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IMMUNOPROTEOMIC ANALYSIS OF *Mycobacterium tuberculosis* AND NONTUBERCULOUS MYCOBACTERIA TOWARDS THE DISCOVERY OF POTENTIAL UNIVERSAL OR SPECIES SPECIFIC BIOMARKERS

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IMMUNOPROTEOMIC ANALYSIS OF *Mycobacterium tuberculosis* AND NONTUBERCULOUS MYCOBACTERIA TOWARDS THE DISCOVERY OF POTENTIAL UNIVERSAL OR SPECIES SPECIFIC BIOMARKERS

by

AIMI ZAFIRA BT RAZMAN

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Table of contents

Contents **Page**

Acknowledgements ..... **ii**

Table of contents ..... **iii**

List of tables ..... **ix**

List of figures ..... **xi**

List of presentation ..... **xv**

List of abbreviations ..... **xvi**

Abstrak ..... **xviii**

Abstract ..... **xx**

Chapter 1: General introduction **1**

1.1 Historical perspectives and the importance of tuberculosis in present day 1

1.2 Epidemiology of tuberculosis 1

1.3 Epidemiology of tuberculosis in Malaysia 3

1.4 The genus of *Mycobacterium* 4

1.4.1 Classification 4

1.4.2 Bacteriology of mycobacteria 7

1.5 Mode of transmission 8

1.6 Clinical manifestations 9

1.6.1 Tuberculosis 9

1.6.2 NTM infections 11

1.7 Pathogenesis 13

1.7.1 Pathogenesis of tuberculosis 13

1.7.2 Pathogenesis of NTM infections 15

1.8 Differential diagnosis 15

1.9 Antimicrobial treatment and prognosis 16

1.10 Laboratory diagnosis 19

1.10.1 Direct microscopic examination of clinical specimens and routine culture isolation 19

1.10.2 Tuberculin skin test 20
1.10.3 Radiology 21
1.10.4 Immunodiagnosis of tuberculosis 21
1.10.5 Molecular techniques for diagnosis and species differentiation 23
1.11 Problem statements 24
1.12 Rationale for the initiation of the present study 25
1.13 The overall objectives of the present study 27

Chapter 2: Characterization and comparative analysis of WCP profile of MTBC and NTM by SDS-PAGE 28
2.1 Introduction 28
2.1.1 The specific objectives of the study 29
2.2 Materials and methods 30
2.2.1 Preparation of common buffers and media 31
2.2.2 Bacteria culture preparation and maintenance 31
2.2.3 Safety considerations 32
2.2.4 Extraction of the WCP 32
2.2.5 Assay for total protein content 35
2.2.6 SDS-PAGE analysis of the WCP 35
2.2.7 Analysis of the WCP profile 36
2.3 Results 37
2.3.1 SDS-PAGE protein profiles of the WCP 37
2.3.2 Dendrogram of WCP profiles 40
2.4 Discussion and conclusions 42

Chapter 3: Immunoproteomic characterization of WCP of MTBC and NTM species by WB assay 46
3.1 Introduction 46
3.1.1 The specific objectives of the study 49
3.2 Materials and methods 50
3.2.1 General purpose reagents and stock solutions 51
3.2.2 Preparation of the WCP 51

3.2.3 Serum specimens for WB analysis 51
3.2.4 SDS-PAGE separation of the WCP of MTBC and NTM species..... 52
3.2.5 Western blot analysis of the WCP of MTBC and NTM species..... 52
3.2.6 The WB reactive band analysis 54
3.3 Results and discussion..... 55
3.3.1 Characterization of antibody isotypes responses in PTS against WCP of MTBC and NTM species..... 55
3.3.1 (a) Immunoreactive bands recognized by PTS in WCP of MTBC species..... 56
3.3.1 (b) Immunoreactive bands recognized by PTS in WCP of NTM species 62
3.3.2 Characterization of antibody isotypes responses in PHS against WCP of MTBC and NTM species..... 69
3.3.2 (a) Immunoreactive bands recognized by PHS in WCP of MTBC species..... 69
3.3.2 (b) Immunoreactive bands recognized by PHS in WCP of MTBC species..... 75
3.3.3 The WB analysis of IgM, IgG and IgA antibody isotypes responses in the individual sera of TB patient 81
3.3.3 (a) The WB analysis of individual sera of TB patient against WCP of MTBC species 82
3.3.3 (b) The WB analysis of individual sera of TB patient against WCP of NTM species..... 87
3.3.4 The WB analysis of IgM, IgG and IgA antibody isotypes responses in the individual sera of healthy subjects 92
3.3.4 (a) The WB analysis of individual sera of healthy subject against WCP of MTBC species 92
3.3.4 (b) The WB analysis of individual sera of healthy subject against WCP of NTM species..... 98
3.4 Conclusions..... 103

Chapter 4: Immunological characterization of the WCP separated by preparative isoelectric focusing and analytical polyacrylamide focusing 109

4.1 Introduction 109

4.1.1 The specific objectives of the study 113

4.2 Materials and methods 114

4.2.1 General buffers and solution 115

4.2.2 Extraction of the WCP for liquid phase preparative IEF in Rotofor system 115

4.2.3 Human sera 115

4.2.4 Rotofor fractionation of the WCP by liquid phase preparative IEF of Rotofor system 116

4.2.5 Flat bed PAGE IEF electrophoresis 117

4.2.5 (a) Casting polyacrylamide IEF gel 117

4.2.5 (b) Analytical PAGE IEF gel electrofocusing of the 20 Rotofor fractions 120

4.2.5 (c) Electrophoretic transfer of protein separated in the analytical PAGE IEF gel 122

4.3 Results 123

4.3.1 Fractionation of the WCP of *M. tuberculosis* H37Rv and *M. abscessus* by liquid phase preparative IEF electrofocusing in Rotofor system 123

4.3.1(a) Preparative and analytical separation of WCP of *M. tuberculosis* H37Rv 123

4.3.1 (b) Preparative and analytical separation of WCP of *M. abscessus* 127

4.3.2 Analysis of PAGE IEF immunoblot of the Rotofor fractions of the WCP of *M. tuberculosis* H37Rv by preparative Rotofor system 130

4.3.3 Analysis of PAGE IEF immunoblot of the Rotofor fractions of the WCP of *M. abscessus* by preparative Rotofor system 134

4.4 Discussion and conclusions 138

Chapter 5: The identification of immunogenic proteins of *M. tuberculosis* H37Rv and *M. abscessus* by MALDI-TOF MS **144**
5.1 Introduction 144
5.1.1 The specific objectives of the study 146
5.2 Materials and methods 147
5.2.1 General buffers and solutions 148
5.2.2 Extraction of the WCP for liquid phase preparative IEF in Rotofor system 148
5.2.3 Analysis of antigenic proteins in the Rotofor fractions by MALDI-TOF 148
5.3 Results 151
5.3.1 Preparative PAGE IEF gel analysis of immunoreactive bands detected in the Rotofor fractions F1 and F16 of *M. tuberculosis* H37Rv and fractions F1 and F3 of *M. abscessus* 151
5.3.2 Bioinformatics analysis of excised protein bands from preparative PAGE IEF 153
5.4 Discussion and conclusions 163

Chapter 6: General conclusions and future directions **168**
6.1 General conclusions 168
6.2 Future directions 174

References **175**

APPENDICES
Appendix A
Appendix B
Appendix C
Appendix D
Appendix E
Appendix F

Appendix G

Appendix H

List of Tables

Tables **Page**

Table 2.1 The summary of WCP protein profiles from various mycobacteria species used in the study 39

Table 3.1 Summary of the overall view of humoral immune response in the IgM, IgG and IgA of PTS against the antigens in the WCP of MTBC species analyzed by WB assay 61

Table 3.2 Summary of the overall view of humoral immune response in the IgM, IgG and IgA of PTS against the antigens in the WCP of NTM species analyzed by WB assay 68

Table 3.3 Summary of the overall view of humoral immune response in the IgM, IgG and IgA of PHS against the antigens in the WCP of MTBC species analyzed by WB assay 74

Table 3.4 Summary of the overall view of humoral immune response in the IgM, IgG and IgA of PHS against the antigens in the WCP of NTM species analyzed by WB assay 80

Table 3.5 Summary of WB analysis response of IgM, IgG and IgA antibody isotypes reactivity in individual sera from TB patient against the WCP of MTBC species 86

Table 3.6 Summary of WB analysis response of IgM, IgG and IgA antibody isotypes reactivity in individual sera from TB patient against the WCP of NTM species 91

Table 3.7 Summary of WB analysis response of IgM, IgG and IgA antibody isotypes reactivity in individual sera from healthy subject against the WCP of MTBC species 97

Table 3.8 Summary of WB analysis response of IgM, IgG and IgA antibody isotypes reactivity in individual sera from healthy subject against the WCP of NTM species 102

Table 4.1 Summary of pH value of 20 Rotofor fractions of *M. tuberculosis* H37RV 126

Table 4.2 Summary of pH value of 20 Rotofor fractions of *M. abscessus* 129

Table 5.1 Summary of identified proteins of immunoreactive bands from fraction F1 and F16 of *M. tuberculosis* H37Rv 156

Table 5.2 Summary of identified proteins of immunoreactive bands from fraction F1 and F3 of *M. abscessus* 160

Table 5.3 Summary of the identified proteins of excised immunoreactive bands from fractions 1 and 16 of *M. tuberculosis* H37Rv and fractions 1 and 3 from *M. abscessus* based on the functional categories 162

List of Figures

Figures **Page**

Figure 1.1 The flowchart illustrates the workflow of experimental procedures involved in chapter 1.....26

Figure 2.1 The flowchart illustrates the workflow of experimental procedures involved in chapter 2.....30

Figure 2.2 The procedure of the whole cell protein (WCP) extraction of MTBC and NTM species.....34

Figure 2.3 Coomasie brilliant blue (CBB) stained profile of WCP extracted from *M. tuberculosis* strains and various NTM species separated by 10% SDS-PAGE 38

Figure 2.4 Dendrogram analysis of WCP of mycobacteria species separated from SDS-PAGE.....41

Figure 3.1 The flowchart illustrates the workflow of experimental procedures involved in chapter 3.....50

Figure 3.2 The WB analysis of the serum IgM antibody isotype reactivity against the SDS-PAGE separated antigens from the WCP of MTBC species probed with pooled TB sera (PTS).....58

Figure 3.3 The WB analysis of the serum IgG antibody isotype reactivity against the SDS-PAGE separated antigens from the WCP of MTBC species probed with pooled TB sera (PTS).....59

Figure 3.4 The WB analysis of the serum IgA antibody isotype reactivity against the SDS-PAGE separated antigens from the WCP of MTBC species probed with pooled TB sera (PTS).....60

Figure 3.5 The WB analysis of the serum IgM antibody isotype reactivity against the SDS-PAGE separated antigens from the WCP of NTM species probed with pooled TB sera (PTS).....65

Figure 3.6 The WB analysis of the serum IgG antibody isotype reactivity against the SDS-PAGE separated antigens from the WCP of NTM species probed with pooled TB sera (PTS).....66

Figure 3.7 The WB analysis of the serum IgA antibody isotype reactivity against the SDS-PAGE separated antigens from the WCP of NTM species probed with pooled TB sera (PTS).....67

Figure 3.8 The WB analysis of the serum IgM antibody isotype reactivity against the SDS-PAGE separated antigens from the WCP of MTBC species probed with pooled healthy sera (PHS) 71

Figure 3.9 The WB analysis of the serum IgG antibody isotype reactivity against the SDS-PAGE separated antigens from the WCP of MTBC species probed with pooled healthy sera (PHS) 72

Figure 3.10 The WB analysis of the serum IgA antibody isotype reactivity against the SDS-PAGE separated antigens from the WCP of MTBC species probed with pooled healthy sera (PHS) 73

Figure 3.11 The WB analysis of the serum IgM antibody isotype reactivity against the SDS-PAGE separated antigens from the WCP of NTM species probed with pooled healthy sera (PHS). 77

Figure 3.12 The WB analysis of the serum IgG antibody isotype reactivity against the SDS-PAGE separated antigens from the WCP of NTM species probed with pooled healthy sera (PHS). 78

Figure 3.13 The WB analysis of the serum IgA antibody isotype reactivity against the SDS-PAGE separated antigens from the WCP of NTM species probed with pooled healthy sera (PHS). 79

Figure 3.14 The representative results from the WB analysis of the serum IgM antibody isotypes reactivity against the SDS-PAGE separated antigens from the WCP of *M. tuberculosis* H37Rv and *M. bovis* BCG probed with individual TB sera 83

Figure 3.15 The representative results from the WB analysis of the serum IgG antibody isotypes reactivity against the SDS-PAGE separated antigens from the WCP of *M. tuberculosis* H37Rv and *M. bovis* BCG probed with individual TB sera 84

Figure 3.16 The representative results from the WB analysis of the serum IgA antibody isotypes reactivity against the SDS-PAGE separated antigens from the WCP of *M. tuberculosis* H37Rv and *M. bovis* BCG probed with individual TB 85

Figure 3.17 The representative results from the WB analysis of the serum IgM antibody isotypes reactivity against the SDS-PAGE separated antigens from the WCP of *M. avium*, *M. abscessus* and *M. gordonae* probed with individual TB sera 88

Figure 3.18 The representative results from the WB analysis of the serum IgG antibody isotypes reactivity against the SDS-PAGE separated antigens from the WCP of *M. avium, M. abscessus* and *M. gordonae* probed with individual TB sera.....89

Figure 3.19 The representative results from the WB analysis of the serum IgA antibody isotypes reactivity against the SDS-PAGE separated antigens from the WCP of *M. avium, M. abscessus* and *M. gordonae* probed with individual TB sera 90

Figure 3.20 The representative results from the WB analysis of the serum IgM antibody isotypes reactivity against the SDS-PAGE separated antigens from the WCP of *M. tuberculosis* H37Rv and *M.bovis* BCG probed with individual healthy subject sera.....94

Figure 3.21 The representative results from the WB analysis of the serum IgG antibody isotypes reactivity against the SDS-PAGE separated antigens from the WCP of *M. tuberculosis* H37Rv and *M.bovis* BCG probed with individual healthy subject sera.....95

Figure 3.22 The representative results from the WB analysis of the serum IgA antibody isotypes reactivity against the SDS-PAGE separated antigens from the WCP of *M. tuberculosis* H37Rv and *M.bovis* BCG probed with individual healthy subject sera.....96

Figure 3.23 The representative results from the WB analysis of the serum IgM antibody isotypes reactivity against the SDS-PAGE separated antigens from the WCP of *M. avium, M. abscessus* and *M. gordonae* probed with individual healthy subject sera 99

Figure 3.24 The representative results from the WB analysis of the serum IgG antibody isotypes reactivity against the SDS-PAGE separated antigens from the WCP of *M. avium, M. abscessus* and *M. gordonae* probed with individual healthy subject sera 100

Figure 3.25 The representative results from the WB analysis of the serum IgA antibody isotypes reactivity against the SDS-PAGE separated antigens from the WCP of *M. avium, M. abscessus* and *M. gordonae* probed with individual healthy subject sera 101

Figure 4.1 Characterization of the WCP by combined fractionation in liquid phase preparative IEF and analytical gel phase IEF followed by IEF immunoblotting 112

Figure 4.2 The flowchart illustrates the workflow of experimental procedures involved in chapter 4 114

Figure 4.3 Preparation of analytical PAGE IEF gel. The figure shows the arrangement of glass plate and casting tray used for preparation of the IEF gel for analytical isoelectric focusing analysis of the rotofor fractions. 119

Figure 4.4 Analytical Polyacrylamide gel IEF electrofocusing. 121

Figure 4.5 CBB stained protein profiles in the analytical PAGE-IEF gel analysis of the 20 rotofor fractions obtained from the fractionation of the WCP of *M. tuberculosis* H37Rv in the preparative IEF 125

Figure 4.6 CBB stained protein profiles in the analytical PAGE-IEF gel analysis of the 20 rotofor fractions obtained from the fractionation of the WCP of *M. abscessus* in the preparative IEF 128

Figure 4.7 Analytical PAGE IEF immunoblot analysis of the WCP of *M. tuberculosis* H37Rv fractionated in preparative Rotofor system (pH gradient 3 to 10) and probed with PTS 132

Figure 4.8 Analytical PAGE IEF immunoblot analysis of the WCP of *M. tuberculosis* H37Rv fractionated in preparative Rotofor system (pH gradient 3 to 10) and probed with PHS 133

Figure 4.9 Analytical PAGE IEF immunoblot analysis of the WCP of *M. abscessus* fractionated in preparative Rotofor system (pH gradient 3 to 10) and probed with PTS. 136

Figure 4.10 Analytical PAGE IEF immunoblot analysis of the WCP of *M. abscessus* fractionated in preparative Rotofor system (pH gradient 3 to 10) and probed with PHS 137

Figure 5.1 The flowchart illustrates the workflow of experimental procedures involved in chapter 5 147

Figure 5.2 The list of excised antigenic protein bands from *M. tuberculosis* H37Rv and *M. abscessus* with designated label. 150

Figure 5.3 CBB stained preparative PAGE IEF gel analysis of the antigenic bands of selected Rotofor fractions 152

List of Presentation

Oral presentation :

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List of Abbreviations

AP	Alkaline phosphatase
CBB	Coomassie brilliant blue
E	Ethambutol
H	Isoniazid
HIV	Human immunodeficiency virus
IEF	Iso-electric focusing
IgM	Immunoglobulin M
IgG	Immunoglobulin G
IgA	Immunoglobulin A
kDa	kilodalton
MAPIA	Multi Antigen Print Immuno Assay
MALDI TOF MS	Matrix Assisted Laser Desorption/Ionization- Time of Flight Mass Spectrometry
MDR-TB	Multi-drug resistant tuberculosis
MTB	*Mycobacteria tuberculosis*
MTBC	*Mycobacteria tuberculosis* complex
MOTT	Mycobacteria other than tuberculosis
NC	Nitrocellulose membrane
NTM	Nontuberculous mycobacteria
PAGE IEF	Polyacrylamide gel iso-electric focusing
PCR	Polymerase chain reaction
PHS	Pool healthy sera
PTS	Pool tuberculosis patient sera
pI	Iso-electric point
R	Rifampicin
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TB	Tuberculosis
TBS	Tris-buffered saline
WCP	Whole cell protein
WB	Western blot

Z	Pyrazinamide

ANALISA IMMUNOPROTEOMIK *Mycobacterium tuberculosis* DAN MIKOBAKTERIA BUKAN TUBERKULOSIS KE ARAH PENEMUAN BIO-PENANDA YANG BERPOTENSI UNIVERSAL ATAU SPESIFIK KEPADA SPESIS

ABSTRAK

Analisa immunoproteomik keseluruhan sel protein (WCP) *Mycobacterium tuberculosis* dan mikobacteria bukan tuberculosis (NTM) telah dijalankan bagi mengenalpasti kehadiran bio-penanda yang berpotensi spesifik kepada spesis atau universal yang wujud di dalam mikobacteria. WCP daripada beberapa spesis mikobacteria telah dipisahkan menggunakan SDS-PAGE dan diikuti dengan WB bagi mengesan tindak balas imun antibodi isotip IgM, IgG dan IgA di dalam serum kumpulan pesakit tuberculosis (TB), kumpulan subjek yang sihat dan serum setiap individu tersebut. Reaksi WB dapat dibahagikan kepada 4 bahagian; bahagian (1) atas 67 kDa ; (2) 67 kDa – 32 kDa ; (3) 30 kDa, 28kDa dan 26 kDa; dan (4) bawah 25 kDa. Berdasarkan keputusan WB, dua spesis telah dipilih iaitu *M. tuberculosis* H37Rv dan *M. abscessus* untuk kajian selanjutnya. Kedua-dua spesis tersebut telah ditulenkan dan dipecahkan menggunakan sistem persediaan Rotofor IEF dan dicirikan dengan kaedah analitikal poliakrilamida gel IEF (PAGE IEF) diikuti dengan kaedah blot imun. Berdasarkan keputusan blot imun, 2 pecahan daripada setiap spesis; pecahan F1 dan F16 *M. tuberculosis* H37Rv dan pecahan F1 dan F3 *M. abscessus* dipilih untuk pencirian bagi mengenalpasti protein-protein dengan kaedah Spektrometri Jisim Matrix-Assisted Laser Desoption-Ionization Time of Flight (MALDI-TOF MS). Dengan menggunakan kaedah tersebut, sebanyak 4 jalur dipotong daripada gel

persediaan PAGE-IEF pecahan *M. tuberculosis* H37Rv (dikodkan sebagai : T1B1, T1B2, T16B1 and T16B2) dan sebanyak 7 jalur bagi pecahan *M. abscessus* (dikodkan sebagai : A1B1, A1B2, A2B3, A3B1, A3B2, A3B3 and A3B4). Kaedah MALDI-TOF mengarahkan kepada identifikasi 21 peptida *M. tuberculosis* H37Rv dan 8 peptida *M. abscessus.* Setiap satu peptida daripada *M. tuberculosis* H37Rv mengarahkan kepada identifikasi protein yang berbeza dengan pelbagai kategori fungsi. Selain daripada itu, 3 peptida *M. abscessus* mendedahkan kepada identifikasi protein yang sama walaupun daripada jalur yang berbeza di dalam pecahan F3. Keputusan dari kajian ini juga menunjukkan protein reaktif imun yang dikenalpasti tidak pernah dilaporkan sebagai antigen di dalam mana-mana kajian sebelum ini, walaupun antigenisiti protein ini telah dapat dilihat dan disahkan di dalam blot imun PAGE IEF. Oleh itu, analisa selanjutnya diperlukan kerana protein-protein yang dikenalpasti ini mempunyai potensi sebagai bio-penanda untuk diagnosis TB dan juga bagi penghasilan vaksin yang lebih baik untuk TB.

IMMUNOPROTEOMIC ANALYSIS OF *Mycobacterium tuberculosis* AND NONTUBERCULOUS MYCOBACTERIA TOWARDS THE DISCOVERY OF POTENTIAL UNIVERSAL OR SPECIES SPECIFIC BIOMARKERS

ABSTRACT

The immunoproteomic analysis of whole cell protein (WCP) of *Mycobacterium tuberculosis* complex (MTBC) and non–tuberculous mycobacteria (NTM) was carried out to identify the potential species specific and cross genus conserved species biomarkers present in the mycobacterial species. The WCP of *Mycobacterium* species were separated using SDS-PAGE and followed by Western blot assay to detect IgM, IgG and IgA immune response in pooled sera of TB patients and healthy subjects as well as the individual sera. The immunoreactive bands in WB assay were divided into 4 region of antigens; (1) above 67 kDa; (2) 67 kDa – 32 kDa; (3) 30 kDa, 28kDa and 26 kDa; and (4) below 25 kDa. Based on the WB results, the two species selected for further investigation were *M. tuberculosis* H37Rv and *M. abscessus*. The WCP of these species were purified and fractionated by preparative rotofor isoelectric focusing (IEF) system and characterized by analytical polyacrylamide gel (PAGE IEF) followed by immunoblotting. Based on the immunoblotting results, 2 fractions from each species were selected. These include fraction F1 and F16 of *M. tuberculosis* H37Rv and fraction F1 and F3 of *M. abscessus*. The proteins from the selected fractions were subjected to identification by MALDI-TOF MS. Employing the approach, a total of 4 bands were excised from preparative PAGE-IEF gel of *M. tuberculosis* H37Rv's fractions (encoded as: T1B1, T1B2, T16B1 and T16B2) and a total of 7 bands from *M. abscessus*'s fractions (encoded as: A1B1, A1B2, A2B3, A3B1, A3B2, A3B3 and

A3B4). The MALDI-TOF led to the identification of 21 peptides from *M. tuberculosis* H37Rv and 8 peptides from *M. abscessus.* Each single peptides from *M. tuberculosis* H37Rv led to the identification of different proteins with various functional category. On the other hand, 3 peptides from *M. abscessus* revealed the identification of the same protein as the peptides from the bands in the particular fractions were fragmented during MALDI-TOF. The results from this study also showed the identified immunoreactive proteins has yet to be reported in other previous studies but the antigenicity of these proteins were observed and confirmed in this study. Thus, further analysis need to be done as the identified proteins from this study hold the potential as biomarkers for TB diagnosis and also for improved vaccine development for TB.

Chapter 1

General Introduction

1.1 Historical perspectives of mycobacterial diseases

Major mycobacterial diseases in human are tuberculosis (TB) and leprosy. Leprosy is a localized infectious disease confined to skin and caused by *Mycobacterium leprae* (Portaels, 1995; Steingart *et al*., 2007). On the other hand, TB is a systemic infectious disease of multi organ systems. However, pulmonary TB is most frequently implicated. The description of *Mycobacterium tuberculosis* started in 1826 followed by its discovery in 1882 by Robert Koch. Another early discovery of mycobacterial disease, leprosy was discovered by Hensen in 1873 (Schuster, 1995; Sato and Frantz, 2005). The identification of non-tuberculous mycobacteria (NTM) started shortly after that but often abandoned during the period. However, it manage to draw attention due to the increasing reports of infections caused by NTM (Portaels, 1995; Schuster, 1995; Moore *et al*, 2010; Mirsaeidi *et al*., 2014).

1.2 Epidemiology of tuberculosis

TB remain as one of the second leading cause of death in developing countries together with malaria and AIDS. The World Health Organization (WHO) declared TB as a global health emergency in 1993 due to the dramatic changes in the magnitude of the problem (Wangdi and Gurung, 2012; Ejeta *et al*., 2013). It was also reported by WHO in 2009 there were almost 13.7 million people infected with TB and compared to other

infectious disease, TB kills more young people and adults (Jassal and Bishai, 2010). In addition, it was estimated that out of 2 billion individuals infected with TB, only 10% develop active TB while the remaining 90% individuals may develop latent TB (Demissie *et al*., 2006). The resurgence and increasing number of cases of TB may have related to several factors including the emergence of multi- drug resistant TB (MDR-TB), HIV epidemic, failure to comply to antibiotic treatment, ineffective control programs, population growth and as well the migration of people from countries with high prevalence of TB (Coker, 2004; Nissapatorn *et al*, 2007; Alavi and Alavi, 2013).

Raviglione and team (1995) stated that global notifications rate per 100 000 populations were increased in the WHO regions such as African, the East Mediterranean, the Southeast Asian and the Western Pacific (Raviglione *et al*., 1995). By comparing the data obtained between 1984 to 1986 and 1989 to 1991 has shown notifications rate increment of 20.8% compared to the latter (Werf and Antoine, 2015). In addition, this trend was also followed by the developing countries worldwide. However, the regions such as American and European showed the decreasing in average number of cases.

In 2005, it has been reported that Asia; Bangladesh, China, India, Indonesia and Pakistan contributed to 49% of new TB cases worldwide (Kaufmann and Helden, 2008). This was supported by the recent report by Glaziou *et al*., (2015) that higher proportions of estimated cases in 2012 which majority of the cases come from Southeast Asia and 27% from Africa.

In Africa, HIV had the greatest impact on the mortality of TB (Glazio *et al*., 2013). According to Dye (2006), the African region was reported to have the highest estimation of incidence rate which is 356 per 100 000 population per year. Improper management of TB control as well as the disruption of political, social and economical explained the resurgence of TB in 1990s (Dye, 2006; Glazio *et al*., 2013).

1.3 Epidemiology of tuberculosis in Malaysia

Historically back in 1940s and 1950s, TB was the leading cause of death due to infectious disease in Malaysia (Iyawoo, 2004). Since 1950s the enormous effort and strategy have been done in order to eradicate the disease. The chemotherapy only available in late 1950s when TB has become the major cause of morbidity and mortility (Iyawoo, 2004). In addition, National Control TB Program (NTP) was launched in 1961 and play as an important role for TB management and control. The efforts gave a successful outcome as the disease significantly declined and it was believed in 1980s, TB was no more a major threat in Malaysia (Shanmuganathan and Subramaniam, 2013). However, the notifications rate per year keeps increasing in these past 20 years. Among all of the notified TB cases in Malaysia, 10% are contributed by the influx of immigrant population from the neighbouring countries especially Indonesia, Myanmar, Bangladesh and India (Iyawoo, 2004; Shanmuganathan and Subramaniam, 2013). In addition, according to the data in Tuberculosis Information System (TBIS), Ministry of Health, the incidence rate of active TB among health care personnel was higher than general population from 2003 t0 2006 which is 73.4 - 77.7 per 100,000 and 60.3-62.6 per 100,000 respectively (Rafiza *et al*.,2011). The state of Sabah was reported with highest burden of TB with

the estimated incidence rate 131 per 100, 000 populations and this followed by Federal Territory, state of Sarawak and state of Penang (Iyawoo, 2004; Ali *et al*., 2015).

1.4 The genus of *Mycobacterium*

1.4.1 Classification

The identification and description of tubercle bacilli by Robert Koch began in the late 1800s. In 1896, Lehmann and Neumann identified Mycobacteria and proposed the genus *Mycobacterium* and renamed the tubercle bacilli as *Mycobacteria tuberculosis* (Collins *et al.*, 1982; Shinnick and Good, 1994). The genus was placed in family of *Mycobacteriaceae*, order of *Actinomyceteles* and class of *Actinomycetes* (Shinnick and Good, 1994). Taxonomically, mycobacteria belong to the genus *Mycobacterium* which one of the oldest defined genus and single genus within the family of Mycobacteriaceae (Rastogi *et al.*, 2001; Saviola and Bishai, 2006). The genus currently comprises of 163 mycobacteria species and 13 subspecies which includes the complex group of *M. tuberculosis* (MTBC) and environmental mycobacteria group according to list of bacteria species names with approved names (www.bacterio.cict.fr/m/mycobacterium.html) (Zivanovic *et al*., 2014).

In addition, due to the similar characteristics with *Nocardia* and *Corynebacterium*, additional criteria were developed to differentiated these groups which includes Gram staining, growth rate, penicillin resistance, percentage of guanine-cytosine (GC) content, mycolic acid profiles and arylsulfatase production (Shinnick and Good, 1994). Hartmans and others reported, the differentiation of these three genera by

mycolic acid content can be done through chemical differentiation ester pyrolysis product of the mycolic acid esters, in combination with the identification of the major menaquinone present in the plasma membrane (Hartmans *et al*.,2006). The unique characteristic of the species in the genus is their ability to synthesis mycolic acids with additional oxygenated functions such as methoxy, keto, epoxy and carboxylic. In *Mycobacterium,* the species usually contains 60-90 carbon atoms which are cleaved at C22 to C26 fatty acid methyl by pyrolysis (Shinnick and Good, 1994). On the other hand, species of *Norcardia* and *Corynebacterium* contains 44-60 and 22-36 carbon atoms which cleaved at C14 to C18 and C8 to C18 fatty acid methyl, respectively (Hartmans *et al*.,2006). The presence of this oxygenated mycolates has made it as the criteria for including an organism in the genus *Mycobacterium* (Frebault and Portaels, 1992).

Following Robert Koch discovery, the other type of tubercle bacilli were discovered such as bovine types, avian type and marine type. Within the genus *Mycobacterium* itself, there are number of complex group were formed eg: *M. tuberculosis* complex (MTBC) and *M. avium* complex (MAC) (Saviola and Bishai, 2006). The species were assigned in particular group based on their genetically high degree of similarities and the formation of similar syndrome of the disease. In MTBC group, it comprised of closely related *Mycobacterium* species such as *M. tuberculosis, M. bovis, M. bovis* BCG, *M. africanum, M. microti* and *M. canetti* which are the etiologic agents for tuberculosis in mammals and human (Medie *et al*., 2011). Taxonomically, despite sharing >95% of DNA-DNA homology with one another among the species in complex group, the members of MTBC differ in their host range and pathogenicity (Shinnick and Good, 1994; Niemann *et al*., 2000). For example, *M. tuberculosis* and

M. bovis can be distinguish through the biochemical test in clinical laboratory (Good, 1985).

The atypical mycobacteria previously referred as environmental mycobacteria, mycobacteria other than tubercle (MOTT) or opportunistic mycobacteria, though the term non-tuberculous mycobacteria (NTM) is preferred (Koh *et al*., 2002). After the discovery of this group of mycobacteria, several numbers NTM were found to cause disease in humans (Hartmans *et al*., 2006). Thus, the identification and classification are necessary.

The first classification of NTM was proposed by Timpe and Runyon in 1954 based on the growth rates, colony morphology and chromogenicity (Koh *et al*., 2002; Marusic and Jankovic, 2012). In the first classification, Runyon proposed a classification of NTM into 4 major groups. The four groups consists of photochromogens, schrotochromogens, non-chromogens and rapid grower group (eg: *M. fortuitum*) (Goods, 1985). The first 3 groups are considered slow growers which requiring a time to grow in culture as *M. tuberculosis* (Rastogi *et al.*, 2001). The slow grower species are further differentiated according to their ability to produce yellow pigment (Koh *et al*., 2002). The growing duration of slow grower mycobacteria usually takes 15-30 days while rapid grower mycobacteria takes 2-7 days to grow on the culture medium before the sufficient growth is achieved (Shinnick and Good, 1994; Saviola and Bishai, 2006).

1.4.2 Bacteriology of Mycobacteria

Mycobacteria are non-motile rod shaped bacterium with <5µm in length, acid-alcohol fast, aerobic and composed of complex cell envelope. The cell envelope consists of a plasma membrane, a peptidoglycan layer, a hydrophobic thick mycolate layer and outer lipids (Brennan and Nikaido, 1995; Barry *et al*. 1998; Osoba, 2004; Marrakchi *et al*., 2014). Mycobacteria are not classified either Gram positive or Gram negative due to the high lipid content on the cell wall which resist decolorization of acid-alcohol. Hence, known as acid-fast bacteria (Leung, 1999).

Mycobacterium species have been isolated from diverse habitats of origin including mammalian and non-mammalian habitat. For instance, soil, fresh, salt water, drinking water system and dust usually imply to the NTM species. Most of the NTM species are environmental organism which are not associated with infections and disease (Wang and Behr, 2014). However, the pattern changed since 1980 when the outbreak of AIDS starting in 1982 and infections of *M. avium* were reported in AIDS patients (Narang 2008; Falkinham, 2009). The fact that NTM species sharing habitats with human and animals is clearly major determinant in disease acquisition (Falkinham, 2009). Unlike NTM, *M. tuberculosis* is an obligate pathogen of human which adapted well in the host and no known environmental reservoir (Wang and Behr, 2014). *M. tuberculosis* have the ability to survive and replicate in human macrophages. Thus, make the macrophages as its major habitat inside the host (Danelishvili *et al*., 2014).

Mycobacterium species are usually subculture on the egg-based medium eg: Lowenstein Jensen (conventional standard media) or Middlebrook media. The bacterium grow at 37°C and produce typical small, rough or smooth and buff colour colony morphology on the culture media usually after 2-4 weeks of incubation. On the other hand, as aforementioned in Runyon classification, NTM species can be distinguished based on the production of colour pigment and growth rate (Johnson and Odell, 2014). Among the rapid grower species of NTM, they usually form bright yellow or orange coloured colonies subjected to carotenoid pigments and familiarity with Runyon classification remains useful for presumptive laboratory identification of possible NTM pathogens. However, positive identification of NTM species are largely based on the biochemical test and other molecular techniques (Ioachimescu and Tomford, 2010).

1.5 Mode of transmission

TB is an airborne disease and mode of transmission is from human-to-human via air droplet. Since the habitat of MTBC species are in the host of human and animal, transmission occurs through the inhalation of the air droplet nuclei containing the bacilli which are typically expelled and suspended in the air when a patient with active TB coughing or sneezing. The primary route of infection involves the lungs due to the small size. The bacilli can remain suspended in the air for several minutes to hours. Thus, the bacteria can be transmitted from one person to another and the risk of getting infected is high for the individuals with close contact to the patient with active TB (Ahmad, 2011; Shakri *et al.*, 2012).

In addition, the risks of infection depend on several factors such as closeness of contact, inhaled bacillary load and the immune status of the potential host. There are also several reported cases of tuberculosis in animal species which lived close and prolonged contact with humans. The infection of *M. tuberculosis* in the animal usually identified in cattle (Ocepek *et al*., 2005**;** Romero *et al*., 2011).

1.6 Clinical manifestations

1.6.1 Tuberculosis

The stages of TB may develop differently for each patient according to their status of immune system. The stages of progress of the disease include latency, primary disease, primary progression of the disease and extrapulmonary disease (Knechel, 2009). At the stage of latency, infected individuals are asymptomatic. The viable bacilli may remain dormant and survive for years or decades. As the immune system of the person start to weaken, the disease may start to reactivate. An individual that has co-infection with human immunodeficiency virus (HIV) may show the prominent progression towards active disease stage. Besides that, other factors such as old age, malnutrition, uncontrolled diabetes mellitus, sepsis, renal failure, smoking, chemotherapy and organ transplantation that lead to diminishing in immunity may trigger the reactivation of the bacilli towards the active stage of the disease (Knechel, 2009).

At the stage of primary disease, the person may not show any symptoms and the diagnostic tests are the only evidence in order to confirm the disease. At this point, the bacilli that has been implanted in the alveoli will spread from the lungs through the

lymphatic circulation which later on may cause pleural effusion (Smith, 2003; Knechel, 2009). It may remain small and in some cases, it may resolve by itself or it also may become large to induce symptoms fever, pleuritic chest pain and dyspnea (Smith, 2003; Knechel, 2009).

The next stage of the disease is a primary progressive disease or active disease which develops in only 5 % to 10% of persons that exposed to *M. tuberculosis.* At this stage, the patients may start showing non-specific symptoms such as fatigue, malaise, weight loss, fever and night sweats. Wasting has been recognized as the classical feature of TB due to the reduction in appetite and lead to a decreased in energy intake. Wasting also usually comprises loss of fats and lean tissue. Thus, decreased muscle mass may contribute to the fatigue (Paton *et al.*, 2004). Coughing is a common symptom of the disease even though initially it is non-productive, later on it may advance to a productive cough with purulent sputum. In addition, pleurisy or inflammation of the pleural surface may occur due to rupture of a subpleural space caseous focus in the lung into the pleural space resulted from the hematogeneous dissemination or the release of bacteria from subpleural of caseous focus in the lungs (Ferrer, 1997; Smith, 2003; Jeon, 2014).

Knechel reported extrapulmonary disease occurs in more than 20% of immunocompetent individual and the risk in getting extrapulmonary disease increase with immunosuppression (Knechel, 2009). Extrapulmonary TB, involving multi organ system usually arise from the dissemination of the bacilli from lung which is the primary site of infection. High percentage of extrapulmonary TB was reported in the USA (1964-1989) compared to the developing countries (Smith, 2003). Besides

that, cases reported in the literature showed that organ systems associated with extrapulmonary TB are varies in a mixed population within a country. In the related study in England showed 20% of the TB patients originated from Europe had extrapulmonary TB which involved lymph node, bone and joints while 90% are associated with genitourinary. On the other hand, 60% out of 45% extrapulmonary TB in patients originated from India subcontinent showed association with lymph node, bones and joints (Yates and Grange, 1993).

Miliary TB is another form of the extrapulmonary TB that is due to septicemia. The dissemination of the bacilli will involves other multiorgan. Miliary TB can be difficult to diagnose due to the nonspecific signs such as fever, weight loss and weakness (Knechel, 2009). Another fatal form of extrapulmonary TB is where the infection takes place in the central nervous system and it may result in meningitidis. Tubercular meningitidis is fatal in most cases if it is not treated. Headaches and mental confusion are the common manifestation of the disease. Another part of the body that may involve in the case of extrapulmonary TB includes bones, joints, pleura and genitourinary system.

1.6.2 NTM infections

NTM species are frequently isolated from environment and often harmless to human. However, they also may responsible in causing opportunistic disease in the subjects presenting with various predisposing condition (Tortoli, 2009). The four distinct clinical syndromes of NTM infections are progressive pulmonary disease, superficial lymphadenitis, disseminated disease and skin and soft tissue infection (Griffith, 2015).

The progressive pulmonary disease takes place in the lungs because it can easily be infected through inhalation of aerosolized mycobacteria. Among HIV-negative patients, the manifestation range from asymptomatic to cavitary disease while X-ray may reveal fibrosis, upper lobe cavitation, nodular or parenchymal opacity, and pleural thickening (Tortoli, 2009). The most affected population is elderly patients with predisposing pulmonary conditions such as obstructive pulmonary disease, bronchiectasis, pneumoconiosis or cancer. On the other hand, the radiographic image in AIDS patients are usually normal with rapid progression. The immunocompromised patients have low CD4 lymphocytes count. The use of anti-retroviral treatment has reduced the frequency of mycobacterial pulmonary disease in HIV-positive patients (Tortoli, 2009). The superficial lymphadenitis are common among the children which most of the cases affected cervical lymph nodes and caused by *M. avium* complex (MAC) and *M. scrofulaceum* (Griffith, 2015). On the other hand, disseminated disease develop mostly in severely immunocompromised patients. The two major routes of infection involve respiratory apparatus and gastrointestinal tract. Disseminated MAC was one of the first opportunistic infections detected in AIDS patients (Tortoli, 2009). For skin and soft tissue infection, it usually infect as the results of direct inoculation. The site of infections usually involve hand, elbow, knee and foot which the infections of cutis and soft tissue are characterized by granulomatous lesions developing a few weeks after infection (Tortoli, 2009). The source of infection include contact with contaminated water or infected fish, traumas and surgical wounds (Tortoli, 2009).

1.7 Pathogenesis

1.7.1 Pathogenesis of tuberculosis

The knowledge on the interaction between the host and bacteria is essential in understanding the pathogenesis of *M. tuberculosis*. As an intracellular pathogen, the tubercle bacilli is able to invade the host and successfully replicate within host macrophages (Smith, 2003). In addition, the bacteria also known to resist and evade the immune response of the host and able to modify the immune response of the host. The tubercle bacilli also able to persist within its host in dormant stage by developing granuloma (Smith, 2003). The virulence factors that contribute to the ability of the bacterium to survive in the host has been described based on their function and cellular localization such as secretory proteins, cell wall compartments, enzymes involved in lipid and fatty acid metabolism or in amino acid and purine biosynthesis, compounds required for metal uptake, oxidative stress proteins and transcription regulators (Mukhopadhyay *et al*., 2012).

The acquisition of the infection start through the inhalation of air droplet contains tubercle bacilli and when the bacilli reach and when the bacilli arrived in the alveoli, it will start to interact with the alveolar macrophages through many different receptors (Rock *et al*., 2008). In contrast to other pathogenic bacteria, *M. tuberculosis* has no known as either endotoxins or exotoxins, therefore the host will not show any immediate immune response at the early stage of infection (Kabra *et al.*, 2004; Sharma *et al*., 2005). *M. tuberculosis* is an intracellular pathogen, thus has the ability to survive over initial defenses, it will continue multiply within the alveolar macrophages.

Macrophages will ingest and contained the tubercle bacilli and continuous interaction between macrophages and the bacilli results in the formation of granuloma that keep the tubercle bacilli in a confine region in the lung. The tubercle bacilli in the granuloma are prevented from multiplication, stay dormant and develop into later stage of infection. At this stage, the majority of the infected individuals will remain "healthy" without showing any symptoms However, if the macrophages cannot destroy and inhibit the growth of *M. tuberculosis*, it may begin to multiply rapidly that will lead to the reactivation.

Host pathogen interaction in TB is dominated by cellular immune response. The initial immune process will continue for 2- 12 weeks in which the bacteria will continue to grow until they achieved sufficient numbers to elicit cellular immune response. The immune response at this state commonly can be measured by Mantoux test (Diagnostic Standards and Classification of Tuberculosis in Adults and Children, 2000; Knechel, 2009). Guidry *et al.* (2007) and Harding (2011) reported the persistency of the *M. tuberculosis* to survive in granuloma is contributed by the changes in bacterial metabolism as well as host metabolism which is part of the effector protein and glycolipids of *M. tuberculosis* (Guidry *et al.*, 2007; Harding *et al.*, 2011). The persistency of the bacilli is influenced by various conditions such as hypoxia, nutrient deficiency, acidic pH and inhibition of respiration by nitric oxide. In the state, the bacilli are able to control the growth, metabolic and replicative activity (Druszczynska *et al*., 2012).

1.7.2 Pathogenesis of NTM infections

In contrast to *M. tuberculosis*, there is no evidence of person-to-person transmission of NTM as the diseases caused by NTM are not contagious. The NTM may take advantage and caused disease in individual due to altered local or systemic immunity such as patients with following trauma, lung disease history and immunosuppresion (Good, 1985; Katoch, 2004). In addition, their occurrence in the disease progress may suggests the source from where the organism derived (Good, 1985). The NTM infections by airborne pathway may contribute to respiratory disease. However, pulmonary disease which caused by NTM often mistakenly thought as tuberculosis due to the hardly distinguishable from clinical, radiological and historical aspects (Good, 1985).

1.8 Differential diagnosis

The most common disease that often confused with TB are pulmonary fibrosis, pneumonia, bronchiectasis, fungal disease, pleurisy, neoplasm, sarcoidosis, undetermined cause of granuloma and lung abscess (Bhatt *et al.*, 2012). Chest radiography, positive tuberculin skin test result and detection of the acid-fast bacilli in the sputum remain as common method in diagnosis of TB. However, in any case which the result is lacking, consideration of differential diagnosis is necessary. For example, lung cancer, pulmonary amoebiasis and abscesses of unresolved pneumonia which caused by *Klebsiella pneumoniae* may appear as cavitary lesion on chest radiography. The absence of the acid-fast bacilli on microscopy may suggest to the above causes (Grange and Zumla, 2008).

1.9 Antimicrobial treatment and prognosis

In treating TB, taking medication remains as the cornerstone in TB management but become complex with the emergence of multidrug-resistant TB (MDR-TB) and HIV coinfection with TB (Maher *et al*., 2003). Furthermore, the possibility of successful treatment of the disease are strongly depends on the completion of the prescribed treatment on TB patient. Interruption of the treatment will result on the progression and ongoing transmission of the disease and patients are at highly risk to develop MDR-TB (Maher *et al*., 2003).

Upon discovery of streptomycin in 1943, it became the first effective anti-TB drug in treating TB (Lienhardt *et al*., 2012). Currently, the treatment for a confirmed case of active TB comprised of two phases; initial intensive phase and a continuous phase (Jain *et al*., 2008).

During the intensive phase, a standard anti-TB therapy required 6 months duration of treatment. The first 2 month, patients will receive 3 to 4 drugs; rifampicin (R), isoniazid (H), pyrazinamide (Z) and in some cases ethambutol (E) will be given. In the following 4 month, patients will continue with rifampin and isoniazid (Jain *et al*., 2008). The usage of multiple drugs is necessary to support the elimination of actively replicating bacilli.

The continuation phase involves most patients with pulmonary or extrapulmonary TB. The anti-TB drugs in this phase consists of rifampicin and isoniazid which administered daily in 4 to 7 month duration. In most patients, this phase usually last

for 4 month except for the patient who did not take pyrazinamide in their initial phase of treatment, patients with cavitary pulmonary disease caused by drug-susceptible TB, patients whose sputum culture remains positive at the end of intensive treatment and patients whose receiving isoniazid with rifampicin once in a week but the culture remains positive for *M. tuberculosis* after the intensive treatment (Inge and Wilson., 2008). In the case of TB patient co-infected with HIV, taking anti-TB drugs may reduce the effectiveness of antiviral drugs. In such cases, the patients may stop taking antiretroviral therapy while they complete the short course of TB regimen which includes rifampin. Otherwise, rifampin may be replaced with other anti-TB drugs which is less likely to interfere AIDS medications (Bhowmik *et al*., 2009).

The goal of TB treatment is to minimize the transmission of the bacteria and to cure the individual infected with the disease. Furthermore, an effective treatment depends on the several factors such as the susceptibility of the bacterial strain to anti-TB drugs, the adherence of patients to the TB regimen and also the duration of therapy. In many cases, patients always fail to finish the course of therapy when they start to feel better after some time of the therapy. However, by stopping the treatment too soon may result in resistance of the bacteria to the drugs and lead to the development of MDR-TB (Tsara *et al*., 2009). The resistant strain is more dangerous, difficult to treat and may become fatal and contributed major problem in the TB management. Thus, The World Health Organization (WHO) has developed a program known directly observed therapy short course (DOTS) to help patients in managing and monitor their course of therapy. In this program, a nurse or health care professional will monitor all patients taking the doses of the drugs required in the course of therapy, rather than relying on patients to take the medicine by their own (Chan and Iseman, 2002; Bhowmik *et al*.,

2009). DOTS has been introduced in Malaysia in late 1990s and became part of national TB program. DOTS has improved the cure rate in many countries in but not in Malaysia (Ronaidi *et al*., 2011). The cure rate had declined in 2005 and 2006 from 70% to 48%, respectively although overall the cure rate was 100% from 2000 to 2007 (Ronaidi *et al*., 2011).

Currently, the only available vaccine that has been used widely throughout the world since 1921 is *Mycobacterium bovis*, bacilli Calmette- Gue´rin (BCG) (Colditz *et al*., 1994; Reyn and Vuola, 2002; Bowmik *et al*, 2009). Although it has been administered in order to prevent the disease, the BCG vaccine has been shown to be only effective in preventing childhood TB. Due to this reason, BCG vaccination has been applied to the newborn to confer protection against the serious forms of childhood TB and the efficacy of the vaccine wanes over the period of 10 to 15 years (Dam and Hitz, 1980; Rodrigues *et al*., 1991; and Brandt *et al*, 2002). Furthermore, the efficacy of BCG vaccine is inconsistent in adults and caused a false positive result in tuberculin skin test (TST). Although there are several vaccine candidates which has been demonstrated in animal models which shows an equal to or superior to the BCG vaccine, the trial on human as the subjects is still underway. Due to this factor, the development of an improved and effective vaccine will require a prolonged period of time. In related development, 16 different vaccine candidates are currently under in clinical-phase trials (Achkar *et al*., 2015).

1.10 Laboratory diagnosis

1.10.1 Direct microscopic examination of clinical specimens and routine culture isolation

The most widely applied routine laboratory diagnosis of TB comprised of microscopic examination of the respiratory samples for acid-fast bacilli using Ziehl-Neelsen (ZN) staining method and culture isolation of the organism (Shingadia and Novelli, 2003; Garg *et al.*, 2003; Palomino, 2005; Kashyap *et al*., 2007; Emovon, 2009). The presence of acid-fast bacilli (AFB) in pink colour indicate the presence of tubercle bacilli. The advantages of this method are inexpensive and rapid as the result should be available within 24 hours after specimen collection. Hence, it has been widely used in resource-poor countries (Brodie and Schluger, 2005). In addition, the number of infecting bacilli can be estimated and thus, the extent of an individual infectiousness of the disease can be assessed. However, different species of mycobacteria can appear similar on the AFB staining and will lead to the low sensitivity and specificity result. Thus, AFB smear alone without culture cannot distinguish between *M. tuberculosis* and NTM species (Eichbaum and Rubin, 2002). Another constraint, it required greater than 10^4 bacilli per ml of sputum. In the case of TB among children and HIV patients, an inadequate amount of sputum will lead to the greater failure rate which later on could be mistreated the disease (Ghadiri *et al*., 2008).

To date, in laboratory diagnosis, culture technique remains as a gold standard which definitive diagnosis of TB and other mycobacterial disease relies. The bacteria can be isolated from various clinical samples such as sputum, body fluid, abscess and wound.

However, it is time consuming and due to the slow growth on the Lowenstein-Jensen (LJ) medium, it required a mean of the incubation period for at least 2-4 weeks to yield results (Kidenya *et al*., 2013). This will lead to the delay of the result and treatment of the patients. In-house medium such as Kirchner or various Middlebrook formulations also has been widely used but lack of standardization while semi-automated culture such as BACTEC system is quite expensive (Heifets *et al*., 2000). In addition, it needs the disposal of radioactive material and cannot be used for confirmation of the colony morphology (Emovon, 2009).

1.10.2 Tuberculin skin test

Tuberculin skin test (TST) is a common and oldest diagnostic test in the history of TB diagnosis. In this test, purified protein derivative (PPD) is injected intradermally and the reaction to the injection is read after 48-72 hours by measuring the diameter of induration in the unit of millimeter (Emovon, 2009; Tsara *et al.*, 2009; Lange and Mori, 2010). According to WHO guideline, positive TST is regarded as positive when a person showing induration of >10mm without prior vaccination previously and >15mm for those who had vaccinated with BCG vaccine (Shingadia, 2012). Traditionally, this test was used to diagnose LTBI. However, the limitation of TST is low specificity because it contains a mixture of antigens that may cross react with many *Mycobacterium* species. The false positive result may also occur when a person had exposed to the infection by NTM and when a person had a history of BCG vaccination. In addition, the false negative result may occur as the result of severe TB and immunocompromised patients (Kashyap *et al*., 2007; Tsara *et al.*, 2009).

However, this does not rule out the TB. Further testing is needed for the confirmation of the disease.

1.10.3 Radiology

In conjunction of TST, screening of chest radiography is often used when the TST result may be unreliable. The purpose of chest radiography is to screen if a person develops an active TB (Leung, 1999). In the case of an individual infected with both active TB and HIV, the chest X-ray may show a 'normal' appearance, thus chest X-ray may not be definitive (Burrill *et al*., 2007). In addition, the interpretations are very subjective which required an expertise to do the interpretation and result can be non-specific.

Computing tomography (CT) imaging is more sensitive than conventional chest X-ray and may be helpful in describing and identifying pulmonary TB such as endobronchiol disease, early cavitation and bronchiectasis which follow by pulmonary TB where chest X-rays are normal or unreliable (Shingadia and Novelli, 2003; Lange and Mori, 2010). Furthermore, CT imaging may also helpful in investigating other form of TB such as TB meningitis or tuberculoma (Shingadia, 2012).

1.10.4 Immunodiagnosis of tuberculosis

Generally, the immunological based assay is preferred over the bacteriological method in the diagnosis of TB because it does not depend on the detection of bacteria (Abebe *et al*., 2007). In immunology of TB, it comprises of two categories of method which

is method that measure cellular immune response to detect latent TB infection (LTBI) and method that measure antibodies level response to specific antigens in diagnosing active TB (Davies and Pai, 2008).

A test that measures cellular immune response is known as interferon-gamma release assay (IGRA) which emerges as an alternative to TST. The LTBI are detected by measuring interferon-gamma (IFN-γ) released from the T-cells after the stimulation with TB specific antigens. However, IGRAs like the TST are incapable in discriminating between active TB disease and LTBI cases (Janssens, 2007). Of all the issues above, it should be highlighted that IGRAs were designed to detect the infection of *M. tuberculosis* instead of active TB disease.

On the other hand, a serology test method which is based on antibody-antigen detection measures the antibody level of an individual that response to the particular antigens. It is an alternative approach to support an early conclusion of the laboratory diagnosis of TB. In the past decades inefficient performance of a serological assay has resulted in extensive studies that lead to the better understanding of the humoral and cellular response of TB. There are still some important criteria need to be taken into account in choosing a diagnostic test such as sensitivity, specificity, positive predictive value, cost, labour intensiveness and the availability of the antigen required with acceptable cost.

In this regard, there are a number of reviews on the serology of TB that demonstrating the low sensitivity and specificity of the test. Currently, there are many serological tests have been developed to diagnose TB disease but there are no commercially

available serodiagnostic tests for TB with acceptable sensitivity and specificity for routine laboratory use (Abebe *et al*., 2007). In order to overcome this constraint, researcher suggested that a combination of antigens in the assay may yield the desired level of sensitivity, without affecting specificity (Lyashchenko *et al*., 2000; Abebe *et al.*, 2007; Steingart *et al*., 2009)

In TB serology, many commercialized serological test kits are based on the detection IgG, IgM and IgA antibodies to the specific antigens of MTB. However, the studies showed that IgG antibody response is more promising in diagnosing an active TB in adults and children (Bhatia *et al*., 2003). However, the inconsistency elevation of all three antibody isotypes in active infection making it important to determine the ideal antibody isotypes for developing a reliable assay (Abebe *et al*., 2007).

1.10.5 Molecular techniques for diagnosis and species differentiation

In an attempt to overcome the limitations of conventional method in diagnosis of TB, several studies have been reported regarding the development of molecular method to detect the presence of *M. tuberculosis* in the clinical specimens. Nucleic acid amplification (NAA) technique is a molecular test which is based on the detection of highly specific fragments of nucleic acids, DNA or RNA of *M. tuberculosis* and followed by amplification of the target sequences that indicate the presence of the bacteria in the specimens (Brodie *et al*., 2005; Green *et al*., 2009; Drobniewski *et al*., 2012). The application of NAA may contribute a valuable tool to complement the conventional approach to the diagnosis of TB (Laraque *et al*., 2009). The advantages of NAA are it is highly efficient and hold the great potential in early diagnosis of TB,

particularly in the case of the infection in the certain body part which the progress is very fast while the culture method is time consuming to yield result (Laraque *et al*., 2009). NAA is also very sensitive as it may detect presence of one to two mycobacteria in the specimen. However, due to the highly sensitive even the smallest amount of contaminating DNA can be amplified and may lead to the misinterpretation of the result (Jain *et al*, 2008). In addition, it also may detect nonviable tubercle bacilli and NAA negative result must be interpreted carefully due to the presence of inhibitors of NAA in the samples that contribute to the false negative result (Paramasivan, 2006). Furthermore, NAA assay requires a specialized instrument, trained personnel, costly and it cannot be considered as a rapid test. This approach is still in the research phase and yet to be user-friendly. Thus, NAA assay is a compliment but not a substitute for culture method.

1.11 Problem statements

In TB diagnosis, clinical manifestation of TB is protean and as the result, diagnosis of TB often difficult (Zumla *et al*., 2013). The non-specific symptoms that occur in the patients may not be sufficient to be included as diagnostic criteria. Physical signs are not always present in the early stage of the disease while the absence of the signs does not rule out the disease. Furthermore, TB symptoms can mimic many different diseases especially other pulmonary disease and this remains a diagnostic dilemma (Hammen, 2015). Diagnostically, culture isolation remain as the gold standard for *Mycobacterium* species identification but often time consuming. As from treatment aspect, the emergence of MDR-TB which rapidly develop resistance to the first line drug and monotherapy treatment create another obstacle. On the other hand, the widely