung tissue, lymph nodes or other organs (Thoen et al. 2009). It is the most important zoonotic disease associated with significant economic losses in the animal industries and major human health problems globally, especially in the developing countries (Cosivi et al. 1998, Organization 2009).

Historically, there has always been a strong association between animal and human tuberculosis disease. TB was described in livestock animals since the early 1800s. In 1865, Villemin indicated that infected tuberculous material could be injected from one animal to another in order to cause disease. In 1882, Robert Koch found out the risk of transmitting TB from livestock to humans and in 1902, Ravenel indicated *M. bovis* in a child infected with tuberculous meningitis (Davies 2006). Human tuberculosis is caused mainly by *M. tuberculosis*, although *M. bovis* the etiological agent of tuberculosis in cattle can also be responsible for the human disease, making *M.bovis* an important zoonotic species (Biet et al. 2005) whereas *M. africanum* causes human health problems in tropical Africa region (Cosivi et al. 1998).

2.7. Human tuberculosis caused by *Mycobacterium bovis* infection:

Bovine Tuberculosis (bTB) is defined as a chronic infectious disease in cattle. This disease caused by a specific species of bacteria called *Mycobacterium bovis*. Bovine tuberculosis generally impacts animals such as cattle, but it can practically impact all mammals including humans. It can be transmitted from cattle to humans as well as to other livestock species (Pesciaroli et al. 2014).

The impact of Bovin tuberculosis on human health is considered low in developed and developing countries, which may be based on the rare identification

of *M. bovis* isolates from human patients (Amanfu 2006). The role of *M. bovis* associated tuberculosis in the global epidemic of human tuberculosis is less known and not fully researched in detail. However, eliminating tuberculosis from human society will require this microorganism to be well understood and controlled. It is important to address the lack of knowledge about the impact of *M. bovis* as a contributor to the human tuberculosis epidemic worldwide. Over the decades, the perception of the role of bovine tuberculosis in human disease has changed substantially. Generally, tuberculosis is not widly observed in wild animals, unless when associated to infected domestic animals or humans. Infected animals can be overspill instances where the disease occurs in the wildlife population but can not be maintained. Nevertheless, many species of wild animals were addressed as reservoirs of *M. bovis*, although cattle are the most common reservoir host (Thoen et al. 2009).

The role of bovine tuberculosis as a serious disease in humans has been observed in the Netherland in1940 where the introduction of pasteurization of milk as the justification for a sharp acceleration in the decrease of the disease there (Ruys 1946). The resistance to pyrazinamide in *M. bovis* is a relevant difference between the disease caused by *M. tuberculosis* and the disease caused by *M. bovis* (Konno, Feldmann and McDermott 1967). This could be a consequence of the essential role of pyrazinamide in the short course treatment regimens for tuberculosis treatment. Though, the correlation of treatment outcome in those whose disease is caused by *M. bovis* with those whose disease is caused by *M. tuberculosis* has not been reported. *M. bovis* is pathogenic for humans, but it is less pathogenic than human *M. tuberculosis*. It is probably capable of creating beneficial cases of pulmonary tuberculosis with positive sputum smear, thus it is likely infectious to other humans (LoBue et al. 2004a). The incidence of tuberculosis due to *M. bovis* in a community is a strong indicator for the probability of a positive tuberculin reaction, but it has an effect on morbidity subsequent to infection that is significantly decreased compared to *M. tuberculosis* infection.

2.8. Importance of the disease:

Over the years, the elimination of bTB was a significant problem due to its effect on public health and high economic impact in the production of live stocks. It is estimated that 85% of livestock and 82% of humans in Africa reside in regions where Bovine tuberculosis is either partially controlled or totally uncontrolled (Cosivi et al. 1998). The current expanding prevalence of tuberculosis in humans, especially in immune-compromised humans, has given a lot of interest in the zoonotic importance of *M. bovis* especially in developing countries. The incidence of tuberculosis infection in an uncontrolled environment from animals to makes this important zoonotic disease (Jemal 2016). Bovine tuberculosis control in human is essentially that of the control of the disease in animals and in conjunction with efficient therapy of the disease. Even though the disease is less probable to occur, *M. Bovis* contributes to the worldwide cancer epidemic and must, therefore, be considered in the global plan for its containment. This is extremely important in the current time, when the convergence of *Mycobacterium bovis* and the human-immunodeficiency virus (HIV) has the possibility to exacerbate the situation (Ayele et al. 2004) since cultures are not routinely performed in most developing countries and with increased in tuberculosis burdens, leading to an unknown percentage of cases caused by *M. bovis* in these countries. Given the raised in HIV infection rate in human and persistence of bacteria in cattle, a substantial fraction of tuberculosis in such developing countries could be of bovine origin.

2.9. Epidemiology of *Mycobacterium bovis* Infections:

Bovine tuberculosis (bTB) was first identified in domestic animals, even though its host range is wide and involves most mammalian species which also includes humans. *Mycobacterium bovis* incorporates one of the widest host ranges of all pathogens with a complicated epidemiological model including human, domestic and wild animal interaction infection (Gemechu et al. 2013). The epidemiology of the *M. bovis* Infections has historically been of great interest. *M. bovis* infection and endemic infection have been recorded in different species. The

potential of *M. bovis* to infect such a broad range of species can be ascribed to the distinct transmission pathways in which the organism can be transmitted from animal to animal. Also, the ability of *M. bovis* attributed to the different transmission mode between animals which include inhalation of contaminated aerosols or fomites, oral transmission through ingestion of the organism, and transmission is by transcutaneous transmission of *M. bovis* in rare cases (Francis 1971, Serraino et al. 1999, Grange and Yates 1994). In addition, vertical transmission had been recognized in some species but rarely documented in wildlife under natural conditions which occurs through consumption of milk from infected animal or by contact between infected animals (Morris and Pfeiffer 1995, Phillips et al. 2003, Palmer, Waters and Whipple 2003). In fact, only little information on the *M. bovis* infection rates in domestic animals in developing countries is available. Understanding of the epidemiology of infection within the ecological system which includes both domestic and wildlife animal species is required for effective control of the disease in humans as well as in animals.

2.10. Transmission of tuberculosis caused by *M. bovis*:

The ability of *Mycobacterium tuberculosis* complex associated bacteria to infect a wide range of mammalian species has been ascribed to their different transmission pathways from infected to susceptible hosts (Kaneene and Pfeiffer 2006). There are several pathways of transmission of the tubercle bacilli as shown in **Figure 2.6.**. However, the rates of susceptibility differ with different host species, pathogenic mycobacteria, pathway of exposure, and infectious dose and virulence of the tubercle bacilli strain (Thoen et al. 2009).

The environment serves as a potential reservoir of *M. bovis* and play an important role in indirect transmission in the epidemiology of bovine tuberculosis. Thus, it is important to evaluate it in order to understand the transmission of the disease within the environment. The role of the environment in the transmission of the disease is through providing a site for indirect transmission of tubercle bacilli, particularly *M. bovis* contaminated substrates between cattle and wildlife species.

Transmission of contaminated aerosols by direct inhalation is the most

important pathway of infection in susceptible animal groups which stay in frequent close contact or constrained to infected individuals (Palmer and Waters 2006). Compared to the other transmission pathways, transmission by inhalation pathway needs a lower number or infective dose of organisms (Cassidy 2006). The infected species typically produce aerosols and mucus containing the pathogens when coughing or sneezing. Furthermore, this way of transmission is efficient in intensively controlled livestock that maintained in a narrow area and in free-range animals that keep in familial groups (Goodchild and Clifton-Hadley 2001).

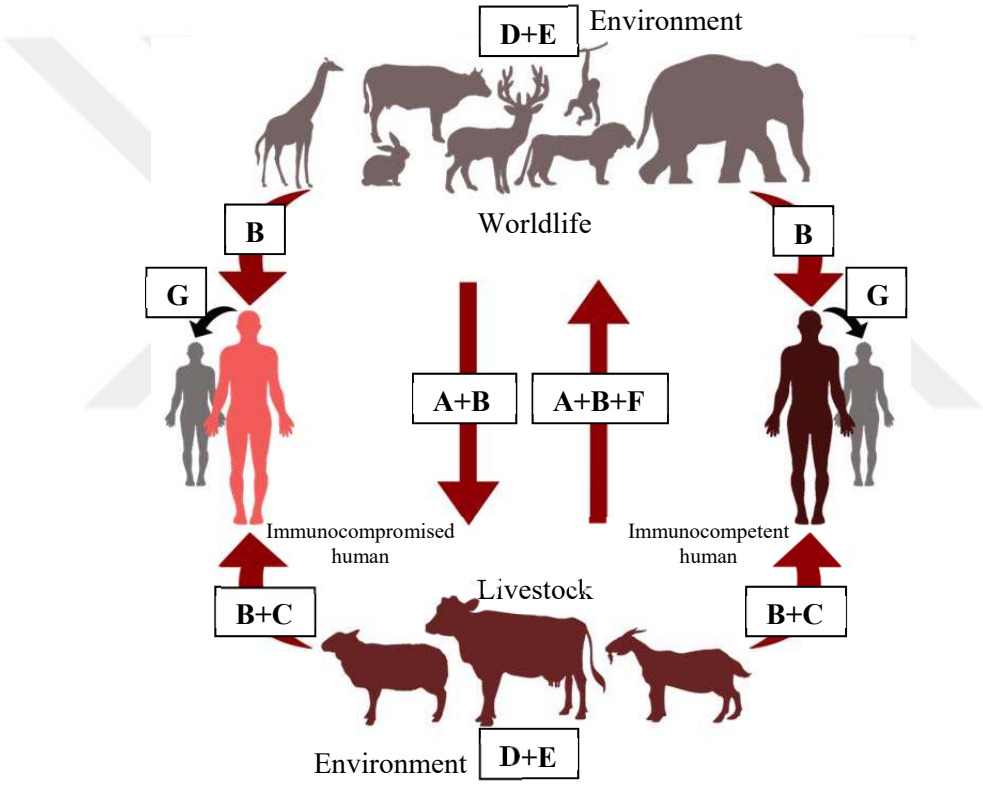

Figure 2.6. Possible pathways of *M. bovis* transmission between environment, livestock, worldlife and human. A: Infection by contaminated materials; B: Infection by aerosol; C: Infection by ingestion of derivative products; D: Vertical transmission; E: Horizontal transmission; F: Infection by predation; G: Human-human transmission.

humans could be infected with *M. bovis* by drinking contaminated milk with resulting in disease commonly manifesting in children as lymphadenitis, another pathway for humans infection is by inhalation of drizzle nuclei produced by sick cattle, which may lead to pulmonary disease (Dankner et al. 1993). In addition, *M. bovis* infection could happen by airborne transmission from sick cattle to abattoir workers and veterinarians (Georghiou et al. 1989, Sauret et al. 1992). In rare cases, humans could be infected with *M. bovis* by direct inoculation (Grange 2001).

There is a potential risk of cattle to human transmission by either ingestion or inhalation since that *M. bovis* is either enzootic or revealed intermittently in much of the developing countries (Cosivi et al. 1998). In addition, overspilling of *M. bovis* from cattle to other wildlife species is another probable cause of human infection. Even though most individuals were reported to have only a latent infection, *M. bovis* was observed to have a positive sputum culture by examination the index animal case (Fanning and Edwards 1991, Wilkins et al. 2003). There is another way of transmission of *M. bovis* which include the transcutaneous transmission from animals to humans who handle infected carcasses (Grange and Yates 1994). Moreover, Humans to cattle transmission is considered to be rare (Van Soolingen 2001).

Human to human transmission of *M. bovis* is considered less efficient than for *M. tuberculosis* (Van Soolingen 2001). Human to human airborne transmission of *M. tuberculosis* is the only way of transmission, therefore it has been considered the primary focus of efforts to control tuberculosis is by public health organizations. However, the ability of *M. bovis* to be transmitted from human to human has been reported for many years but this mode of transmission was considered rare and of doubtful significance (Schönfeld 1982, Kubin et al. 1984, O'Reilly and Daborn 1995). Many recent evidence suggests that human to human transmission of *M. bovis* could be more essential than initially assumed. Several epidemics of multidrug-resistant *M. bovis* has been reported in Europe during the

mid of 1990s (Samper et al. 1997, Guerrero et al. 1997, Blazquez et al. 1997, Rivero et al. 2001). The entirety of the included patients were infected with the human immunodeficiency virus (HIV). Furthermore, HIV positive individuals were also shown a susceptibility to *M. bovis* infection in other studies (Dankner et al. 1993, O'Reilly and Daborn 1995, LoBue et al. 2003).

Tuberculosis and HIV/AIDS both are chronic immunosuppressive diseases, where dual existence affects each other. Immunocompetent individuals infected with tuberculosis have about a ten percent lifetime risk of developing the disease, with half the risk of infection existing within one to two years (Ayele et al. 2004, Hopewell and Bloom 2005). Immunocompromised humans (HIV-positive individuals) with latent tuberculosis are about twenty to thirty times the risk of developing the disease compared to Immunocompetent humans (HIV-negative individuals) at a rate of 8-10% per year (Organization 2011). Moreover, HIV coinfection also raises the risk of recently acquired active disease infection (Whalen et al. 2011). In countries with high levels of *M. bovis* infection in bovine animals and high levels of HIV infection in humans, the ability exists for transmission of *M. bovis* from these animals to immunosuppressed individuals, immediately followed by human to human transmission between immunosuppressed individuals (Cosivi et al. 1998).

Even though the probability of transmission between individuals who are not infected with HIV is likely to be significantly lower, there is evidence that such transmission occurs. Comparative studies between patients infected with pulmonary *M. bovis* and patients infected with pulmonary *M. tubercuosis* indicates that these patient groups were probable to have cavities on chest x-ray films in addition to have positive sputum smears for acid fast bacilli (AFB)(LoBue et al. 2004a, LoBue, LeClair and Moser 2004b)

In general, the overall range of the problem caused by *M. bovis* remains unknown due to the lack of surveillance information of the disease, also the conditions in which a tuberculous animal becomes an effective disseminator of infection still actually undefined.

2.11. Pathogenesis of *Mycobacterium bovis*:

Tubercle bacilli has been identified more years ago. Nevertheless, the pathogenesis of *Mycobacterium bovis* is not well understood as for human tuberculosis and definitive information on it is not available (Thoen et al. 2006). Advancements and results are extrapolated in the field of human tuberculosis using different small animal models of *M. tuberculosis* infection to better understand bovine tuberculosis (Gupta and Katoch 2005, Pollock et al. 2006, Cassidy 2006). Tuberculosis is characterized by progressive development of granulomatous lesions or tubercles in the impacted tissues or organs (Liebana et al. 2008). Tuberculous lesions were observed to be mostly distributed in the respiratory tract and thoracic lymph nodes in infected cattle and humans, mainly in portions of the lungs near to the pleural surface (Thoen and Bloom 1995, Palmer and Waters 2006, Cassidy 2006).

Cattle infected by ingestion of *M. bovis* contaminated water and food often develop primary foci in intestinal tract associated lymph tissues. In a significant proportion of animals, there are also prevailing findings of lesions in the retropharyngeal, submandibular and parotid lymph nodes, indicating potential excretion foci on the upper respiratory tract surface (Corner 1994, Neill et al. 1994). Experimental comparative studies in animals indicate that the nature and extent of the disease differs with the pathway of exposure and dose of organism (Biet et al. 2005). Tubercle bacilli have developed adapted abilities to prevent immune clearance and cause chronic lesions to ensure transmission. Consequently, lesions are evolved as mechanisms to restrict the distribution of the bacillus, thus preventing the early death of the host (Cassidy 2006).

In some individuals, an initial respiratory infection may progress to emerge as following: Subpleural infectious foci rupture into pleural space, resulting in tuberculous pleuritis, intensive caseous pneumonia, extension of the tuberculous focus into a bronchus resulting in intensive endobronchial spread across one or both lungs, tuberculous focus rupture into a pulmonary vessel with haematogenous

spread resulting in acute disseminated disease (McAdams, Erasmus and Winter 1995). This initial infection, which also known as primary tuberculosis could spontaneously resolve for most individuals. Tuberculosis may reactivate months or years after the containment but many patients may stay completely asymptomatic (Thoen et al. 2009).

2.12. Clinical signs and lesions of the disease:

Human tuberculosis as well as bovine tuberculosis are difficult to diagnose based on clinical signs only. The signs of active tuberculosis are sometimes non-specific and primarily pulmonary; fever, soft and chronic cough with or without blood, night sweats, lack of appetite, weight loss, and acute thoracic pain may appear in the patient. If the disease progresses, respiratory problems and progressive loss of function may occur. Cell-mediated by T-lymphocytes is the first and most prevalent immune response which is the cause of the chronic characteristic caseous granulomas (Monaghan et al. 1994). These lesions begin with infectious droplet nuclei forming the primary structure which found mainly in the lungs and thoracic lymph nodes (Neill and Pollock 2000).

The deficiency of the immune system to fight tuberculosis triggers haematogenic spread of species to different areas to cause extra-pulmonary disease (Carrol, Clark and Cant 2001). Haematogenic spread to the brain or meninges leads to tuberculous meningitis (TBM) (Girgis et al. 1998). TBM is the most prevalent type of tuberculosis of the central nervous system with very high morbidity and mortality (Verdon et al. 1996). It is primarily a sign of serious extra-pulmonary tuberculosis in childhood (Uysal et al. 2001).

2.13. Laboratory diagnosis of tuberculosis:

For appropriate treatment and transmission prevention, proper early diagnosis of a patient with tuberculosis is important. Inappropriate diagnosis can lead to improper treatment, thus trigger health problems and raise treatment costs (Kambashi et al. 2001).

2.13.1. Immunological Diagnostic Tests:

The tuberculin skin test (TST) is an in vivo assay that is used for detection latent infection of tuberculosis. This test, also known since 1910 as an intradermal Mantoux test, is one of the earliest diagnostic tests which still used currently (Barnes 2006). However, The validity of the TST has low specificity. The terms tuberculin skin test, Mantoux, TB skin test and PPDs are often interchangeable. Purified protein derivative (PPD) is a culture filtrate of tubercle bacilli that includes more than 200 antigens shared by *M. bovis* BCG and most non-tuberculous mycobacteria (Seibert and Dufour 1948, Huebner, Schein and Bass Jr 1993).

In cattle and other animals, the intradermal tuberculin prepared from the culture filtrate of *M. bovis* by precipitation with ammonium sulfate (NH_42SO_4) or trichloroacetic acid ($C_2HCl_3O_2$) is commonly used for identification of tuberculosis (O'Reilly 1995, Thoen et al. 2009). The reaction to intracutaneously injected tuberculin is the classic example of a delayed cellular hypersensitivity reaction. The reaction is read after 48 to 72 hours. If there are separate papules, the diameter of the biggest papules is measured. When vesiculation appears, the result is recorded independently and considered as positive. When papules coalesce, the diameter of the biggest locus of induration is measured (Clark and Kruse 1990). In general, the mechanism of this test is as following: T-cells sensitized by previous infection are attracted to the skin surface where they produce lymphokines, these lymphokines cause induration to the region through local vasodilatation, edema, fibrin deposition, and induction of other inflammatory cells. The characteristics of the response include: its delayed course, achieving a peak further than 24 hours after application of the antigen; its indurated character; and its occasional vesiculation and necrosis (Nayak and Acharjya 2012). Furthermore, blood tests that also measure cellular immunity such as the gamma interferon (IFN-γ) test was described for monitoring cell-mediated responses. The sensitivity of the IFN-γ assay has significantly higher than the single intradermal tuberculin test (Wood et

al. 1991). In practice, both tuberculin skin and gamma interferon assays were used to maximize the effectiveness of identifying latent tuberculosis and early levels of the disease in immunocompromised hosts (Kim et al. 2009).

2.13.2. Microscopic Diagnostic Tests of *Mycobacterium bovis*:

In the poorest regions of the world, Africa, where the majority of the disease burden lies, the mainstay of diagnosis is microscopy; which can be performed by using either light microscopy with a stain such as "Ziehl-Neelsen" (ZN-stain) or fluorescent microscopy with a stain such as "auramine O" applied on a sputum smear sample. Microscopy of sputum smear allows a rapid and reliable identification of patients with pulmonary tuberculosis where there are between 5,000 and 10,000 bacilli per milliliter of sputum.

Preliminary of *M. bovis* identification is depends on colonial morphology as well as staining characteristics. *M. bovis* as well as all species of mycobacteria are appear as red coccobacilli or short rods with 3 to 4µm long and are assembled in serpentine cords against a blue background by Ziehl-Neelsen staining. **Figure 2.7.** Whereas, the organisms appear as fluorescent rods with a color range from yellow to orange in the fluorochrome procedures to demonstrate the acid-fast tubercle bacilli under the fluorescent microscope (Corner 1994, Collins, Grange and Yates 1997, Marais et al. 2008). **Figure 2.8.**

The reliability of sputum smear microscopy based on the quality of sputum collection, the proper preparation and interpretation of slides (Singhal and Myneedu 2015).

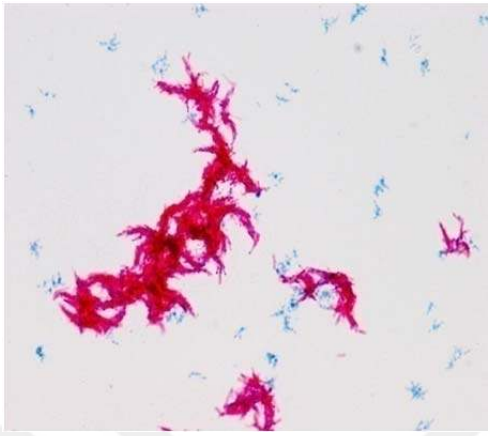

Figure 2.8.AFB serpentine cords ZN-stain (labmed.ucsf.edu)

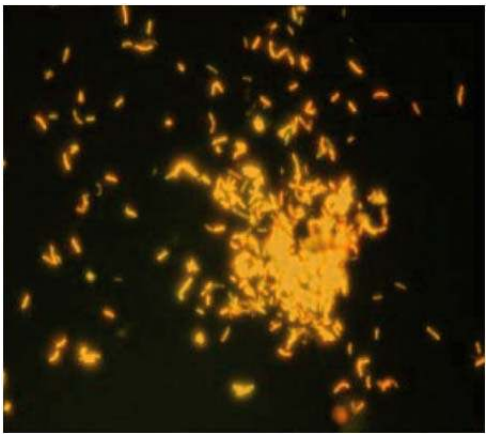

Figure 2.7.AFB with Fluorochrome stained with stain (OĞUZ et al. 2011)

The identification of *M. bovis* using the fluorescent microscopy-based method, though it requires an expensive fluorescent microscope in addition to a dark room, is more sensitive than the other light microscopy-based method with ZN-staining. More recent advances of low-cost fluorescent LED technology has allowed this high sensitive diagnostic device to be introduced into the areas where this technique is most needed (Mizuno et al. 2009).

2.13.3. Culture Test of *Mycobacterium bovis*:

Culture is considered as the "gold standard" for diagnosis of the mycobacterium tuberculosis complex infections; regardless of whether the disease is associated with *M. tuberculosis* or *M. bovis* infection. However, it is not always positive in patients who are treated for tuberculosis. The sensitivity culture test is much greater than microscopy and as little as 10 bacilli per milliliter have been identified (Yeager Jr et al. 1967). The diagnosis is confirmed by growth and isolation of the Mycobacterium on different selective media which are either egg-based such as Löwenstein-Jensen media or agar-based such as Middlebrook 7H10/7H11) or liquid such as Middlebrook 7H9/7H12/Kirshners media.

Stonebrink's medium containing glycerol-free sodium pyruvate may be the best medium. Solid culture media is prepared in tubes or bottles as *M. bovis* is typically present in small numbers, and therefore large inoculums are required to be distribute over the surface of the medium. *Mycobacterium bovis* colonies are expected to appear after 8 weeks minimum (this period can be extended up to 10 or 12 weeks) of incubation at 37^{o}C±1 with or without CO2 (Strong and Kubica 1981).

More recently, it has been shown that automated liquid culture technologies such as MGIT 960 (Becton Dickinson, New Jersey, USA) and MB/ BacT (bioMerieux, France) both increase sensitivity and decrease detection duration compared to solid media. In these systems, growth is measured by radiometric or fluorometric methods (Palomino et al. 2008, Parrish et al. 2009, Sorlozano et al. 2009). These radiometric technique provides results considerably faster than traditional culture, but is often sensitive to fungal contamination, especially when certain animal samples are used, and will grow atypical Mycobacteria or Non-mycobacterial organisms that are not limited by the incorporated antibiotics (Wayne 1984). The culture should be repeated using the retained inoculum with an appropriate decontaminating agent when gross contamination of culture media appears (Zerdo et al. 2014).

The MGIT 960 system utilizes an altered 7H9 broth from Middlebrook and a fluorophore is bound to oxygen in the tube. When the organisms divide and uses the oxygen, this fluorphore is released and detected by the analyzer. The MB/BacT system uses a similar culture media, but the detection method varies from the MGIT 960. A pH-dependent sensor that changes when the pH falls in the presence of increased CO2, which produced by dividing organisms (Palomino et al. 2008, Parrish et al. 2009, Sorlozano et al. 2009).

The disadvantages of the BACTEC method are that this method is more expensive, requires a culture vial reading device, and includes handling of radioisotopes (Ayele et al. 2004). Furthermore, culture should be performed on all

samples in order to provide accurate species identification, drug susceptibility testing and genotyping.

2.13.4. Phenotypic Characteristics-based Diagnostic Tests:

The differentiation of *Mycobacterium tuberculosis* complex (MTBC) subspecies could be accomplished by laboratory tests based on the phenotypic characteristics of the organism. These tests areinclude: colony morphology, growth characteristics on different media and biochemical tests. The colony morphology varies among the *M. tuberculosis* complex subspecies; ranging from dry and rough to flat smooth, domed glossy colonies (Pfyffer 2015). It has been indicated that colony morphology conversion of mycobacteria is correlated with alterations in cell wall content of the organism, including glycolipids (Belisle and Brennan 1989), lipooligosaccharides (LOSs), and mycosides (Daffe et al. 1991).

Biochemical tests comprise isonicotinicacid hydrazide (INH), cycloserine, inhibition of thiophen-2-carboxylic acid (TCH), niacin accumulation, cyclosporinem, nitrate reduction, urease activity, nicotinamidase and pyrazinamidase activity.

*Mycobacterium bovis*is sensitive to isonicotinic acid hydrazide (INH) and thiophen-2-carboxylic acid hydrazide (TCH). Niacin production and nitrate reduction for *M. bovis* are negative while these are positive for *M. tuberculosis*. In the amidase test, *M. bovis* is positive for urease and negative for pyrazinamidase and nicotinamidase (Commission and Committee 2008). There are other tests that can provide more information are include: tween 80 hydrolysis, tellurite reduction, iron uptake and NaCL tolerance. The biochemical tests are often performed on Löwenstein–Jensen (L.J.) media and require up to three weeks to obtain the results (Babady and Wengenack 2012).

Furthermore, although these tests are simple and inexpensive, it considered to be labour intensive, time-consuming and depending on sufficient growth of the organism. In addition, these tests cannot be performed in a routine manner in all

laboratories and the obtained results are not conclusive (Prodinger et al. 2005, Okazaki et al. 2005, Hall and Roberts 2006).

2.13.5. Molecular-based Diagnostic Tests:

The basis of all molecular diagnostic methods is based on the acquisition of genetic mutations over time within particular regions of the MTBC genome, therefore creating unique profiles between isolates. Though there are virtually identical genomes in two bacterial strains of the same species, differences will certainly occur at a few loci. Another molecular epidemiological techniques beyond the standard fingerprinting reference method for *M. bovis* have been described (van Soolingen et al. 1991).

2.13.5.1. Genomic Deletion-typing (RD deletion-typing):

The genomic typing method was developed to differentiate of between the *M. tuberculosis* complex members known to cause TB in humans and animals depending on the deletion typing of various genomic Regions of Difference (RD). Regions of difference (RDs), the genes for antigenic proteins between the genomes of the various members of the MTBC has been identified and the genetic lineage of the complex has been determined by analyzes of the distribution of these variable genome regions in various MTBC strains isolated from different human populations and diverse animal species. Most of these RDs consisted of DNA sequence deletions. Since recombination in this group is not known, a lost sequence of DNA cannot be recovered and the deletion will characterize the lineage indefinitely (Brosch et al. 2002). Host strains animal-adapted from a nested lineage are characterized by the absence of the specific Region of Difference number 9 (RD9) (Smith et al. 2006). This lineages group involves *M. bovis*, *M. africanum* and *M. microti* and they are differentiated from *M. tuberculosis* by the deletion of RD9. Based on the presence (+) or absence (-) of such conserved RD regions, a level of relatedness to the last common ancestor of the MTBC was suggested that the lineages of *M. tuberculosis* and *M. bovis* separated before the *M. tuberculosis* specific deletion TbD1 happened. Therefore, it is obvious that *M.*

bovis can not have been the ancestor of *M. tuberculosis* but, slightly, appears to be descended from *M. tuberculosis* or to have emerged independently (Brosch et al. 2002).

The deletion of the Region of difference number 4 (RD4) distinguishes *M. bovis* and *M. bovis* BCG from other closely related lineages such as seal lineage (*M. pinnipedii*) and goat lineage (*M. caprae*). Identification of hyper-variable regions between the *M. bovis* and *M. tuberculosis* genomes and several loci exist that show significant variability along the bacilli strains have been identified (Gordon et al. 2001). The distribution of Regions of Difference (RDs) allows us to differentiate the five main groups among the *M. bovis* strains. The evolutionary scheme of the proposed evolutionary pathway of MTBC is based on the presence (+) or absence (-) of conserved deleted regions and on sequence polymorphisms in five selected genes **Figure 2.9.**

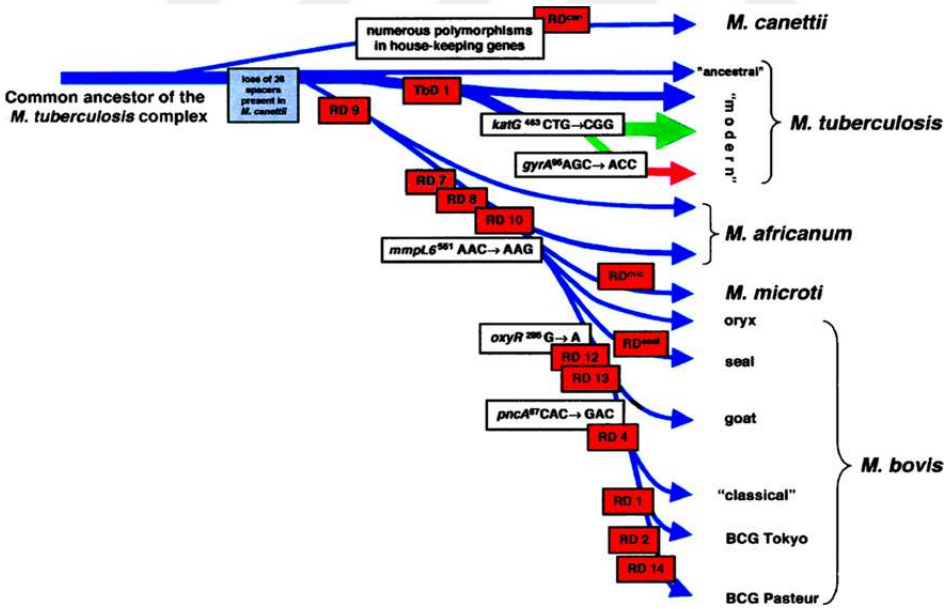

Figure 2.9. Scheme of the proposed evolutionary pathway of the MTBC illustrating successive deletion of DNA in certain lineages (dark/red boxes). The scheme is based on the presence (+) or absence (-) of conserved deleted regions and on sequence polymorphisms in five selected genes (Brosch et al. 2002)

M. bovis BCG lacks a group of genes (Rv3971 to rv3879) including the genes for antigenic proteins, Region of Difference number 1 (RD1) produced by MTBC early during culture growth but with unclear precise action (Arend et al. 2000). *M. tuberculosis* strains are highly conserved for RD1, RD2, RD4,RD7, RD8, RD9, RD10, RD12, RD13, and RD14, and these Regions of Difference (RDs) demonstrate regions that can differentiate *M. tuberculosis* strains separately of their geographical origin and their typing properties from certain other members of the MTBC. In addition, these regions may be associated with the host specificity of *M. tuberculosis* (Brosch et al. 2002). Genomic deletions of the Regions of difference RD4-RD11 between *M. tuberculosis* and *M. bovis*, and the deletion of RD1 due to repeated subcultivation of *M. bovis* strain resulting in *M. bovis* BCG attenuated strain are shown in **Figure 2.10.**

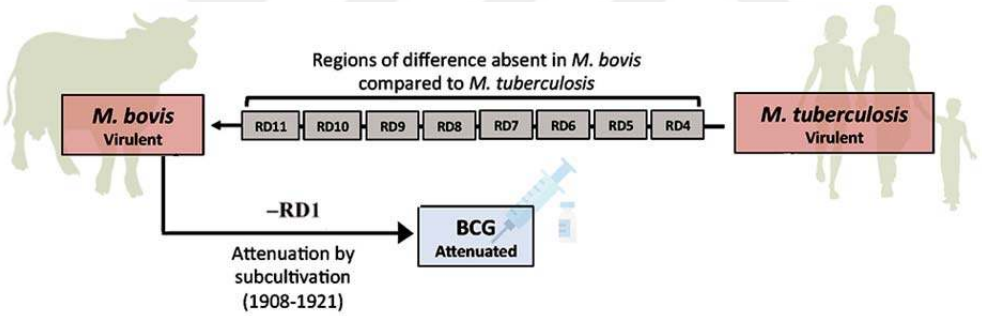

Figure 2.10. Genomic deletions between *M. tuberculosis* and *M. bovis* pathogens. Eight regions of difference (RD) deleted in the *M. bovis* with respect to the *M. tuberculosis*. Repeated subcultivation of *M. bovis* strain for 13 years (1908–1921), according to classical Pasteur's postulates, resulted in attenuation due to loss of RD1, which led to Bacille Calmette-Guérin (BCG) vaccine. This figure has been modified from: (Gonzalo-Asensio et al. 2017)

2.13.5.2. Spoligotyping:

Spoligotyping (spacer oligonucleotide typing) is a PCR-based molecular typing method which has been proposed as an alternative technique to hybridization-based fingerprinting techniques for diagnosis and epidemiology of

tuberculosis (Kamerbeek et al. 1997). Spoligotypingis the first genotypic method which allows detection and differentiation of *M. tuberculosis* complex strains directly from clinical samples. This method is depend on the detection of DNA polymorphisms in the spacer units present within the direct repeat (DR) locus of *Mycobacterium tuberculosis* complex genome. This locus consists of multiple direct repeats (DRs) of 36 bp long separated by DNA sequences of non-repetitive spacers varying in length from 35 to 41 bp (Groenen et al. 1993) **Figure 10.**. This single locus is composed of a series of 43 interspersed spacer sequences (previously identified in laboratory strain H37Rv and *M. bovis* BCG vaccine strain P3) in the genomic DR region of *Mycobacterium tuberculosis* complex (MTBC). The MTBC strains vary in the number of DRs and in the presence or absence of particular spacers. The DR locus contains over 60 such spacers, but aset of 43 spacers were selected as a basis of this method. This RD regions allows differentiation among the strains which have different patterns (Smith and Upton 2012). In the MTBC strains, absence of these spacers may happened due to homologous recombination or transposition of IS*6110* insertion sequences, which is almost invariably present in the DR region (Groenen et al. 1993). For *M. bovis* strains carrying only one copy of this transposable element, IS*6110* is inserted into the direct repeat (DR)inserted between spacer sequences number 24 and 25 (Kamerbeek et al. 1997, Legrand et al. 2001).

Spoligotyping patterns are obtained by PCR amplification for the complete DR locus, allowing for amplification of all spacers in this region. This PCR contains non-and biotinylated primers of the DR region. The amplified products are then reverse hybridized to a set of complimentary spacer oligonucleotides onto a membrane, each corresponds to one of the 43 potential unique spacer DNA sequences within the DR which have been sequenced from a number of the MTBC strains. A second antibody is then used to visualize the patterns, which results in a fluorescent emission (Kamerbeek et al. 1997). The output of the spoligotyping is obtained by determination of binary result (the presence or absence) as a result of the hybridization of each of the 43 consequent sequences.

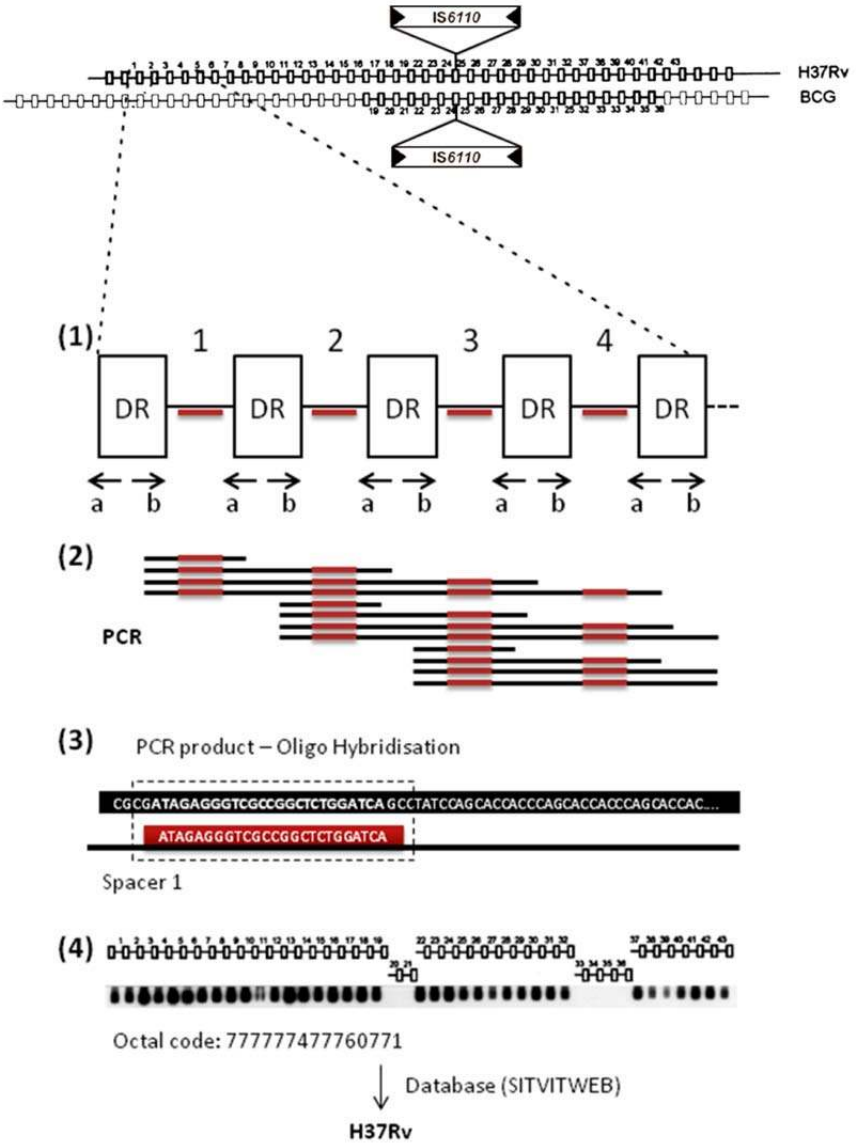

Figure 2.11. Spoligotype determination steps: 1) Direct Repeat locus (DR) structure in *M. tuberculosis* genome. 2) PCR amplification products complementary to the conserved DR region. 3) PCR product-Oligo Hybridization of an amplified DNA fragment onto the membrane comprising spacer sequences probes. 4) Hybridization pattern result and octal code derived. Spoligotypes are derived using the latest largest international spoligotype database (SITVITWEB), (Demay et al. 2012)

The absence of certain spacers is helpful to differentiating certain *M. tuberculosis* and different *M. bovis* strains. The reference *M. tuberculosis* strain H37Rv is characterized by the absence of spacers 20, 21, and 33 to 36, whereas *M. bovis* BCG is characterized by the absence of spacers 3, 9, 16, and 39 to 43 (Kamerbeek et al. 1997). **Figure 2.11.**

Moreover, the international designation database of the recorded spoligotype patterns of *M. bovis* and other RD9-deleted lineages has facilitated the comparison of results from different countries and helps elucidate the distribution and spread of strains, and is now freely available online (Brudey et al. 2006). The international largest database of MTBC genotypes based on spoligotyping is SITVITWEB (SpolDB4) (Demay et al. 2012). There are also another tools have been developed to extract spoligotypes from WGS database (Coll et al. 2012).

Spoligotyping can be applied to trace transmission links and determine transmission patterns such as endogenous reactivations and also detect possible zoonoses with *M. bovis* and other circulating bacilli. In addition, this method requires small amounts of DNA and is less technically demanding and hasthe potential to be used directly on clinical samples such as sputum, without the need forculture, paraffin embedded samples and cell culture lysates (Driscoll, McGarry and Taber 1999). This method also allows identification of new clonal composition of *M. tuberculosis* isolates in a certain country, and tracking of tuberculosis dynamics and immigration-related epidemiological effects (Zozio et al. 2005).

However, spoligotyping alone is not always informative and does not provide sufficient discrimination between *M. bovis* strains, and strains with shared spoligotypes may have variable matched IS*6110* profiles, therefore this method cannot be relied upon alone as a typing method for evolutionary studies of MTBC, but can be combined with other techniques such as VNTR or RFLP (Durr et al. 2000a, Cronin et al. 2001, Bifani et al. 2002, Kremer et al. 2004).

2.13.5.3. IS*6110* Restriction Fragment Length Polymorphism (RFLP):

Insertion sequences (IS) are small transposable (mobile) genetic elements commonly found in most bacterial genomes. IS*6110* is a member of the IS3 family of enterobacterial insertion sequence elements and is considered to be presented in multiple copies specific to the members of the *Mycobacterium tuberculosis* complex (Thierry et al. 1990, Cave et al. 1991). The total number of IS*6110* copies found in the genome varies widely among the various *M. tuberculosis* complex species, ranging from 0 to 26, with most strains having 8 to 15 copies which result in a different MTBC polymorphism. For example, the *M. tuberculosis* genome may carries up to 19 copies of IS6110, whereas *M. bovis* only contains 1 to 5 copies, these elements are distributed among the genome, although there are certain hot spots for their integration (van Soolingen et al. 1991, Flores et al. 2010, McEvoy et al. 2007). Molecular fingerprinting using IS*6110* is based on the cultivation of the organism, genomic DNA extraction, enzyme restriction RFLP, agarose gel electrophoresis, Southern blotting and detection of the IS elements with a labeled probe. Thus, the combination of the restriction enzyme used and the DNA sequence of the probe enables visualization of polymorphisms in the genomic DNA (Van Embden et al. 1993).

The first step of this technique is purification of DNA from culture, followed by digestion of the genomic DNA with the "*PvuII*" restriction enzyme, which cleaves the genome in several fragments, but cleaves IS*6110* only once. The obtained DNA fragments are then electrophoresed on agarose gels, and then transferred onto a nitro-cellulose membrane, where southern blot hybridization is performed. The labeled probe is hybridized complementary to the 3' end of the IS*6110* sequence. Each band in the hybridization pattern, denotes a single copy of IS*6110* surrounded by different lengths of flanking DNA, depending on the distance between the *PvuII* cleavage sites. Finally, a detection system is applied to the membrane which interacts with the probe label, typically producing a luminescent reaction that can be photographed (Durr, Hewinson and Clifton Hadley 2000b, Ei et al. 2016) Figure 2.12.

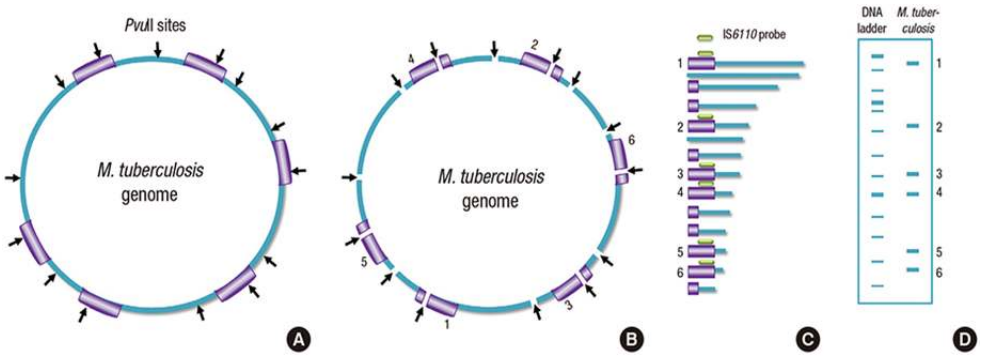

Figure 2.12. Principles of RFLP. (A) *M. tuberculosis* genome with insertion segment IS6110 showing the PvuII cleavage sites. (B) Digestion of the whole genome with PvuII. (C) DNA segments of different sizes after run in the gel are transferred onto a membrane followed by hybridization. (D) Visualized fragments, which represent a single copy of IS6110 surrounded by flanking DNA of different lengths (Ei et al. 2016)

IS*6110*-RFLP, though has a high discriminatory power, it is labour intensive, time consuming and requires a high DNA quality. Furthermore, IS*6110*-RFLP is less useful for detection of *M. bovis* isolates because strains of *M. bovis* only carries1 to 5 copies of IS*6110*. In addition, this element is usually found in the same 1.9-kb *PvuII* fragment of several *M. bovis* strains limiting the differential ability of this method (van Soolingen et al. 1991, Comas et al. 2009).

2.13.5.4. Mycobacterial Interspersed Repetitive Units–Variable Number Tandem Repeat (MIRU-VNTR):

Mycobacterial interspersed repetitive units (MIRUs) are small tandem repetitive DNA sequences usually identified throughout the intergenic region (IR) of the *senX3-regX3* operon of *M. tuberculosis* (Supply et al. 1997). MIRU-typing relies on the polymorphisms identified in 41 VNTRs ranging from 40-100 bp distributed within the genome of the MTBC. These variable number of repeats has been identified at 12, 15 or 24 selected MIRU loci and can be used for genotyping using a PCR-based method. This PCR-based typing technique has been

first described by Frothingham and Meeker-O'Connell, where used for human forensic and paternity testing (Frothingham and Meeker-O'Connell 1998).

The method of MIRU-VNTR typing is performed using PCR amplification of each locus with specific primers complementary to the flanking regions, followed by gel electrophoresis for detection of the amplicons size. The size of the amplicons is directly associated with the number of tandem repeats, which can be determined comparing to a reference of previously known repeat unit. The number of tandem repeats is then converted into a code, in which each digit represents the copies number of each locus (Supply et al. 2000, Mazars et al. 2001). **Figure 2.13.** MIRU loci have been used to distinguish between *M. bovis* BCG and virulent strains of MTBC due to the presence of the 53 bp MIRU of *senX3-regX3* intercistronic region in the other strains and its absence from *M. bovis* BCG strains (Magdalena et al. 1998). In addition, it has been found that one locus from the *M. tuberculosis* genome, MIRU 21, is absent from all *M. bovis* strains (Supply et al. 2000).

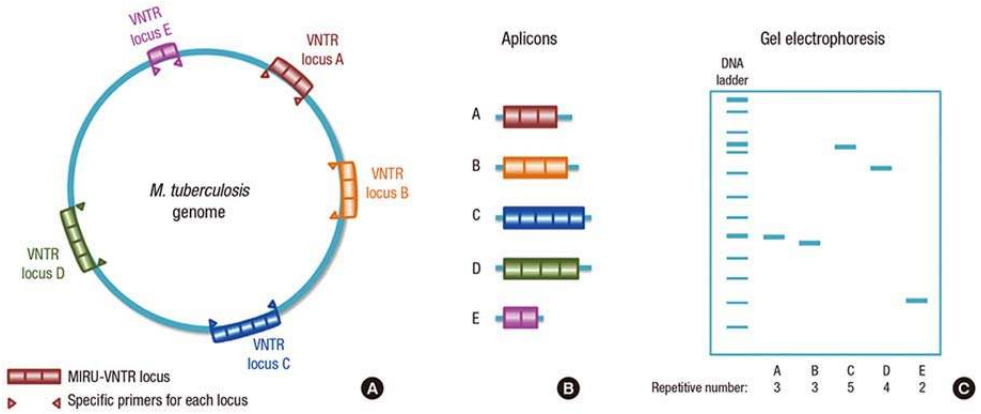

Figure 2.13. Principle of MIRU-VNTR genotyping. (A) MIRU-VNTR loci of different repetitive numbers scattered in *M. tuberculosi*s genome are amplified by specific primers for each locus. (B) Different sizes of amplicons after PCR. (C) Amplicons can be seen after gel electrophoresis with different sizes that reflect the repetitive number of each VNTR locus (Ei et al. 2016)

MIRU-VNTR typing results are intrinsically digital and analysis can be directly applied to culture without the need for DNA purification. In addition, this method is highly discriminative for *M. tuberculosis* and has also the potential to be the method of choice for *M. bovis* typing. VNTR typing has been also used to evaluate *M. bovis* transmission (Roring et al. 2004). MIRU-VNTR markers may be useful for larger population-based studies, and can also provide valuable predictions for classification of the strains (Huang et al. 2013). However, it is not yet standardized and allelic diversity of loci may vary among the MTBC species and in between different countries (Supply et al. 2001).

2.14. Treatment of the disease:

The conventional treatment for tuberculosis is a standard regimen of 6 month course includes isoniazid, rifampin, pyrazinamide, and ethambutol antibiotics. *M. bovis* has been especially frequent in sites suggest to represent primary tuberculosis outside the lung (Kazwala et al. 2001). Although there is an effective medicine for human patients with uncomplicated *M. tuberculosis* infections, extrapulmonary tuberculosis, particularly caused by *M. bovis*, still not has a special cure and is notoriously difficult to treat (Maas, Michel and Rutten 2013). Furthermore, *M. bovis* has an innate resistance to pyrazinamide antibiotics, thus an extended course of antibiotics is required to be used for efficient treatment of *M. bovis* infections. Unfortunately, most of doctors around the world do not trace the source of tuberculosis in patients and thus do not differentiate between *M. tuberculosis* infection and *M. bovis* infection before initiating treatment in human, and it has been indicated that *M. bovis* causes a higher mortality rate than *M. tuberculosis* (Miller and Olea-Popelka 2013).

The incidence of tuberculosis associated with *M. bovis* infection in a community is a strong indicator of the possibility of a positive tuberculin reaction, but it has an effect on morbidity subsequent to infection that is much lower than infection with *M. tuberculosis*.

3. MATERIAL AND METHOD

3.1. Study area and sample collection:

The samples used in this study have been collected from different areas in Çukrova region of south Turkey (Adana, Mersin, Gaziantep, Hatay, Osmaniye, Kilis, Kahramanmaraş) were presented to the Tropical Diseases and Application Center at Çukurova University. **Figure 3.1.** The strains used for this study were randomly selected among the Mycobacterium tuberculosis complex, which include *M. tuberculosis*, *M. bovis* and *M. bovis* BCG and other strains.

Figure 3.1. Map of Turkey showing the study area and the source of samples

3.2. Sample Processing and Cultures:

The clinical samples obtained from different laboratories have been subjected to a series of preparation steps including decontamination of the collected sputum samples from bacterial flora by sodium-hydroxide (NaOH), and then diagnosed for investigation of *M. tuberculosis* complex by conventional methods based on the phenotypic characteristics of *M. tuberculosis* complex, where it have been examined under microscope and differentiated from Non-tuberculosis mycobacterial species (NTM) by Ziehl-Neelsen (ZN) stain based on

colour and colony morphology. *M. tuberculosis* complex samples were tested based on growth characteristics on different media and biochemical tests. *Mycobacterium bovis* is then differentiated from the other species of *M. tuberculosis* complex based on the previous tests and as below:

- Culture-based tests: *M. bovis* cultured on Lowenstein-Jensen (L.J) slants has no growth when using a glycerol-based media and expected to appear after 8-12 weeks using glycerol-free media.

- Biochemical tests: Niacin production and nitrate reduction for *M. bovis* are negative while these are positive for the other species of the complex including *M. tuberculosis*. In the amidase test, *M. bovis* is positive for urease and negative for pyrazinamidase and nicotinamidase.

- MPT64 antigen test.

Consequently, the samples of which expected to be *Mycobacterium bovis* were forwarded to the molecular-based method for further analysis, in addition to other samples of the MTBC in order to determine the effectiveness of our molecular-based test. **Figure 3.2.**

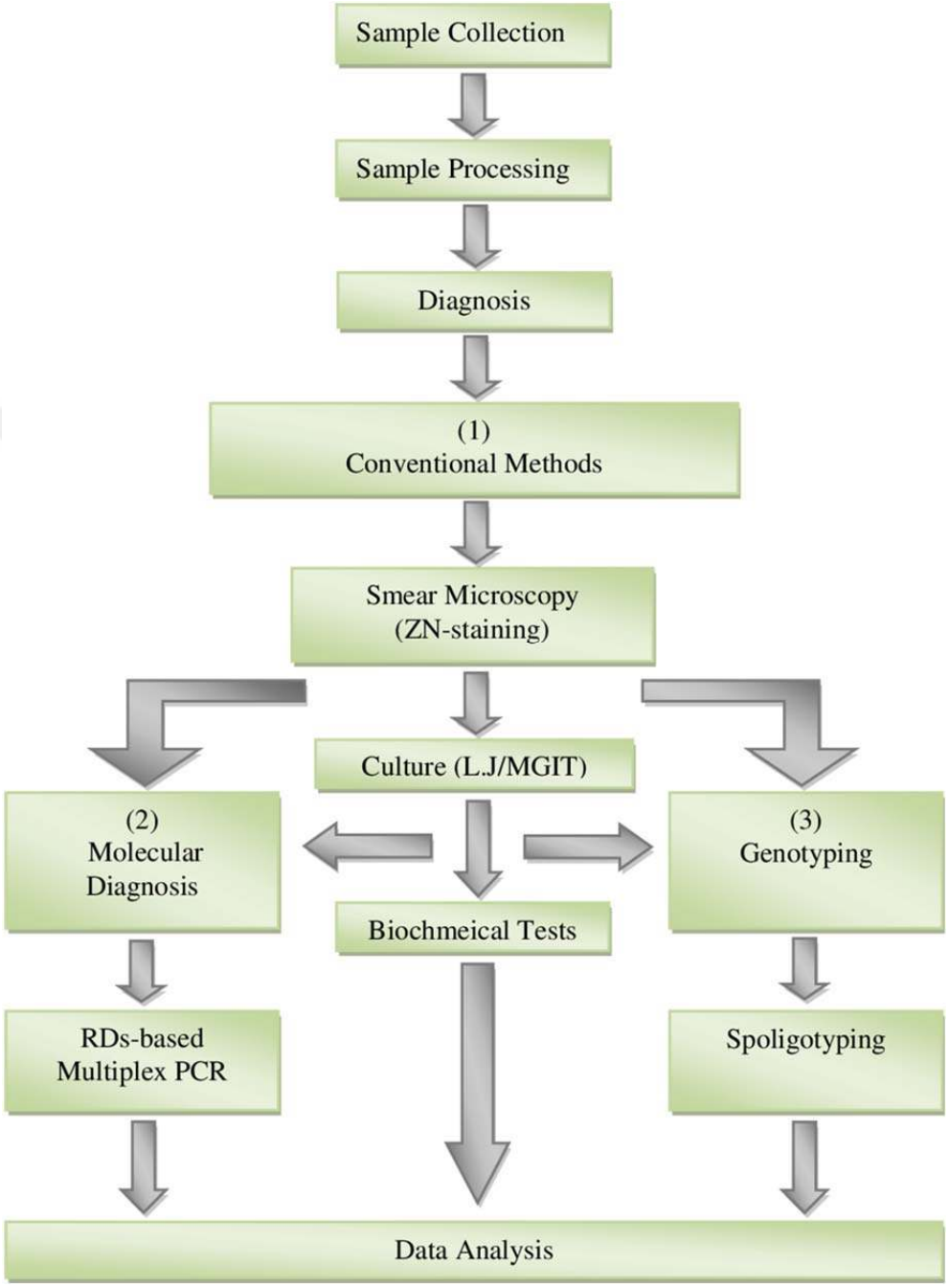

Figure 3.2. Flow diagram of our study procedure.

3.3. Liquid-Culture Inoculation (MGIT-culture):

1) The samples were inoculated into a liquid culture media which is a commercial based system specific for mycobacterial culture known as "BACTEC Mycobacterial Growth Indicator Tube (MGIT)" system and placed in MGIT 960 device.
2) The tubes contain a modified Middlebrook culture broth and a growth supplement; MGIT 960 SIRE Supplement (Oleic acid, dextrose, catalase, bovine albumin, polyoxyethylene stearate) added to each tube. Growth of mycobacteria depletes the oxygen present in the medium by a layer containing fluorophore at the bottom of each MGIT tube where the reduction in oxygen level activates this matter. (MGIT kit, BACTEC)
3) The Bactec MIGIT reads every tube in the instrument once each hour and compared the readings with other tubes taken previously. If the degree of fluorescence has changed significantly, the tube has been reported as positive, this usually occurred between 10-22 days. The tubes which remain negative for 6-8 weeks has been reported as negative.

3.4. Genomic DNA Extraction:

The DNA were extracted from the samples using Mickle method (cell disruptor apparatus) with the following procedure:

1) For each sample, an amount of 0.5 mL from the liquid-culture tube has been transferred to 1.5 mL eppendorf tube.
2) The samples were subjected to heat inactivation process at 80°C by VWR Digital Heat Block for 30 minutes.
3) The samples were spin at 12,700 rpm inside centrifuge for 15 minutes.
4) The supernatant has been discarded and 500 µl of TE (Tris-EDTA) buffer added then pipetted (Tris is the main buffering component. EDTA binds divalent cations such as Ca+2 and Mg+2 since these ions help maintain the integrity of the cell membrane, eliminating them with EDTA destabilizes

the membrane).

5) The samples were spin again at 12,700 for 15 minutes.
6) The supernatant has been discarded and 250 µl of TE (Tris-EDTA) buffer added then pipette.
7) A volume of ~100µl of acid washed glass beads (SIGMA) added and the samples were settled in Mickle cell disruptor apparatus for 2 minutes.
8) The samples were spin again at 12,700 rpm inside centrifuge for15 minutes.
9) The aqueous (upper) layer has been transferred to a fresh 1.5 mL eppendorf tube.
10) The DNA samples were preserved at – 20°C.

3.5. Molecular Diagnosis:

3.5.1. PCR Amplification:

The extracted DNA samples were used for multiplex PCR of three mixes where each mix contained different mixed three or four primers mixed together as well as single-plex (single target) PCR for testing any unclear band(s) on the gel after electrophoresis. All PCR tests were performed in duplicate and one of the samples was spiked with positive control DNA (extracted from H37Rv reference *M. tuberculosis* strain) to look for an inhibition in addition to negative control (a sample spiked with nuclease-free water).

3.5.2. Primer Design and Target gene/locus:

The primer pairs targeting each specific locus of *M. tuberculosis* complex were designed and BLAST search was performed to confirm its specificity. The primer pairs used in this study are listed in **Table 3.1.**

Table 3.1. List of the primer pairs used in this study (SIGMA Genosys)

Primer Type and Target Locus	Primer Name	Nucleotide sequence
16SrRNA	16SrRNA(F)	5' ACG GTG GGT ACT AGG TGT GGG TTT C 3'
	16SrRNA(R)	5' TCT GCG ATT ACT AGC GAC TCC GAC TTC A 3'
Rv0577	Rv0577(F)	5' ATG CCC AAG AGA AGC GAA TAC AGG CAA 3'
	Rv0577(R)	5' CTA TTG CTG CGG TGC GGG CTT CAA 3'
IS1561' (Rv3349c)	IS1561'(F)	5' GCT GGG TGG GCC CTG GAA TAC GTG AAC TCT 3
	IS1561'(R)	5' AAC TGC TCA CCC TGG CCA CCACCA TTG ACT 3'
Rv3877/8 (RD1)	Rv3877/8(F)	5' CGA CGG GTC TGA CGG CCA AAC TCA TC 3'
	Rv3877/8(R)	5' CTT GCT CGG TGG CCG GTT TTT CAG C 3'
Rv1510 (RD4)	Rv1510(F)	5' GTG CGC TCC ACC CAA ATA GTT GC 3'
	Rv1510(R)	5' TGT CGA CCT GGG GCA CAA ATC AGT C 3'
Rv1970 (RD7)	Rv1970(F)	5' GCG CAG CTG CCG GAT GTC AAC 3'
	Rv1970(R)	5' CGC CGG CAG CCT CAC GAA ATG 3'
Rv2073c (RD9)	Rv2073c(F)	5' TCG CCG CTG CCA GAT GAG TC 3'
	Rv2073c(R)	5' TTT GGG AGC CGC CGG TGG TGA TGA 3'
Rv3120 (RD12)	Rv3120(F)	5' GTC GGC GAT AGA CCA TGA GTC CGT CTC CAT 3
	Rv3120(R)	5' GCG AAA AGT GGG CGG ATG CCA GAA TAG T 3'
Rv1257c (RD13)	Rv1257c(F)	5' GGT GGC GAG CTG GAA TTC GTG AGA CAT TAC 3
	Rv1257c(R)	5' CAT CGC CGA GGA GCG GAA TCT GAT GAT 3'
Rv1773 (RD14)	Rv1773(F)	5' TGA CAA CGA ATC AAC GAA CC 3'
	Rv1773(R)	5' TCA GCA CTG ACA GCC TGA AC 3'

All of the primers used in this study were prepared according to the manufacturer procedure (SIGMA Genosys) and then diluted to final concentration of 25%.

3.5.3. Multiplex PCR Test:

In this study, we have used the method of Multiplex PCR where the primer pairs have been combined in different mixes, in order to reduce time and effort and also material consumption used in different steps of PCR amplification and analysis (Master mix, agarose, etc.).

3.5.4. Primer Mixing of Multiplex PCR:

The set of primer pairs used in this study were grouped in different mixes depending on the length of the expected bands (target products) for avoiding bands interference. Primer-BLAST tool was used to know the length of the expected product based on nucleotide sequence of each primer. The group mix of different primer pairs are listed in **Table 3.2.**

Table 3.2. List of the group mix of different primer pairs

Mix Number	Primer Pairs	Product Legnth (bp)
1st Mix	RD7	1116
	IS1561'	943
	RD9	600
	RD12	404
2nd Mix	RD4	1033
	Rv0577	786
	16SrRNA	543
3rd Mix	RD14	1521
	RD1	999
	RD13	456

3.5.5. Standardization of Multiplex PCR:

The multiplex PCR was standardized using different combinations of primer concentrations of three/four primer pairs as shown in **Table 3.3.**

Table 3.3. Combinations of different concentrations of primer pairs used in standardization of multiplex PCR

Mix Number	Primer Pairs (F+R)	Combination Pairs			
		1	2	3	4
1st Mix	RD7	0.15	0.25	0.50	0.75
	IS*1561'*	0.25	0.25	0.25	0.50
	RD9	0.15	0.25	0.50	0.25
	RD12	0.25	0.25	0.25	0.50
2nd Mix	RD4	0.15	0.25	0.50	0.75
	Rv0577	0.25	0.25	0.25	0.50
	16SrRNA	0.15	0.25	0.50	0.75
3rd Mix	RD14	0.15	0.25	0.50	0.75
	RD1	0.25	0.25	0.25	0.50
	RD13	0.15	0.25	0.50	0.75

The same concentration which used for both Forward (F) and Reverse (R) of each primer in all combinations and the difference in concentration was only between different primer pairs.

3.5.6. Optimization of Multiplex PCR:

The experiment was duplicated for testing the optimal combination of concentrations of primer pairs mixes and to check the analytical sensitivity of the

multiplex PCR test. For the first mix, the combination of concentrations of the third combination (0.50:0.25:0.50:0.25) gave the best result and showed highest sensitivity when performed by gel electrophoresis, and the second combination (0.25:0.25:0.25) gave the best result and showed highest sensitivity for the second mix, and the third one (0.50:0.25:0.50) gave the best result and showed highest sensitivity for the third mix, thus this combinations of concentrations were optimized and used accordingly in the final experiment. Each combination was checked with same concentrations of *M. bovis* (or *M. bovis* BCG) DNA template.

3.5.7. Multiplex PCR Test:

3.5.7.1. Multiplex PCR mixture additions for the 1st Mix:

1) 2X PCR Master mix (Thermo Scientific)*: 12.5 µl
2) Nuclease-free H_2O: 2.5 µl
3) DMSO: 1 µl
4) Primer pairs mix:

RD7 Forward: 0.25 µl

RD7 Reverse: 0.25 µl

IS*1561'* Forward: 0.50 µl

IS*1561'* Reverse: 0.50 µl

RD9 Forward: 0.25 µl

RD9 Reverse: 0.25 µl

RD12 Forward: 0.50 µl

RD12 Reverse: 0.50 µl

5) DNA template: 5 µl

All of these additions were added and mixed in same eppendorf tube with final volume of 25 µl for each sample.

* Composition of the PCR Master Mix (2X):

0.05 U/µL Taq DNA polymerase, reaction buffer, 4 mM MgCl2, 0.4 mM of each dNTP (dATP, dCTP, dGTP and dTTP).

3.5.7.2. Multiplex PCR mixture additions for the 2nd Mix:

1) 2X PCR Master mix (Thermo Scientific) : 12.5 µl
2) Nuclease-free H_2O: 4.75 µl
3) DMSO: 1.25 µl
4) Primer pairs mix:

RD4 Forward: 0.25 µl

RD4 Reverse: 0.25 µl

Rv0577 Forward: 0.25 µl

Rv0577 Reverse: 0.25 µl

16SrRNA Forward: 0.25 µl

16SrRNA Reverse: 0.25 µl

5) DNA template: 5 µl

All of these additions were added and mixed in same eppendorf tube with final volume of 25 µl for each sample.

3.5.7.3. Multiplex PCR mixture additions for the 2nd Mix:

1) 2X PCR Master mix (Thermo Scientific) : 12.5 µl
2) Nuclease-free H_2O: 4 µl
3) DMSO: 1 µl
4) Primer pairs mix:

RD14 Forward: 0.50 µl

RD14 Reverse: 0.50 µl

RD1 Forward: 0.25 µl

RD1 Reverse: 0.25 µl

RD13 Forward: 0.50 µl

RD13 Reverse: 0.50 µl

5) DNA template: 5 µl

All of these additions were added and mixed in same eppendorf tube with final volume of 25 µl for each sample.

3.5.7.4. Multiplex PCR Conditions:

The annealing temperature of each primer was calculated using NEB Tm Calculator tool (New England BioLabs), where most of the annealing temperatures of all primers were around 60°C except for RD14 where it was 50°C. However, the annealing temperature at 60°C for all primers showed the efficiency the same as the calculated ones when it tested individually. Therefore, the annealing temperature was standardized at 60°C for all primers according to this optimization. Consequently, the samples of different mixes could be placed on the same thermal cycler (Applied Biosystems Veriti™) with same conditions as shown in **Table 3.4.**:

Table 3.4. PCR program for Multiplex PCR used in this study

Step	Process	Temperature	Time	Cycles
1	Initial denaturation	94°C	5 min.	1
2	Denaturation	94°C	1 min.	25
	Annealing	60°C	1 min.	
	Elongation	72°C	1 min.	
3	Final elongation	72°C	10 min.	1

3.5.8. Single-plex PCR Test:

The unclear result band(s) on the gel after electrophoresis were subjected to Single-plex (single target) PCR test to confirm Multiplex PCR results.

3.5.8.1. Single-plex PCR mixture additions:

2X PCR Master mix (Thermo Scientific) : 12.5 µl

Nuclease-free H_2O: 6 µl

DMSO: 1 µl

Primer pairs:

Forward Primer: 0.25 µl

Reverse Primer: 0.25 µl

DNA template: 5 µl

All of these additions were added and mixed in same eppendorf tube with final volume of 25 µl for each sample.

3.5.8.2. Single-plex PCR Conditions:

The PCR conditions used for Single-plex PCR test are listed in **3.5.**

Table 3.5. PCR program for Single-plex PCR used in this study

Step	Process	Temperature	Time	Cycles
1	Initial denaturation	94°C	5 min.	1
2	Denaturation	94°C	1 min.	25
	Annealing	60°C	1 min.	
	Elongation	72°C	1 min.	
3	Final elongation	72°C	10 min.	1

3.6. Gel Electrophoresis:

The PCR products and DNA molecular-weight marker (50 bp DNA ladder) were electrophoresis on 2% agarose gel. The gel was run at 110 V for 40 minutes and the results were visualized using UV light inside transilluminator and gel photos were captured by the camera of the transilluminator.

3.7. Genotyping:

3.7.1. Spoligotyping:

3.7.1.1. Amplification of spacer DNA:

i. Principle of amplification:

Amplification of 43 spacers between the direct repeats (DRs) is accomplished using the primer DRa (biotinylated at 5 end) and the primer DRb, which originally described by Kamerbeek et al. (1997), and enable to amplify the whole DR region. Both primers were dissolved in 1.5 ml demineralized distilled water. Primer DRa is stored at 4°C and DRb is stored at 20°C. The primers with their sequences are listed below:

DRa: 5'-GGT TTT GGG TCT GAC GAC- 3' (biotinylated at 5′ end)

DRb: 5'-CCG AGA GGG GAC GGA AAC-3'

ii. PCR mixture additions for Spoligotyping:

1) 2X PCR Master mix (Thermo Scientific) : 12.5 µl
2) Nuclease-free H_2O: 6 µl
3) DMSO: 1 µl
4) Primer pairs:

DRa Primer: 0.25 µl

DRb Primer: 0.25 µl

5) DNA template: 5 µl

Two positive controls were used which containing 5µl of DNA from standard strain *M. tuberculosis* H37Rv and *M. bovis* BCG while 5µl of sterile distilled water was used as a negative control.

iii. PCR Conditions of Spoligotyping:

The PCR conditions used for Single-plex PCR test are listed in **Table 3.6.**

Table 3.6. PCR program for spoligotyping used in this study

Step	Process	Temperature	Time	Cycles
1	Initial denaturation	95°C	5 min.	1
2	Denaturation	94°C	1 min.	40
	Annealing	55°C	1 min.	
	Elongation	72°C	45 sec.	
3	Final elongation	72°C	10 min.	1

iv. Preparation of solutions:

The following solutions were used in spoligotyping and prepared as bellow:

- **0.5M EDTA (pH 8.0, autoclaved) :**
  - ✓ EDTA : 46.53 g
  - ✓ dH_2O : 250 mL
  - ✓ pH : 8.0

- **20mM EDTA (pH 8.0) :**
  - ✓ 0.5M EDTA : 40 mL
  - ✓ dH_2O : 960 mL

- **20X SSPE (pH 7.4, autoclaved):**
  - ✓ 0.2M Na_2HPO_4 : 28.39 g/L
  - ✓ 3.6M NaCl : 210.24 g/L
  - ✓ dH_2O : 960 mL
  - ✓ 0.5M EDTA : 40 mL

- **2X SSPE :**
  - ✓ 20X SSPE : 100 mL
  - ✓ dH_2O : 900 mL

- **10% SDS :**
  - ✓ dH_2O (60°C warm) : 250 mL
  - ✓ SDS : 25 g

- **2X SSPE / 0.1% SDS :**
  - ✓ 20X SSPE : 100 mL
  - ✓ 10% SDS : 10 mL
  - ✓ dH_2O : 890 mL

- **2X SSPE / 0.5% SDS solution :**
  - ✓ 20X SSPE : 100 mL
  - ✓ 10% SDS : 50 mL
  - ✓ dH_2O : 850 mL

- **1% SDS :**
  - ✓ 10% SDS : 100 mL
  - ✓ dH_2O : 900 mL

- **10X TE :**
  - ✓ Tris HCl : 15.764g (100mM)
  - ✓ 0.5M EDTA : 20 mL (10mM)
  - ✓ dH_2O : 980 mL

v. Hybridization with PCR product and detection:

a) Principle of hybridization

The PCR products were hybridized to a membrane containing 43 spacer-oligonucleotides by reverse line blotting. Oligonucleotides are linked to an activated membrane in parallel lines. PCR products are hybridized perpendicularly to the oligonucleotide lines. After hybridization the membrane was incubated in streptavidin-peroxidase which binds to the biotin labeled PCR products and detected by enhanced chemiluminescence (ECL) system. The peroxidase present on the streptavidin catalyzes the reaction which results in emission of light and was detected on a stretch film as the following procedure:

1) All buffer solutions used to prepared the membrane were pre-warmed to the required temperature and quantities as bellow:
   - 80 mL 2X SSPE / 0.1% SDS : 60°C
   - 160 mL 2X SSPE / 0.5% SDS : 60°C
   - 160 mL 2X SSPE / 0.5% SDS : 42°C
   - 160 mL 2X SSPE : room temp.
2) 20 mL of PCR product was added onto 150mL of 2X SSPE/0.1% SDS.
3) PCR products were heat-denatured at 99°C for 10 minutes, then and cooled on ice immediately.
4) While denaturing the PCR products, the membrane (ISOGEN) was washed with 80ml 2X SSPE / 0.1% SDS at 60°C for 5 minutes.
5) The membrane and a sponge pad (Immunetiks Plastic Cushion PC200) were placed into the miniblotter (Miniblotter-3024), the slots of the miniblotter were perpendicular to the oligonucleotides and in line to the membrane.
6) Residual liquid in the slots was aspirated by a vacuum pump.
7) Slots were filled with diluted hot PCR product then the miniblotter was placed inside the oven (FINEPCR-combi-SV120) for 60 minutes at 60°C

in horizontal position for hybridization.

8) The samples were aspirated from the slots and the membrane was removed from the miniblotter using plastic collets.
9) The membrane was washed twice with 80 mL 2X SSPE / 0.5% SDS at 60°C for 10 minutes.
10) The membrane was placed into a plastic bag (file).
11) 1.25 mL of streptavidin-alkaline phosphatase conjugate (500U/mL) (Promega) was added onto 5 mL of 2X SSPE / 0.5% SDS, the mixture was poured into a plastic bag (three sides sealed by heating), the air bubbles were removed and the fourth side were closed and then incubate at 42°C for 45-60 minutes inside water bath.
12) The membrane was incubated twice with 80 mL 2X SSPE / 0.5% SDS for 10 min at 42°C,
13) The membrane rinsed with 80 mL 2XSSPE for 5 minutes with shaking at room temperature.
14) 5 mL ECF substrate (Attophos AP Flourescent Substrate, Promega) was added onto the membrane, then this bag was preserved in dark for 60 minutes.
15) The membrane wrapped with stretch film and exposed in the appropriate illumination cassette (450nm) for 20 seconds (QUANTUM-ST4 3020-WL/BLUE/20M).

The presence or absence of spacers was visualized as a radioactive squares. *M. tuberculosis* H37Rv and *M. bovis* BCG were used as positive controls in each run.

vi. Regeneration of the membrane :

The hybridized PCR product was dissociated from the membrane in order to regenerate the membrane for the next hybridization experiment. The membrane

was washed twice with 1% SDS at 80°C for 30 min, and then with 100 mL 20 mM EDTA pH 8, for 15 min at room temperature. The membrane was stored in 20 mL of 20mM EDTA at 4 °C until use (sealed in plastic file, to avoid dehydration of the membrane).

3.7.1.2. Result Analysis of Hybridization:

The standard spoligotyping assay was performed by using a membrane. In this membrane, each one of the 43 spacers showed either a radioactive spot (indicating the presence of the spacer) or not (indicating the absence of the spacer). To simplify recording, the band pattern was converted to a series of "■" and "□" where these signs indicate:

■ : The spacer is present.

□ : The spacer is absent.

This series of "■" and "□" gives a binary code of a single spoligotype which consists of 43 digits as shown in Figure 4.6.

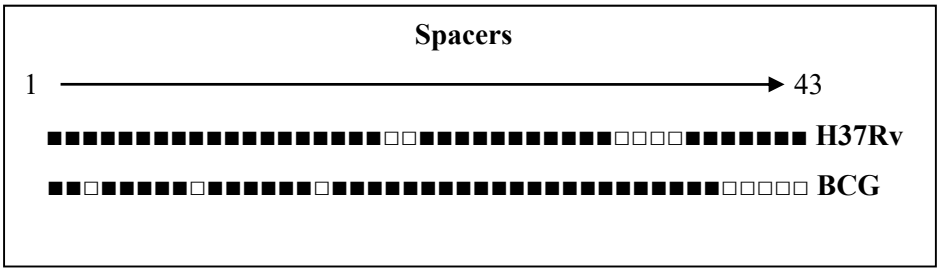

Figure 3. 3. Blotting representations of spoligotype patterns (H37Rv and BCG)

The spacers 20–21 and 33-36 are absent in *M. tuberculosis* H37Rv strain and the spacers 3, 9, 16 and 39-43 absent in *M. bovis* BCG strain. These strains were used as control for spoligotyping.

3.7.2. Spoligotyping Strain Clustering Analysis:

Spoligotypes in a binary code were entered into a Microsoft excel database and into an international database named SITVIT2 of Pasteur Institute of Guadeloupe (largest worldwide database of genotyping markers for *M. tuberculosis*) which is an updated version of the previously released of the fourth International Spoligotyping Database (SpolDB4).

SITVIT2 database (available online) contains genotypic data for the reference strains representing the major MTBC lineages. The spoligotyping in a binary format was converted to an octal code as shown in **Figure 4.7.** for comparison with the codes in the SITVIT2 database, these databases can be queried using data generated for genotyping methods and a strain lineage can be assigned.

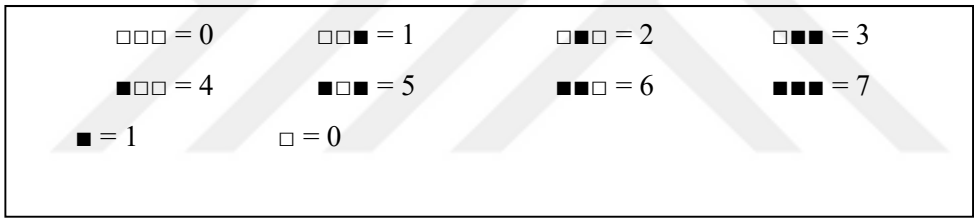

Figure 3. 4. The octal code key used to evaluate spoligotyping results

In order to assign designations like shared-types (ST) which are also known as spoligotype international type (SIT), binary outcomes comparison was submitted to the SITVIT2. It provides SIT numbers or orphan status to uploaded strains. When two or more isolates were present in the database with identical profiles, SIT number was assigned. If not, it was deemed an orphan strain. Lineage, family and sub-family were assigned to strains according to the supplemental updated SpolDB4 profiles. The existence of the SITVIT2 database means that spoligotypes can be used to make global comparisons between strains.

3.8. Data and statistical analysis :

The Statistical Analysis System- SAS (2012) program was used to effect of difference factors in study parameters. Chi-square test was used to significant compare between percentages results of this study.

4. RESULTS AND DISCUSSION

4.1. RDs-based Multiplex PCR

The results among all clinical samples collected from different areas of Cukurova region were subjected to Multiplex PCR based on the region of differences genes between different species of *M. tuberculosis* complex in order to differentiate *M. bovis* species from *M. bovis* BCG species.

4.1.1. Region of Difference Typing

The obtained results were compared to the pervious related studies according to the present or absent of these RDs to ascertain their usefulness as part of an MTBC PCR typing panel. This method allows identification of *Mycobacterium tuberculosis* complex subspecies and associated lineages.

For this study it was necessary to determine the presence of mycobacterial DNA. Species-specific nucleotide polymorphisms in the 16SrRNA gene have been used for detection of Mycobacterium species and differentiate it from Mycobacterium other than tuberculosis species (MOTT) (Springer et al. 1996, Turenne et al. 2001). For these matter, the 16SrRNA designed primer pair were successful in amplifying a DNA fragment from some mycobacterial isolates of our samples (total of 43 isolates) but it were failed in amplifying DNA fragment from another (total of 5 isolates).

In general, the RDs-based Multiplex PCR with the prepared mixes was failed in amplifying some of the obtained samples, this could be due to a contamination source in these samples (*n=6*), these samples have been excluded in further result analysis.

The Rv0577 gene is restricted to the MTBC subspecies, thus the *presence* of the Rv0577 gene differentiates Mycobacterium tuberculosis complex (MTBC) subspecies from Mycobacterium other than tuberculosis (MOTT) species (Huard et al. 2003). In our study, Rv0577 was specifically and consistently amplified from all

MTBC subspecies and strains tested (*n=43*) whereas none of the MOTT strains (*n=5*) showed a PCR fragment as shown in Table 4.1. This indicated that Rv0577 is a genotypic marker for the MTBC and that it can be used to distinguish the MTBC subspecies from MOTT species (Huard et al. 2003).

The Rv2073c (RD9) gene is restricted to *M. canettii* and *M. tuberculosis* species, thus the *presence* of this gene differentiates *M. tuberculosis* and *M. canettii* from the other MTBC subspecies (Brosch et al. 2002). In our study, Rv2073c (RD9) gene was presented in some tested isolates (*n=14*) these isolates have been distinguished as *M. tuberculosis* (*n=9*) and as other species which will be discussed later on (*n=5*). Whereas this gene was deleted from the other MTBC strains (*n=30*) as shown in Table 4.1.

The Rv1970 (RD7) gene differentiates *M. tuberculosis*, *M. africanum* subtype II, and "*M. canettii*" from the other MTBC subspecies. In the previous studies, PCR analysis revealed RD7 to be *present* in all of the tested *M. tuberculosis* and "*M. canettii*" strains, as well as some *M. africanum* isolates, but not in the evaluated *M. microti*, *M. bovis*, *M. bovis BCG*, and *M. caprae* strains (Brosch et al. 1998, Gordon et al. 1999a, Mostowy et al. 2002). In this study, RD7 primers were generated to amplify Rv1970 gene, and the PCR results were positive for *M. tuberculosis* (*n=9)* and in some isolates (*n=2*) which will be discussed later on. Table 4.1.

Absence of the IS*561'* element differentiates *M. microti* from the other MTBC subspecies. In the previous studies, it has been indicated that PCR amplification for the transposase pseudogene fragment IS*1561'* (Rv3349c) was positive for all MTBC isolates tested except for their single *M. microti* (OV254) strain, and they postulated that it might prove to be a *M. microti*-specific marker (Gordon et al. 1999b). In our study, primers to the IS*1561'* element were generated and the collected MTBC strains were evaluated. The IS*1561'* PCR fragment was found to be present in all of the evaluated MTBC isolates (*n=33*) but was absent in some isolates which are belongs to *M. microti* and other strain which will be

discussed later on Table 4.1.

The Rv3120 (RD12) gene differentiates *M. bovis*, *M. bovis* BCG, *M. caprae*, and "*M. canettii*" from the other MTBC species. RD^{can} is a unique "*M. canettii*"- specific deletion that partially overlaps with RD12, both of which include the gene Rv3120 (Brosch et al. 2002). In our study, RD12 primers were generated to amplify within Rv3120 gene. A PCR fragment of the correct size was detected from the evaluated *M. tuberculosis* (*n=9*) and in some isolates (*n=4*) which will be discussed later on. Table 4.1.

The Rv1257c (RD13) gene differentiates *M. bovis*, *M. bovis* BCG, *M. caprae* from the other MTBC species. Specific deletion (*Absence*) of this region in these species distinguishs it from the other MTBC subspecies (Kremer et al. 1999). In our study, RD13 primers to the Rv1257c gene were generated and the collected MTBC strains were evaluated. The Rv1257c PCR fragment was found to be present in all *M. tuberculosis* strains (*n=9*) as well as in a new strain (*M. tuberculosis* RD^{Rio}) which will be discussed later. Table 4.1.

Absence of the Rv1510 (RD4) gene differentiates *M. bovis* and *M. bovis* BCG from the other MTBC subspecies. In the previous studies, it has been indicated that PCR primers generated to the RD4 locus could distinguish *M. bovis* (strict sense) and *M. bovis* BCG from the rest of the MTBC subspecies (Behr et al. 1999, Brodin et al. 2002, Gordon et al. 1999a). In our study, RD4 primers were generated to Rv1510 in order to confirm *M. bovis* subspecies identity and therefore this amplification experiment was unexpectedly successfully amplified this region and RD4 gene was presented in all *M. bovis* BCG strains (*n=16*). This unexpected case will be discussed later on. However, one of *M. bovis* BCG strains showed RD4 deletion. Table 4.1.

Absence of the Rv3877 (RD1) gene differentiates *M. bovis* BCG from the other MTBC subspecies. The RD1 locus was found previously to be selectively absent in all *M. bovis* BCG isolates and, therefore, the presence of RD1 has been shown to be a good negative marker for *M. bovis* BCG (Behr et al. 1999, Gordon et

al. 1999a). In our study, RD1 primers were generated to amplify within Rv3877 gene and the collected MTBC strains were evaluated. The Rv3877 PCR fragment was found to be absent in all of *M. bovis* BCG strains (*n=20*) and, therefore, this specific deletion in region of difference 1 provided a good was a good marker for *M. bovis* BCG identification. Table 4.1.

Absence of the Rv1773 (RD14) differentiates *M. bovis* BCG-Pasteur from the other MTBC subspecies including *M. bovis* and *M. bovis* BCG (classical/Tokyo). In the previous studies, it has been indicated that Rv1773 gene is a transcriptional repressor deleted from BCG-Pasteur (Alexander and Behr 2007). In our study, The Rv1773 PCR fragment was found to be present in all *M. bovis* BCG strains except in one isolate where it was belong to *M. bovis* BCG-Pastuer. Table 4.1.

IS*1561'* Deletion in *M. tuberculosis*; this case has occurred in sample number 8, where PCR failed in amplifying IS*1561'* locus resulted in a negative on the gel for this region. This case has been previously defined as RD^{Rio} (Brazil) Wild-type strain of *M. tuberculosis* and this deletion in IS*1561'* gene emerging as a determinant of biological diversity. These strains were also found in Latin American, Europe and Africa (Gibson et al. 2008).

In addition to these common results, we have found some uncommon results among MTBC strains such as the positive result for the RD4 region in some samples, this case does not exist in the *M. bovis* strains according to the previous studies; it is supposed that there was a mutation of an insertion event occurred in this region led to this new profile of *M. bovis* BCG, which named as *M. bovis* BCG^{RD4+}. Moreover, there are other results that need to be confirmed by another set of flanking primers for testing the deletion of these regions in further studies. Table 4.1.

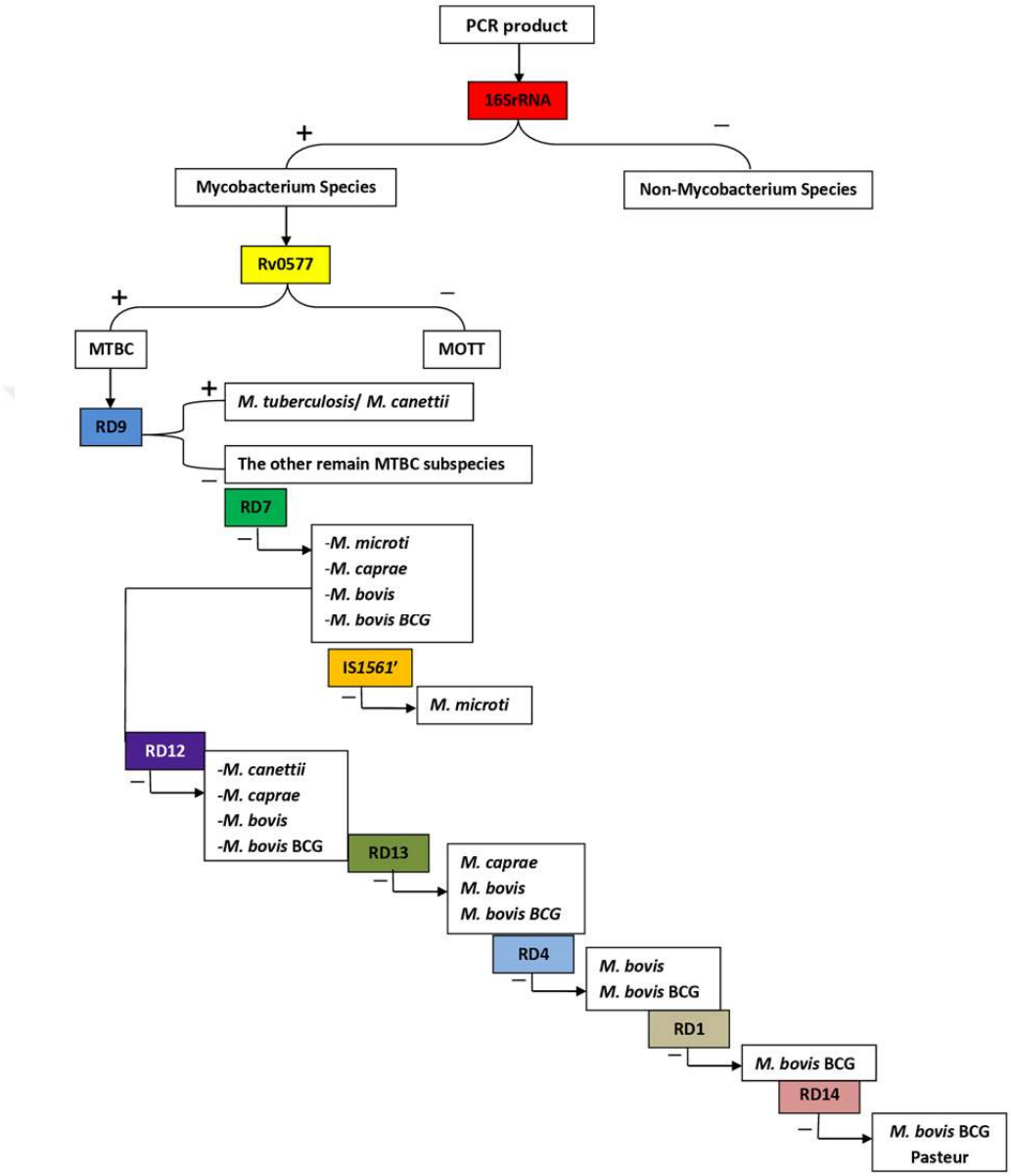

Figure 4.1. Summarized scheme for the genes and the RDs used in our study

4.1.2. Multiplex PCR Results and Data Analysis:

4.1.2.1. Guidelines for investigation the results obtained:

1) The captured photos of the gel under the transilluminator have been “color inverted” and “contrast increased” for a sharp view of the bands.
2) The obtained “double well line” gel photos have been separated into two separate photos “single well line” for giving enough area to the samples numbers and the RDs for each sample.
3) We have constructed the best scheme to demonstrate the gene and RDs locus for our multiplex PCR results. The RD regions in each constructed scheme have been ordered according to the size of the expected bands (bigger to lower), and each sample took 3 wells in the gel each well contained different mixed primers (4 or 3) with a total of 10 primers for each sample, as below:

	Primer type/ target locus	Size (bp)
1st mix	**RD7**	1,116
	IS1561	943
2nd mix	**RD9**	595
	RD12	404
3rd mix	----------	----------
	RD4	1,033
	Rv0577	786
	16SrRNA	543
	----------	----------
	RD14	1521
	RD1	999
	RD13	456

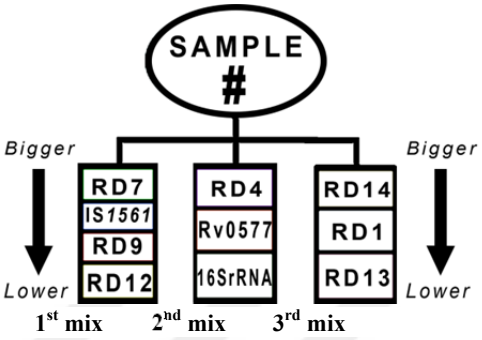

Figure 4.2. Scheme demonstrating the gene and RDs locus for our multiplex PCR

5) Explanation of the signs on the right of the RD regions:

⊕: The band on the gel is Positive.

⊖: The band on the gel is Negative.

±: The band on the gel is unclear; either exist or not. To confirm this result, the samples with +/– result has been subjected to a "Single-Plex" experiment.

6) Marker and Controls (+ and –) have the below scheme. However, there are No Controls for the samples 25 to 48 were used for giving a space to additional samples in the experiment since the controls have been tested in the previous experiments and all of the samples were had the same conditions (same PCR mixture, etc.).

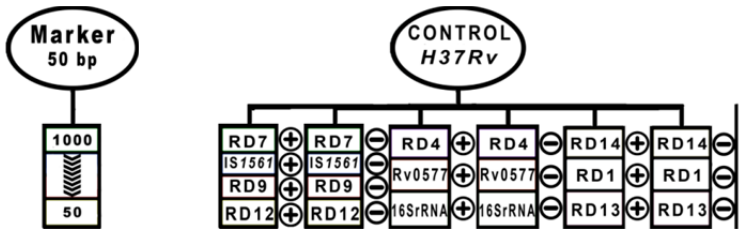

Figure 4.3. Scheme of marker and controls

7) The results of the samples have been arranged in a table under each photo and the expected mycobacterial strain has been written in a table as well as in a box above the samples.

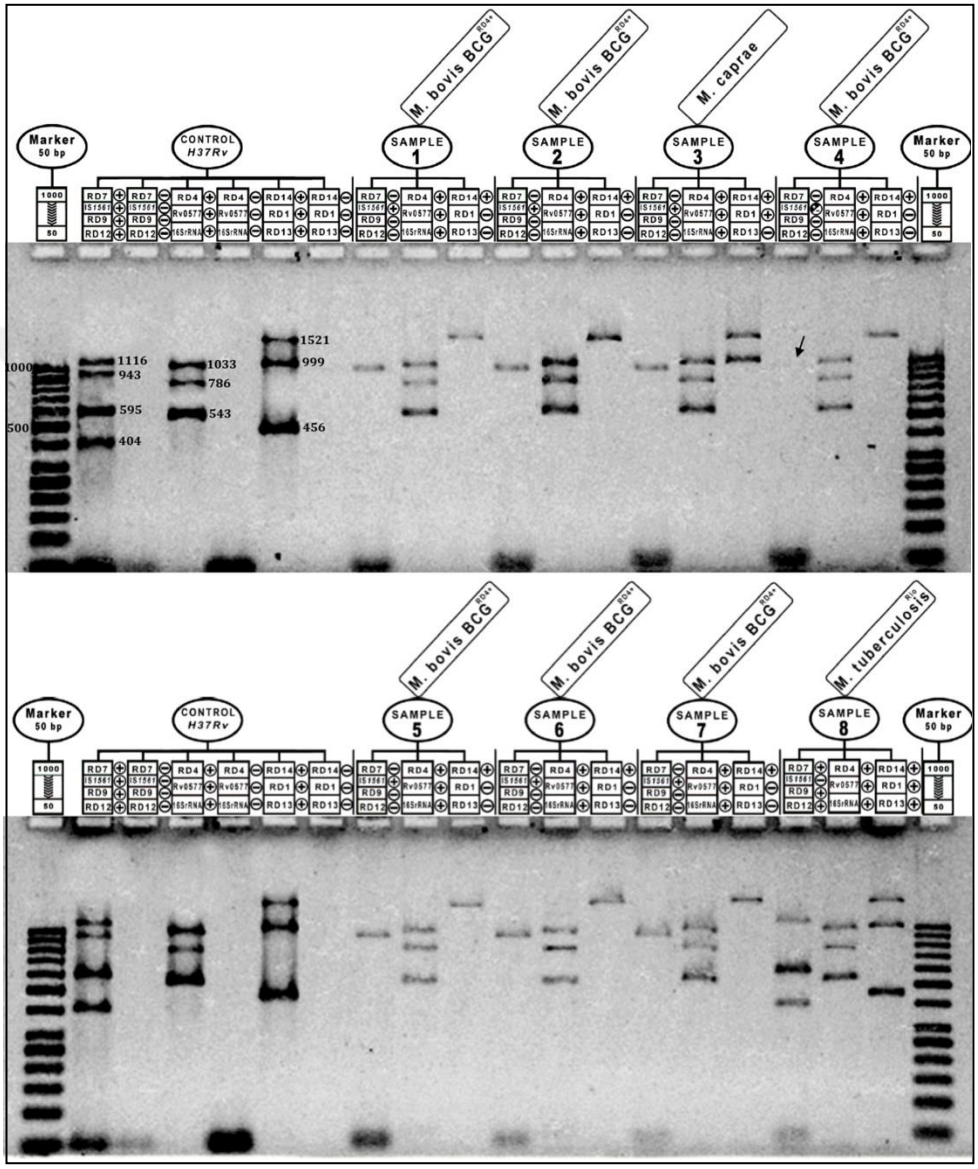

Figure 4.4. Multiplex PCR amplification results visualized on 2% agarose gel for the samples 1 to 8 in addition to positive and negative controls. 50 bp DNA ladder was used

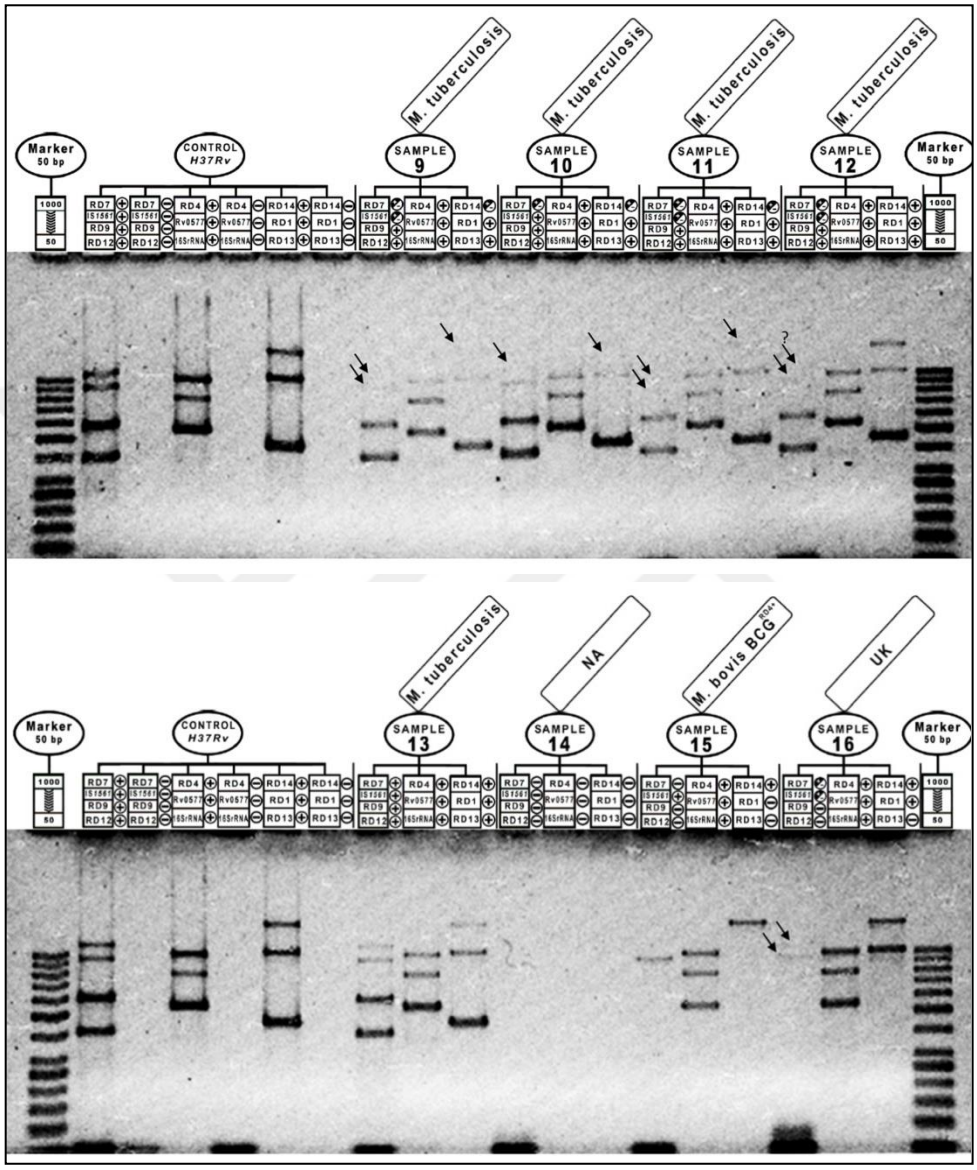

Figure 4.5. Multiplex PCR amplification results visualized on 2% agarose gel for the samples 9 to 16 in addition to positive and negative controls. 50 bp DNA ladder was used

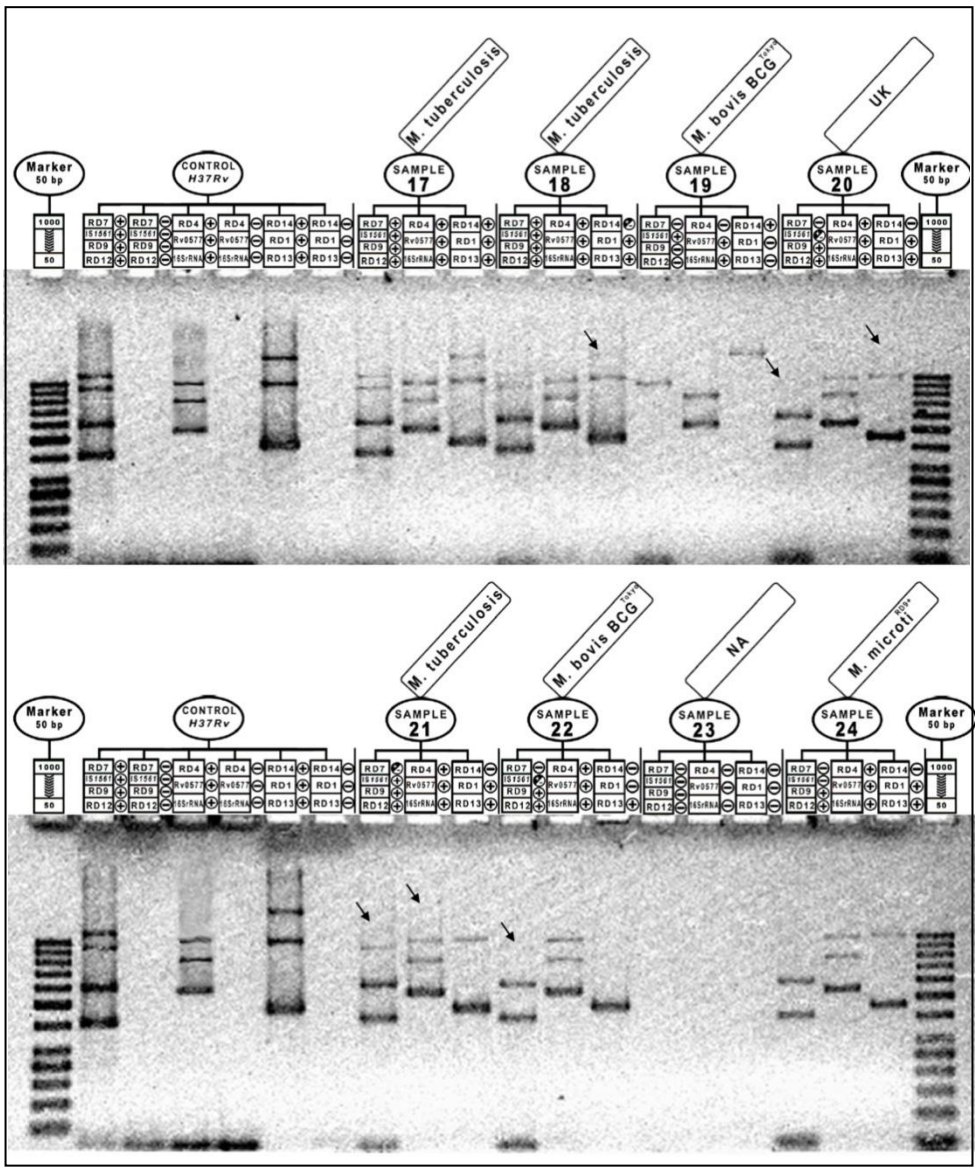

Figure 4.6. Multiplex PCR amplification results visualized on 2% agarose gel for the samples 17 to 24 in addition to positive and negative controls. 50 bp DNA ladder was used

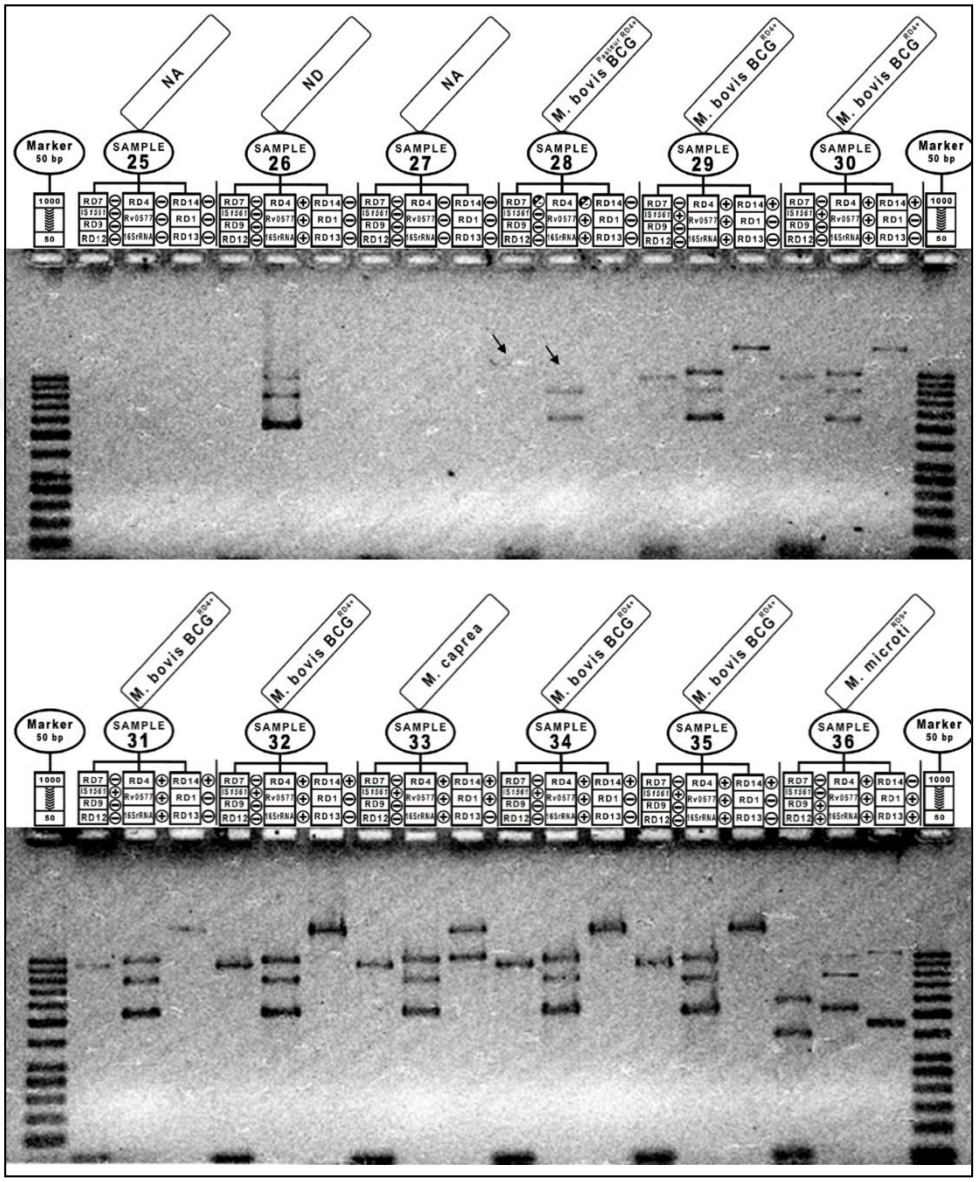

Figure 4.7. Multiplex PCR amplification results visualized on 2% agarose gel for the samples 25 to 36 in addition to positive and negative controls. 50 bp DNA ladder was used

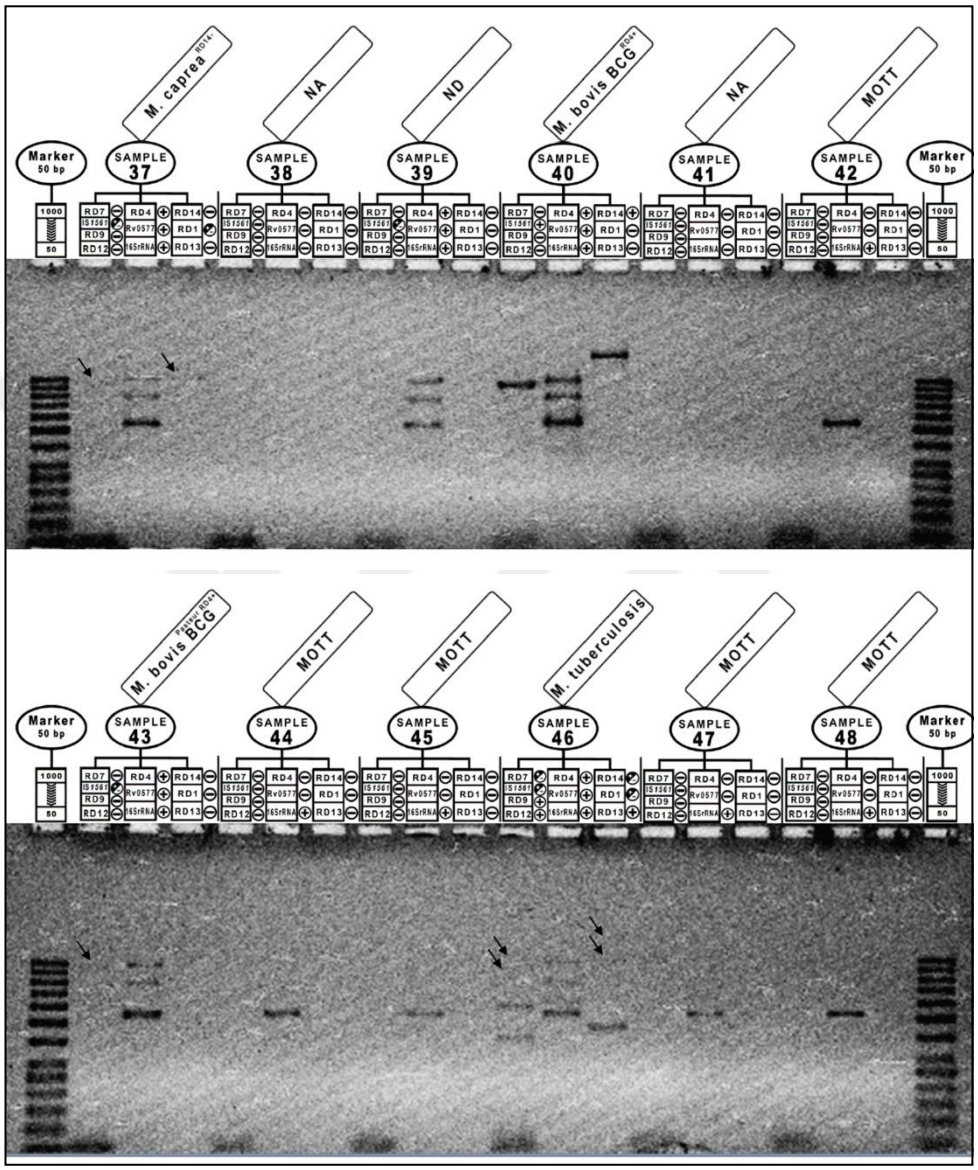

Figure 4.8. Multiplex PCR amplification results visualized on 2% agarose gel for the samples 37 to 48 in addition to positive and negative controls. 50 bp DNA ladder was used

Table 4.1. Multiplex PCR results for the primer mixes

Sample	RD-7	IS*1561*	RD-9	RD-12	RD-4	Rv057	16SrR	RD-14	RD-1	RD-13	Strain
H37Rv (+)	+	+	+	+	+	+	+	+	+	+	**Positive Control**
H37Rv (–)	–	–	–	–	–	–	–	–	–	–	**Negative Control**
Sample(1)	–	+	–	–	+	+	+	+	–	–	***M. bovis* BCG^{RD4+}**
Sample (2)	–	+	–	–	+	+	+	+	–	–	***M. bovis* BCG^{RD4+}**
Sample (3)	–	+	–	–	+	+	+	+	+	–	***M. caprae***
Sample (4)	–	+/-	–	–	+	+	+	+	–	–	***M. bovis* BCG^{RD4+}**
Sample (5)	–	+	–	–	+	+	+	+	–	–	***M. bovis* BCG^{RD4+}**
Sample (6)	–	+	–	–	+	+	+	+	–	–	***M. bovis* BCG^{RD4+}**
Sample (7)	–	+	–	–	+	+	+	+	–	–	***M. bovis* BCG^{RD4+}**
Sample (8)	+	–	+	+	+	+	+	+	+	+	***M. tb* RD^{Rio}**
Sample (9)	+/-	+/-	+	+	+	+	+	+/-	+	+	***M. tuberculosis***
Sample (10)	+/-	+	+	+	+	+	+	+/-	+	+	***M. tuberculosis***
Sample (11)	+/-	+/-	+	+	+	+	+	+/-	+	+	***M. tuberculosis***
Sample (12)	+/-	+/-	+	+	+	+	+	+	+	+	***M. tuberculosis***
Sample (13)	+	+	+	+	+	+	+	+	+	–	***M. tuberculosis***
Sample (14)	–	–	–	–	–	–	–	–	–	–	**NA**
Sample (15)	–	+	–	–	+	+	+	+	–	–	***M. bovis* BCG^{RD4+}**
Sample (16)	+/–	+/-	–	–	+	+	+	+	+	–	**UK**
Sample (17)	+	+	+	+	+	+	+	+	+	+	***M. tuberculosis***
Sample (18)	+	+	+	+	+	+	+	+/-	+	+	***M. tuberculosis***
Sample (19)	–	+	–	–	–	+	+	+	–	–	***M. bovis* BCG^{Tokyo}**
Sample (20)	–	+/-	+	+	+	+	+	+/–	+	+	**UK**
Sample (21)	+/-	+	+	+	+	+	+	+/-	+	+	***M. tuberculosis***
Sample (22)	–	+/-	+	+	+	+	+	–	–	+	***M. bovis* BCG^{Pasteur}**

Table 4.1. Multiplex PCR results for the primer mixes –*continued*-

Sample (23)	–	–	–	–	–	–	–	–	–	–	NA
Sample (24)	–	–	+	+	+	+	+	–	+	+	***M. microti*** $^{RD9+}$
Sample (25)	–	–	–	–	–	–	–	–	–	–	**NA**
Sample (26)	–	–	–	–	+	+	+	–	–	–	**ND**
Sample (27)	–	–	–	–	–	–	–	–	–	–	**NA**
Sample (28)	+/-	+/-	–	–	+/-	+	+	–	–	–	***M. bovis*** **BCG** $^{PasteurRD4+}$
Sample (29)	–	+/-	–	–	+	+	+	+	–	–	***M. bovis*** **BCG** $^{RD4+}$
Sample (30)	–	+	–	–	+	+	+	+	–	–	***M. bovis*** **BCG** $^{RD4+}$
Sample (31)	–	+	–	–	+	+	+	+	–	–	***M. bovis*** **BCG** $^{RD4+}$
Sample (32)	–	+	–	–	+	+	+	+	–	–	***M. bovis*** **BCG** $^{RD4+}$
Sample (33)	–	+	–	–	+	+	+	+	+	–	***M. caprae***
Sample (34)	–	+	–	–	+	+	+	+	–	–	***M. bovis*** **BCG** $^{RD4+}$
Sample (35)	–	+	–	–	+	+	+	+	–	–	***M. bovis*** **BCG** $^{RD4+}$
Sample (36)	–	–	+	+	+	+	+	–	+	+	***M. microti*** $^{RD9+}$
Sample (37)	–	+/-	–	–	+	+	+	–	+/–	–	***M. caprae*** $^{RD14–}$
Sample (38)	–	–	–	–	–	–	–	–	–	–	**NA**
Sample (39)	–	+/–	–	–	+	+	+	–	–	–	**ND**
Sample (40)	–	+	–	–	+	+	+	+	–	–	***M. bovis*** **BCG** $^{RD4+}$
Sample (41)	–	–	–	–	–	–	–	–	–	–	**NA**
Sample (42)	–	–	–	–	–	–	+	–	–	–	MOTT
Sample (43)	–	+/-	–	–	+	+	+	–	–	–	***M. bovis*** **BCG** $^{Pasteur\ RD4+}$
Sample (44)	–	–	–	–	–	–	+	–	–	–	**MOTT**
Sample (45)	–	–	–	–	–	–	+	–	–	–	**MOTT**
Sample (46)	+/-	+/-	+	+	+	+	+	+/-	+/-	+	***M. tuberculosis***
Sample (47)	–	–	–	–	–	–	+	–	–	–	**MOTT**
Sample (48)	–	–	–	–	–	–	+	–	–	–	**MOTT**

Sign: +, region is present; –, region is deleted, NA :No amplification, ND: No data, UK: Unknown.

4.2. Single-plex PCR Results and Data analysis:

The single-plex PCR results for the target loci of unclear band(s) which has not detected by Multiplex PCR test are shown in **Table 4.1.** with bold font and also separated as the following table. **Table 4.2.**

Table 4.2. Single-plex PCR results for the single pair primers

Sample	RD-7	IS*1561'*	RD-4	RD-14	RD-1
Sample (4)	NT	+	NT	NT	NT
Sample (9)	+	+	NT	+	NT
Sample (10)	+	NT	NT	+	NT
Sample (11)	+	+	NT	+	NT
Sample (12)	+	+	NT	NT	NT
Sample (16)	–	+	NT	NT	NT
Sample (18)	NT	NT	NT	+	NT
Sample (20)	NT	+	NT	–	NT
Sample (21)	+	NT	NT	+	NT
Sample (22)	NT	+	NT	NT	NT
Sample (28)	+	+	+	NT	NT
Sample (29)	NT	+	NT	NT	NT
Sample (37)	NT	+	NT	NT	+
Sample (39)	NT	–	NT	NT	NT
Sample (46)	+	+	NT	+	+

NT: Not tested for this region.*

4.3. Spoligotyping Results:

This part of the study was carried out previously at CU Tropical Diseases Research and Application Center laboratory by our research group on the same isolates. The results of our RDs-based Multiplex PCR method has a correlation with the typical spoligotypes of *M. tuberculosis* and *M. bovis* strains. The results also were compared with *Mycobacterium bovis* Spoligotype Database (Mbovis.org) and MIRU-VNTR*plus* website. The figure below indicate the different spoligotype patterns of *M. tuberculosis*, *M. bovis*, and BCG strains. Figure 4.9.

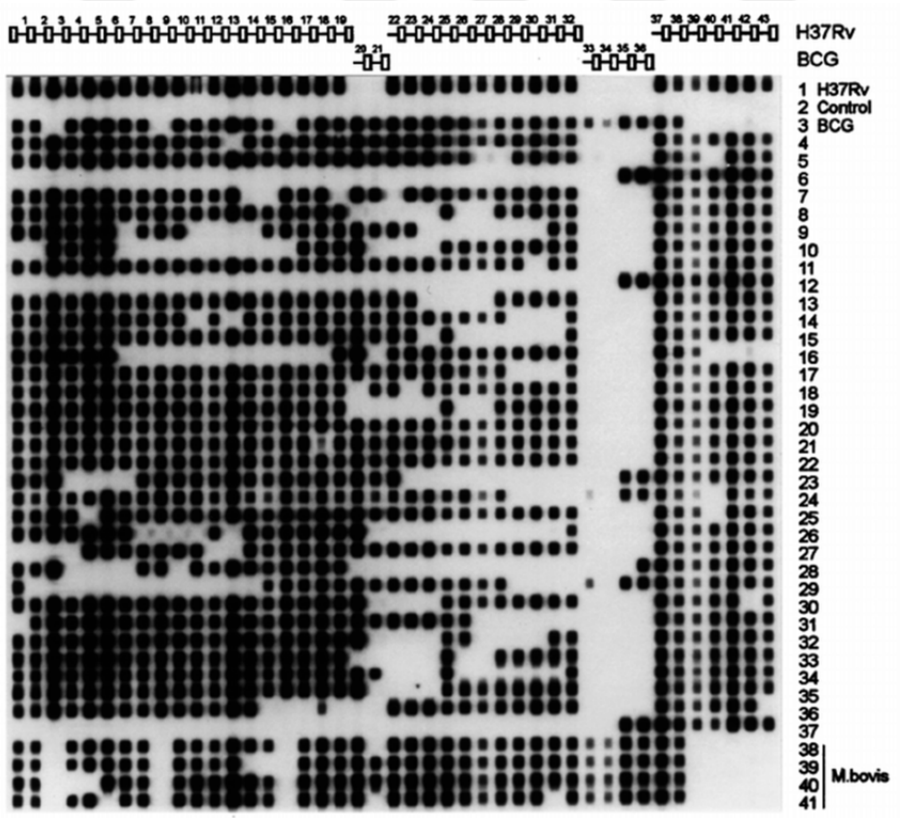

Figure 4.9. Hybridization patterns (spoligotypes) of amplified mycobacterial DNAs of *M. tuberculosis* and *M. bovis* strains. This figure is from Kamerbeek et al's work (Kamerbeek et al. 1997)

Spoligotype patterns were successfully identified in most of the isolates which showed unique patterns. However, some isolates failed to show any pattern probably due to lack of enough DNA or test limitations. In addition, spoligotyping was not carried out on all the isolates due to the lack of some resources in our laboratory.

5. CONCLUSION AND RECOMMENDATION

This study aimed to determine the species and strain lineages of MTBC in Cukurova region. In addition, information was generated on the dynamics of tuberculosis spread by *M. bovis*. In addition, an initiative will be taken into establishing a database for *Mycobacterium bovis* infections as a source of tuberculosis and to raise awareness about the public health risks associated with the presence of *M. bovis*-associated TB infected human in Turkey. The RDs-based Multiplex PCR is an enhanced technique over current phenotypic and molecular-based determination methods of MTBC subspecies and sublineages. Though, the ability for independent strain-specific deletions that overlap other subspecies-specific or lineage-specific LSPs is an intrinsic concern of RDs-based PCR targeting techniques for the differentiation of MTBC subspecies (Huard et al. 2003). In this study, the emerging of RD regions resulted in uncommon isolates with novel strain-specific RDs-based Multiplex PCR profiles or rare outliers that give the RDs-based Multiplex PCR profile of another MTBC subspecies as described before by Huard et al (Huard et al. 2003).

Moreover, for this work we tried to develop an IT-tool (RDs-tool) based on the presence or absence of the RDs and other segments, which could enable the rapid and accurate differentiation of MTBC members especially within *M. bovis* and *M. bovis* BCG strains without the need to review the previous studies and articles. Currently, it has been recognized that different genotypes of *M. tuberculosis* complex are predominantly in different geographical regions of the world. It has also been widely considered that variation from strain to strain can have major phenotypic consequences, which include virulence, transmissibility and treatment response. These variations will impact future treatments, drugs and vaccines and thus on product design and the management of patients. Therefore, further regional studies are extremely important in order to determine the entire range of strains of MTC most implicated in diseases worldwide. Therefore it is

clear that molecular-based techniques should be recommended in reference mycobacteriological laboratories for the MTBC speciation tests. Nonetheless, molecular genotyping has indicated that the lineages of *Mycobacterium tuberculosis* are geographically restricted and associated with specific ethnic populations. The predominant cause of TB in Rio de Janeiro, Brazil, was a certain genotype of the LAM lineage (Lazzarini et al. 2007). A single novel long sequence polymorphism (>26.3 kb) was characterized by these strains and designated RD^{Rio}. This strain with the genomic deletion in IS*1561'* region was failed to be amplified in our panel of PCR targets this locus for one sample. However, an extension work for this research will be specifically testing the flanking regions of the RD^{Rio} locus in order to confirm this genetic deletion. The identification of RD^{Rio} strains in our region contributes to the ongoing intercontinental dissemination of this important genotype.

The RDs-based Multiplex PCR technique could also play a role in determining the best treatment approach for particular cases in areas with a high incidence of tuberculosis where the co-infection of HIV makes the diagnosis of tuberculosis more difficult and/or where the differentiation from non-tuberculosis mycobacteria species is of the extreme importance. The RDs-based Multiplex PCR will play a role in the rapid identification of *M. bovis* and BCG strains and also in the diagnosis of *M. bovis* BCG dissemination as a complication of BCG vaccination or immunostimulation against bladder cancer (Scully 1998, Jouanguy et al. 1996). Furthermore, the RDs-kit may also contribute to major public health investigations including *M. bovis* transmission from cattle to humans (O'Reilly and Daborn 1995). The RDs-kit results support the theory that the accumulation of LSPs is an important evolutionary mechanism of the MTBC subspecies which generates genetic and biological diversity. It also indicated that other *M. tuberculosis* LSPs exist and that these LSPs could provide an additional means of assigning genetic relatedness to *M. tuberculosis* strains.

Lastly, in the comparison between spoligotyping method with the molecular diagnostic method using RDs-based Multiplex PCR, this method provides a new promising technique and allows rapid detection and alternative to other molecular methods.

However, the results of the RDs-based Multiplex PCR technique must be tested in further studies by other methods in order to confirm the uncommon strains which could be a result of mutation events. This could be achieved by using other sets of flanking primers which will be generated to amplify within the deleted DNA fragments and thus we can confirm these new strains and include it among the MTBC subspecies.

5.1. RD-kit and RD-tool:

5.1.1. RD-kit:

This RDs-based Multiplex PCR kit enabled the rapid and accurate differentiation of MTBC members within a period of one working day. The method described is significantly faster and simpler than conventional mycobacteriological methods, which would have taken weeks to achieve the same level of classification. We demonstrate that this kit can be beneficial in the veterinary and human health arenas. Its application could make surveillance studies feasible, particularly in settings with high HIV prevalence. Further potential utility includes its use in routine laboratories for the assessment of high-risk cases. Finally, this kit could serve an essential function in the differentiation of MTBC members and detection of transmission in captive and other animal populations.

5.1.1.1. RD-kit mixture composition and procedure:

1) DNA sample will be applied to the RDs-kit which contains different combinations of primers and required enzymes. The RD regions in each

tube have been mixed according to the size of the expected bands with avoiding bands interference. The concentration of each primer pair in this mix was standardized as of which has been optimized in this study (page 57).

2) each sample will take 3 wells in the gel so that each well will contain a different mix of primers with a total of 10 primers for each sample, as in Figure 5.1.

Table 5.1. Optimpized concentrations of primer pairs used in RDs-kit

Mix Number	Primer Pairs (F+R)	Optimized Combination
1st Mix	RD7	0.50
	IS*1561'*	0.25
	RD9	0.50
	RD12	0.25
2nd Mix	RD4	0.25
	Rv0577	0.25
	16SrRNA	0.25
3rd Mix	RD14	0.50
	RD1	0.25
	RD13	0.50

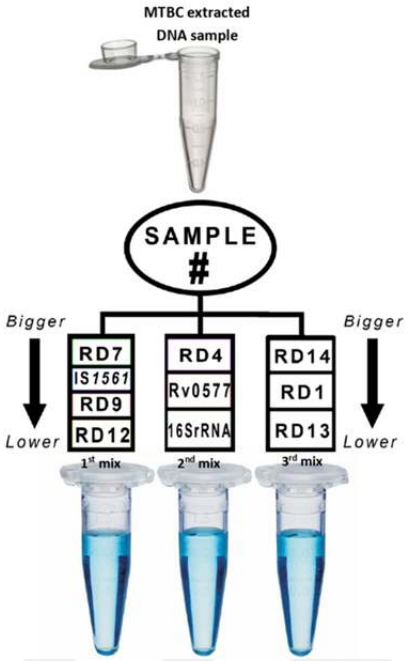

Figure 5.1. RD-kit of different mixes and combinations of RDs.

3) The below scheme will be used for detecting the bands on the captured picture of the gel and the result of each sample for the RDs will be recorded (+/–).

5.1.2. RD-tool:

The RDs tool will play an important role in rapid diagnosis of the mycobacterium tuberculosis complex subspecies depending on the presence and absence of the specific RD regions using the Multiplex RD-based PCR kit as described below and with based on the evolutionary tree of the Mycobacterium tuberculosis complex.

The RDs tool simply will depend on the stored database for the expected result entered before and based on the positive (+) and negative (–) probabilities corresponds with the evolutionary tree of the MTBC for the genes and regions we used as summarized below but without the need to follow the map as the regular procedure.

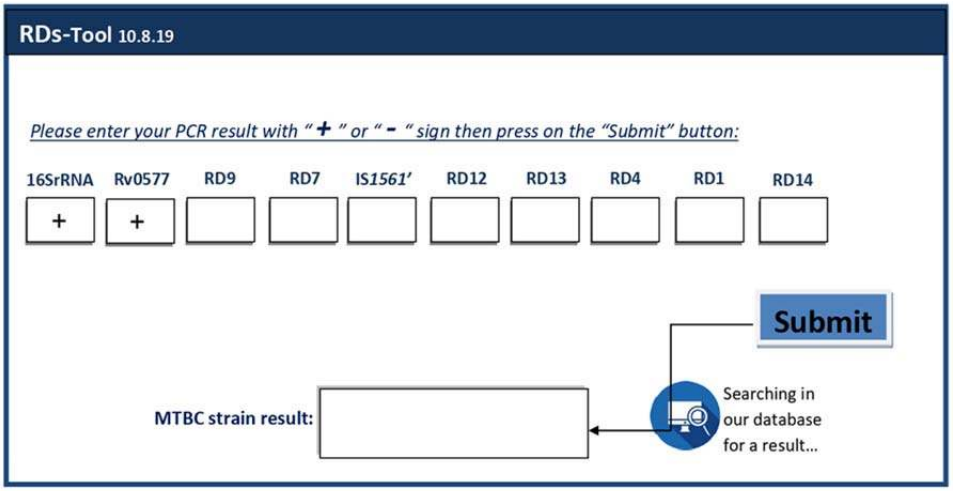

Figure 5.2. Virtual screen of RDs-Tool.

This tool were designed by using one of the programming languages as an "software tool" and it also be designed using a web design language to be submitted as an "Online tool" to the related scientific websites. We can also increase the RDs number for this tool by adding the remain RD regions to include all the MTBC subspecies and associated lineages in order to increase its discriminatory power.(RDs-tool:Test.programmer-corner.com)

5.1.2.1. RD-tool Programming:

The principle of the RDs-tool is depending on the different probabilities of the presence or absence of these regions (+ or –). However, the result must be according to the collective (sum) of these probabilities not individually (odd).

Example:

if **" 16SrRNA"** is + *and* "**Rv0577"** is + *and*……*and*……..…*then*>>>output: "*M. tuberculosis*"

Table 5. 2. Principle of the RDs-tool is depending on the different probabilities

if (,*and* ,*and* ,*and* ,*and* ,*and* ,*and* ,*and* ,*and* ,*and*) *is* (+ *or* -) *then*>>>Output is↓

16SrRNA	Rv0577	RD9	RD7	IS*1561'*	RD12	RD13	RD4	RD1	RD14	MTBC subspecies or MOTT species
+	+	+	+	+	–	+	+	+	+	*"Mycobacterium canettii"*
+	+	+	+	+	+	+	+	+	+	*Mycobacterium tuberculosis*
+	+	–	+	+	+	+	+	+	+	*Mycobacterium africanum* subtype II
+	+	–	–	+	+	+	+	+	+	*Mycobacterium africanum* subtype I
+	+	–	–	–	+	+	+	+	+	*Mycobacterium microti*
+	+	–	–	+	–	–	+	+	+	*Mycobacterium caproe*
+	+	–	–	+	–	–	–	+	+	*Mycobacterium bovis*
+	+	–	–	+	–	–	–	–	+	*Mycobacterium bovis* BCG
+	+	–	–	+	–	–	–	–	–	*Mycobacterium bovis* BCG- Pasteur
+	–	–	–	–	–	–	–	–	+	*Mycobacterium abscessus/ Mycobacterium avium* complex

if the results (+/-) other than these data, *then*>>>output: " No data", since that there are no data about such results/strains in the previous studies.

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CURRICULUM VITAE

My name is Mahmoud SHOMAN. I was born on January 23rd, 1993 in Zarqa, Jordan. There, I have obtained my Baccalaureate (BAC) Diploma in 2011.

Due to my ongoing interest in applied natural science and researches related to Genetic Engineering since high school, I got admitted to a Bachelor Program in Biotechnology at The Hashemite University of Jordan where I acquired my BSc in January, 2016. After graduation and work for 9 months in the field of Microbiology, I felt that I need to arm myself with requisite knowledge which a graduate program can furnish, so I enrolled to a Master program in Biotechnology at the Institute of Natural and Applied Sciences of Çukurova University, with specializing in Medical Microbiology.

Since the first year of my Master study, and to further nourish my research interest, I have joined The Tropical Diseases Research and Application Center, the regional TB research center and I chose it as the place of my thesis research. During my work there, I have gotten a glimpse of the rigorousness of lab work along with the anxiety of anticipation, the pure exhilaration of a positive result. This has further strengthened my dream and desire to be a part of a laboratory where cutting edge research is performed.

My ultimate aim is to utilize the knowledge and training in the field of Biotechnological research to work in a Research laboratory where I will look to hone and perfect my practical skills before eventually starting my own Research Center. I hope, in the future, to find treatments for chronic and incurable diseases.