# DEVELOPMENT OF A NUCLEIC ACID AMPLIFICATION TEST FOR THE DETECTION OF *MYCOBACTERIUM TUBERCULOSIS*

PRIYATHARISNI KANIAPPAN

UNIVERSITI SAINS MALAYSIA

2015

# DEVELOPMENT OF A NUCLEIC ACID AMPLIFICATION TEST FOR THE DETECTION OF *MYCOBACTERIUM TUBERCULOSIS*

**By**

**PRIYATHARISNI KANIAPPAN**

**Thesis submitted in fulfillment of the requirements for the degree of Master of Science**

**UNIVERSITI SAINS MALAYSIA**

**OCTOBER 2015**

## ACKNOWLEDGEMENTS

First and foremost, I would like to express my gratitude to my supervisor, Professor Dr. Tang Thean Hock, for providing me the opportunity to pursue MSc. Degree under his supervision. His guidance, patience and constructive criticism have always been supportive at all times during my work. I am very deeply indebted to my field supervisor, Datin Dr Ganeswrie Rajasekaram, Hospital Sultanah Aminah Johor Bahru for her advice and continuous support and all insights.

I thank the Infectomic Cluster, Advanced Diagnostic Laboratory (AMDI) and Microbiology Unit of Hospital Sultanah Aminah for providing the bacterial cultures, which has contributed significantly to this work. I am thankful to the Ministry of Health (MOH) for awading me scholarship 'Hadiah Latihan Persekutuan'. I am grateful to the management staffs of AMDI, USM who rendered me with valuable experiences outside of the laboratory. I would also like to thank the family of Infectomic Cluster, AMDI, in particular Mdm Siti Aminah, Dr. Hoe Chee Hock, Dr. Citartan Marimuthu, Ms. Lee Li Pin, Ms. Thiviyaa Othaya Kumar, Ms. Nitya Ravichandran and Mr. Emmanuel Jayaraj Moses for their assistance and support.

A huge thank you to my other half, Mr. Nageswaran Muniandy for understanding my ambition and my inner need to pursue higher education. Last but not least, I am grateful to the Almighty God for all His guidance, blessings, lessons and 'companionship' when I was away from my family to pursue my MSc. Words alone can't explain the degree of gratitude towards my family for showering me with love, care and motivations for me to strive and complete this work successfully. This thesis would not have been possible without their supports. My prayers for my late friend and housemate Ms.Shailaja Balasubramaniam Menon whom is with angels right now. Lastly, I offer my sincerest thanks to my friends and to those who supported me in any respect during the completion of the project.

# TABLE OF CONTENTS

Page

## LIST OF TABLES

## LIST OF FIGURES

## LIST OF ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| A | Adenine |
| bp | Base pair(s) |
| BLAST | Basic Local Alignment Search Tool. |
| Megablast | Mega Basic Local Alignment Search Tool |
| C | Cytosine |
| °C | Degrees Celsius |
| CFU | Colony Forming Unit |
| dATP | Deoxyadenosine triphosphate |
| dCTP | Deoxycytidine triphosphate |
| $ddH_2O$ | Double-distilled water |
| dGTP | Deoxyguanosine triphosphate |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxyribonucleotide triphosphate |
| dsDNA | Double-stranded DNA |
| dTTP | Deoxythymidine triphosphate |
| EDTA | Ethylenediaminetetraacetic Acid |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| et al. | and others |
| EtBr | 3, 8-diamino-5-Ethyl-6-phenyl phenanthridinium Bromide |
| g | Gram |
| G | Guanine |

| | |
|---|---|
| *GAPDH* | Glyceraldehyde-3-phospate-dehydrogenate |
| HCl | Hydrochloric acid |
| H | Hour (s) |
| IAC | Internal Amplification Control |
| $KH_2PO_4$ | Potassium phosphate |
| KCl | Potassium chloride |
| kDa | Kilodalton |
| L | Litre |
| LB | Luria Bertani |
| M | Mol/Liter, molar |
| mg | Milligram |
| $MgCl_2$ | Magnesuim Chloride |
| min | Minute (s) |
| mL | Milliliter |
| mM | Millimolar |
| mRNA | Messenger RNA |
| mPCR | Multiplex Polymerase Chain Reaction |
| NaCl | Sodium chloride |
| NaOAc. $3H_2O$ | Sodium acetate trihydrate |
| NaOH | Sodium hydroxide |
| ng | Nanogram |
| $NH_2HPO_4$ | Sodium hydrogen phosphate |
| nt | Nucleotide (s) |

| | |
|---|---|
| OD | Optical Density |
| npcRNA | Non-protein-coding gene |
| PBS | Phosphate buffered saline |
| PCR | Polymerase Chain Reaction |
| pg | Picogram |
| RBS | Ribosomal Binding Site |
| RNA | Ribonucleic acid |
| RNase | Ribonuclease |
| RPM | Revolutions per minute |
| rRNA | Ribosomal RNA |
| RT | Room temperature |
| sec | Second (s) |
| SDS | Sodium dodecyl sulfate |
| siRNA | Small interfering RNA |
| sRNA | Small RNA |
| ssDNA | Single-stranded DNA |
| T | Thymine |
| TAE | Tris–Acetic Acid–EDTA |
| Tris | Tris-(Hydroxymethyl)-Aminomethane |
| Tris-HCl | Tris-(Hydroxymethyl)-Aminomethane Hydrocloride |
| tRNA | Transfer RNA |
| UV | Ultraviolet |
| V | Volt (s) |

| | |
|---|---|
| v/v | Volume per volume |
| XLD | Xylose Lysine Deoxycholate |
| µL | Micro liter |
| µM | Micro molar |
| % | Percentage |
| β | Beta |

PEMBANGUNAN UJIAN AMPLIFIKASI ASID NUKLEIK BAGI TUJUAN PENGESANAN KUMAN *MYCOBACTERIUM TUBERCULOSIS*

## ABSTRAK

Batuk kering ialah salah satu penyakit berjangkit yang lazim di Malaysia, yang disebabkan oleh patogen bawaan udara, *Mycobacterium tuberculosis*. Disebabkan sifat jangkitan penyakit ini, pengesanan diagnostik berasaskan *M. tuberculosis* adalah penting untuk pengurusan penyakit yang berkesan, pemantauan, kajian epidemiologi serta untuk pengurusan rawatan yang tepat dan cepat. Kaedah-kaedah diagnosis semasa bergantung kepada calitan mikroskop dan pengkulturan. Bagaimanapun kaedah-kaedah ini tidak spesifik atau mengambil masa yang lama. Ini menyebabkan perhatian diberikan terhadap kaedah pengesanan *M. tuberculosis* berdasarkan asid nukleik yang memerlukan sasaran asid nukleik yang sesuai dijadikan sebagai penanda diagnostik. Satu kelas asid nukleik, yang merupakan RNA-bukan-pengekodan-protein, ialah kelas RNA yang tidak diterjemahkan kepada protein tetapi memainkan peranan dalam melaksanakan fungsi pengawalseliaan tertentu. Ia juga menunjukkan potensi untuk dimanfaatkan sebagai penanda bio diagnostik. Analisis berasaskan bioinformatik mendedahkan kehadiran 140 jujukan yang berpotensi sebagai penanda diagnostic. Salah satu daripada jujukan tersebut, npcTB 6715, khusus untuk *M. tuberculosis* dipilih sebagai penanda diagnostik yang berpotensi dan diuji. Sebagai ujian pengesanan berasaskan asid nukleik paling mantap, asai PCR dipilih sebagai strategi pilihan untuk menguji potensi npcRNA ini untuk pengesanan diagnostik *M. tuberculosis*. Multiplex PCR (mPCR) yang dibangunkan, menggabungkan gen IS*6110* dengan npcTB 6715, di mana penggunaan

npcRNA ini mampu mengesan *M. tuberculosis* yang tidak mempunyai gen IS*6110*, sekaligus mampu mengelakkan keputusan negatif palsu. mPCR ini menunjukkan had pengesanan sebanyak 10 pg. Evaluasi mPCR dengan 500 kultur *Mycobacterium* menunjukkan nilai-nilai 98.4%, 96.1%, 98.6% dan 95.4% masing-masing untuk kepekaan, kekhususan, Nilai Ramalan Positif dan Nilai Ramalan Negatif. Nilai-nilai yang diperolehi mencadangkan potensi diagnostik mPCR, sebagai ujian diagnostik untuk mengesan *M. tuberculosis* dan menyokong potensi diagnostik npcTB 6715.

**DEVELOPMENT OF A NUCLEIC ACID AMPLIFICATION TEST FOR THE DETECTION OF *MYCOBACTERIUM TUBERCULOSIS***

## ABSTRACT

Tuberculosis is one of the common infectious diseases in Malaysia, caused by the airborne pathogen, *Mycobacterium tuberculosis*. Due to the infectious nature of the disease, diagnostic-based detection of *Mycobacterium tuberculosis* is vital for effective disease management, surveillance, epidemiological purpose and for the administration of a prompt treatment. Current diagnostic methods rely on smear microscopy and culture method; however these strategies suffer from several pitfalls. This shifts the attention towards the nucleic-acid based detection of *M. tuberculosis*, which requires the identification of suitable nucleic acid target as the diagnostic marker. One novel class of nucleic acid, which is the non-protein-coding RNAs, is a class of RNA that is not translated into protein but perform certain regulatory functions, suggesting the potentiality to be harnessed as a diagnostic biomarker. Bioinformatic-based analysis revealed the presence of 140 npcRNA genes, one of it, npcTB 6715, which is specific for *M. tuberculosis* is chosen as the potential diagnostic marker. As the most established nucleic acid-based detection platform, PCR assay is chosen as the platform of choice to test the potentiality of this npcRNA for the diagnostic-based detection of *M. tuberculosis*. The PCR assay developed also incorporates IS*6110* as the counterpart of npcTB 6715, whereby the usage of the npcRNA is able to detect the IS*6110*-deficient strains of *M. tuberculosis*, averting false-negative results. The multiplex PCR assay developed showed a detection limit of 10 pg. Evaluation with 500 Mycobacterium

cultures demonstrated values of 98.4%, 96.1%, 98.6% and 95.4%, respectively for the sensitivity, specificity, Positive Predictive Value and the Negative Predictive Value. The values obtained suggested the diagnostic potentiality of the mPCR, as a promising ASSURED' diagnostic test for the specific detection of *M. tuberculosis* and corroborated the diagnostic potentiality of the npcTB 6715.

# CHAPTER 1:

# INTRODUCTION

## 1.1. Mycobacteria

Mycobacteria are aerobic, non-motile, non-sporulation rod shaped microorganisms, GC rich nucleotide containing microorganism with size range of 2-4μm (length) and 0.5-0.2μm (wide). Classified under actinobacteria phylum, mycobacteria are further categorized into the family of Mycobacteriaceae. There are over a hundred species of *Mycobacterium*, which are genetically closely related as reported by16S rRNA sequencing studies (Tortoli, 2006). Generally, mycobacteria can be divided into three major categories (Figure 1.1): *Mycobacterium tuberculosis complex* (MTBC), non-tuberculosis mycobacteria (NTM) and *Mycobacterium leprae* (*M. Leprae*) (Jagielski et al., 2014).

## 1.2 Non Tuberculous Mycobacteria (NTM)

NTM are ubiquitous in the environment. Most of the NTM are non-pathogenic organisms which are known to colonize water sources, soil, dust particles, and food supplies. NTM can be cultivated on common liquid and solid culture media. Depending on the growth period, they are divided into rapidly growing mycobacteria such as

*Mycobacteria fortuitum, Mycobacteria chelonae*, and *Mycobacteria abscessus* as well as slow growing species such as *Mycobacteria avium, Mycobacteria kansasii*, and *Mycobacteria marinum*. About 160 different species of NTM have been identified.

### 1.3 *M. leprae*

One of the earliest identified slow growing NTM is the *M. leprae*. *M. leprae* causes leprosy (Hansen's disease) in human (Cole et al., 2001). *M. leprae* is an intracellular, pleomorphic and acid-fast bacterium. It is an aerobic bacillus (rod-shaped) surrounded by the characteristic waxy coating unique to mycobacteria. *M. leprae* is different from other mycobacteria in terms of the arrangement in smear, as it is arranged in parallel chains, just like cigarettes in a pack. Leprosy infections are mainly focused on the skin and peripheral nerves. Therefore, their diagnoses are largely established based on skin and neurologic examination of the patient.

### 1.4 *M. tuberculosis complex* (MTBC)

*MTBC* is a group of closely related mycobacterial species consisting of *M. tuberculosis, M. bovis, M. africanum, M. microti*, *M. canetti*, *M. caprae*, *M. pinnipedii* and *M. mungi* and *M. suricattae*.

*M. bovis,* the main cause of TB in bovine animal is known to cause cross species infection from bovine to humans through consumption of contaminated milk (Cosivi et al., 1998). Originating from the African sub-continent, *M. africanum* represents another major cause of TB especially in Sub-Saharan Africa. However, reports on the presence of *M. africanum* have emerged in countries other than Sub-Saharan Africa such as England, Spain, and the United States (Desmond et al., 2004; Grange and Yates, 1989; Remacha Esteras et al., 2003). On the other hand, *M. microtii* is a rare pathogen that usually infects rodents and shrews (Cavanagh et al., 2002). *M. canetti* causes infection in humans although the prevalence of this subspecies in not common. Out of the many mycobacterial species of the MTBC, *M. tuberculosis* is the most common causative agent of TB among humans.

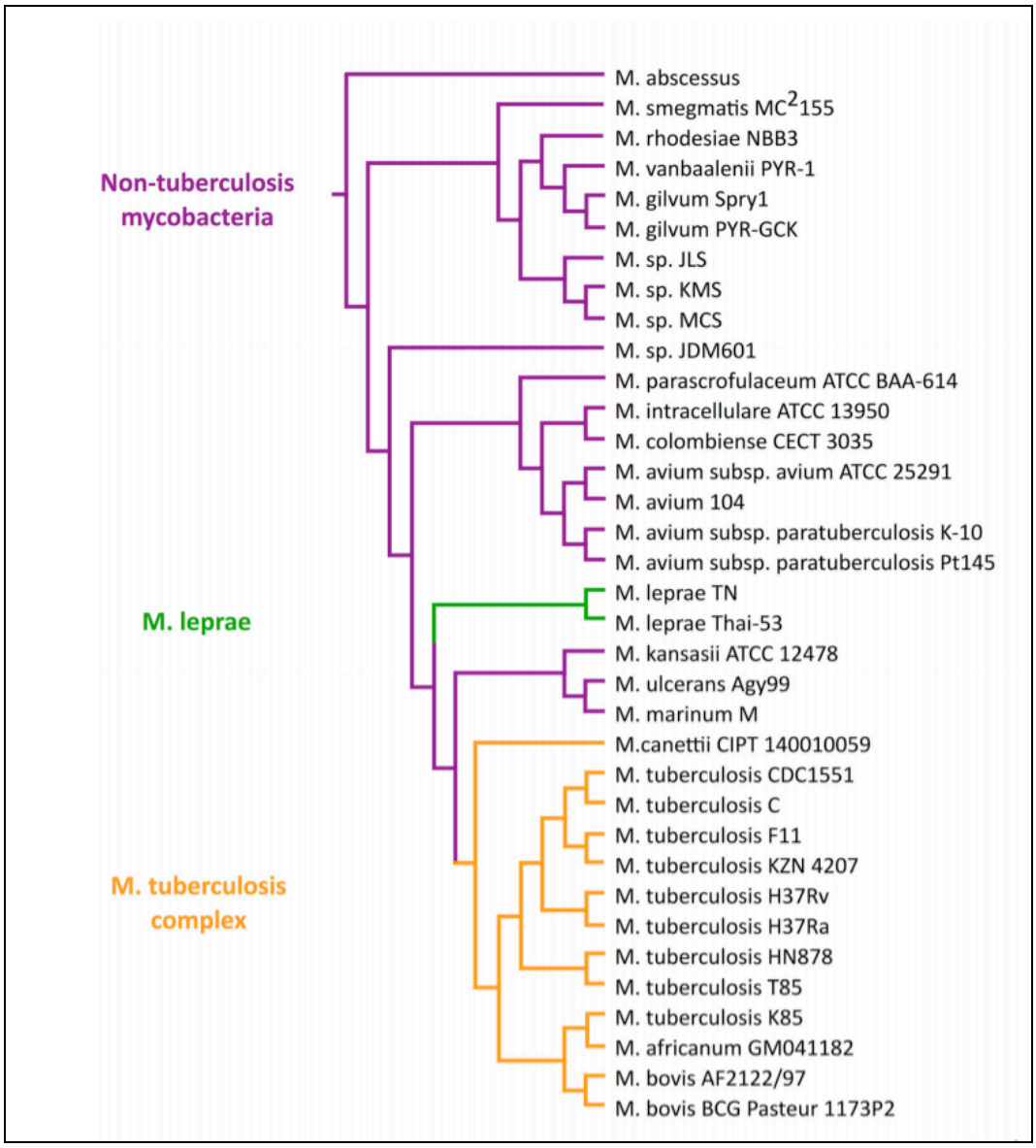



Figure 1.1 Phylogenetic lineage of Non-Tubercoulosis Mycobacteria, *M. leprae* and *M. tuberculosis complex* [Adapted from (Haning et al., 2014)].

## 1.5. Mycobacterium tuberculosis

*M. tuberculosis* is a non-motile, slender, straight, or slightly curved bacillus. It is an obligate aerobic organism; hence it can only survive in an oxygen-containing environment. *M. tuberculosis* bacilli are 2-4 micrometers in length and 0.2-0.5 μm in width. The generation time of *M. tuberculosis*, in synthetic medium or infected animals is approximately 24 hours (Ginsberg and Spigelman, 2007). The slow growing feature of *M. tuberculosis* contributes to the chronic nature of the disease, which requires a prolonged treatment. In addition, the compositional complexity of its cell wall could also account for the nature of the disease, which represents a formidable impediment for researchers and health care system.

### 1.5.1. Cell wall of *M. tuberculosis* confers pathogenesis and acid fast property

The cell wall of *M. tuberculosis,* is composed of peptidoglycan, arabinogalactan and mycolic acids covalently linked to each other (Figure 1.2) (Chatterjee, 1997). Due to the presence of mycolic acids, the primary stain used for gram staining does not penetrate well into the cell wall, making it unsuitable for gram staining (Washington, 1996). As an alternative, acid-fast staining can be used to stain *M. tuberculosis.* The stain forms a complex with the mycolic acids, thereby resist decolorization by acid containing decolorization agent (Cheesbrough, 2006). These mycolic acids are also responsible for the pathogenesis of TB (Forrellad et al., 2013).

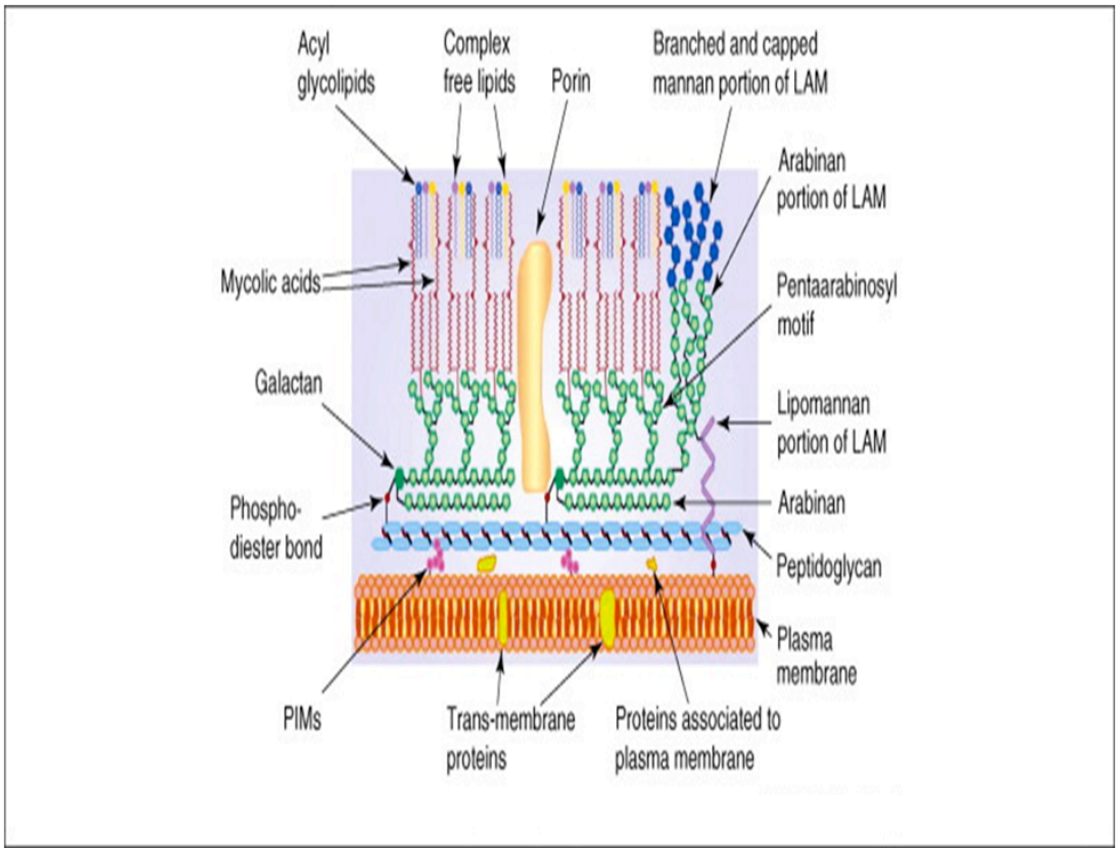



Figure 1.2: Mycobacterial cell wall [Reproduced from (Medjahed et al., 2010)].

### 1.5.2 Transmission and immune response upon TB infection

TB infection spreads via droplets in the air expelled by an infected person during common daily acts such as sneezing, coughing, laughing, and even talking. The amount of bacilli present in the droplet, UV light exposure, ventilation factor and aerosolization occurrence plays important role in transmission (American Thoracic Society and CDC, 2000). The spread of TB is enhanced by factors such as rapid increase in global travel, malnutrition, overcrowding, and co-infection of HIV and TB.

TB infection is a tug-of-war between the host immune system and the pathogen's virulence. The first mechanism triggered upon the infection is the physical defense mechanism, which sets to expel any foreign molecules from invading the human body. Likewise, when a person inhales droplets containing *M. tuberculosis* bacilli, they are trapped in the upper respiratory. Following this, the mucus secreting Goblet cells on the respiratory tract would secrets mucus, trapping the pathogen and pushing it upwards to be expelled from the system. However, in some cases, the *M. tuberculosis* bacilli that managed to elude this physical defense system travels to the alveoli in the lungs and is predisposed to another mechanism known as the innate immune system. In this defense system, alveolar macrophage surrounds and phagocytes the *M. tuberculosis*, attempting to destroy the bacilli.

At this stage, destruction of the bacilli is reliant on the interplay between immunity of the host and the virulence of the bacilli itself (van Crevel et al., 2002). As

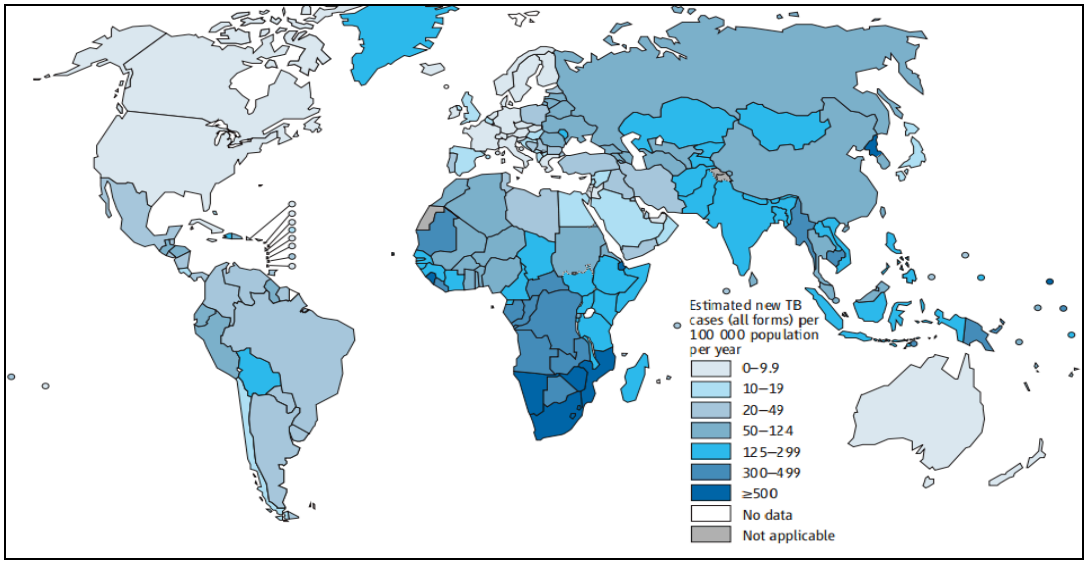
such, in immuno-compromised individual, viable *M. tuberculosis* that infects lungs cause pulmonary tuberculosis. The classic clinical symptoms of TB is productive cough that produces thick cloudy sputum for more than 2 weeks, chest pain, shortness of breath, hemoptysis, fever, chills, night sweats, loss of appetite and sudden weight loss. Apart from residing in the alveolus, viable *M. tuberculosis* can also travel to other tissue via the lymphatic system causing extrapulmonary TB (Dale et al., 2003). In addition, the infected individual also has the potential to incur infection on other individuals. In contrast, in the case of Latent TB, the infected person is not responsible for any transmission of TB. Only 5-10% of latent TB infection would develop into active TB.

### 1.5.3 Epidemiology of Tuberculosis

World Health Organization (WHO) estimated that 8.6 million people were newly infected by TB and 1.3 million deaths occurred due to TB infection in 2012 (WHO, 2013). Out of the 8.6 million TB cases, 2.9 million cases were women and 0.5 million cases occurred among children. Over 400000 deaths and 74000 deaths were women and children, respectively. Nearly 1 million deaths and 0.3 million deaths occurred among non-HIV infected individuals and HIV infected individuals, respectively. Global surveillance data indicated that most of the cases in 2012 occurred in Asia (58%) and the African region (27%) [Figure 1.3(A)]. Additionally, 22 countries around the world have been identified as 'High Burden' for TB and among them are the Asian countries such as India, China, Indonesia, Thailand, Bangladesh, and Vietnam.

In Malaysia, according to the Ministry of Health Malaysia (MOHM), TB is one of the major 5 communicable diseases for the past 15 years (MOHM, 1997-2013). TB cases in Malaysia have steadily increased since 2005 and tend to rise further. In 2012, TB incidence rate including among HIV infected individuals was estimated at 24000 (MOH, 2012). Mortality rates have been between 4 and 6% during the years from 1990 to 2011 [Figure 1.3(B)]. Approximately 1414 deaths were reported and occurred among non-HIV infected individuals in 2012, with a mortality rate of 4.9% (WHO, 2014). Due to this alarming rate of TB infection, MOHM aims to strengthen the case detection and provide prompt treatment to TB infected individuals in Malaysia. In line with this, The National TB Control Program is currently implementing 'National Strategic Plan to Control TB, 2011-2015' to enhance all activities to reduce the burden of TB in the country (MOH, 2012). An effective diagnostic method for efficient management of the disease is also part of the endeavor to alleviate the problem of TB in the country.

(A)




(B)

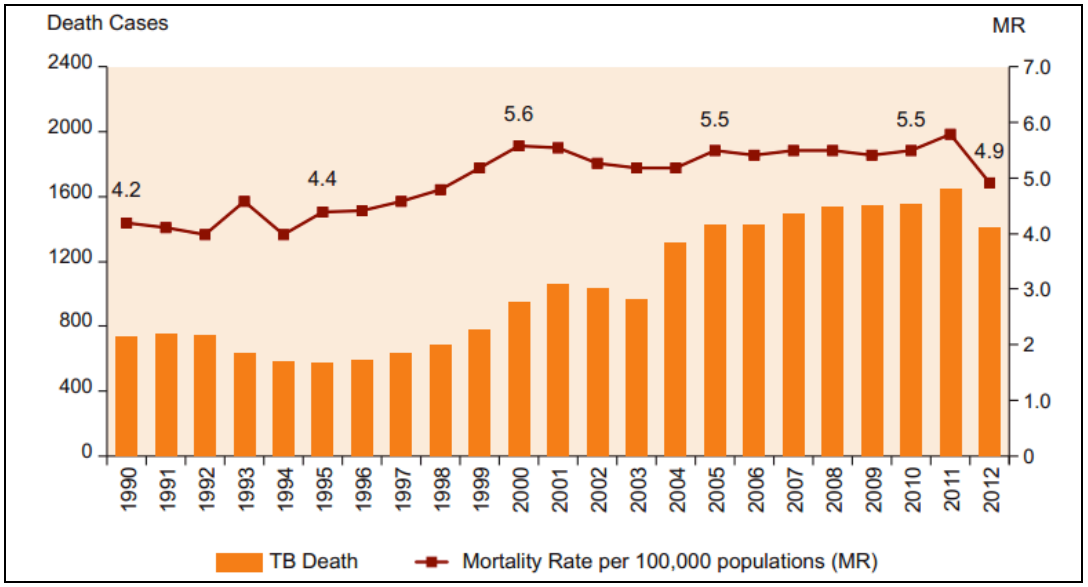



Figure 1.3 (A): Estimated new TB rates for year 2012 (Adapted from Global tuberculosis Report 2013) (B): TB Mortality Rate in Malaysia, 1990-2012 (Adapted from Ministry of Health Malaysia Annual Report 2012).

## 1.6 Diagnostic of Tuberculosis

The very first step towards controlling TB infection is the suitable diagnostic method. This added with the infectious nature of TB, particularly which of the pulmonary TB, stresses the need for a fast and accurate diagnostic strategy. To date, a potpourri of diagnostic methods were devised, ranging from simple microscopy examination to intricate molecular methods. These methods vary in terms of duration, sensitivity, simplicity, or even specificity.

### 1.6.1 Smear Microscopy

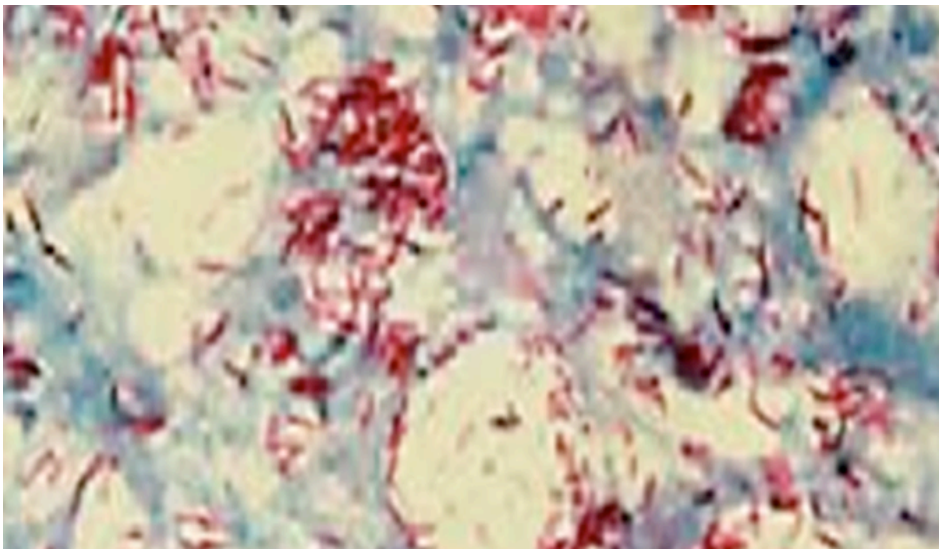
Smear microscopy represents the most common method for TB diagnosis. The microscopy method is easy, rapid, in-expensive and could identify individuals with TB at the infectious stage (Aber et al., 1980, Huebner et al., 1993). Due to the low cost, most low and middle-income countries utilize smear microscopy with Ziehl-Neelsen (ZN) staining as the primary tool to diagnose TB (Eyangoh et al., 2008). Samples, commonly sputum, were smeared directly on the slides without prior processing and are subjected to ZN staining. The acid-fast bacteria mycobacteria appear bright red with blue background following ZN staining [Figure1.4 (A)]. WHO has recommended 2 or 3 sputum samples collected for laboratory examination (WHO, 1998).

Investigation of Acid Fast Bacilli (AFB) yield in serial sputum examination reported that 85.8% of TB cases were detected in the first sputum sample while an

increment of 11.9% and 3.1% were reported consecutively for the second and third samples (WHO, 2009). However, the shortcoming of smear microscopy is that it has low sensitivity and specificity; whereby the detection is limited only to 10,000 bacilli in 1 mL of sample. This hampers diagnosis, especially for HIV-associated TB cases and among children who are unable to provide quality sputum samples. Furthermore, the presence of *M. tuberculosis* cannot be confirmed with smear microscopy alone.

The disadvantages associated with conventional compound microscope can be possibly addressed by the usage of Flourescence Microscopy (WHO, 2011). This microscope method relies on acid-fast fluorochrome stain such as auromine O or auramine-rhodamine instead of normal acid fast stain. Image visualization is actualized via powerful light source such as halogen, high presure mercury vapour lamp or Light-emitting Diod (LED). *Mycobacterium* fluoresces against dark back ground as shown in Figure1.4 (B). FM increases sputum positive detection by 10 to 37% (Annam et al., 2009, Hooja et al., 2011, Laifangbam et al., 2009). The application of FM consumes lesser time than conventional microscopy, as the former uses lower power objective lense (usually 25x) than that of the latter (usually 100x) (Kivihya-Ndugga et al., 2003; Steingart et al., 2006). Furthermore, florochrome staining method is relatively easier than Ziehl-Neelsen method (Holst et al., 1959). Although FM clearly has advantages over conventional microscopy, its use is limited by the high cost and it requires skillful personnel. Moreover, cases such as incorporation of florochrome dye into the inorganic objects and the ability of the dead cells to be stained with florochrome stain result in false positivity (Steingart et al., 2006; Washington, 2006).

(A)



(B)

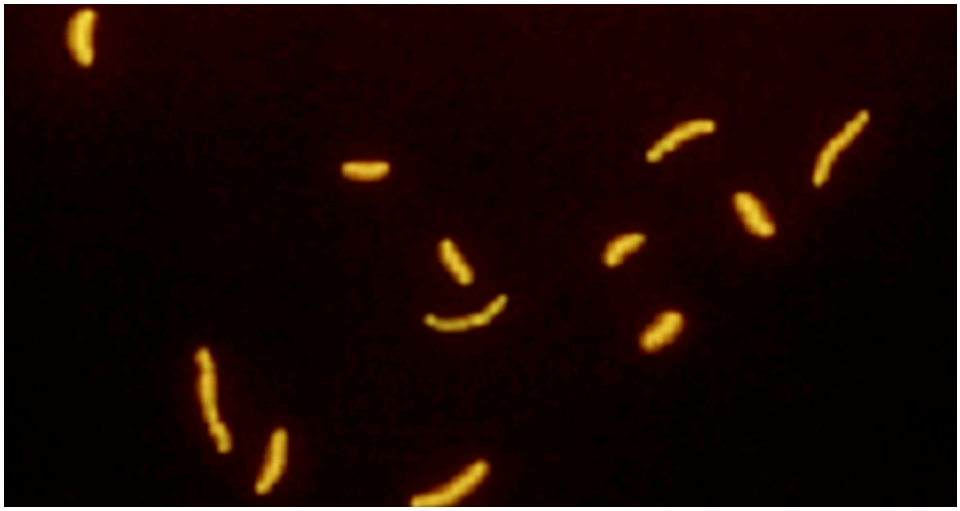


Figure 1.4: (A) Ziehl-Neelsen Stain (B) Auramine-rhodamine stain. The acid-fast bacteria mycobacteria appear bright red following Ziehl-Neelsen staining whereas in Auramine-Rhodamine staining the bacillus appears in orange colors (extracted on 15 December 2014 from www below).

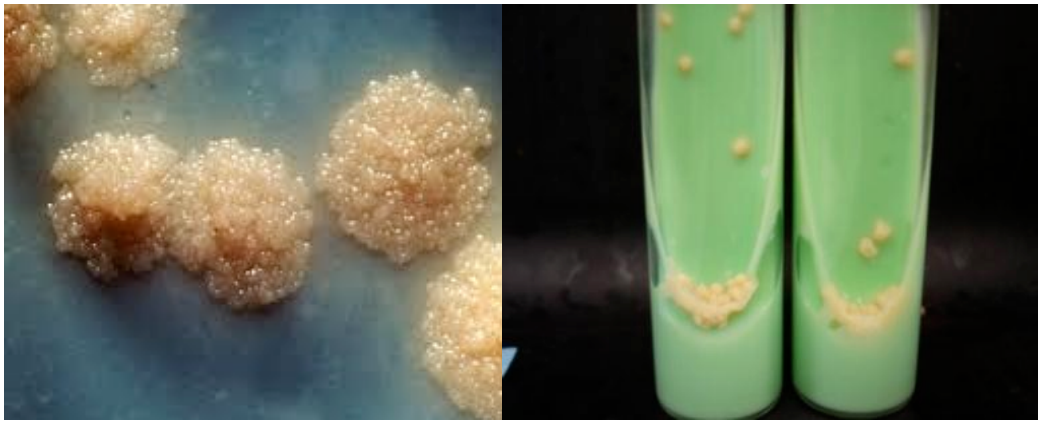
http://www.digitalpathology.uct.ac.za/topics/classiccasesoftb/post_pulmonarytb.html).

### 1.6.2 Culture method

Culture method is considered as the 'gold-standard' for TB diagnostic. Firstly, sputum samples, are decontaminated and concentrated to maximize the recovery of *M. tuberculosis*. The digestion and decontamination method recommended by WHO is the Modified Petroff method (WHO, 1998). Finally, the decontaminated sample will be concentrated by centrifugation prior to the inoculation of the sediment into the culturing media. Cultivation of the *M. tuberculosis* requires egg-enriched media containing glycerol, asparagine and agar or liquid medium supplemented with serum or bovine albumin to grow (WHO, 1998). Most commonly used solid culture media to cultivate *M. tuberculosis* is Lowenstein-Jensen (Rageade et al., 2014). However, due to the slow-growing nature of *M. tuberculosis*; the conventional culture method takes a period of 6-8 weeks prior to the reporting of the result. Figure 1.5 (A) and (B) shows *M. tuberculosis* colonies grown on Lowenstein-Jensen media.

The slow-growing property that results in the delay of reporting *M. tuberculosis* cases can be possibly addressed with liquid culture using radiometric medium. The commercial liquid culture (Mycobacterium Growth Indicator Tube (MGIT), developed by Benton Dickinson, USA, was endorsed by WHO in 2007 (Boehme et al., 2013). Studies have reported that MGIT culture extensively reduces the time of detection to as low as 5 to 12 days and increases recovery chances of mycobacteria from clinical sample from 10 to 15%. The liquid culture system reportedly reduces number of false negativityby 50% (Chan et al., 2008; Hillemann et al., 2006; Satti et al., 2010).

However, contamination, cost and complexity of liquid culture system hinders its usage especially in low income countries (Boehme et al., 2013).



(A) (B)

Figure 1.5: (A) *M. tuberculosis* colony is buff to yellow in colour with rough and wrinkle appearance, (B) *M. tuberculosis* colony on Lowenstein-Jensen media, which takes 2-6 weeks to get visual colony growth (extracted on 15 Dec 2014 from www below).

http://www.microbeworld.org/component/jlibrary/?view=article&id=6641

### 1.6.3 Nucleic acid-based detection of *M. tuberculosis*

The caveats associated with microscopy and culture method, which is time-consuming and non-specific draws the attention towards the direct detection of *M. tuberculosis* sample. In tandem with this, Nucleic Acid Amplification Tests (NAAT) are harnessed as the diagnostic tool for the direct detection of *M. tuberculosis*. NAAT provides faster, more accurate results compared to the conventional biochemical based tests, allowing timely identification of active TB. Generally, NAAT is carried out in several steps. Firstly, the sample containing *M. tuberculosis* cells are lysed and the lysed samples are further processed to remove proteins or other unwanted compounds. Subsequently, specific nucleic acid sequences of *M. tuberculosis* are amplified to expedite detection by fluorescence or lateral flow assay (Boehme et al., 2013). The most common nucleic acid amplification method is the Polymerase Chain Reaction (PCR).

## 1.7 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is a method first conceived in the 1980's by Kary Mullis. PCR is used extensively in the field of molecular biology due to its specificity. The aim of PCR is to exponentially synthesize a predeterminated DNA sequence *in vitro* by using short oligonucleotides (termed primers). The first step in PCR is the denaturation process, whereby the reaction mixture containing DNA templates are heated at high temperature (90-95$^{o}$C) for 30 sec to 5 min. This is followed

by the annealing step, the process at which both the forward and reverse primers anneal to their complementary sequences on the template DNA under the temperature between 40-60$^{o}$C. Next, is the elongation step, by which the temperature is raised to 72$^{o}$C to allow the enzyme *Taq Polymerase* to carry out synthesis of new copies of sequences in the presence of magnesium ion, and excess amounts of dNTPs. This amplification, which occurs exponentially, allows easy detection of the amplicon, for example by visualization after running PCR product on the gel electrophoresis. The most important criterion prior to the development of a PCR assay is the identification of the suitable target for amplification.

### 1.7.1 Non-protein coding RNAs (npcRNA) -Potential Diagnostic marker of PCR-based detection of *M. tuberculosis*

Non-protein coding RNAs (npcRNA) are RNA transcripts capable of performing specific functions but are not translated into protein. One cut off size-based subgroup of npcRNAs, small RNAs, having the size range between 20-300 nts, participate in various cellular processes; housekeeping, virulence, pathogenesis, regulation of DNA replication, transcription, and mRNA stability (Bartel, 2004; Huttenhofer et al., 2005). To date, a large number of sRNA were identified and characterized (Gopinath et al., 2010; Storz, 2002, Washietl et al., 2005; Yong et al., 2013; Zhou and Xie, 2011). Moreover, some of these molecules have been suggested as molecular markers of genetic diseases and cancer (Calin et al., 2004; Costa, 2005; Esteller, 2011; Hall and Russell, 2005; Meng et al., 2013; Ren et al., 2013;

Rozhdestvensky et al., 2007, Tang et al., 2005). One example is the MALAT1 sRNA that is reported as markers to determine the overall survival of patients (Zheng et al., 2014).

On the other hand, infectious agents such as *Helicobacter pylori* (Sharma et al., 2010) and *Vibrio cholera* (Livny and Waldor, 2010; Raabe et al., 2011) have been the subjects of immense interest towards the discovery of sRNAs. Previous studies carried out by our group have led to the discovery of 97 sRNAs from the human pathogen *Salmonella* Typhi, a causative agent of salmonellosis (Chinni et al., 2010). Recent study by Nithya et al., (2015) also have demonstrated the potentiality of the sRNA genes as the diagnostic marker for the differentiation of Salmonella species. Despite the growing evidence that sRNAs can serve as biological markers for human diseases, investigation into the use of small sRNAs as targets for the diagnosis of infectious agents has not been explored exhaustively.

### 1.7.2 IS*6110*, the widely employed gene target for PCR-based detection of *M. tuberculosis*

Amongst the available gene targets, IS*6110*, remains the most popular target for laboratory-developed tests against *M. tuberculosis* (Balamurugan et al., 2006; Chaidir et al., 2012; Deshpande et al., 2007; Maurya et al., 2011; Mokrousov et al., 2006; Mokrousov et al., 2003; Nandagopal et al., 2010). IS elements are segments of DNA that can insert at multiple sites in a target molecule (Mahillon and Chandler, 1998). In

*M. tuberculosis*, one particular IS element, IS*6110* with the length of 1.36 kbp has 28-bp terminal inverted repeats (TIR) at its ends (Figure 1.6). *IS6110* is associated with the adaptation to the host, activation of genes during infection, evolution and is involved in activation of downstream genes with an orientation-dependent activity promoter (Alonso et al., 2013). IS*6110* is present in multiple copies usually up to 6-25 times copies in the *M. tuberculosis* genome incontras with *M. bovis*, which contains only one copy (Philipp, 1996).

Its high specificity to MTBC, the presence in multiple copies and stability in the genome of *M. tuberculosis* renders IS*6110* an ideal biomarker. Moreover location of an IS*6110* copy in a specific strain can be used for rapid identification and the differentiation of that particular strain amidst the other strains (Millan-Lou et al., 2012). IS*6110* has been extensively used in diagnostic and epidemiology study of *M. tuberculosis*. de Lassence and colleagues stipulated that the utilization of IS*6110* in PCR is more sensitive than other identification protocol (de Lassence et al., 1992). Other studies, utilizes IS*6110* gene detecting in *M. tuberculosis* with various success rates (Barani et al., 2011, Chaidir et al., 2012; Kulkarni et al., 2012). However, absence or low copy of IS*6110* in certain strains of *M. tuberculosis* suggests that an additional target(s) could be amalgamated with IS*6110* to elevate the efficiency and accuracy of the PCR-based diagnostic assay.

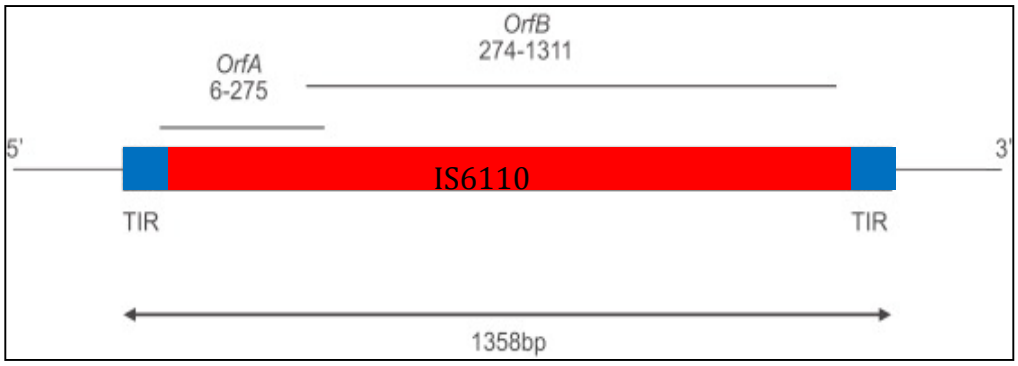



Figure 1.6: Diagram of IS*6110*. Diagram illustrates the position of the Terminal Inverted Repeats (TIR) and the two open reading frames orfA and orfB; two partially overlapping reading frames [Reproduced from (McEvoy et al., 2007)].

## 1.8. Research objectives

In concert with the effort to test the potentiality of the npcRNA genes as the potential diagnostic markers for *M. tuberculosis* detection, the incorporation of IS*6110* as the counterpart in PCR amplification is viewed to be able to ward off false negative results derived from IS*6110*-deficient *M. tuberculosis* strains.

Thus, the initial aim of the project was to identify npcRNA genes that could act as the potential diagnostic markers for *M. tuberculosis* detection. The identified target is then coupled with IS*6110* towards the development and optimization of multiplex PCR. Collectively, the objectives are as follows;

1. Identification of npcRNA genes specific for the detection of *M. tuberculosis* via BLASTn analysis Software.
2. Development and optimization of the multiplex PCR using the identified npcRNA genes and IS*6110* as the targets. The parameters are:-
    i) Optimization of annealing temperature
    ii) Optimization of primer concentration
    iii) Incorporation of Internal Amplification Control
    iv) Optimization of deoxyribonucleotide triphosphate
    v) Optimization of $MgCl_2$
    vi) Determination of the specificity of mPCR
    vii) Determination of the sensitivity of the mPCR

3. Evaluation of the mPCR assay

i) Validation of the developed mPCR with culture positive samples

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Materials

### 2.1.1 Chemicals

Chemicals and reagents used in this study were of analytical grade and are listed below:

| Manufacturer | Chemicals |
|---|---|
| Amresco (USA) | Sodium acetate; Sodium hydroxide; Potassium chloride anhydrous. |
| Bio basic inc (Canada) | EDTA; PCR primers. |
| Bio-Rad (Hercules,USA) | Ethidium bromide (10mg/mL); Sodium deodecyl sulfate (SDS) |
| Biotools (Canada) | 10X PCR buffer; Magnesium chloride ($MgCl_2$) ; dNTP mix; DNA polymerase. |
| Laborotarious Conda (Madrid,Spain) | LB agar (lennox); LB broth (lennox), |
| Merck (Darmstadt,Germany) | Middlebrooke 7H9, Middlebrooke 7H10 |
| | Ethanol; Acetic acid (glacial) 100%; Sodium hydroxide; Glycerol |
| Invitrogen ® (Carlsbad,CA) | Ultra-pure Tris |
| Promega (Madison,USA) | 100bp DNA ladder; Agarose powder |

### 2.1.2 Culture and bacterial strains

Bacterial strains used in this study are from the stock collection, obtained from the Department of Microbiology & Parasitology, School of Medical Sciences, USM and Infectomic Cluster, Advance Medical and Dental Institute (AMDI), USM.

| Bacterial Strains | Description | Source |
|---|---|---|
| ***Mycobacterium tuberculosis* H37Rv** | Virulent laboratory strain | AMDI |
| ***Mycobacterium tuberculosis* H37Ra** | Attenuated tubercle bacillus closely related *M. tuberculosis* H37Rv | AMDI |
| ***Mycobacterium avium*** | Non tuberculosis Mycobacterium | AMDI |
| ***Mycobacterium abscessus*** | Non tuberculosis Mycobacterium | AMDI |
| ***Mycobacterium fortuitum*** | Non tuberculosis Mycobacterium | AMDI |
| ***Mycobacterium fortuitum*** | Non tuberculosis Mycobacterium | AMDI |
| ***Mycobacterium gordonae*** | Non tuberculosis Mycobacterium | AMDI |
| ***Mycobacterium scroferaceum*** | Non tuberculosis Mycobacterium | AMDI |
| ***Mycobacterium intracellulare*** | Non tuberculosis Mycobacterium | AMDI |
| ***Mycobacterium kansasii*** | Non tuberculosis Mycobacterium | AMDI |