ISOLATION OF RNA APTAMERS SPECIFIC TOWARD 16 kDa *Mycobacterium tuberculosis* ANTIGEN PROTEIN

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ISOLATION OF RNA APTAMERS SPECIFIC TOWARD 16 kDa *Mycobacterium tuberculosis* ANTIGEN PROTEIN

by

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LIST OF SYMBOLS AND ABBREVIATIONS

°C	Degree Celsius
α	Alpha
µg	Microgram
µL	Microlitre
µM	Micromolar
Acr	Alpha crystalline
AGE	Agarose gel electrophoresis
AIDS	Acquired Immune Deficiency Syndrome
ALISA	Aptamer-Linked Immunosorbent Assay
AMD	Age-related macular degenaration
APS	Ammonium persulphate
AuNP	Gold nanoparticle
bp	Base pair (s)
BSA	Bovine serum albumin
BCG	Bacille Calmette-Guérin
cDNA	Complementary DNA
C	Cytosine
$C_3H_4N_2$	Imidazole
CFP-10	10 kDa Culture Filtrate Protein
CO_2	Carbon dioxide
CT	Computed tomography
CXR	Chest X-ray
dH_2O	Distilled water

dNTP	Deoxyribonucleotide trisphosphate
dsDNA	Double-stranded DNA
DC	Dendritic cell
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DOTS	Directly observed therapy
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
ELONA	Enzyme-Linked Oligonucleotide Assay
EMSA	Electrophoretic Mobility Shift Assay
ESAT-6	6 kDa Early Secretory Antigenic Target
FDA	Food and Drug Administration
FRET	Fluorescence resonance energy transfer
g	Gravitational acceleration
g	Gram
G	Glycine
H_20	Water
HCl	Hydrochloric acid
HIV	Human Immunodeficiency Virus
HRP	Horseradish peroxidise
HSP	Heat shock protein
HTS	High-throughput screening

IFN--γ	Interferon gamma
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IGRAs	Interferon Gamma Release Assays
IMAC	Immobilized metal affinity chromatography
IL	Interleukin
IPTG	Isopropyl β-d-1-thiogalactopyranoside
kb	Kilobase
kDa	KiloDalton
K_d	Dissociation constant
KCl	Potassium chloride
KH_2PO_4	Monopotassium phosphate
L	Litre
LAM	Lipoarabinomannan
LAMP	Loop-mediated isothermal amplification
LB	Luria-Bertani
LPA	Line probe assay
LTBI	Latent Tuberculosis Infection
mA	Milliampere
mg	Milligram
mL	Millilitre
mM	Millimolar
M	Molar
MDR-TB	Mutitidrug-resistant tuberculosis
MFE	Minimum-free energy

M.tb	*Mycobacterium tuberculosis*
MRI	Magnetic resonance imaging
MTBC	Mycobacterium tuberculosis complex
ng	Nanogram
NaAOc	Sodium acetate
NaCl	Sodium chloride
NaH_2PO_4	Monosodium phosphate
NaOH	Sodium hydroxide
Na_3PO_4	Sodium phosphate
NK	Natural cell
NTM	Nontuberculous mycobacteria
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffer saline
PBST	Phosphate buffer saline + Tween 20
PCR	Polymerase chain reaction
PKR	Protein kinase RNA-activated
PPD	Purified protein derivative
QD	Quantum dot
QFT-Gold	QuantiFERON-tuberculosis Gold
rpm	Rotation per minute
RE	Restriction enzyme
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Reverse transcription-PCR

ssDNA	Single-stranded DNA
SDS	Sodium Dodecyl Sulphate
SELEX	Systematic Evolution of Ligands by Exponential Enrichment
SPR	Surface Plasmon Resonance
T_a	Annealing temperature
TA	Tris-acetate
TAE	Tris-acetate-EDTA
TAR	Trans-activation response
TB	Tris-borate
TBE	Tris-borate-EDTA
TBM	Tuberculous meningitis
TE	Tris-EDTA
TNF-α	Tumor necrosis factor alpha
TST	Tuberculin skin test
v/v	Volume per volume
V	Voltage
VA-RNA	Virus-associated RNA
VEGF	Vascular endothelial growth factor
w/v	Weight per volume
WHO	World Health Organisation
XDR-TB	Extensively drug-resistant tuberculosis

PEMENCILAN RNA APTAMER YANG KHUSUS MENGIKAT TERHADAP PROTIN ANTIGEN 16 kDa *Mycobacterium tuberculosis*

ABSTRAK

Tuberkulosis (TB) adalah sejenis penyakit berjangkit tertua yang disebabkan oleh *Mycobacterium tuberculosis* (*M.tb*) yang memberi kesan ke atas manusia. Terdapat beberapa diagnosis makmal yang tersedia untuk pemeriksaan TB seperti kaedah pengkulturan media, mikroskopi calitan kahak dan pengamplifikasian asid nukleik untuk mendiagnosis pesakit TB aktif. Walau bagaimanapun, kaedah-kaedah tersebut tidak dapat mengesan jangkitan TB laten (LTBI). Walaupun Esei Pengesanan Interferon Gamma Terbebas (IGRAs) telah diperkenalkan untuk mendiagnosis LTBI, namun demikian ujian ini merupakan ujian makmal yang mahal dan memerlukan proses makmal yang banyak. Ujian ini juga tidak dapat diakses oleh negara-negara miskin yang mempunyai bebanan kes TB yang tinggi kerana kos dan tidak mempunyai kemudahan membuat diagnosis secara terus. Oleh itu, teknologi pendiagnosan secara terus yang baharu sangat diperlukan untuk menambah baik diagnosis LTBI yang sedia ada agar lebih menjimatkan dan senang dilaksanakan terutamanya kepada negara-negara yang tidak mempunyai kemudahan fasiliti yang baik. Sehingga kini, aptamer telah mendapat banyak perhatian kerana ciri-cirinya yang dikenalpasti mampu menyerupai fungsi antibodi yang mengikat sasaran dengan nilai pengikatan dan spesifikasi yang tinggi. Protin 16 kDa daripada *M.tb* telah dipilih sebagai sasaran utama kajian kerana kepentingannya dalam TB laten dan sifatnya yang imunodominan. Oleh itu, kajian ini dijalankan bagi memencil dan mencirikan aptamer RNA yang spesifik ke atas protin 16 kDa. Dengan menggunakan kaedah Pengkayaan Ligan Secara Eksponensial Melalui Evolusi Sistematik

(SELEX), RNA aptamer yang spesifik ke atas protin 16 kDa telah berjaya dipencilkan dan dicirikan. Aptamer yang dipencilkan telah diklusterkan mengikut jujukan homologi nukleotidanya, manakala struktur sekunder, motif dan analisa *G-quadruplex* telah dijalankan dengan menggunakan perisian atas talian secara percuma. Dalam kajian ini, esei pelekatan (EMSA) dengan menggunakan kaedah gel elektroforesis agarosa untuk mengenalpasti pengikatan dan nilai pemalar pemisahan (Kd) untuk setiap RNA aptamer yang dipencil. Daripada lima kluster aptamer RNA telah dipencilkan, kluster TB_APG01 didapati mempunyai jujukan nukleotida dengan kekerapan yang paling tinggi (14/104) dan nilai Kd 6.428±4.97 μM, namun demikian kluster TB_APG04 telah dikenalpasti sebagai aptamer yang paling kuat kerana mempunyai nilai Kd yang paling rendah (3.935±1.60 μM) walaupun hanya mempunyai 2/104 klon. Secara kesimpulannya, kajian ini telah berjaya memencilkan RNA aptamer yang mengikat kepada protin 16 kDa dan berkemungkinan boleh digunakan untuk tujuan pendiagnosan laten TB secara terus atau sebagai alat pengimejan.

ISOLATION OF RNA APTAMERS SPECIFIC TOWARD

16 kDa *Mycobacterium tuberculosis* ANTIGEN PROTEIN

ABSTRACT

Tuberculosis (TB) is an old, infectious disease scourge caused by *Mycobacterium tuberculosis* (*M.tb*) that affects human. There are several laboratories diagnostic methods available for TB screening such as sputum smear microscopy, culture and nucleic acid amplification that can diagnose patients with active TB. However, the methods are not for detection of latent TB infection (LTBI). Despite the advent of Interferon Gamma Release Assay (IGRAs) for diagnosis of LTBI, the test is expensive and laborious. The test is also not accessible for poor countries that have high TB burden due to cost and lack of rapid point-of-care setting. Thus, a new point-of-care technology is urgently needed to improve the current LTBI diagnosis to more economical and easily implemented technology especially for low resource settings countries. So far, aptamers have received considerable attention due to its properties that could imitate function of antibody against the target with high specificity and affinity. The 16 kDa protein of *M.tb.* was selected as a prime candidate for this study due to its importance in immunodominant property and tuberculosis latency. Thus, this study was aimed to isolate and characterise the RNA aptamers against the 16 kDa protein. By performing Systematic Evolution of Ligands by Exponential Enrichment (SELEX) method using nitrocellulose membrane, the RNA aptamers against 16 kDa protein were successfully isolated and characterised. The isolated aptamers were then clustered together based on their nucleotide homology sequences, while the secondary structure, motif sequence and G-quadruplex analysis were identified using free online software. Electrophoretic

Mobility Shift Assay (EMSA) was performed using agarose gel electrophoresis to determine the binding and the dissociation constant (Kd) value for each isolated RNA aptamer. Out of five isolated clusters of RNA aptamers, the cluster (TB_APG01) was had the nucleotide sequences with the highest frequency clones (14/104) and Kd value 6.428±4.97 μM, however the cluster TB_APG04 was recognized as the strongest aptamer with the lowest Kd value (3.935±1.60μM) although only have 2/104 clones. As a conclusion, this study was successfully isolated the RNA aptamers that bound to the 16 kDa protein and maybe useful for direct LTBI diagnosis or as imaging tool.

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CHAPTER 1

INTRODUCTION

1.1 Research Background

The discovery of several laboratory diagnosis of tuberculosis infection (TB) had one of the most critical impacts on society since earlier treatment will give hope to the TB patients and prolonged their lifespan. Nevertheless, the impingement of the disease can be distressing even today, particularly in poor regions that suffering high burdens of TB since most of the introduced TB diagnostics were uneconomical, not a point-of-care settings and need to be conducted by highly technical expertise that lead to undermine the effort of the global health department to combat the mortality rate of TB worldwide.

Contempt of all advanced technology has been brought out for TB diagnostic, a new tool for TB diagnosis that is feasible and implemented easily is needed to be grown to improve the recurring limitations of the other methods. Hence, the idea of generating aptamers against specific target proteins in *Mycobacterium tuberculosis* (*M.tb*) for TB diagnosis is one of the first steps for producing low cost, rapid and precise diagnostic tools.

This study is aimed to isolate and characterise the RNA aptamers against the 16 kDa protein by performing Systematic Evolution of Ligands by Exponential Enrichment (SELEX) method using nitrocellulose membrane-based technique.

1.2 The Rationale of the Study

The burden of tuberculosis (TB) is one of the major public health concerns nowadays that affected humankind leading to various kinds of TB treatment, and diagnostic tools have been evolved in order to cope with the high mortality of TB patients annually. Besides, the efforts for control of TB have been severely hampered by the emergence of drug resistance (Nachega and Chaisson, 2003) since it raises the possibility for coming back to an era which drugs are no longer beneficial (Gandhi *et al.*, 2010). Thus, in order to treat the suspected infected patients earlier, the fast and reliable technique needs to be developed to diagnose the patients with latent TB because failure to identify and treat latently infected individuals will allow the reactivation of *M.tb* and the chain of transmission to continue becomes active TB disease.

Several laboratory diagnoses of TB infection have been widely used, including detection of antibody, nucleic acid amplification, microscopy, culture and also biochemical characteristics. The sputum smear microscopy, culture-based methods and rapid molecular tests (Xpert® MTB/RIF assay) are the diagnostic tests for TB that have been endorsed by the World Health Organisation (WHO, 2019). However, all this tests have limited specificity, time-consuming which takes 2-3 weeks for detection under optimal condition, relatively expensive for poor country where TB is most prevalent, and the technology is still too complex to be feasible for TB control programs respectively (Drobniewski *et al.*, 2003; Srivastava *et al.*, 2013). Therefore, there is a need to develop strictly accurate, simple to use and new point-of-care tool technology for diagnosing TB.

Aptamers are a `chemical antibody′ that used as detecting agents which is easily implemented that offers much more advantages and exciting features such as selected entirely *in vitro* process, non-immunogenic and can be easily modified which set them apart from the antibodies (Meyer *et al.*, 2011). Besides, an aptamer also has shown high strength of binding affinity in a wide range of applications and the type of target molecules are unlimited which is a convincing substitute for an antibody (Song *et al.*, 2012).

The reliable biomarkers as a mark of detection were needed to ascertain the exact diagnosis of latent TB (LTBI). The 16 kDa antigen protein also known as heat shock protein (HspX) or Acr protein (α-crystalline protein homolog) have been highlighted to be desirable targets for a new diagnosis of LTBI and have been reported significance to its latency (Siddiqui *et al.*, 2011). Thus, with the importance of the 16 kDa protein as a biomarker for LTBI as mentioned above, it is convincing to choose the antigen as a binding ligand to isolate RNA aptamers in this study.

Last but not least, the aptamer is believed will enhancing TB diagnostic especially in low resource setting and developing countries as it is cheap and thermal stable. In addition, aptamer also has a potential to replace the use of drug and monoclonal antibody or help in enhanced the efficacy of drug and targeted delivery therapeutic.

1.3 Objectives of the Study

1.3.1 General Objective

The general objective of this study is to isolate and characterise the potential RNA aptamers as a detection agent that can bind specifically to 16 kDa antigen protein of *Mycobacterium tuberculosis* for better LTBI diagnosis.

1.3.2 Specific Objectives

The specific objectives are as follow:

1. To prepare RNA pool for RNA aptamers selection against 16 kDa antigen protein of *Mycobacterium tuberculosis*.
2. To isolate, identify the sequences and predict the secondary structure of the potential RNA aptamers candidates after selection with that 16 kDa antigen protein of *Mycobacterium tuberculosis*.
3. To evaluate the binding affinity by determining the dissociation constant (Kd) value between the selected RNA aptamers with the 16 kDa antigen protein of *Mycobacterium tuberculosis*.

CHAPTER 2

LITERATURE REVIEW

2.1 Epidemiology of Tuberculosis

Human tuberculosis (TB) is a daunting public health challenge that leads significantly to illness and affecting millions of lives around the world (Chang *et al.*, 1996). TB has been reported by the World Health Organisation (WHO) as one of the top ten causes of mortality from a single infectious agent compare to HIV/AIDS. Generally, a relatively small proportion (5-10%) of the estimated individual infected annually with TB will progress into TB disease throughout their lifetime. However, the probability is much higher among HIV patient and people with malnutrition and diabetic problem (WHO, 2018). Globally, it has been estimated that 10.0 million people (range, 9.0-11.1 million) affected by the TB disease with accounted for 57 %, 32 % of women and 11 % of children in 2018 but the highest burden is in men (WHO, 2019)

The severity of national epidemics cases relative to population (incident rate) varies widely among country as can be seen in **Figure 2.1.** Most of the countries affected in 2018 were South-East Asia and Africa which reached the incident rates above 300 per 100 000 population. The lowest incident rates usually found predominantly in high-income countries including Western Europe, United State of America, New Zealand, Japan, Canada and Australia. In these countries, the incidence rate per 100 000 population is less than 10 incidences (WHO, 2019). In Malaysia, the death rate due to TB in 2018 is 6.6 per 100 000 population, which Selangor recorded the highest cases, 5071 cases followed by Sabah, 5008 cases as reported by Datuk Dr Noor Hisham Abdullah, the Health Director General in BERNAMA on March 26, 2019. Similar to other developing countries, TB cases in

Malaysia are clustered in populated density, poor housing conditions or environment and low socioeconomic status, also driven by the influx of foreign immigrants (Rasam *et al.*, 2019). In general, the prevalence of the disease can be due to the increasing number of HIV/AIDs patients, the emergence of multi-drug resistant TB, congregational transmission, and the high influx of foreign labors from high TB burden (Brennan, 1997).

Besides, the problems of TB remained unsolved due to the existence of drug-resistant TB (558 000 people) is resistant to the most effective first-line drug which is rifampicin (RR-TB). The multidrug-resistant TB (MDR-TB) patients have been developed due to the mycobacteria that show high-level resistance to both isoniazid and rifampicin, with or without resistance to other anti-TB drugs (Ormerod, 2005). Another issue is the prevalence of extensively drug-resistant TB (XDR-TB) identified as MDR-TB with additional resistance to any fluoroquinolone and at least one of three injectable drugs used for TB treatment: amikacin capreomycin, kanamycin (Parsons *et al.*, 2011). It occurs due to the diagnosis that depends on confirming isolated organism's drug susceptibility pattern, which is often either very limited or non-existent in many countries with high burden of disease and only possible in rich-resource settings. Drug-resistance should be strongly suspected in persons in TB treatment failure cases (Ormerod, 2005).

Until now, an attenuated strain of *Mycobacterium bovis* termed Bacillus Calmette-Guerin (BCG) is the only vaccine present and available for prevention of TB (Tang *et al.*, 2014) and the BCG immunization is considered effective in children, providing defense against severe and disseminated tuberculosis by 80% (Trunz *et al.*, 2006) but has limited or largely ineffective on adult pulmonary TB

(Reyes *et al.*, 2013b). About 45-85% of mortality in HIV infected patients related with the TB delay diagnosis and initiation of appropriate medication more than 3 weeks after the symptom presentation (Devi *et al.*, 2003). Therefore, an early diagnosis of TB or latent TB (LTBI) identification will help in the better management and control of this disease.

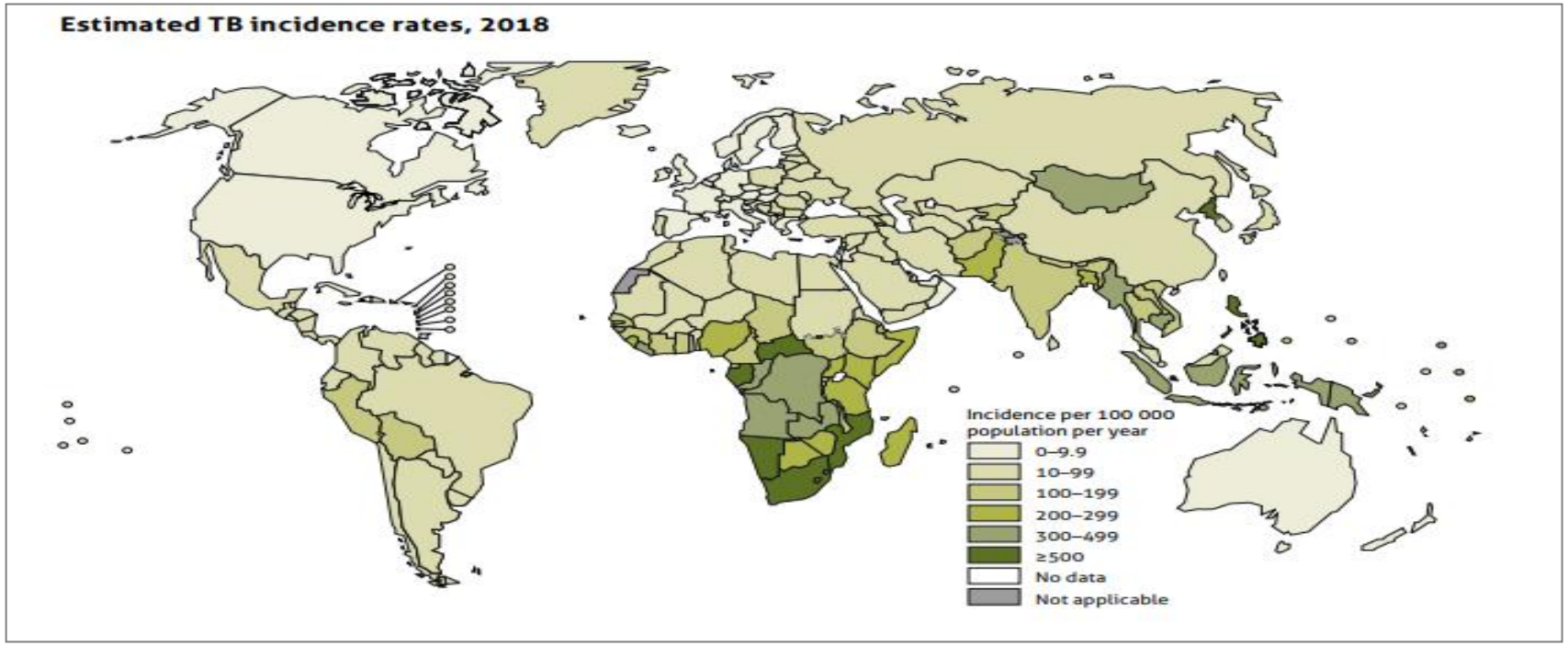

Figure 2.1 The map of an estimated TB incidence rates in 2018 worldwide. Source: (World Health Organisation, 2019).

2.1.1 An Aetiology of Tuberculosis

The aetiology agent of TB in human is *Mycobacterium tuberculosis* (*M.tb*). The *M.tb* is a Gram-positive bacteria (Goulding *et al.*, 2004) species in the family Mycobacteriaceae that was first discovered by a German physician, Robert Koch in 1882 and a most well-known species, infecting the human population and also able to transmit the infection to animals that have contacted with the human. It is categorized as the group of Mycobacterium tuberculosis complex (MTBC) together with *Mycobacterium canettii*, *Mycobacterium africanum*, *Mycobacterium microti*, *Mycobacterium bovis*, *Mycobacterium caprae* and *Mycobacterium pinnipedii* that are genetically very similar (Forrellad *et al.*, 2013b; Murray *et al.*, 2015). The genome of *M.tb* comprises of 4,411,529 base pairs, consists of 4,000 genes, and has very high cytosine and guanine content that is expressed in the protein's biased amino acid content (Cole *et al.*, 1998). The bacterium is around 0.5 μM in diameter, 1-4 μM in length and it is an aerobic intracellular pathogen (Welin, 2011).

The characteristics features of the tubercle bacillus include slow growth, dormancy, complex cell envelope, intracellular pathogenesis and genetic homogeneity. It takes typically 24 hours of doubling time for *M.tb* generated in synthetic medium or infected animal (Ducati *et al.*, 2006). The state of dormancy in which bacillus remains latent within infected tissue may indicate its vital metabolic activities shutdown and survives in its host for a long time by operating certain key activities which are essential for its survival (Jee *et al.*, 2018). By deciphering *M.tb* whole genome sequence done before by Cole and his colleagues, the dormancy is believed to be entirely a physiological phenomenon (Cole *et al.*, 1998) but, it is now thought to be a well programmed event and regulated by number of factors such as hypoxia, known to be one of the main strength behind this phenomenon while small

heat shock protein, 16 kDa is crucial for the maintenance of dormancy in *M.tb* (Jee *et al.*, 2018) as discussed later in this chapter. However, as immunity becomes weaker due to immune suppression or ageing, the dormant bacteria reactivate and often cause disease outbreak many decades after the initial infection (Cole *et al.*, 1998).

The cell envelope of *M.tb* that have a G+C rich contents of 65.6% that contains an additional layer beyond the peptidoglycan that is exceptionally rich in unusual lipids, glycolipids and polysaccharide (Kolattukudy *et al.*, 1997; Cole *et al.*, 1998). The arrangement structure of the lipids and glycolipids on the exterior mycobacterial cell envelope's surface passively confers upon the bacilli properties such as impermeability to toxic macromolecules, a strong resistance to degradation by host enzymes, and inactivation of reactive oxygen and nitrogen derivatives (Korf *et al.*, 2005). Several cell wall structures of *M.tb* have been identified as representing a pathogen-associated molecular patterns (PAMP), including the glycolipid lipoarabinomannan (LAM), soluble tuberculosis factor, and lipopeptides which are essential for pathogen survival (Korf *et al.*, 2005).

Besides, the macrophages are critical both allowing and in linking the host to innate and adaptive immunity. It facilitates the activation and recruitment of T cells to contain the mycobacteria within granulomas in the lung and even can replicate within the harsh environment of macrophages, sequestered in poorly acidified phagosomes that do not fuse with lysosomes. The *M.tb* also can evade macrophages bactericidal function by inhibiting interferon (IFN)-γ-mediated signalling and has been reported to interfere with multiple signalling pathways, antigen presentation and transcriptional responses within the macrophage, however the mycobacterial genes that involved are largely unknown (Rengarajan *et al.*, 2005).

Once the bacterium is taken up in the form of infected droplets released from an infected individual's lungs, typically by coughing and sneezing, a *M.tb* is transmitted promptly. **Figure 2.2** and **Figure 2.3** show illustrated of the formation of the granuloma and infection process of *M.tb***.** Upon inhalation, the resident alveolar macrophages and dendritic cell (DC) phagocytose the bacterium and generate signals for the induction of the cell-mediated immune response. However, the mycobacteria may persist and subvert the killing mechanism to allow replication as intracellular parasites. The initially infected cells release pro-inflammatory cytokines resulting in more DC, neutrophils and monocytes being recruited from the blood stream and the infected DC become triggered and migrate to the local lymph node, thereby activating specific T cells. The cytokines IL-12 and IL-18 from the infected cells induce Natural Killer (NK) cell activity, and NK cells in turn produce IFN-γ, which stimulates macrophages to produce Tumour Necrosis Factor alpha (TNF-α) and microbicidal substances (Keane *et al.*, 1997; Korbel *et al.*, 2008).

The granuloma is formed through cytokine and chemokine signalling. In granuloma, macrophages are further differentiating into extracellular matrix protein and epithelioid cells or foamy outer cuff of fibroblasts. Thereby, the bacilli are to be contained for years even decades, persisting in a dormancy or slowly-replicating state until the granuloma fails due to immunosuppression and is unable to prevent the growth (Russell *et al.*, 2009; Forrellad *et al.*, 2013a). The mechanism that would reduce the ability for macrophages to function as protected sanctuaries for TB as it coincides with the reduction of bacterial growth in lung and the onset of adaptive immunity and may be significant benefit to the host. Conversely, killing of alveolar macrophages may benefit the mycobacteria by reducing phagocyte numbers and probably interfering with cell-mediated immunity induction. In active disease, the

granuloma centre become caseous, restraining necrotic macrophages in the lung that form advanced TB cavities. When the structure rupture, contagious bacilli spill into the airways, allowing new individuals to inhale (Keane *et al.*, 1997; De Chastellier, 2009).

The pulmonary TB (lungs TB) is the primary site of infection, resulting in symptoms such as chronic bloody coughs, night sweats and weight loss which are the most severe clinical manifestation of *M.tb* infection. However, it also able to spread the infection to more distant sites in the body (extrapulmonary TB), such as peripheral lymph nodes, kidneys, brain and bones and this extrapulmonary TB can manifest itself as pericarditis, meningitis, or spinal TB (Donoghue, 2009). The development of new treatments, vaccines and diagnostic tools to help prevent or control this pandemic require a better understanding of the mechanisms involved in mycobacteria's interaction with the host and the virulence factors (Forrellad *et al.*, 2013a).

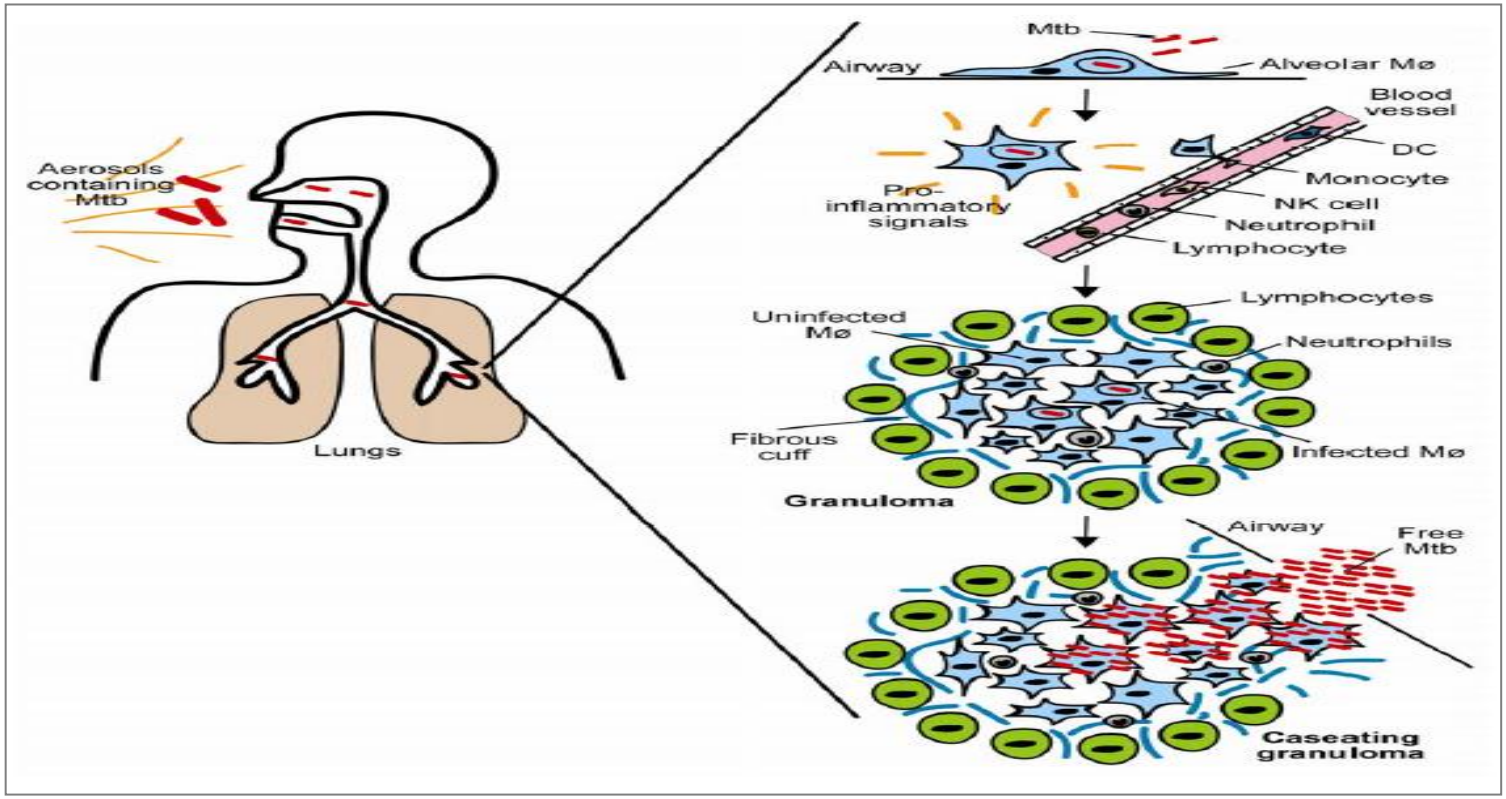

Figure 2.2 The diagram granuloma mechanism formation after the *M.tb* infection in a human.

The resident alveolar macrophages (M_0) phagocytose inhaled bacteria causing a pro-inflammatory response and recruitment of cells of innate and adaptive immune systems, and the formation of granuloma. The bacilli can contain within the structure until immune controls fails which lead to the rupture of granuloma and another *M.tb* is spilled into the airways. Source: (Russell, 2007)

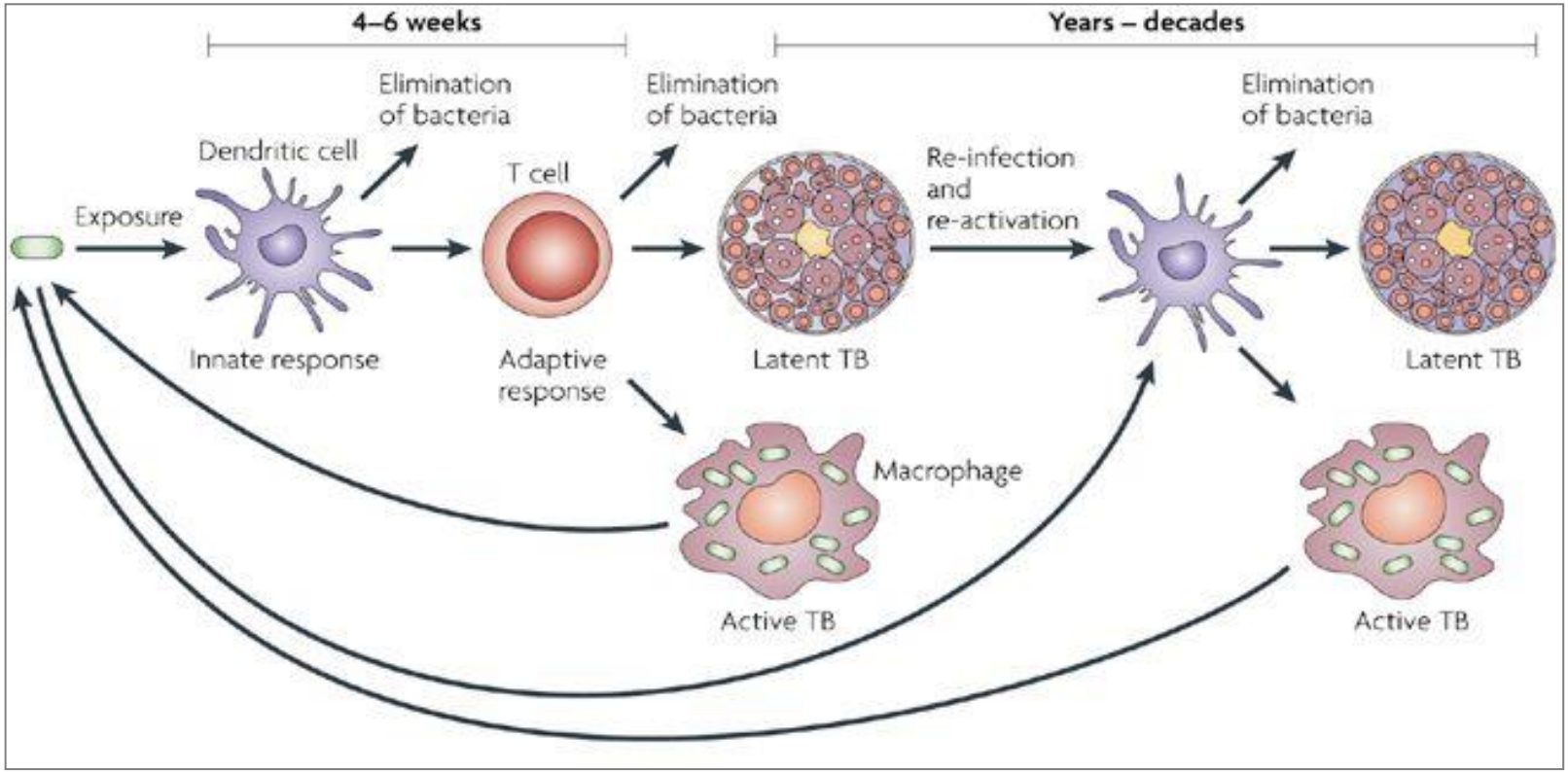

Figure 2.3 The summary of *M.tb* infection process from an early infection until development of active TB and LTBI.

The life cycle of *M.tb* once inhaled by a human can last for 4-6 weeks while the stage of latent TB can stand for years even decades. The failure of immune response of the host causing the LTBI becoming an active TB when the bacteria start to re-infect and re-activate Source: (https://sites.google.com/site/mycobacteriumtbstudy/home/life-cycle-of-organism).

2.1.2 Diagnosis of Tuberculosis

Inhalation of contaminated droplets from an infected person with TB can be either: 1) eradicated by immune system of the host; 2) contained as latent infection; 3) progress towards the active disease. Less than 10% of infected individuals experience active TB throughout their lifetime (Gengenbacher and Kaufmann, 2012) and another approximately 90% individuals are effective in suppressing the infection and remain in latent state, identified as a state of persistent immune response to *M.tb* antigens stimulation without clinically manifested active TB evidence (Ilievska-Poposka *et al.*, 2018). An individual with LTBI has no symptoms of active TB and does not feel ill upon infected with the *M.tb*. Nevertheless, the risk of developing active TB in immuno-compromising situations such as patients with co-infection with human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) or chronic inflammatory diseases or diabetes/obesity is increased (Barry *et al.*, 2009).

Several diagnostic assays and techniques were available for detection of the *M.tb* in LTBI or active TB stage. The techniques include chest x-rays, sputum smear microscopy, culture, rapid molecular tests, tuberculin skin test, IFN-γ release assays, phage amplification assays and others. The chest X-ray (CXR) is a rapid imaging technique that identifies abnormalities of lungs and is used to diagnose thoracic cavity conditions including the airways, lungs, ribs, heart and diaphragm., The CXR has been one of the primary tools for TB screening historically, which had high sensitivity mainly for pulmonary TB. However, CXR has low specificity, although some CXR abnormalities are rather specific to pulmonary TB (e.g. cavities), many CXR abnormalities that are consistent with pulmonary TB are also seen in several

other lung diseases (WHO, 2016). Therefore, another test is required to accompany to CXR diagnosis, as relying solely on CXR for TB diagnosis will lead to overdiagnosis and underdiagnosis (Koppaka and Bock, 2004).

Sputum smear microscopy test is another technique that usually used for the *M.tb* identification. Despite it low cost technique, it has major drawback such as poor sensitivity which is estimated at 50% (Rotherham *et al.*, 2012), time consuming, poor specificity and false-positive/negative results (He *et al.*, 2016). This technique also is not applicable for HIV-coinfected patients and for children because of the decreased pulmonary bacillary loads in these patients. Additionally, in resource-poor countries, there are many smear microscopy laboratories but it is single room and understaffed with poorly maintained microscopes and some of these laboratories lack of clean water supplies and electricity (Parsons *et al.*, 2011).

Another available technique for diagnosis is by culturing the *M.tb* bacteria in the lab. The culture-based method is the most sensitive to detect *M.tb.* in infected patients however, it requires more developed laboratory resources and it can take up to 12 weeks to provide the results and high standard of technical competence personnel is needed (Rotherham *et al.*, 2012; WHO, 2018). Even the most rapid culture technique such as radiometric liquid culture (BACTEC) been used, it still requires an average of 13 days to become positive and potential risk of cross contamination is high (Piersimoni *et al.*, 2001).

Rapid molecular tests have been introduced to overcome sputum microscopy and culture-based identification technique such as Loop-mediated Isothermal Amplification (LAMP), Xpert MTB/RIF, GenoType Line-Probe Assays (LPA), *FluoroType MTB* and others (Eddabra and Benhassou, 2018). Among all, Xpert®

MTB/RIF assay (Cepheid, USA) is the only rapid test currently approved by World Health Organisation (WHO) as the first-line diagnostic that can provide fast result within 2 hours and has much accuracy than sputum smear microscopy (WHO, 2018). However, it is costly especially to low and middle income countries that wish to implement this technology is the cost. This technology may incur US$9.98 per sample even after the announcement of a global price reduction for Xpert MTB/RIF cartridges (Schnippel *et al.*, 2013; Van Rie *et al.*, 2013) which not affordable by the poor countries. In addition, it has been estimated that the sensitivity of a single Xpert test is only 62-79% to detect *M.tb* in all negative smear microscopy result which is inadequate, although it is substantially better than sputum smear microscopy technique (Schnippel *et al.*, 2013).

However, all the rapid test is not for LTBI diagnosis and treatment indications are based on epidemiological, clinical, laboratory and patient acceptance criteria (Cohn *et al.*, 2000). The identification of LTBI offers an opportunity for initial treatment and prevent latent infection reactivation which lead to active disease, particularly in those with compromised immune systems (Ilievska-Poposka *et al.*, 2018) and early treatment of LTBI reduces risk of progressing to future disease by 75-90%, hence identifying individuals with LTBI has both individual benefits and substantial societal (WHO, 2008). The *in vivo,* century old tuberculin test (TST) and the *ex vivo,* interferon-γ release assays (IGRAs) are the two techniques that widely used to detect LTBI and previous TB infection (Zhou *et al.*, 2017).

The TST test or Mantoux test based on principles of deferred oversensitivity to recruit T cells memory to the site on an intradermal injection of purified protein derivative (PPD) of *M.tb.* The test is limited in use by its poor specificity due to

cross-reactivity with previous BCG vaccination and non-tuberculous mycobacteria (NTM), (Fu *et al.*, 2009; Zhou *et al.*, 2017). The other limitations of the TST include the necessary of inter-observer variability, scheduling a reading visit and the boosting phenomenon. In immuno-compromised patients, young people and the elderly, the false-negative results may be common (Cohn *et al.*, 2000).

The IGRAs are designed as an aid to TST for the LTBI diagnosis due to their logistical advantages and improved specificity over TST (Herrera *et al.*, 2011). The IGRAs, specifically the QuantiFERON-TB Gold test (Cellestis) and T-SPOT. TB test (Oxford Immunotec) have been developed to detect and quantify the *in vitro* IFN-γ releases from T cells stimulated by TB-specific antigen in blood samples (Pollock *et al.*, 2014). The Cellestis uses an enzyme-linked immunosorbent assay (ELISA) to measure the amount of IFN-γ produced, and the QFT-Gold in-tube (GIT), recent version of the QuantiFERON enables blood to be collected directly into pre-coated tubes to reduce sample processing and facilitate incubation. While T-SPOT TB assay uses an enzyme-linked immunospot assay to measure the number of IFN-γ-producing cells on pre-coated plates.

Both assays are available with a positive control (phytohaemagglutinin) and use highly specific antigens of *M.tb* such as CFP-10 and ESAT-6 which are absence in any BCG strain or in most environmental mycobacteria, thus providing specificity over TST (Herrera *et al.*, 2011). The IGRAs, despite its high specificity, it is expensive and required particular expertise, that presents a barrier in resource-limited settings (Pollock *et al.*, 2014).

Overall, both TST and IGRAs are valuable screening tool for LTBI detection however, it should be noted that neither of these immune-based tests performs with

optimum sensitivity in individual who are immuno-compromised or able discriminating between active and past disease from latent infection, which is a major issue in TB endemic areas (Goletti *et al.*, 2016; Zhou *et al.*, 2017)

Therefore, TB control strategy recommended by WHO is Directly Observed Treatment and Short-course drug therapy (DOTS) programs which is the most successful treatment strategy available for TB control and one of the most rapidly expanding and effective intervention since of the 1990s (Aziah, 2004). To ensure compliance, the patients are observed when they take their medication since non-compliance is a major contributor to the antibiotic resistance development (Dye and Williams, 2010). The TB delayed diagnosis may enhance the infection transmission, increase the risk of death, and maybe the reason why TB incidence has not decreased significantly, hence there is a crucial need for new, simple, sensitive and rapid point-of-care diagnosis as well as quality assurance programs, investments in laboratory infrastructure and well-trained personnel.

2.1.3 Biomarkers for TB Diagnosis

Specific biomarkers are important prerequisite for the development of diagnostic reagents or improved available vaccines for TB disease. The mycobacterial antigens such as MPT64, Mtb81, 38 kDa, MPT48, ESAT-6, CFP-10, 16 kDa and others have been evaluated for their serodiagnosis potential for both latent TB and active TB (Wu *et al.*, 2011).

One of the major *M.tb* antigens is a 24 kDa secretory protein, also known as MPT64. The SD MPT64TB Ag Kit, a new rapid immunochromatographic test kit was evaluated for rapid identification of *M.tb.* for detection of the antigen isolated

using mouse monoclonal MPT64 antibody (Kumar *et al.*, 2011). The previous report also reconfirm that the test is a simple and cost-effective method for differentiating *mycobacterial* cultures as *Mycobacterium tuberculosis* complex (MTBC) from non-tuberculous *mycobacteria (*NTM). However, if the test results are negative, alternative specific identification tests need to be carried out to confirm the MTBC and NTM isolates (Shenoy and Mukhopadhyay, 2014; Sharma *et al.*, 2015).

Besides, Mtb81 protein, identified by serological proteome analysis has appears to be a potential antigen for the serodiagnosis of TB, especially for patients co-infected with HIV (Hendrickson *et al.*, 2000). Nevertheless, the use of single antigen alone for TB diagnosis has not been able to achieve sufficiently high sensitivity and specificity at the same time. Therefore, it is worth pursuing to evaluate the response of antibodies to multiple *M.tb* antigens to recognize superior antigens and their possible combinations for serological diagnosis of TB (Yan *et al.*, 2018) which is certainly time-consuming and less productive.

Since many studies suggest that the response of the antibody to *M.tb* antigens is diverse among individuals, thus the antibodies detection against a single antigen normally has a low sensitivity for TB diagnosis, particularly in bacterium negative cases. Therefore, another approach for evaluating antibodies against 38 kDa antigen protein and other major antigens was introduced. The data suggest that characterization of antibodies against 38-kDa, MTB48, and CFP-10/ESAT-6 fusion antigens can increase the specificity and sensitivity for active TB diagnosis. Moreover, compared to single antigen, the positive antibody responses rates to two or more antigens were significantly higher (Wu *et al.*, 2011). Nonetheless, additional

efforts should be made for serological detection of smear- or culture-negative infections (Lodes *et al.*, 2001).

Besides, a report have been shown that a Camel/Llama Heavy Chain monoclonal antibody (VHH) is demonstrated specific and exclusively recognized the 16 kDa heat shock protein. The 16 kDa protein had been shown to bind in immobilized lysate in direct ELISA tests and soluble antigen by surface plasmon resonance (SPR) analysis (Trilling *et al.*, 2011). However, the isolation of the antibody may require the use of larger animals that need to be regularly immunize with 2-3 immunogens at the same time and re-use the animal is after waiting at least 6 months in case of failure of antibody isolation. It also required the selection of the bio-hazardous bacteriophage which is greatly outweighed by the ability to screen generate and control the resulting VHHs rapidly and reliably (Bever *et al.*, 2016) which is inconvenient process and time-consuming.

2.1.3 (a) Aptamers for TB Diagnosis

Due to some limitations encountered by antibodies for TB detection using several significant biomarkers, aptamers have been generated to substitute the use of antibody against *M.tb.* antigens. For example, a functional ssDNA aptamers (CE24 and E15) that specific bound to ESAT6-CFP10 (CE) protein had been developed that showed the highest specificity and binding affinity and further detected using ELONA for both proteins in serum samples from active pulmonary TB patients, extrapulmonary TB patients and healthy donors. The report was convinced with a good correlation was observed between aptamer-based ELONA and T-SPOT TB assay. However, some optimisations were required and large sample tests to further

validate their aptamer-based method of detecting antigens in human samples (Tang *et al.*, 2014).

Another ssDNA aptamers were selected for detection of *M.tb* secreted protein MPT64 that showed a good specificity and sensitivity values (90% and 91.3% respectively), and be less likely to give false positive results. The recent study did not compare the binding mechanism of aptamer-MPT64 with antibody-MPT64 because of size differences and steric hindrance (Sypabekova *et al.*, 2017). However, this study is need to be improved in case of low signal occurs by labelling with nanoparticles or fluorescent reporting molecules for effective detection of TB and do another validation for specificity and sensitivity test from non-tuberculous mycobacteria and bacillus Calmette-Guérin strains culture samples.

Recently, a DNA aptamer against HspX have been isolated as an alternative diagnostic reagent for accurate tuberculous meningitis (TBM) diagnosis. Aptamer H63 SL-2 M6 showed interaction against HspX with high specificity and affinity (Kd ∼9.0×10−8 M). By using Aptamer Linked Immobilized Sorbent Assay (ALISA), H63 SL-2 M6 significantly distinguished between cerebrospinal fluid specimens (CSF) from TBM and non-TBM subjects. Nevertheless, a larger study involving a multiple age group and ethnicity is needed to validate these findings and determine the diagnostic efficacy of aptamer-based rapid test in the clinical setting (Dhiman *et al.*, 2018). In addition, the used of DNA aptamers have several disadvantages as discussed in the **section 2.2.1 (a).**

In latent infection, low numbers of bacteria are presence usually and the physical microenvironment in which bacteria survive is thought to involve restricted access to nutrients, oxygen, and low pH will induces a massive up-regulation of the

production of this protein to adapt to low-oxygen or nutrient deficit dormancy, which is associated with a thickened cell envelope, providing evidence that 16 kDa as potential targets of biomarkers of LTBI (Wilkinson *et al.*, 1998; Demissie *et al.*, 2006; Zhang *et al.*, 2015).

The 16 kda heat shock protein (HSP) is encodes by Rv2031c/*hspX*/*acr* (Yuan *et al.*, 1998; Jee *et al.*, 2018) and it is belongs to the α-crystallin super-family of heat shock proteins (HSPs) and has been variably referred to as sHsp 16, Hsp.16.3, Acr and MPT63 (Devi *et al.*, 2002). Though it has been classified as HSP family, it is not a heat induced protein as name implies but it is hypoxia induced oxygen (Yuan *et al.*, 1996). The important of 16 kDa protein is represented by the fact that deficiency of this protein deteriorates the *M.tb* tolerance to anaerobiosis. Thus, it is invoked as a potential candidate facilitating the pathogen's survival during hypoxia, a situation close to latency (Mushtaq et al., 2015) that becomes a key aspect of the active participation of the bacterium and the pathogen's lifestyle in the formation of granulomas (Hu *et al.*, 2006).

The identification of 16 kDa antigen protein as the major membrane protein proposes that the protein does not function purely cytoplasmically (Lee *et al.*, 1992). The exact cellular location is unknown till date, although peripherally associated with inner membrane and occurred outside of the cell wall (Jee *et al.*, 2018). It was also not found in culture supernatant (Verbon *et al.*, 1990) as concordance with the study by Trilling *et al* on Camel/Llama Heavy Chain monoclonal antibody (VHHs) against 16 kDa protein. The ELISA signal of the VHHs to culture medium or whole cells of the *M.tb* could be hardly observe, while strong signal occurred in cell lysate that proved 16 kDa protein is not released into culture supernatant. Thus, lysis of

bacteria will be require before detection in sputum due to this subcellular localization (Trilling *et al.*, 2011).

The 16 kDa protein exhibited its chaperonin property by preventing the thermal denaturation of alcohol dehydrogenase *in vitro* (Yuan *et al.*, 1996). The modulation of its chaperone activity by exposing its hydrophobic surface for interaction with denaturing protein and the protein structure will highly stable and flexible, thus highly adapted for its function (Yang *et al.*, 1999). In addition, its chaperone activity is exclusively temperature dependant (Fu and Chang, 2004) and even pre-incubation at 1000°C resulted in enhanced chaperone like activity without altering the surface hydrophobicity (Jee *et al.*, 2018).

The 16 kDa is indispensable for growth of the bacillus as a study on gene knockout suggested that it is an inevitable requirement for *M.tb* normal growth during infection (Yuan *et al.*, 1998). The deletion of *hsp*X gene resulted in bacterial growth enhancement while its overexpression delayed the growth of bacilli during early phase of mice infection which associated to latency phase (Hu *et al.*, 2006). Moreover, it is the most prominent protein in the extract of *M.tb* during latency (Yuan *et al.*, 1998). It was found to be involved in the thickening of cell walls, therefore indirectly facilitating dormancy since the thickened cell wall helps the bacilli to withstand the hostile environment during infection (Cunningham and Spreadbury, 1998).

In addition, the 16 kDa protein could be used as a marker for LTBI, even in a BCG vaccinated population (Siddiqui *et al.*, 2011) as it has been found that T-cells can respond to this antigen in order to distinguish between latently infected and BCG vaccinated individuals (Geluk *et al.*, 2007). A study has also been reported on