PROTEIN ANALYSIS AND CHARACTERIZATION OF RECOMBINANT CLONE MYT272-3 TOWARDS TUBERCULOSIS VACCINE

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2017

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THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

INSTITUTE OF BIOLOGICAL SCIENCES
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UNIVERSITY OF MALAYA
KUALA LUMPUR

2017

UNIVERSITY OF MALAYA

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Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"):

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ABSTRACT

Tuberculosis (TB) infection is well-known for millennia and has been well apprehended since the end of 19th century. Robert Koch discovered its causative agent, *Mycobacterium tuberculosis* in 1882. TB vaccine has been in used for about a century, and antibiotics have been in place and utilized for more than six decades. They all work, yet 1.5 million deaths were recorded in 2014 due to TB which made it the leading infectious killer disease globally. Bacillus Calmette-Guérin (BCG), a live attenuated vaccine and is currently the most widely used. It has variability in effectiveness ranging from 0-80%, hence an urgent need for a better vaccine candidate is of paramount important. The recombinant clone (pET30a / Myt272-3 clone) constructed in Molecular Bacteriology and Toxicology Laboratory of University of Malaya was screened for its stability, and was found stable. The recombinant clone was transformed into BL21 (DE3) pLysS strain of *Escherichia coli*. The Myt272-3 protein was successfully expressed in pET30a / Myt272-3 clone. The molecular weight of the protein was found to be approximately 10.58 kDa as determined by SDS-PAGE and conformed to the MW computed by Expert Protein Analysis System (EXPASY MW bioinformatics tool). Protein BLAST (Basic Local Alignment Search tool) bioinformatics analysis indicated 81% homology with Phenolpthiocerol synthesis polyketide synthase I PpSA of *Mycobacterium tuberculosis,* MALDI-TOF analysis further validated the homology of the protein. The concentration of protein was determined by detergent-compatible method of protein assay. The protein was purified by both Nickel based (Nickel-nitrilotriacetic acid, Ni-NTA) and Cobalt based (Dynabeads®) affinity chromatographic techniques. Recently, computational biology approaches are found very useful for organizing and understanding huge data leading to the new field called immunoinformatics. Bioinformatics software were used to analyze

the protein sequence for predictions of allergenicity, antigenicity, major histocompatibility complexes, I and II binding and B-cell epitope binding. Moreover, toxicity of epitopes was predicted via toxicity predictive tool. These predictive findings serve as a practical guide towards *Mycobacterium tuberculosis* peptide vaccine design and development.

ABSTRAK

Jangkitan batuk kering (TB) sudah terkenal selama beribu-ribu tahun yang lalu dan juga telah disedari sejak akhir abad ke-19. Pada tahun 1882 Robert Koch telah menemui agen penyebab batuk kering adalah disebabkan oleh *Mycobacterium tuberculosis*. Vaksin TB telah digunakan selama kira-kira satu abad, dan antibiotiknya pula telah mendapat tempat dandigunakan lebih daripada enam dekad. Semuanya berkesannamun pada tahun 2014 tercatat 1.5 juta kematian disebabkan oleh TB, telah menjadikannya suatu penyakit-pembunuh berjangkit terkemuka di peringkat global. Bacillus Calmette-Guérin (BCG), merupakan vaksin hidup yang telah dilemahkan sepenuhnya dan kini paling banyak digunakan. Tahap keberkesanannya yang pelbagai 0-80%, telah menjadikannya satu kemestian segera bagi mencari calon vaksin yang lebih baik. Klon rekombinan (pET30a/Myt272-3) yang telah dibuat di Makmal Bakteriologi Molekul dan Toksikologi Universiti Malaya, telah diujisaring terlebih dahulu bagi penentuan kestabilannya. sebelum dipindahkan ke dalam *Escherichia coli* strain BL21 (DE3) pLysS. Protein Myt272-3 telah berjaya dihasilkan dalam klon pET30a/Myt272-3. Berat molekul protein yang didapati adalah lebih kurang 10.58 kDa ditentukan melalui analisis SDS-PAGE dan selaras dengan berat molekul yang dikira dengan menggunakan alat bioinformatik EXPASY MW. Analisis bioinformatik BLAST Protein menunjukkan bahawa 81% homologi dengan sintesis fenoltioserol polikitida synthase I PpSA *Mycobacterium tuberculosis*, di mana ianya juga disahkan melaui analisis MALDI-TOF. Kepekatan protein telah ditentukan oleh kaedah detergen serasi asei protein. Protein dibersihkan dengan menggunakan dua teknik afiniti kromatografi iaitu berdasarkan nikel (Ni-NTA) dan kobalt (Dynabeads®). Baru-baru ini, pendekatan komputional biologi didapati amat berguna bagi mengatur dan memahami data yang begitu banyak ke arah suatu bidang baru yang dikenali sebagai imunoinformatik. Perisian bioinformatik telah digunakan untuk menganalisis turutan protein bagi jangkaan-jangkaan alahan , antigenisiti, kompleks

utama histokompatibiliti, ikatan I dan II dan ikatan epitop B-sel. Tambahan lagi, ketoksikan epitop telah diramalkan melalui alat ramalan ketoksikan. Penemuan ramalan ini menjadi panduan praktikal ke arah pembentukan dan pembangunan peptida vaksin *Mycobacterium tuberculosis*. Penemuan hasil-hasil kajian ramalan juga mempunyai implikasi bagi pembentukan dan pembangunan vaksin.

ACKNOWLEDGEMENTS

In the name of Allah, the most Gracious and the most Merceful. All praise are due to Him for giving this opportunity and granting me the ability to advance up to this level successufully.

This work wouldn't have been reach up to this level with the support from my Suppervisors. I would like to express my sincere appreciation to my supervisors, Prof. Dr. Salmah Ismail and Dr Teoh Teow Chong for their guidance, patience and encouragement throughout the duration this study.

My deepest appreciation goes to my beloved parents (Hajiya Zainab and Alhaji Usman Dijami) for their support and prayers throughout my life, their prayers really made this journey very successful one. I also acknowledge the support rendered by my elder brother, Alh Baba Usman, my siblings, friends and other members of my family.

I would like to extend my gratitude to my wife, Amina Ahmad Abubakar for her patience and prayers toward the success of this journey. My appreciation also goes to my kids (Usman, Khadija and Bashir), your prayers highly appreciated.

I would also like to express my deepest appreciation to the management of Modibbo Adama University of Technology, Yola, Nigeria and the Tertiary Education Trust fund (TETFUND) for awarding me a scholarship for this programme. I am also greatful to the former Vice Chancellor of Modibbo Adama University of Technology, Yola, Nigeria in person of Prof Bashir Haruna Usman for his support and encouragement.

My sincere gratitude goes to Mr Kelvin and all members of Molecular Bacteriology and Toxicology Laboratory, University of Malaya for their support. Finally, I would also like to acknowledge the University of Malaya for supporting this research through IPPP grant (grant no.PG238-2014B).

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AIDS	:	Acquired immunodeficiency syndrome
BCG	:	Bacillus Calmette-Guérin
BLAST	:	Basic Local Alignment Search tool
CO_2	:	Carbon dioxide
CBB	:	Coomassie brilliant blue
CFP	:	Culture filtrate protein
^{o}C	:	Degree celcius
pH	:	Degree of acidity and alkanility
DC	:	Dentritic cell
dATP	:	Deoxyadenosine Triphosphate
dCTP	:	Deoxycytidine triphosphate
dGTP	:	Deoxyguanosine Triphosphate
DNA	:	Deoxyribonucleic acid
dTTP	:	Deoxythymidine triphosphate
DOTS	:	Directly Observed Treatment, Short-course
EM	:	Environmentam mycobacteria
ELISA	:	Enzyme linked Immunosoebent Assay
EDTA	:	Ethylenediaminetetraacetic acid
EXPASY	:	Expert Protein Analysis System
HIV	:	Human immunodeficiency virus
HLA	:	Human leukocyte antigens
IVET	:	*In vivo* expression technology

IL	:	Interleukin
INF-γ	:	Interferon-gamma
IPTG	:	Isopropyl β-D-1-thiogalactopyranoside
kb	:	kilo base pair
kDa	:	kilo dalton
LB	:	Luria-Bertani
MALDI-TOF:		Matrix assisted laser desorption ionization-time of flight
ml	:	mililitre
mM	:	milomolar
MHC	:	Multi Histocompatibility Complex
MDR	:	Multidrug-resistant
MW	:	Molecular weight
Mtb	:	*Mycobacterium tuberculosis*
nm	:	nanometre
Ni-NTA	:	Nickel-nitrilotriacetic acid
ORF	:	Open reading frame
OD	:	Optical density
PAGE	:	Polyacrylamide gel electrophoresis
PCR	:	Polymerase chain reaction
PPD	:	Purified protein derivative
RNI	:	Reactive nitrogen intermediate
ROI	:	Reactive oxygen intermediate
RD	:	Region of difference
STM	:	Signature tagged mutagenesis

SDS	:	Sodium dodecyl sulfate
SSI	:	Statens serum institute
FAO	:	The United Nations Food and Agriculture Organization
TSS	:	Transformation storage solution
TAE	:	Tris-acetate-EDTA
TB	:	Tuberculosis
TBVI	:	Tuberculosis Vaccine Initiative
TNF	:	Tumor necrosis factor
µl	:	micolitre
TEMED	:	N, N, N, N – tetramethylethylenediamine
V	:	Volt
WHO	:	World Health Organisation

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CHAPTER 1: INTRODUCTION

1.1 Introduction

Tuberculosis (TB) is an infectious disease affecting human being that is established for millennia and has been well-known since the end of 19th century. Robert Koch, discovered the pathogenic organism, *Mycobacterium tuberculosis* (*Mtb*) in 1882. TB vaccine is been administered for about a century, and still its drugs (antibiotics) have been in place and utilized for more than six decades (McMillen, 2015). All these work yet, TB is considered a leading infectious disease going by the number of death of 1.5 million recorded in 2014. (Wallis et al., 2016). TB is a bacterial disease and still positioned as a major global health problem. Despite reported declining incidence in recent years, tuberculosis remains a leading killer among infectious disease in the world, seconding only to Human immunodeficiency virus (HIV) (Yuen et al., 2014). It is also partly as a result of the emergence of multidrug-resistant TB (MDR-TB) and extensively drug resistant TB (XDR-TB) strains as well as co-infection with Human immunodeficiency virus (Fang et al., 2015). According to World Health Organisation (WHO) report, an estimated figure of 8.6 million people had developed TB disease in 2012. Out of that, 1.3 million death was recorded due to the disease with 320 000 deaths among HIV infected individuals (WHO, 2013). *Mtb* is accountable for more human mortality than any other single pathogen (Luca & Mihaescu, 2013). The targets of Stop Tuberculosis Partnership include bringing down the world burden of TB by 50% by the year 2015 compared with the levels recorded in 1990 and eradicating the disease as a global threat by 2050 (WHO, 2011). Bacille Calmette-Guérin (BCG) obtained from *Mycobacterium bovis* is still considered the currently available vaccine candidate against TB (Delogu et al., 2014). The major downside of BCG vaccine is that it gives protection against childhood forms of disease but not to adulthood form. Due to variability in its efficacy there is an urgent need and concerted global effort to create a better vaccine with maximal efficacy to

reduce the thread of tuberculosis. The variable effectiveness of BCG vaccine is due to differences in the strains used for vaccination as well as considerable batch to batch variations (Keyser et al., 2011). The quest for an effectual vaccine plan for TB has become a worldwide research prime concern where varieties of avenues including Bacille Calmette-Guérin, attenuated *Mycobacterium tuberculosis*, DNA based vaccines and recombinant peptide/protein antigen subunit vaccines are used (Mir et al., 2009). Co-infection of tuberculosis with HIV is one of the factors responsible for rising in TB cases (Kaufmann, 2011).

Vaccines are considered one of the most majestic success stories achieved in the field of medicine (Kaufmann et al., 2014). The excellent vaccine against tuberculosis should be side effects free, non-living, safe for injection to immunocompromised individuals, protective against subsequent infection and also gives protection against endogenous reactions of post exposure (earlier infection).

It is well established that genetic variations occur among bacterial strains within a single species. Deletions and rearrangements have been reported in different associates of the *Mycobcteruim tuberculosis* complex and have been related to attenuation and loss of virulence (Sarojini et al., 2011). The whole genomic sequence of the Laboratory strain (H37Rv) of *Mtb* published by Strewart Cole and his colleagues in 1988 provided a headway in TB research (Cole et al., 1998). The laboratory reference strain of *Mtb* contains 14 regions of difference (RD1-14), with size ranging from 2-12.7 kd as identified by differential hybridization arrays, these regions were not found in Bacille Calmette-Guérin. In parallel, six region known as regions of deletions (RvD1-5) and *Mtb* specific delition 1 (TBD1) were absent in the genome of H37Rv strain in relation to other members of *Mtb* complex (Behr et al., 1999; Brosch et al., 2002; Gordon et al., 1999). Recombinant proteins and/or overlapping of synthesis peptides evaluations are among the

approaches applied for immunological character study of the predicted proteins specific to *Mtb*. Several proteins of *Mtb* with antigenic properties were identified through this approach, including those encoded by genes found in RD 1 with abilities for specific TB diagnosis and/or new vaccines development, such as Open Reading Frame protein (ORF14), early secretory antigenic target (ESAT6), culture filtrate protein 10 (CFP10), PE, PPE proteins (Mustafa, 2005). It has been reported that RD1 region contain key virulent genes including ESAT6, MW 6 kDa and CFP10, MW 10 kDa (Rastogi & Sola, 2007). PE and PPE are also interesting families of protein genes found in *Mtb*. They are termed PE and PPE because of the motifs of Pro-Glu (for PE) and Pro-Pro-Glu (for PPE) located near the N-terminal of the protein. PE and PPE play an essential role in the survival as well as multiplication of *Mtb* in a varying environments (Portillo et al., 2007). The ORF-14 is also expressed as secreted antigenic protein, which is encorded by region of difference (RD1) of *Mycobacterium tuberculosis* (Amoudy et al., 2007).

In vaccine design, epitopes are attracing interest to both basic biomedical and clinical researchers due to their enomous potentials. In past, vaccine development solely rely on biochemical and immunological methods that are very expensive and time-taking. These methods include phage display library, overlapping peptides, NMR, Enzyme linked Immunosoebent Assay (ELISA), radioimmunotheraphy, western blotting technique, immunofluroscence, structural study via X-ray crystallographic analysis of antigen/antibody as well as application of random mutations and serial passage in attenuation of wild type pathogenic organism. With the advancement of epitope predictive tools now, protein of interest are narrowed and there is drastic decrease in the number of wet experiments thereby lessen the time taken in vaccine design. (Yang & Yu, 2009). A new pattern of designing vaccine is now emerging, sequel to important discoveries in immunology coupled with development of bioinformatics software/tools for prediction of T-cell epitope from primary protein sequences (De Groot et al., 2002).

This could be linked to the fact that, new technologies were developed due to sequencing of entire bacterial genomes (Scarselli et al., 2005), including that of H37Rv strain of *Mtb*, a well-established laboratory reference strain (Cole et al., 1998). A new branch of immunology called epitope mapping focuses on the choosing the most potential epitope capable of serving as a prospective targets toward production of epitope-based immune-preparations (Ahmad et al., 2016). The choosing of suitable target epitope with the capability of inducing a protective B-cell dependent immune response is also vital for vaccine development (Ahmad et al., 2016). New approaches are now available as a result of advent in computer and informatics that smooth the way for vaccine research and development. Immunoinformatics is directed toward addressing immunological problems using mathematical and computational approaches. Numerous immunoinformatics techniques have developed and utilized for predictions of T-cell and B-cell immune epitopes since 1980s (He et al., 2011).

1.2 Aim and objectives

This research work is aimed at achieving the following objectives

1. To produce, analyze and purify the 6X His-tagged protein from bacterial recombinant clone Myt272-3.
2. To compute the allergenicity and antigenicity of the protein by a Algped and Vaxigen software, respectively.
3. To determine computationally the Multi Histocompatibility Complexes (MHC) I and II binding by Propred server and B-cell epitope binding of the protein by Immune epitope database.
4. To predict the toxicity of the MHCs peptide sequences and B-cell epitopes using *in silico* approach.

CHAPTER 2: LITERATURE REVIEW

2.1 History of tuberculosis

According to Gutierrez and her colleagues, it has been concluded that an early ancestor of *Mtb* was present as far back as 3 million years ago in East Africa and they suggested that it might have affected early hominids at that time (Prasanthi & Murty, 2015). The presently known members of *Mtb* complex were originated common hypothetical ancestors about 15,000 - 35,000 years back (Kremer et al., 2005). In Egypt, tuberculosis was documented more than 500 years back, distinctive skeletal abnormalities of TB like characteristic Pott's deformities was observed in Egyptian mummies (Daniel, 2006). TB was named as phthisis by Hippocrates (2,470- 2,367 BP) "to waste away", and in England it was termed "consumption" having the same meaning (El-Najjar et al., 1996). In 1680, the Dutch physician known as Franciscus Sylvius demonstrated that phthisis was as a result nodules (small, rounded knots), which he termed as turbercles (Ducati et al., 2006; El-Najjar et al., 1996). Gore- lamo Fracastoro (1483-1553) was the first to identify the contagious nature of TB (El-Najjar et al., 1996).

The transmissibility of TB was first demonstrated experimentally in 1843 by Philipp Klencke of Berlin (1813–1881) which served as a proof of the infectiousness of tuberculosis. Klencke considered TB as tumour and he experimentally shown his findings by injecting intravenously material from a turbercle into rabbits leading to the death of animals of TB (Herzog, 1998). A further step was made by Jacob Henle (1809-1885), he demonstrated TB infection in human was related to animal disease. Observation was made on TB transmission from cat to human, then from inoculated human samples to rabbits (Cambau & Drancourt, 2014)

Understanding of tuberculosis pathogenicity began in 19th century. Advancement on this work was carried out by Jean-Antoine Villemin in the year 1865 where he indicated

transmissibility of *Mtb* infection. Identification of *Mtb* as causative agent of TB was done by Robert Koch (Daniel, 2006; Saran & Das, 2012). Koch was a recipient of 1905 Novel price in Medicine and Physiology for his contribution to discover the cause of TB (Daniel, 2005). The allergy test for TB diagnosis in children by Clemens von, a pediatrician was published in 1907 by Vienna Medical Weekly (Lange & Rieder, 2011).

2.2 Mycobacteria and *Mycobacterium tuberculosis* complex

Mycobacteria are nonspore forming, nonmotile, weak gram positive in nature, acid-fast with straight or slightly curved rods structural appearance microscopically. They have length ranging from 1.0 to 4.0 µm and wide ranging 0.3 to 0.6 µm (Sakamoto, 2012). *Mtb* is one of the members of the family Mycobacterium; other members of the family are non-tuberculous mycobacteria. Mycobactria are members of family known as Mycobacteriaceae characterized by having high lipid content. They possess high level of waxes called mycolic acid (Salman, 2015). Mycobacteria fall within the order known as Actinomycetales (Sakamoto, 2012). The most prevalent agent of the complex is *Mycobacterium tuberculosis*; *Mycobacterium africanum* and *Mycobacterium bovis* are other members, Identification of more than 160 species of non-tuberculous mycobacteria has been done and species are classified based on growth as rapid growers, which grow in culture media within one week or slowly growers, that grow after one week and not all of the members are considered pathogenic to human beings (Ribeiro & Goldenberg, 2015).

Mycobacterium tuberculosis complex is the group of related and associated species and subspecies including *M. africanum, M. bovis, M. bovis* bacillus Calmette-Guérin, *M. canettii, M. microti, M. mungi, M. pinnipedii* and *M tuberculosis,* among these members The most common and well known pathogenic agent of human TB is *Mtb* while *Mycobacterium bovis* is a causal agent of TB in both humans and animals (Kim et al.,

2013). *Mycobacterium africanum* and *Mycobacterium canettii* are in close relation to *Mycobacterium tuberculosis*, they also leads to TB and are isolated from Africa patients, *Mycobacterium caprae* is the only strain isolated from goats while *M. microti* is pathogenic agent in rodent isolated from rodents belonging to Microtus genus and other related genera which can also lead human disease in immunocompromised individuals (Forrellad et al., 2013).

Mycobacterium tuberculosis complex are aerobic (oxygen requiring organisms) bacteria (Pfyffer, 2007). Level of genetic differences at the nucleotide is very low in members of *Mtb* complex and species have more than 99.9 percent homology in their genomic sequence, they possessed identical 16S ribosomal gene sequences, the size of genome of these species is approximately 4.4 million base pairs with higher GC content (about 65%) (Costa et al., 2014). It has been shown that members of *Mtb* complex have developed from a common ancestor through successive deletions or insertion of DNA leading to the current pathogenicity differences and speciation of Mycobacterium. Analysis of genome has been basic for these studies which assisted to recorganise 14 regions of differences (14-RD1-14). These regions of differences are found in in the H37Rv, the reference labolotary strain of *Mtb* but absent in BCG strain of *Mycobacterium bovis*, thus assisting to indicate genes associated toward pathogenicity. In contrast, 6 regions, called deletion 1 to 5 (RvD1-5) and *Mtb* specific deletion 1 (TbD1) are absent in H37Rv strain genome in relation to other members. *Mycobacterium canettii* possess all the regions; RD, RvD and TbD1 which as a result considered the most strongly related to genome of the bacilli's ancestor. *Mycobacterium africanum* stains mainly of West africa origin are deficient of region of defference (RD9), while East african strain lack RD3 but have RD9 preserved, in *M. microti* special region, called RDmic and regions RD7 to RD10 are absent. The "Classical *M. bovis*", the most common *M. bovis* strain isolated from the bovines in the following countries Argentina, the Netherland, Spain and United kingdom and also from

humans, indicated considerable number of RD deletions, with regions RD4 to RD12 and RD13 absent (Forrellad et al., 2013).

2.2.1 Morphology and structural features of *Mycobacteruim tuberculosis*

Mtb are immotile, noncapsulated and nonsporing organisms that exist in straight or slightly rod shape arranged separately or in group. They have length of 1-4u and wide ranging between 0.2 to 0.8u (Gupte, 2012). Variation of morphology (pleomorphism) in *Mtb* has also been reported which is classified into two; rod, V, Y- shaped, buds or branched are frequently seen during exponential growth phase, the second category include oval, round, L-forms, spore like that are seen infrequently under stress (Velayati & Farnia, 2011). Transformation of TB bacilli in latent stage of infection from rod-shaped, acid-fast cells with size ranging from 1.5 to 3.0 µm size into non acid-fast oval form with size 0.1 to 0.3µm was reported (Velayati et al., 2012). The structural illustration of *Mtb* is shown in Figure 2.1.

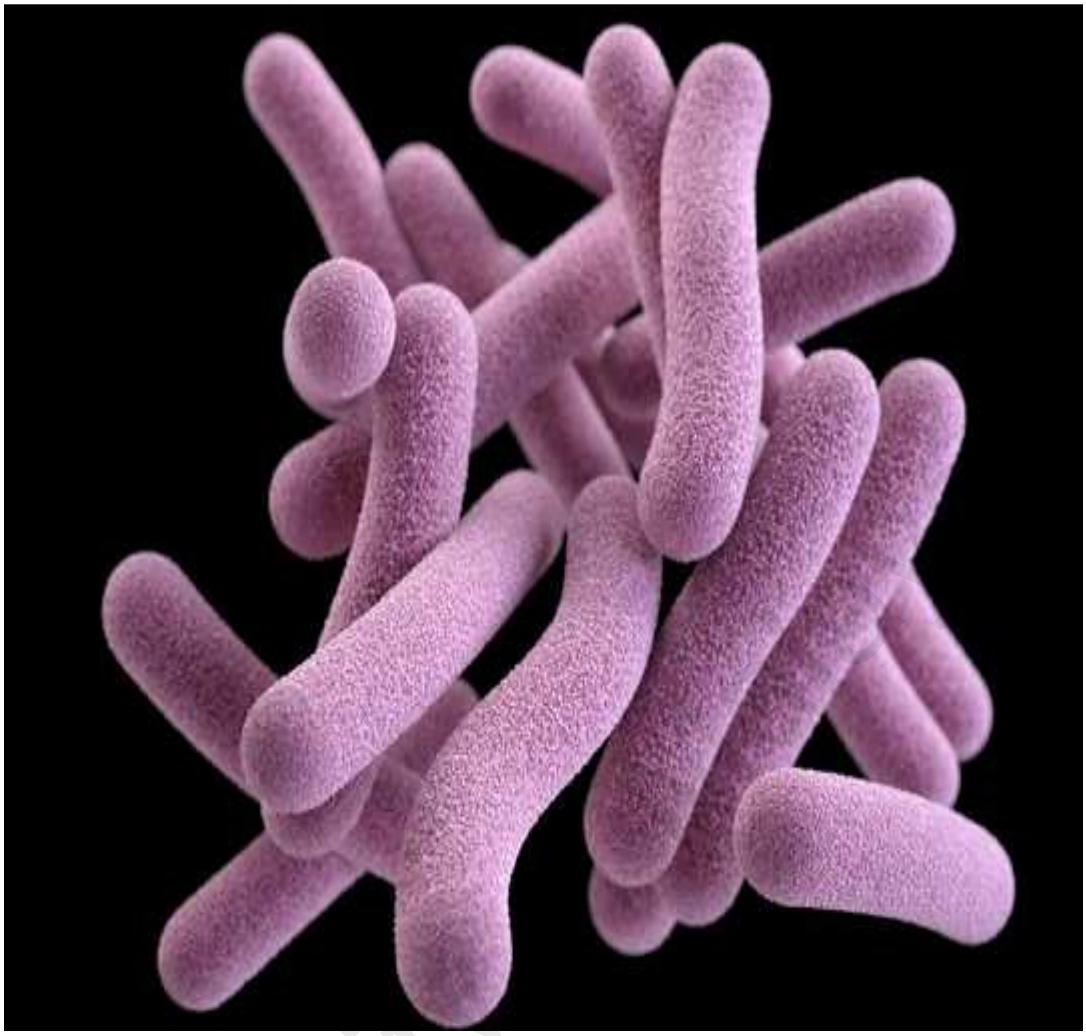

Figure 2.1: Structural illustration of *Mycobacterium tuberculosis*

Mtb in rod shaped and are arranged in group. (Centre for Disease Control and Prevention, with permission).

2.2.2 Growth requirements of *Mycobacteruim tuberculosis*

Macrophages are essential to host protection against microbes, but their antimicrobial roles are avoided by intracellular pathogens. *Mtb* has successfully harnessed macrophages as its prime place *in vivo* (Rengarajan et al., 2005). The turbercle bacillus is considered prototrophic in that has ability to build its components from basic elements carbon and nitrogen; it is also considered heterotrophic as it utilize already made organic compounds for carbon and energy source, for they are metabolically active (Velayati & Farnia, 2011).The ability of tubercle bacilli to adopt to environmental changes during the course

of infection made it a successful pathogen. Generally, temporary lifestyle of *Mtb* is determined by quality of nutrition and other physical parameters. These changes involve; nutrient distress, deficient oxygen, pH, temperature, salinity and many stressful conditions (exogenous) (Velayati & Farnia, 2011).

A comparative analysis on the growth rate of *Mtb* was carried over a period of 6 years on three defferent media including, Lowenstein-Jensen, American Trudeau society and Middlebrook 7H10 with growth rate of 82%, 79% and 56% respectively. The low positive growth rate is attributed to the decreased carbon dioxide (CO_2) enrichment (Liu et al., 1973). An atmospheric CO_2 level ranging from 5 – 10% was reported to be essential to support culture growth, at all events during the early phase of incubation (Barrera, 2007). The growth of *Mtb* is stimulated markedly with an increased CO_2 content (Schaefer et al., 1955). The growth of tubercle bacilli in salt solutions can be achieved with glycerol (as a source of carbon) while amino acid, asparagine and ammonium ions (as source of nitrogen) and micronutrients. *Mtb* can metabolise glycerol into pyruvic acid, whereas *M. bovis* cannot, as gonome sequence analysis of revealed that all genes needed for production of pyuvic acid were defective in *M.bovis.* Trace elements are also required for functional or structural role in cell wall. Iron in form insoluble ferric salt is needed in the environment as it is incorporated into the cell wall, Magnesium is also required by *Mtb* as its deficiency frequently decrease virulency. In addition, oxygen is needed by tubercle bacillus. Naturally, bacillus grow well in tissues such lung that has high oxygen partial tension. *M. bovis* on the other hand is termed as microaerophilic as it grows better at a decreased oxygen tension. Multiplication of *Mtb* is limited to conditions by warm-blooded animals, they grow at about temperature of 37ºC and a neutral pH (Barrera, 2007).

2.2.3 Nature of *Mycobacteruim tuberculosis* cell wall

The cell wall of mycobacterial is made up of inner portion and outer layer which surround the plasma membrane. The outer section is composed of both proteins and lipids. The lipids are frequently and freely in association with the cell wall, having some short- and long-chained fatty acids complementing the long and short chains present in the inner portion. The outer part is associated with the lipid-linked polysaccharides consisting of lipomannan, lipoarabinomannan, phthiocerol-containing lipids like phthiocerol dimycocerosate, cord factor (dimycolyl trehalose) and sulpolipids (Hett & Rubin, 2008).

The presence of a wide arrangement of complex lipids and lipoglycans on the cell surface of *Mtb* made it unique among the bacterial pathogens, these lipids are considered very vital in pathogenesis of *Mtb* (Forrellad et al., 2013). The hydrophobic nature and the present of complex cell wall consisting of the following arabinogalactan, peptidoglycan, fatty acid (mycolic acids) and glycolipids layered on top of the plasma membrane critically made *Mtb* to escape the immune system of the host (Fukuda et al., 2013). The structural composition of *Mtb* cell wall compose of peptidoglycan, a cross-linked network linked to arabinogalactan via a linker unit, and which in turn is acylated to peptidoglycan at its distal end with mycolic acids. The unusual low permeability of the cell wall and also resistance to commonly used antibiotics were accounted due to this unique cell wall structure (Alderwick et al., 2007). The basic structural components of *Mtb* cell wall is shown in Figure 2.2 below.

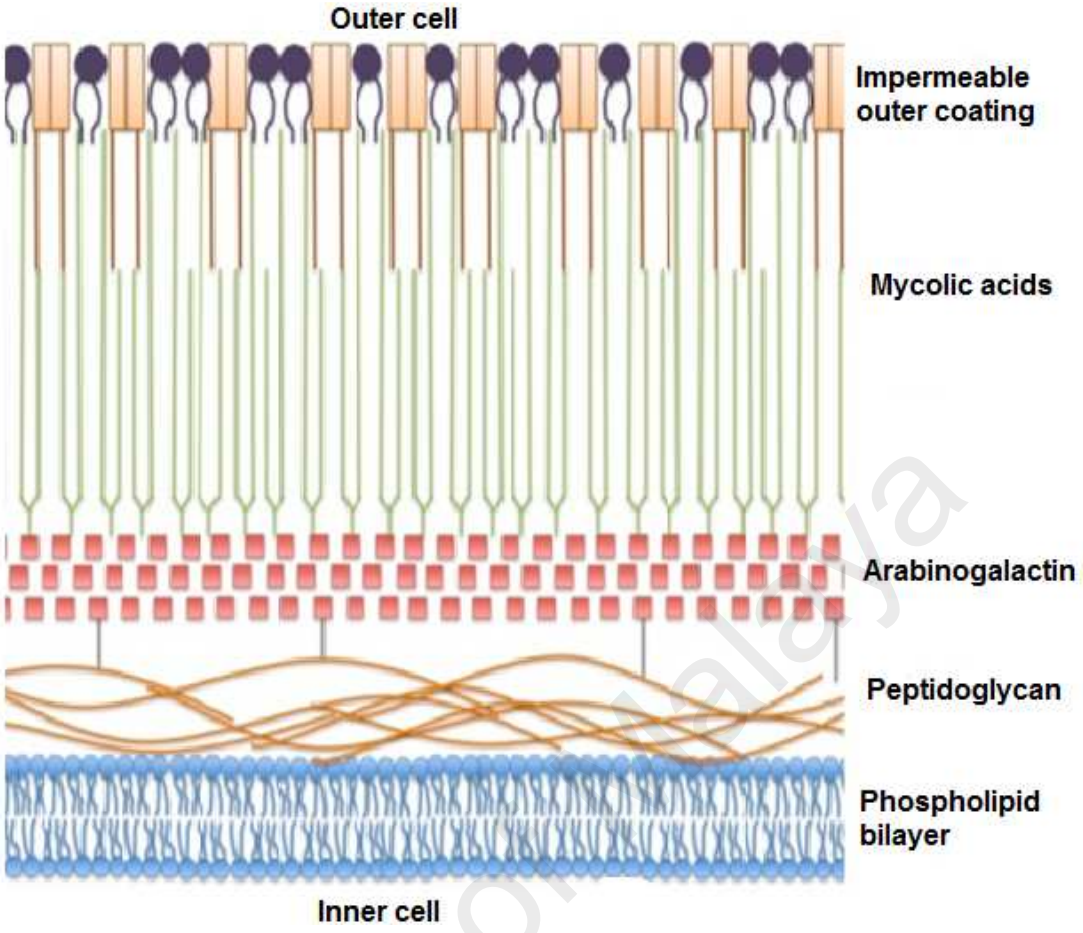

Figure 2.2: The basic structural illustration of *Mtb* cell wall

The cell of *Mtb* is composed of four components including mycolic acids (the outer layer) that sorrounds arabinogalactan and peptidoglycan (inner layers). These surround the phospholip bilayer component of the cell (Smith, 2011). (With permission)

2.3 Disease and treatment

To combat the effect of TB and spread of the resistant forms of the disease (MDR-TB and XDR-TB) new treatment strategies are required, there is urgent clinical need to discover potent agents that can reduce the time taken of MDR and XDR disease treatment with a success rate compared to that of present treatments for drug-susceptible TB (Pethe et al., 2013). Early diagnosis of TB and immediate therapy respond is required for a successful TB control, delay in diagnosis of tuberculosis is sufficiently great toward

prognosis of disease at the individual level and also spread at community level and the progressive rate of the tuberculosis epidemic (Storla et al., 2008).

The central challenge faced in TB drug development and control is drug tolerance, most form of tuberculosis need at least 6 months for eradication of slowly killed, drug-tolerant *Mycobacterium tuberculosis* populations and minimize risk of recurrence of disease (Walter et al., 2015). This duration of treatment burdens health system as well as patients and serve as contributory factor for medication non-adherence, leading to advance TB transmission and acquired drug resistance (Lipsitch & Levin, 1998; Walter et al., 2015). Treatment of TB by using multiple drug is usually highly effective to clear the infection (Lipsitch & Levin, 1998).

The TB control strategy recommended internationally, which was later termed Directly Observed Treatment, Short-course (DOTS) was lunched in 1994. The components of DOTS are commitment from government, detection of cases, standard short-course chemotherapy, regular drug supply system and a monitoring system to supervise and evaluate the program (Montoro & Rodriguez, 2007). DOTS is the present method used for TB treatment; this strategy involves combination of the following drugs rifampicin, isoniazid, pyrazinamide and ethambutol. These four drugs are given over a period ranging from 6-9 months. Due to the significant adverse effects as well as long regiment of these drugs, it is often causes discontinuation of treatment by patients leading to non-compliance of drug and establishment of MDR (Singh et al., 2014).

2.3.1 Multi drug resistant

TB control is facing a great problem of resistance of the organisms to tuberculosis drugs which threatens the global progress made as far TB control, drug resistance originate as a result of improper utilization of drugs (antibiotics) while treating drug susceptible organisms, Multidrug-resistance TB is referred to as resistant to isoniazid and

rifampicin (the most commonly drugs in use) in the common four drug regiment (Bhembe et al., 2014). In TB-infected individuals, majority of the bacteria will be killed even with an inadequate treatment but this will also permit the growth of the few that are resistant within the mycobacterial population that are arising due to spontaneous mutation. Therefore, a population that are entirely resistant to an unaccompanied drug emerges, and with persistent in inadequate treatment among this population, small portion which have mutated develop resistant further to drugs. Therefore, resistance established to one drug may lead to resistance to two drugs and therefore becoming resistance to many drugs in sequential manner (Faustini et al., 2006). *Mycobacterium tuberculosis* strains resisting anti-microbial treatments, involving multidrug-resistant tuberculosis strains (with combined resistance to both isoniazid and rifampicin) have been reported worldly (Ani et al., 2009). A study was carried out between 2000 and 2006 in Mato Grosso do Sul, Brazil in order to ascertain the drug resistance profile of *Mtb*, high degree of resistance was reported which compromise the efforts toward TB control in the area with acquired multi-drug resistance 12.7 times greater than the was the initial multi-drug resistance, showing that the previous utilization of drug treatment is the responsible indicator of the resistance (Marques et al., 2010).

2.4 Pathoginesity of tuberculosis

Pathogenicity is involving several factors and microorganism to satisfy cardinal conditions. For pathogenic microorganism such as mycobacteria that lack direct access to the host tissue, the bacilli must be capable to colonize the mucous surface, gain access to the host cells, proliferate in the environment of host tissue, interfere with the defense mechanism of the host and brings about host's tissue damage (Daffe & Etienne, 1999). Varieties of factors take part in the pathogenesis of TB, virulence factors such as catalase-peroxidase enzyme, glycolipids, mycolic acid, sulfatides and lipoarabinomannan produce by *Mtb* are associated with its intracellular survival. These products can assist in

granuloma formation, they also help to develop resistance against host oxidative response and also brings about cytokines toxicity in animal models. (Ribeiro & Goldenberg, 2015)

Mtb is an intracellular pathogen that has greater liking to lung tissue which is rich in oxygen supply. The most important source of tuberculosis is the patients that developed pulmonary TB and infection is initiated by inhalation of droplet nuclei particles of size ranging from 1-5 um diameter (Ahmad, 2010). When infection is established by attacking of bronchi and pulmonary alveoli, than the next line of action is dissemination of the bacilli particles from the lumen of the alveoli into the blood thereby spreading the infection to the upper lung and organ systems (Mehta et al., 2006). After inhalation of mycobacteria they multiply in the pulmonary epithelium or macrophages, within 2- 4 weeks of infection many TB bacilli are attacked and destroyed by immune system (Fisher & Harvey, 2013). *Mycobacterium tuberculosis* has a unique characteristic of growing in immunologically activated macrophages and also remains viable within the host for decade (Fisher & Harvey, 2013).

2.4.1 Latent tuberculosis infection

Latent state of TB infection represent an equilibrium state whereby the host controls the infection but unable to clear it successfully , thereby leading the survival of *Mtb* at relatively constant low levels (Fallahi-Sichani et al., 2011). Latent form of tuberculosis infection is characterized due to the occurrence of immune responses to *Mtb* infection without indication of clinical evidence of active tuberculosis (Getahun et al., 2015). In latent stage of TB infection, the pathogenic microorganism is contained by the active interplay of immune cells in an infected individuals (Kaufmann & Gengenbacher, 2012). The containment breakdown as a result of immunedeficiency, this explains the dramatic deteriorating of the worldwide tuberculosis situation after showing up and spread of HIV in the 1980s. HIV infected individual has about 10% risk of active TB development

within the first year of coinfection whereas an individual with latent form of tuberculosis has an estimated 10% risk of developing TB in lifetime (Kaufmann & Gengenbacher, 2012; Kaufmann et al., 2010). Clemens von Pirquet was the first person to describe the concept of latent *Mycobacterium tuberculosis* infection and who also carried out a test known as tuberculin test using Koch's tuberculin (mycobacterial antigens crude mixture). He came out with a proposed definition describing a child with no symptoms of pulmonary or extrapulmonary tuberculosis but indicated a positive skin reaction upon administration of tuberculin(DRuSZCZYńSKA et al., 2012). Healthy individuals with latent tuberculosis infection or dormant stand a 10 percent lifetime risk that dormancy of the mycobacteria will be broken to an active and thereby causing TB and persons with HIV and latent form of TB co-infection have a risk for TB reactivation of 10 per cent per annum (Garfein et al., 2010). Latently infected individuals cannot transmit TB, but the disease can be developed and become infectious with a life-time risk of roughly 5–15% (Sotgiu et al., 2015).

2.4.1.1 Turberculin skin test of TB

Turberculin skin test was the only diagnostic technique used for the detection of latent form of TB infection until the beginning of this century (Trajman et al., 2013). After mycobacterial infection, proliferation of T lymphocytes occur and they become sensitizes. Upon tuberculin injection into skin lymphocytes are stimulated as a result series of events happen leading to delayed-type hypersensitivity response. This response is termed "delayed" due to the fact that reaction becomes noticeable only after 24-48 hours (Huebner et al., 1993). The purified protein derivative (PPD) which is obtained from cultures of *Mtb* is the most widely used turberculin. In turberculin test is apparent when T-cells sensitized by preceding infection are recruited to the skin site where lymphokines are release. Induration of skin via local vasodilatation, fibrin deposition,

edema and recruitment of other inflammatory cells to that area are induced by lymphokines (Nayak & Acharjya, 2012).

Other technique used is interferon-gamma (INF-γ) release assay that is capable of detecting the immune response specifically to *Mtb* antigens that are absent in BCG. This test works on the principle that the T cells of a person that acquired TB will respond by releasing INF-γ upon restimulation with *Mtb* antigens (Trajman et al., 2013).

2.4.2 Reactivation of latency

World health organization reported that approximately 2 to 3 million people are latently infected with *Mtb* globally, and about 5- 15 percent will suffer from TB reactivation during their life time (WHO, 2015). Therefore, global prevention of tuberculosis infection is directly influenced by the treatment of the latent TB infection, presently the study of latent TB infection depends heavily upon screening for high-risk populations and on strategies involve for the treatment of disease.

2.5 Transmission of tuberculosis and immune response

Tuberculosis infection is transmitted by respirable small droplets released as a result of forceful expiratory maneuvers like sneezing, coughing (Konstantinos, 2010). The mode of entry of the *Mycobacterium tuberculosis* into the body is through respiratory tract by inhalation of small droplet nuclei of size ranging from 1-2 um or less than that which can pass into lower respiratory tract (Schluger & Rom, 1998) . In the early stage of tuberculosis infection, the causative pathogen is engulfed by immune cells, intracellular replication occurs, and the bacteria-laden immune cells may cross the alveolar barrier to affect number of tissues and organs (Ahmad, 2010).

Alveolar macrophage is the site where *Mtb* infection is believed to take place at the initial step. The mycobacterial replication occurs inside the macrophage and triggers

cytokines which is responsible for initiation of the inflammatory response in the lungs. The granuloma is form upon migration of macrophages and lymphocytes to the infection site (Flynn & Chan, 2001). Alveolar macrophages engulf mycobacteria when they enter the lung, where *Mtb* reproduces and inhibit macrophage killing mechanisms. Despite inhibitory influence of *Mtb*, infected macrophages secrete chemokines and cytokines, leading to the recruitment and activation of many immune cell populations to the lung (Monin & Khader, 2014) The infection is excluded by the granuloma so as to avert spreading to the uninfected parts of the lungs as well as other tissues. It also play a vital role in gathering the immunological response directly at the location of infection. In a persistently infected host, the granuloma is maintained, possibly as a result of chronic immune cells stimulation, and form the basis for a TB lesion. It has been reported that live bacilli was isolated from granulomas in the lungs of individuals with clinically inactive TB, which is an indication that the organism can persist in a granulomatous lesion for years (Noss et al., 2000).

The innate immunity activation depend on recognition of *Mtb* components of the cell wall as mycolic acid, mannan, petidoglycans through toll-like receptors (Matucci et al., 2014). Mycobacterial antigen recognition, macrophages and dendritic cells (DCs) activation as well as other cells involved in innate immunity needs toll-like receptors (Saraav et al., 2014). The bacilli is engulfed by macrophage through phagocytic receptors, of which the complement and mannose receptors serve an important role (Behar et al., 2011). The immune cells enclose the pathogenic bacteria in the first stage of tuberculosis infection, intracellular multiplication occurs, and the bacteria-overloaded cells may traverse the alveolar barrier affecting other tissues and organs (Ahmad, 2010). Survival of *Mtb* in macrophages is achieved by inhibiting acidification of the phagosomal complement and also by inhibition of the fusion of the phagosome with lysosomes (Behar et al., 2011). The cell-mediated immunity is effectively involved in regulating *Mtb*

limitation in granulomatous lesions of the lungs, usually without eliminating the bacteria which prevail in latent stage (Kozakiewicz et al., 2013). Besides macrophages, it is appreciated that DCs also play a role as an important intracellular niche for *Mtb* (Madan-Lala et al., 2014). DCs are key regulators of adaptive immunity and are potent antigen presenting cells (Lambrecht et al., 2013). DCs have the unique ability of migrating to draining lymph node from the site of infection and afterwards recruit T cells of infection where they effectively activate the acquired immune response (Morris et al., 2013).

When cell-mediated immune responses are playing a crutial role of defense against intracellular pathogens, so also, the extracellular counterparts which are best monitored by B cells and humoral immune (Chan et al., 2014). Protective immunity against *Mtb* depends critically on T lymphocytes due to its intracellular lifestyle (Kaufmann, 2013). In tuberculosis, cellular responses are the mediators of both pathogenesis and protection, which involves primarily interactions phagocytes of macrophages lineage and lymphocytes (Mustafa et al., 2011). Production of cytokines like INF-γ and tumor necrosis factor (TNF) establish protective immune responses against *Mycobacterium tuberculosis* infection, both cytokines activate macrophage toward *Mtb* control (Gopal et al., 2014). The cytokines serve an essential role in controlling mycobacterium growth by expression of reactive nitrogen and oxygen (Andersen & Woodworth, 2014). The *Mtb* immune response consists of great number of different cell kinds including T cells, neutrophils, B-cells and natural killer cells and the roles played by CD4 T helper 1 cells is best understood (Ottenhoff & Kaufmann, 2012). Other interleukin (IL) producing T lymphocytes such as CD8 T lymphocyte and CD4 cells likely take part in protective immunity (Andersen & Kaufmann, 2014). After infection, *Mtb* stimulates both CD4 and CD8 T cells and other immune cells, secretion of INF-γ dominated a strong type 1 immune response (Nikolova et al., 2013). During adaptive immune response to *Mtb* infection, CD4 cells are the primary source of INF-γ which are required the survival of

the host during both phases of acute and the chronic infection (Green et al., 2013). The need for INF-γ in immune protection of tuberculosis is well established both in animal models and human (Desvignes et al., 2012). INF-γ is the key cytokine in human and also in mice, whose role is to activate bactericidal actions in the host cell, macrophage (Sharma et al., 2014). It has been reported that individuals with genetic deficiency in the INF-γ receptor were more likely to be infected with mycobacterial (Wareham et al., 2014). Several studies carried out revealed an increased susceptibility to mycobacterial diseases in INF-γ-deficient mice and also in human having INF-γ receptor abnormalities (Madan-Lala et al., 2014). INF-γ is expressing protein peptides cathelicitin and defensin-β 2 which are delivered to *Mtb* phagosomes via vitamin D dependent pathway (Andersen & Woodworth, 2014), defensins and a single cathelicidin, LL-37 are major groups of host defensive peptides in human, susceptibility to infectious diseases including tuberculosis has been reported due to alteration in the synthesis of these molecules (Andersen & Woodworth, 2014; Rivas-Santiago et al., 2013; Rivas-Santiago et al., 2009).

Autophagy is a quality and quantity control process in cytoplasm through which consituents of cytoplasm sequestered into double membrane organelles known as autophagosomes and conveyed for degradation and elimination to lysosomes (Deretic, 2010). Autophagy has been recorganised with an antimicrobial function against pathogen (Deretic, 2014). It is one of the principal innate immune defense mechanisms utilised by infected macrophages to destroy *Mtb*. Pathogens are engulfed upon macrophages stimulation, their activation results in the immune-related GTPase M (IRGM1) production responsible for stimulation of INF-γ which serves as a key cytokine in inducing autophagy (Longhi et al., 2016). Survival of *Mtb* in machrophages is achieved by inhibition of phagolysosome biogenesis. Blockage of the mycobacterial phagosome is overcomes upon induction of autophagy (Vergne et al., 2006). In addition, autophagy delivers to *Mtb* conventional anti-*Mtb* antimicrobial peptides like cathelicidin via fusion

with lysosomes. It also generate a mixture containing neo-antimicrobial peptides called cryptides and deliver to *Mtb* (Bradfute et al., 2013).

2.5.1 Reactive nitrogen and oxygen intermediate

During the process of TB infection, *Mtb* must device a means to manage the diverse host-mediated stresses, specifically, the antibacterial characteristic of macrophages. Macrophages synthesise reactive oxygen and nitrogen intermediate species that are antimicrobial (Voskuil et al., 2011). There are established evidence for the formation of reactive in mammalian phagocytes, oxygen intermediates (ROI) are produced by the phagocyte oxidase (phox) whereas nitrogen intermediates (RNI) are formed by nitric oxide synthase (NOS) (Shiloh & Nathan, 2000). Bacterial DNA, lipids and proteins are damage by RNIs, RNIs are important in containment of infection and for killing mycobacteria due to their toxicity. Moreover, nitric oxide also produce S-nitro glutathione that is toxic to bacteria when reacted with glutathione (Longhi et al., 2016). In the presence of oxygen, an enzyme associated with macrophage functions in tuberculosis called nitric oxide synthese-2 catalyse the metabolism of L-arginine into L-citrulline and nitric oxide, which is taking part in killing the intracellular pathogen (Duque-Correa et al., 2014). This process also lead to generation of nitric oxide radicals or subsequent intermediates (Bogdan et al., 2000)

2.5.2 Humoral immunity

Humoral immune responses are also vital for protection from *Mtb* in addition to the pivotal role of cellular immunity in the control of *Mtb* infection (Feris et al., 2016). Although there were believe previously that antibodies had little defensive role against intracellular pathogens, that view had changed in recent decades (Achkar et al., 2015; Casadevall & Pirofski, 2006). Protection is mediated by antibodies to intracellular pathogens via different mechanisms extending from classical functions like opsonization

and activation of complement to nonclassical functions like modulation of the inflammatory host response and signaling through Fc receptors (FcR) (Achkar et al., 2015; Casadevall & Pirofski, 2006)

2.6 *Mycobactrerium tuberculosis* genome

Isolation of *Mycobacterium tuberculosis*, H37Rv was done in 1905 and represent the most widely used strain employed in TB research, *Mycobacterium tuberculosis*, H37Rv is the best-characterized strain of *Mtb*. Determination and analysis of the complete genomic sequence of H37Rv strain has improved understanding of its biology for therapeutic intervention (Cole et al., 1998)**.** The following three experimental action plans can be carried out using a complete genomic sequence; the experimental plans include *in silico* analysis, functional genomics and pengenomic technique. Moreover, four steps are involved for these three strategies including selecting genes from the entire genome, cloning and expression of the genes selected, recombinant proteins purification and *in vitro* and *in vivo* analysis to identify the suitable defensive candidates (Movahedi & Hampson, 2008). The H37Rv genome consist of 4,411,529 base pairs (bp) with about 4,000 genes, the large proportion of the coding ability of *M. tuberculosis* are dedicated for synthesis of enzymes taking part in lipolysis and lypogenesis (Cole et al., 1998).

Out of 3924 OFRs identified as a result of the original annotation of the *Mtb* H37Rv genome, about 60% were assigned to specific metabolic functions, the remaining OFRs were classified as conserved hypothetical proteins, approximately 25% or encoding proteins without known function, ~16% (Slayden et al., 2013). The genome of *Mtb* is mostly coding for protein enzymes participating in lipolysis (responsible for survival of mycobacterial inside its host) and lipogenesis (for the synthesis of cellular envelop). Mtb posses about 250 enzymes catalyzing fatty acid metabolism (Ducati et al., 2006). It has been reported that based on the genome sequence, it is clear that *Mtb* has potentiality of

switching one metabolic pathway to another which include aerobic (like oxidative phosphorylation) and anaerobic pathway like nitrate reduction. This adjustability is helful for survival of *Mtb* in an adjusting environments within the human host which range in the lung alveolus that is riched in high oxygen tention to microaerophilic/anaerobic states within the tuberculous granuloma (Portillo et al., 2007).

2.7 Disease burden

The WHO report on TB published in March, 2009, reported an estimated figure of 9.27 million tuberculosis cases globally in the year 2007 (Donald & Van Helden, 2009). Modeling studies carried out estimates that, about 101.7 million new cases and 17.9 million TB related death will occur in southern Asia alone from 2015 through 2050 if there is no new vaccine or other measures toward eliminating TB (Beresford & Sadoff, 2010). In 2008, South Africa was ranked as third-highest country with TB burden, and in 2007 South Africa carried approximately 25% of the world burden of HIV-associated tuberculosis cases (Wood et al., 2011). In 2011, an estimated figure of 8.7 million new of TB was reported, 13 per cent of which are co-infection with HIV. Number of death due to TB infection was 1.4 million (with about 430, 000 among HIV infected and 1 million among HIV- negative). In addition, TB is considered one of the leading killers of women as 200, 000 and 300,000 deaths were reported among HIV-positive and HIV-negative women respectively in 2011 (WHO, 2012). In 2012, 58% of world's TB cases were accounted collectively by South-east region and western pacific region where The African region had approximately one quarter of world's cases, India and China recorded a highest number of cases with 29% and 12% of the global total respectively. South Africa and Swaziland had the highest incidence per capita (WHO, 2013). The targets set by Millenium Development Goals to curb and reverse TB incidence by 2015 was achieved globally, in 22 countries with high TB burden as well as in all WHO regions. About 1.5% cut down in average incidence rate was observed per annum between 2000 to 2013. It has

also been reported, mortality due to TB fell by about 45% between years 1990 and 2013 (WHO, 2014). According to WHO report of 2015, an estimated 9.6 million people were reported to have fallen sick due to TB worldwide in 2014 of which 1.0 million were children, 3.5 million women and 5.4 million men. Out of the 9.8 million new cases reported globally,the global incidence rate of TB in 2014 is shown on Figure 2.3 global as reported by WHO, 15% were HIV infected individuals. In the same year, 1.5 million death due to TB was reported (0.4 million HIV-positive while 1.1 million were HIV-negative) (WHO, 2015). World's estimated HIV prevalence in new and deteriorate TB cases, 2014 is shown in Figure 2.4.

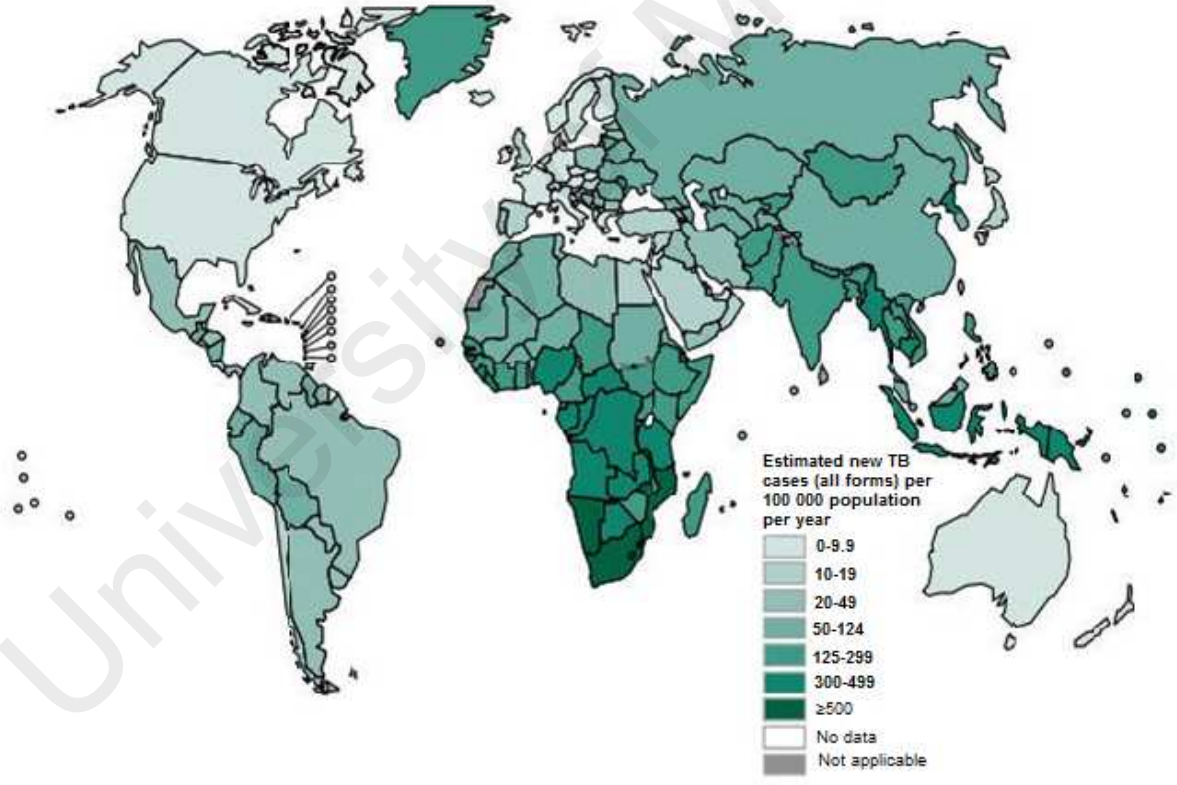

Figure 2.3: Estimated rates of TB incidence 2014

The TB incident cases in relation to the global population size differ widely among countries as shown. With permission from WHO.

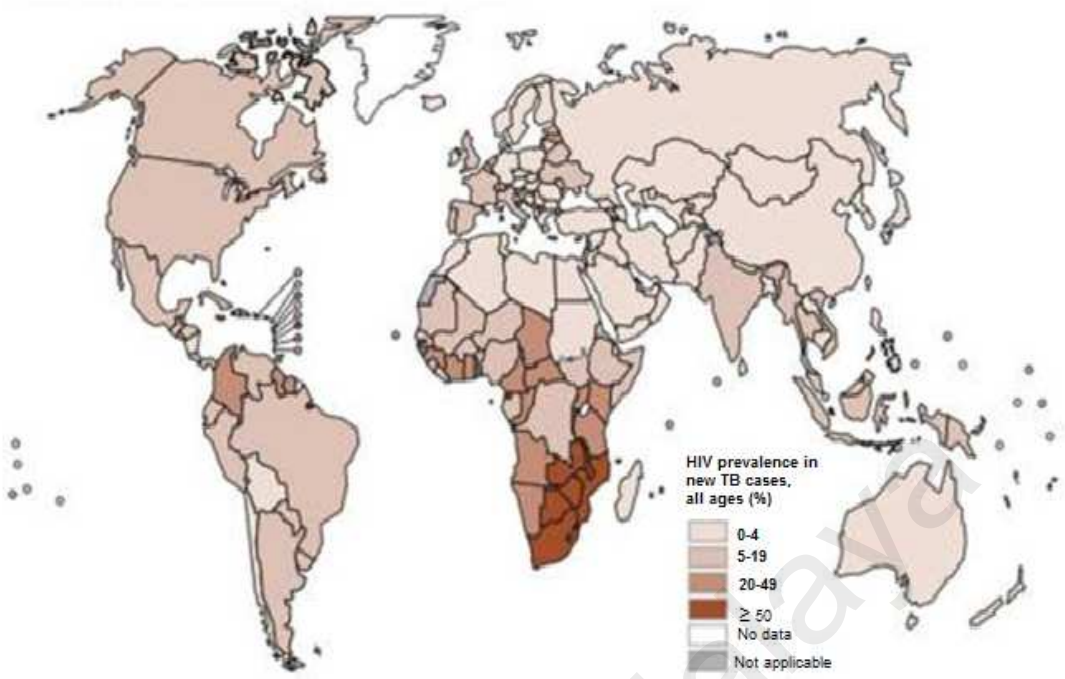

Figure 2.4: Estimated HIV prevalence in new and deteriorate TB cases 2014

The tuberculosis cases coinfected with HIV was highest in the nation in the Afrcan region. More than 50% TB co-infection were reported in parts of south Africa. With permission from the WHO.

2.7.1 Epidemiology of turberculosis in Malaysia

In Malaysia TB was considered one of the cause of death in the early 1940s and 50s, the chemotherapeutic measures of TB was available in the late 1950s. In Malaysia, the National TB Control Programme (NTP) was launched 1961 due to tuberculosis effects on morbidity and mortality (Iyawoo, 2004). TB is considered one of the major health threat in Malaysia, which accounted over 24,000 cases reported per annum since 2013 (Philip et al., 2016). TB is considered number one killer disease among the people in relation to diseases such as heart diseases or diabetes (Mokhtar & Rahman, 2015) . A study was carried over a period of eight years (2000-2007) of TB in patients admitted in University of Malaya Medical Centre. Out of 131 cases reported, 48% were Malay, 22% Chinese, 17% Indians and others were 18% (Ibrahim, 2010). Ismail et al. constructed a

model from the Malaysian national TB data (1990-2014) and the model was used for comparative analysis with the observed data obtained within similar years, further more underrepresented cases were quantified. Their studies revealed an increased shift of TB cases ranging between 14,032-22,260 determined from 1990 to 2014 with incidence rate per annum between 1.0–5.5 per cents than the reported national observed cases ranging between 11,702-24,711, having similar incidence rate per annum. Their findings further indicated underrepresentation of TB in Malaysian observed data with cases ranging between 13.11% to 13.49% (Ismail et al., 2016)

2.8 Tuberculosis vaccines

Development of more effiaccious vaccine for TB prevention than *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) is one of the greatest priorities of tuberculosis research. Reseach and development serves as one out of three pillars set by WHO TB strategy that will play a vital role towards accelerating the decrease in TB incidence and mortality needed to meet the global TB targets to decrease TB death by 95% and to decrease new infection by 90% between years 2015-2035 (Evans et al., 2016).

The administration of TB vaccines can be done principally in three different stages of disease/infection. Administration of preexposure vaccines is carried out prior to infection with *Mycobacterium tuberculosis*. The currently used BCG vaccine, the viable recombinant vaccine candidates and most subunits vaccines that will be used as BCG boosters have been made as prexposure vaccines (Andersen & Kaufmann, 2014). Currently, Clinical trials of most TB vaccines are focused toward either replacement to BCG or serving as booster vaccines after vaccination with prime BCG (Gopal & Khader, 2013). Aftermath to the failure in some few years back of MVA85A (a subunit vaccine that utilize the modified vaccinia virus Ankara to express 85A antigen), there was no new, pronounced TB vaccine going into clinical testing. Sixteen (16) potential TB vaccines

candidate are in clinical trial which are categorized into priming vaccines, boosters to prime candidates and immunotherapeutic once (Schito et al., 2015). Most of these vaccines candidates are subunit, opted for antigens derived from *Mycobacterium tuberculosis* are engineered into a recombinant viral vectors and expressed, then administered or are given while combined with adjuvant as protein/adjuvant vaccines (Whole Mycobacteria Cell Vaccines for Tuberculosis Summary, 2015). The developmental pipeline of new TB vaccines are shown in Table 2.1.

Table 2.1: The developmental pipeline trend for new vaccines against tuberculosis

TB vaccine	Vaccine Type/Strategy	Sponsors	Reference
MTBVAC Phase I	Live-attenuated vaccine/priming vaccine	University of Zaragoza, Biofabri,The *Tuberculosis* Vaccine Initiative (TBVI)	(Montagnani et al., 2014)
VPM1002 Phase I	Recombinant live/prime	Max Planck, Vakzine Projekt management GmbH, The Tuberculosis Vaccine Initiative (TBVI)	(Hawkridge & Mahomed, 2011)
Ad5 Ag85A Phase I	Viral-vectored vaccine Prime booster	McMaster University, Supported by Tianjin Cansino Biotech. Inc	(Ocampo, 2015)
M72 + AS0 Phase IIb	Protein and adjuvant/prime booster	GlaxoSmithKline, Aeras	(Frick, 2015)
MVA85A Phase I	Attenuated *Mycobacterium tuberculosis* strain	The *Tuberculosis* Vaccine Initiative (TBVI), Zaragoza, Biofabri	(Ahsan, 2015)
CrucellAd35+ MVA85A Phase I	Viral vector/ Prime booster	Crucell, Oxford University, Aeras	(Frick, 2015)
Hybrid 1 + IC31 Phase I	Recombinant protein/Prime-boost	Statens Serum Institut / Tuberculosis Vaccine Initiative /Intercell	(Jefferys, 2012)
Hybrid 4 + IC31 Phase I	Recombinant and adjuvant	Statens Serum Institut (SSI), Tuberculosis Vaccine Initiative (TBVI)	(Kaufmann et al., 2010)
Hybrid 56 + IC31 Phase II	Adjuvanted subunit/Prime-Boost	Statens Serum Institut	(Da Costa et al., 2015)
ChAdOx1 85A + MVA85A Phase I	Viral vector/ Prime-boost	Oxford University	(Frick, 2015)
ID93 + GLA-SE Phase I	Adjuvanted subunit/ Prime-Boost	Infectious Diseases Research Institute	(Da Costa et al., 2015)
DAR-901 Phase I	Mycobacterial-whole cell or Extract	Darmouth, Aeras	(Ahsan, 2015)
TB/FLU-04L Phase I	Viral vector/prime booster	Research Institute for Biological Safety Problem	(Ahsan, 2015)
Mycobacterium vaccae Phase III	Therapeutic/Boost, Post infection	NIH, Aeras, Immodulon	(Hawkridge & Mahomed, 2011)
RUTI PhaseII	Immunotherapeutic/Fragmented *Mtb*	Archivel Farma	(Wingfield & Jefferys, 2012)

2.8.1 Bacillus Calmette-Guérin

In the 1880s, Louis Pasteur discovered the principle and devise by which virulence of a living microbe is deliberately attenuated to produce a successful vaccine candidate. Beginning in 1908, this technique was borrowed by Albert Calmette and Camille Guérin to create TB vaccine. After coincidentally knowing that the virulence of *Mycobacterium bovis* diminished when growth on ox bile, Calmette and Guérin carefully performed 230 passage serially of the single isolate of the organism, adequate for it to lose its capability to cause progressive deadly TB in a variety of animals such as chimpanzees, cows, guinea pigs, horses, rabbits and monkey (Murray, 2004). The BCG vaccine was developed by Camille Calmette and Albert Guerin between 1906 and 1919 at the Pasteur Institute in Paris. An attenuated strain of the original *Mycobacterium bovis* were obtained by these researchers, after it was passed through a culture every three weeks over a 13-year period, a total of 231 times, In 1921 the vaccine produced began to be used in humans, and named Bacillus Calmette Guerin (Pereira et al., 2007). Through continues passage of original BCG strain culture by different recipient laboratories lead to the creation of new substrains the varied genetically. Despite its position as globe's most widely used vaccine, BCG is considered the most controversial vaccine in use presently (Fine, 1995). The clinical effectiveness of is measured on the basis of percentage decrease in disease among people vaccinated which is due to vaccination (Fine et al., 1999). Results from clinical trials indicated that BCG vaccine has no much protective efficacy in adults when administered at birth, a meta- analysis study revealed that BCG vaccine has a protective efficacy ranging from zero to eighty per cent (Evans et al., 2013). The poor efficacy of BCG vaccine could be attributed to several factors including the decline in memory T-cell response, administration of variable strains of BCG, another possible explanation as regard to the established inefficacy is attributed to the limited capability of the vaccine to brings about potent CD8+ T-cell responses when compared to *Mycobacterium*

tuberculosis (Bruffaerts et al., 2014). The waning of T-cell responses in adolescence is explained considering the fact that immunological memory after one BCG vaccination doesn't last for lifetime, and BCG is not considered as secondary boster immunization later in life because its live vaccine (Andersen & Kaufmann, 2014).

Despite variability of efficacies noted with BCG vaccine in human, it has some advantages over new forms vaccines. Firstly, it has long track record of safety, it was also found to be very protective against severe form of childhood TB, its inexpensive vaccine and was found to give protection against leprosy (McMurray, 2003). It is as a result of the above-mentioned reasons that some researchers are at the opinion of using BCG or boosting of its protective capability against adult form of the disease. Life attenuated Bacillus Calmette Guerin is used earlier in life for protection against TB, and is considered one of the most widely used vaccines against pulmonary tuberculosis, majority of people have been vaccinated with BCG, which is not actually a single organism but rather a number of strains that varies in genotypes and phenotypes (Antas, 2013). BCG play a protective role against *Mtb* as it induces CD4+ (T helper type 1) and CD8+ (T cell) responses (Poyntz et al., 2014). BCG gives efficient protection against tuberculosis in newborns, but does not provide prevention of latent infection or reactivation of TB in adults (Mirlekar et al., 2012). BCG is still on used to children in so many countries as part of WHO Expanded program on immunization but surrounded with controversy on its efficacy in protecting adult and adolescent forms of TB (Deng et al., 2011). There is need of both Th1 and Th17 responses for an ideal host protection against tuberculosis, BCG happened to induce Th1 response and failing to induce Th17 response in the lung. Ability of BCG to induced Th1 but failing on Th17 mostly lead for the inferior efficiency of BCG vaccine (Samuchiwal et al., 2014). INF-γ is produced as a result of Th1 response and required for protection, Marchant and co-workers demonstrated that Th1 memory response is induced at birth upon BCG immunization in

a similar way when administered later in life (Marchant et al., 1999). An improved understanding of the reason behind variability of BCG efficacy to such a greater extent is important to inform assessment of new vaccines against tuberculosis that are undergoing clinical trial (Mangtani et al., 2014). BCG has been in used for neonates since 1974, and it provides protection against tuberculosis and tuberculosis meningitis with a 50 % reduced risk of disease development in young children (Bruffaerts et al., 2014). Despite being a safe vaccine, BCG vaccines are considered among the most reactogenic vaccines, reactogenicity depend on vary with different strains and the number of viable bacilli (Bernatowska et al., 2007). Immunocompromised children infected with HIV or immunosuppressed individuals are especially vulnerable to complications of BCG vaccine (Pac et al., 2012). A study was carried out on 349 BCG-immunised patients having severe combined immunodeficiency in 17 countries, the results indicated high rate of complications of BCG vaccine (Principi & Esposito, 2015).

2.8.1.1 Variability in protective effectiveness of BCG

Evaluation of BCG vaccine started formally in 1930s, but the degree of variability between the various results became known in 1950s. More than 75 percent protection was reported in the UK, through trials conducted by the Medical Research Council, whereas US, trial done by public health services reported less than 30 per cent protections. Several other studies and trials demonstrated in the persisted broad range of estimates (Fine, 1995).

It is well accepted that BCG vaccines varies in diverse properties, both in the genetic makeup, mycobacterial strain and physical properties involved in preparations of vaccine (Fine et al., 1999). Variation in BCG stains could lead to different protection level of BCG vaccine (Mangtani et al., 2014). Immune response could be affected due to exposure to other environmental mycobacteria (EM), thereby hindering the BCG

effectiveness. BCG effectiveness > 70 % was reported in population of individuals from the countries located far away from the equator where there is less or no prevalence of EM (Barreto et al., 2006). Significantly decreased BCG efficacy was reported of countries located near to the equator (Principi & Esposito, 2015).

2.8.1.2 Duration of BCG protection

Neonatal BCG protection ranging between 15 to 20 years after vaccination has been reported (Barreto et al., 2005). Duration of BCG protection ranging from 10 to 20 years has been reported in most cases (Moliva et al., 2015). Nguipdop-Djoma et al. demonstrated that BCG vaccination exhibited long-lasting protective ability against TB in a study involving individuals aged ranging between 12-50 years. The vaccination was carried out between the years 1962-1975 on subjects with negative tuberculin skin test. The rates of TB in unvaccinated participants were 3.3 per 1000 persons while 1.3 per 1000 persons in vaccinated participant (Nguipdop-Djomo et al., 2016).

2.8.2 New live attinuated vaccines

Recombinant Bacillus Calmette-Guerin (rBCG30) was the first recombinant tuberculosis vaccine tested on human being developed at University of California, Los Angeles by Dr Horowitz's research co-workers (Beresford & Sadoff, 2010). rBCG30 was found to secret 30 kDa recombinant protein which significantly induce greater protective immunity against TB compared to common BCG in animal model (Sarojini et al., 2011).

The loss of RD1 region is the genetic principal behind BCG attenuation, the region encoding the machinery needed to synthesize and export the major T-cell antigen/virulence factor ESAT-6/CFP-10 (Arbues et al., 2013). The first recombinant BCG was generated by Horwitz *et al.* (Horwitz et al., 2000) and Horwitz & Harth (Horwitz & Harth, 2003). rBCG3 overexpressed antigen Ag85b that induce protection against TB significantly in animals compared with parental BCG, rBCG30 significantly

increased Ag85b-specific T cells that inhibit intracellular mycobacteria (Hoft et al., 2008).

VPM1002 is the second recombinant BGC vaccine candidate (Principi & Esposito, 2015), formed because of two variations on live *Mtb*. The gene encoding for listeriolysin (Hly) from *Listeria monocytogenes* incorporated into the genome of BCG (Montagnani et al., 2014). rBCGUre:CHly* conferred high protection against *Mycobacterium tuberculosis* challenge through aerosol, this improves protection was because of efficient perforation of the phagocyte phagosomal membrane by listeriolysin (Hly) (Moliva et al., 2015), rBCGUre:CHly* is now in the phase of clinical trial due to its enhanced protection against tuberculosis (Orme, 2013). Recombinant rBCGUre:CHly* construct move from endosomes to cytosol due to the activity of Listeriolysin with concomitant deletion of Urease gene. Loss of Urease gene leads to an improved mycobacterial antigen processing via MHC I pathway as well as improved CD8 cytotoxic T cell activity (Svenson et al., 2010). Phase I trials of VPM1002 carried out in both South Africa and Europe were successful and the results provided an effective immunogenicity and safety data (Tang et al., 2016).

BCG::ESAT-L28A/L29S improved BCG strain with modifications at amino acid residues Leu^{28}-Leu^{29} of the ESAT molecule showed strong attenuation in mice and high protective efficiency both in mouse and guinea-pig vaccination-infection models (Bottai et al., 2015). Wang et al. (2012) constructed three recombinant BCG strains that overexpressed immunodominants antigens of *Mycobacterium tuberculosis*, Ag85B (rBCG::85B), Ag85A (rBCG::85A). Both recombinants (rBCG::AB) provided stronger and longer-lasting protection compared to the BCG containing vector without insert pMV261(rBCG::261) using mice.

In January, 2013 MTBVAC entered phase I clinical trial and it is the live-attenuated *Mycobacterium tuberculosis* vaccine that entered phase I trial. it is a derivative of attenuated strain SO2 obtained by insertion of a kanamycin-resistance cassette in the phoP (phoP is a transcription regulator) gene of *Mtb* transcription, mutation of phoP causes lack of expression of several genes including ESAT6, a virulence factors (Montagnani et al., 2014). In preclinical studies it was found that MTBVAC showed safety and biodistribution profiles the same as BCG and indicated superior protection (Arbues et al., 2013). The satisfactory safety of MTBVAC could be explained based on the following factors, lack of front-line lipids, loss of ESAT-6 expression and down-expression of the PhoP regulon, essentially for pathogenicity and virulence of *Mycobacterium tuberculosis* (Arbues et al., 2013). Highly attenuated MTBVAC could be potential vaccine for population with high-risk immunosuppression due to inactivation of an additional gene generated repeated protein (Erp) (Ahsan, 2015).

2.8.3 Subunit and viral vector-based vaccines

Subunit vaccines are more advantageous compared to whole pathogen vaccines in that they more stable with long shelf live and do not change back to a pathogenic state (Doytchinova & Flower, 2008).

Live-based vaccines are not products of choice by most manufactures because of safety considerations especially in immunosuppressed individuals and technical challenges on reproducibility (Girard et al., 2005). The main reasons for developing recombinant protein-based vaccine are as follows: they develop less reactogenic, considered more potent, safer, and better characterized vaccines (Unnikrishnan et al., 2012). *Mtb* secretes proteins during *in vitro* growth, one of the possible ways of improvement toward tuberculosis vaccines involve use of such proteins secreted. Some of these proteins are immunogenic, these proteins or their agreeing genes could serve as a major part of either

DNA-based vaccine or subunit vaccine. Identification of antigens secreted in the culture fluid is important for establishing protective immune response against TB (Sarhan, 2010). Several studies carried out showed promising results for DNA vaccination against tuberculosis. DNA vaccines express different *Mtb* antigens; these include Ag85A, Ag85B, ESAT-6, MTP-64, PstS-3 and 65kDa heat-shock protein, these proteins all found effective inhibiting the growth of *Mtb* infected mice (Yu et al., 2008).

Li et al. (2014) reported a recombinant adenovirus (Ad5-CEAB) expressing *Mtb* antigens Ag85A, Ag85B, CFP10 and ESAT6 proteins combined in a mixture. Ad5-CEAB resulted in a strong antigen-specific immune response as well as heightened humoral responses with a dramatically antigen-specific serum immunoglobulin (IgG).

2.8.3.1 Viral-vector vaccine

Ad5Ag85A is a viral vectored adenovirus serotype 5 vector vaccine expressing Ag85A developed by Mcmaster University and supported by Tianjin CanSino Biotechnology Inc. The vaccine went through Phase I trial in 24 Canadian adult, 12 from BCG naïve and 12 from previously BCG-vaccinated, healthy adult, there was no vaccine-related serious adverse effect recorded. Ad5Ag85A had immunogenic in both groups with stimulation of polyfunctional T-cell responses, but found more effectively boosted both CD4+ and CD8+ T-cell immunity in a group previously-immunised compared to BCG-naïve group, which is reassuring its further clinical development for serving as a booster vaccine candidate after BCG priming (Ocampo, 2015).

A phase I trial involving MVA85A combined with Crucell Ad35 (Crucell Ad35 + MVA85A) was carried out among 40 adult participants at Oxford University (Frick, 2015). Research Institute for Safety Problems and the Research Institute on Influenza, Russia developed a recombinant influenza vaccine called TB/FLU-04L is developed composed of influenza virus strain A/Puetro Rico/8/34 H1N1 and *Mtb* antigens Ag85A

and ESAT6. Phase IIa trial is being planned for this vaccine candidate whereas a phase I trial was completed (Ahsan, 2015). ChAdOx1.85A is another adenovirus vaccine that expresses *Mtb* antigen Ag85A, a phase I clinical study is testing the safety of ChAdOx1.85A vaccination along and in fusion with MVA85A in adults vaccinated with BCG in the United Kingdom (Frick, 2015).

2.8.3.2 Subunit Adjuvant

Adjuvants includes compounds, molecules/macromolecular complexes capable of boosting the potency and lastingness of specific immunological response to antigens (Reed et al., 2009)**.** The major hindrance in developing vaccines against bacteria has been attributed to lack of adjuvant that adequately stimulate cell mediated immunity (Ottenhoff et al., 2010). It is therefore essential to administer subunit vaccines with an adjuvant to enhance immune responses to subunit vaccines. The following adjuvants approved for human use includes Aluminum salts, the AS03/04 and MF59, they are primarily promoters of a humoral or Th2 rather than Th1 response (Ottenhoff et al., 2010).

H1+IC31 is a subunit adjuvant vaccine developed by the Statens Serum Institute, TBVI and Intacell. it's a hybrid of ESAT6 and Ag85B antigen with IC31, the components of the adjuvant system are oligodeoxynucleotide ODN1a and the cationic protein polyaminoacid KLK (Ahsan, 2015). Reither et al. (2014) evaluated vaccine candidate, H1/IC31in 48 patients infected with HIV and the results showed durable Th1 immune responses.

Hybrid 4 + IC31 vaccine has a fusion of *Mtb* antigens (Ag85B and TB10.4) with adjuvant IC31 owned by Valneva. In 2014, a three-arm phase IIa study was announced by Aeras in order to determine the safety and immunogenicity of H4+IC31 and BCG revaccination in approximately 1000 BCG-immunized, non-HIV adolescents South Africans (Frick, 2015). Hybrid 4 + IC31 adjuvant vaccine effectively boosted and

lengthened immunity induce by BCG, leading to enhanced protection against *Mtb* due to domination of immune response by INF-γ, TNF-α, IL-2 or TNF-α, IL-2, CD4+ cell (Billeskov et al., 2012).

Hybrid 56 + IC31, a protein adjuvanated vaccine composed of H56, a fusion protein consisting of Ag85B, ESAT6 and Rv2660c (latency-associated protein), incorporated with the adjuvant IC31 (Montagnani et al., 2014). Hybrid 56 + IC31 subunit adjuvant vaccine showed an ability to control late-stage of TB infection and contains latent stage of tuberculosis (Lin et al., 2012).

Thacher et al. (2014) evaluated M72/AS01 candidate vaccine on 37 HIV-infected adults on cART (combination anti-retroviral therapy) in Switzerland in a ratio 3:1:1 to vaccine, adjuvant (AS01) and saline placebo. The vaccine was found to be immunogenic with induction of persistent CD4+ T-cell responses specific to M72. M72/AS01 candidate vaccine is a recombinant fusion protein obtained from the following *Mtb* proteins (*Mtb*32A and *Mtb*39A) together with the AS01 adjuvant (Gillard et al., 2016).

Infectious Disease Research Institute came up with ID93 vaccine which is the most recent tuberculosis entering clinical trial and was designed to target both forms of active and latent tuberculosis (Andersen & Kaufmann, 2014). IDR93 is a protein/adjuvant vaccine that combined four novel sets of antigens including Rv2608, Rv3619, Rv3620, and Rv1813 in addition to the adjuvant (synthetic MPL formulated in a glucopyranosyl lipid stable emulsion), preclinical studies with mice revealed that, IDR93vaccine was found to be protective almost at BCG levels and in guinea pigs, combination of ID93 with BCG reduced mortality rate (Andersen & Kaufmann, 2014).

2.8.4 Immunotherapeutic vaccines

The main targets of therapeutic vaccines design are to prevent latent infection or to reduce needs of chemotherapy (Ruiz Manzano & Vilaplana, 2014). RUTI is one of the therapeutic vaccines made of detoxified, fragmented *Mtb* cells delivered in liposomes. Previous study reveals that RUTI has shown its efficacy in controlling latent form of TB infection in mice and guinea-pigs with inducting a combined Th1/Th2/Th3 polyantigenic response after a short period of chemotherapy (Cardona, 2006). Nell et al. (2014) evaluated by RUTI vaccine in a placebo-controlled clinical trial involving 95 patients infected latently with TB, RUTI vaccine showed its immunogenicity. A phase 1 clinical trial (randomized, placebo-controlled) of RUTI vaccine was carried out in Spain using healthy white males without history of tuberculosis infection and prior BCG vaccination to find out tolerability and immunogenicity of four RUTI doses (ranging between 5 μg and 200 μg). After completion of the trial in October, 2008 result showed that all the doses tolerable, but moderate pain noted with higher doses, 35 days after vaccination four specific antigens were traced (Whole Mycobacteria Cell Vaccines for Tuberculosis Summary, 2015). A phase II trial of RUTI showed immunogenicity and safety in both HIV-infected and non infected individuals with latent TB infection. A plan for a phase IIa trial in order to acertain the safety and immunogenicity of RUTI in MDR-TB patients is on the way (Kaufmann et al., 2017).

Mycobacterium vaccae was developed as an immunotherapeutic vaccine initially by inactivation of the whole cell strain of *M. vaccae* (McShane, 2011). In a phase III trial, variable INF-γ and humoral responses were induced by *Mycobacterium vaccae*, according to CD4+ T-cells count, HIV viral load and previous TB treatment (Montagnani et al., 2014). In Tanzania, vaccination with multiple-dose series of *Mycobacterium vaccae* to HIV-positive adults immunized with BCG at childhood is associated with significant protection of HIV associated TB. These result revealed that vaccination with

M. vaccae game plan towards HIV- associated tuberculosis prevention as no detrimental effect on CD4 cells observed (Von Reyn et al., 2010). In 2001, *M. vaccae* was approved for sale in China for immune therapeutic purpose against TB, it appeared to provide a measurable improvement in some geographical settings but not in other places; the inconsistency the lead to doubt on its efficacy by some researchers (Ocampo, 2015).

2.9 Emergence of computational biology and bioinformatics

Application of computers and the Internet have develop into an integral part of different research areas in science and technology. Application of these tools in tackling problems in molecular biology lead to an emergence of new displines called computational biology or bioinformatics (Ilyas et al., 2011). One of the most important developments in the biomedical sciences today is the emergence of bioinformatics as the science by which biological data are manage and analyze using advanced computing techniques is one of the vital developments in the biomedical sciences (Shachak et al., 2007). Bioinformatics is often designated as being an infant area, but during the early years of 1960s computers became known as an important tools in the field of molecular biology, a decade prior to DNA sequencing became possible (Hagen, 2000). From the late years of 1980s forwards, the term bioinformatics typically has been employed to refer to computational approaches towards comparative study/analysis of genome data. Nevertheless, the concept was originally more broadly defined as the field of study of informatic processes in living system (Hogeweg, 2011). The development the field in the early 1990s served as a case study of a scientific burst, in that the desires and requirements of the area grow rapidly into a different direction, due to availability data and DNA sequencing technology (Ouzounis, 2009).

2.10 Protein identification by Matrix assisted laser desorption/ionization-time of flight (MALDI TOF) and database searching

In proteomics studies proteins are identified from gels after electrophoretic analysis (Mortz et al., 2001). In proteomic studies protein identification requires either of these two techniques namely; Tandem Mass spectrometry that is relatively costly and Matrix-Assisted Laser Desorption Ionization Time of Flight mass spectrometry which is significantly cheaper, followed by peptide search in databases. The signifacntly cheaper method can be used in a situation whereby genone sequence of an organism under investigation is available (Millares et al., 2012). MALDI technique is employed to ionized and volotized an intact proteins, and then presented to a mass analyser (Aebersold & Mann, 2003). Proteolysis is frequently the critical step involved in proteins preparation for analysis using mass spectrometry. In most digestion procedure, trypsin is the ideal reagent used in digestion protocols because it is powerful, predictable, cheap, and yield peptides agreeable to mass spectrometry. Typical digestions analysis take place in solution or in gel for a period of 16 - 24 hour at a temperature condition of 37°C. In many cases this step proceed overnight, for convenient sample processing. Enhanced methods of trypsin involving digestion in mixed aqueous/organic solvent medium been investigated (Remily-Wood et al., 2009).

Mascot, a peptide fragment fingerprinting software is a large cross-species package used for identification of protein (McHugh & Arthur, 2008). Data generated is compared with the theoretical patterns of peptides fragments obtained from the sequences incorporated within the Mascot package (Bringans et al., 2008). The main limitation associated with this method is its limited usefulness where the protein sequences are unknown. This inflicts a specific limitation on many directions of investigations, mainly in biodiscovery setting where the organisms are often lacking satisfactory genomic and proteomic coverage (Bringans et al., 2008).

2.11 Computational analysis/*in silico* analysis in vaccine design

The recent development in the fields incuding bioinformatics, proteomics, immunoinformatics, structural biology and others have driven to vaccinomic (Nandy & Basak, 2016). Genomics has reorganized vaccine research. The complete genomic sequencing of a virulent microorganism has led to the screening for the most probable protective antigen *in silico* (computational screening) before carrying out the confirmatory experiment. This could be achieved by approach known as reverse vaccinology (Doytchinova & Flower, 2007). Identification of portions in microorganisms capable of triggering of immune response is the initial step toward new vaccine development (Monterrubio-López, 2015). The systematic identification of prospective antigen within a pathogen based on computational/*In silico* analysis of the pathogen's genome could be achieved by the application of reverse vaccinology together with advanced molecular biology technology. Many Bioinformatics software can be used to identify outer membrane or surface- associated proteins, lipoproteins and signal proteins (Doytchinova & Flower, 2008). The new vaccine developments begins with identification of unique part of virulent microorganism responsible of inducing a protective immune response (Monterrubio-López, 2015). The bioinformatics software/tools development along with progress in recombinant DNA technology together with the knowledge on the genetics of the pathogenic organism and the host immunological responses will bring about new vaccine candidate against diseases that currently have no or few control measures in just few years via *in silico* predictions to particular targets (Soria-Guerra et al., 2015). Application of bioinformatics in immunology is known as immunoinformatics, this area of study is widely accepted which assist in new vaccine design through B and T-cell epitopes as well as MHC identification (Rahman Oany, 2015). Identification of B-cell is essential in development of diagnostic test and in initial step in design of vaccine,

in the same way potent immune response depends on binding specificity as well as variety of antigen binding to HLA alleles (Barh et al., 2010).

2.11.1 Introduction to reverse vaccinology (RV)

In 1995, Craig Venter published the result of entire genome of first free living organism, *Haemophillus influenza* based on sequencing and assembly (Fleischmann et al., 1995). This advancement provided an avenue beyond Pasteur's rules, where information derived from the genome allows vaccine design computationally through an approached termed "reverse vaccinology" (Sette & Rappuoli, 2010). In reverse vaccinology vaccine development is based genomic sequence of the pathogenic organism, from the genome information, a catalog of all antigenic proteins which can be expressed by the pathogen is obtained (Rappuoli, 2000). Once the genomic sequence of an organism is acquired, identification of all hopeful proteins that could be expressed is possible. Several software and package programs are used for identification of all ORFs that constitute the sequence with capability of expressing the majority of proteins. Reverse vaccinology is the next step, whereby several antigenic and physicochemical characteristics that have been related with acceptable antigens are determined. Bioinformatics approaches are utilised to select the protein(s) with the best characteristics for screening via *in vitro* and *in vivo* methods, so as to prove its safety and immunogenicity (María et al., 2017).

The first and successful candidate upon which the concept of reverse vaccinolology was applied is *Neisseria meningitidis* serogroup B (MenB) (Rappuoli, 2000). Later the concept of reverse vaccinology was applied to other pathogenic organisms including *Streptococcus agalactiae, Streptococcus pneumoniae, Streptococcus pyogenes*, and pathogenic *E. coli* (Seib et al., 2012).

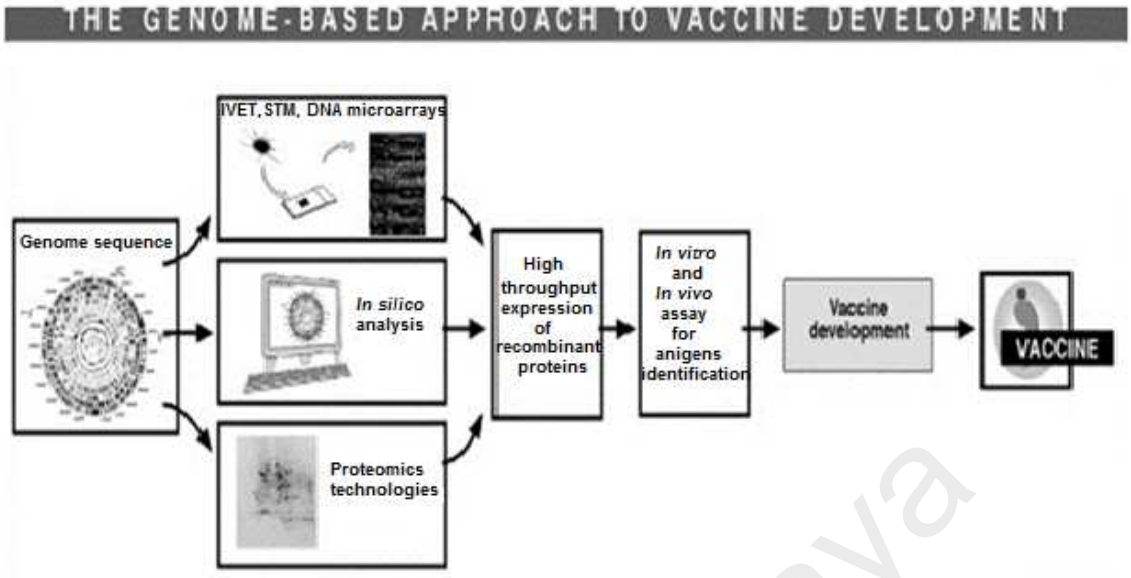

Figure 2.5: The genome-based approach toward vaccine development

Well built technologies such as genomic sequencing computational analysis (*in silico* analysis), proteomics, DNA microarrays, *In vivo* expression technology (IVET) and signature tagged mutagenesis (STM) have transformed the manner at which bacterial phathogenesis and vaccine design is studied. Figure 2.5 illustrates steps involved in vaccine development via genome-base approach (Adu-Bobie et al., 2003). Vaccine (with permission).

2.11.2 *In silico* prediction of allerginecity

Allergy encompasses a series of reactions that are complex in nature and both intrinsic and extrinsic factors are resposible for the establishment of the disease and activating of the symptoms. Allergens induced Type I hypersensitive reaction that cause specific IgE antibodies or as a result of cross-reactivity between homologous allergens that are common and of different sources (Saha & Raghava, 2006).

The IgE antibody response is commonly triggered by allergens as antigens. Allergen-specific B cells produce immunoglobulin (IgE) driven by allergen-specific Th2 cells. The IgE is capable of binding to the high-affinity surface receptor FcεRI found on basophils, activated eosinophils mast cells and. Open activation, stored mediators from these cells

are released, which in turn resulting to inflammation and tissue damage with diverse of symptoms (Dimitrov et al., 2013). Assessment of allergenicity is considered one of the important steps in the development of peptide vaccine because when the peptide vaccine is administered, the human body detects it as a foreign substance. This could cause inflammation, indicating an allergic reaction (Oany et al., 2014). The guidelines for evaluating the potential allerginecity of protein was developed by The United Nations Food and Agriculture Organization (FAO) and the World Health Organization (WHO) (FAO/WHO, 2003). Based on the guidelines, protein is said to be potential allergen if it possess either an identity of 6 to 8 consecutive amino acids or >35 per cent total sequence similarity over a window of 80 amino acids compared with the known allergens (Dimitrov et al., 2014). However, according Stadler and Stadler made a claim that rule involving the 35% over 80 residues might be too conventional, considering allergenic cross-reactivity typically needs greater 70% identity across the complete protein (Stadler & Stadler, 2003). According to Codex Alimentarius commission, recommendations were made on various tests involved in allergenic examination of proteins; including sequence similarities with familiar allegens, source of gene, IgE binding and protein stability. These recommedations were made in view of uncertainties associated with various tests based on FAO/WHO 2001 recommendations (Fiers et al., 2004; Saha & Raghava, 2006).

2.11.3 *In silico* prediction of antigenicity

Identification of antigenic proteins with the ability to trigger a significant humoral immune system response is essential toward addressing fundamental problems in immunology, virology and bacteriology. It is also of paramount important in practical applications ranging from diagnostic to vaccine design (Magnan et al., 2010). Protein with immunoprotective ability can be predicted *in silico* using Vaxijen server (Doytchinova & Flower, 2008). Immunogenicity of whole proteins is predicted by

VaxiJen with accuracy levels ranging from 70% to 97%. It covers bacterial, fungal, parasite, tumor and viral (Patronov & Doytchinova, 2013).

Most approaches for identification of antigens employ sequence alignment. This is challenging for some reasons. Obviously, some proteins are lacking sequence similarity, even though they may have similar biological characteristics and structures. The sequence antigenicity could be encoded in a subtle and unclear way not changeable to direct identification using sequence alignment. Due to the lack of similarity to antigen of known origin, the discovery of accurately novel antigens will be upset. These limitations of alignment-dependent approach was overcome with the proposed and first aligment-free technique of antigen prediction via VaxiJen server (Doytchinova & Flower, 2007).

2.11.4 Major histocompatibility I and II binding predictions

Major histocompatibility complex (MHC) proteins (glycoproteins), also called human leukocyte antigens (HLA) (Dimitrov et al., 2010). In cellular immune response to pathogen, two important T-cell responses are taking place; one of which is mediated by Major histocompatibility complex I (MHC I) that is restricted to CD8+ Cytotoxic T lymphocytes while the second response is mediated by MHC II restricted Th response (Zhang, 2013). MHC molecules bind to self-peptides or foreign peptides derived from proteins related with the cell under study which is achieve by a stable, receptor-like condition.(Maffei & Harris, 1998). Protein-derived peptides associated with MHC I are recognize by CD8+ T cells while those associated with MHC II are recognize by MHC class II (Blum et al., 2013). The endogenous peptide antigens are presented to CD8 T cells by MHC I, the intraction of the complex formed by MHC I and peptide with the CD8 and T receptor generate a signal to T cell thereby killing a MHC-bearing cell (Kwame & Lawrence, 2012). The major function MHC class II molecule is to present processed antigen which are primarily derived from the exogenous sources to CD4 T

cells. Therefore MHC are essential in antigen-specific immune response initiation (Holling et al., 2004).

Due to the host immune response complexity against tuberculosis as well as genetic restrictions inflicted by the MHC, it is likely that effectual subunit vaccine having multiple epitopes regions will be needed for the broad coverage of heterogeneous population (Mir et al., 2009). Specific identification of peptides capable of binding specifically to MHC molecules is of paramount important for understanding the mechanism of immune response, as well as boosting the immunogenic epitopes. Epitope that binds to multiple variants MHC molecules are important candidates for vaccine development. Epitopes derived vaccines are regarded as a safe and potent way against infectious diseases (Zhang et al., 2012). Recently, algorithms were developed for prediction of affinity of protein derived peptide sequences for diverse MHC class I and II variants (McNamara et al., 2010). Data-based prediction of T-cell epitopes is most valuable amongst existent prediction technologies. Prediction of T-cell epitope depends primarily upon the expectation of peptide-binding to MHC molecules (Reche et al., 2014). In this ProPred1 server only 9mers peptide are predicted, 10mers or 8mers peptide lengh are not predicted which as result 8mers or 10mers binders could be possibly missed (Singh & Raghava, 2003).

2.11.5 Antibody binding prediction

The minimal structural determinant that an antibody reorganizes is known as an epitope an antibody. Understanding of the molecular basis of immunity as well as autoimmunity relies on the proper identification of epitope of an antibody. In addition, it can also help in designing of that trigger alike antibodies in a vaccine (Sela-Culang et al., 2015). B-cells epitopes are components of proteins with antigenic properties, which are recognized by antibodies. B-cells epitopes identification can be used as the initial step for

vaccine design as well as in establishment of diagnosis test (Barh et al., 2010). B-cells epitopes give information for the synthesis of peptide that trigger cross-reaction immunoglobulins, thereby supporting toward synthetic peptide-vaccine development (Saha & Raghava, 2004)

2.11.6 Toxicity of peptide predictions

In silico methods for toxicity prediction of peptides helps in facilitating of better therapeutic peptides design in addition to cost and time saving (Gupta et al., 2013). The success of peptide base therapy is sometimes obstructed by toxicity, an ideal peptide should possess high antigenicity but less or no toxicity (Parvege et al., 2016). Hence, the need of toxicity screening. Peptide toxicity can categorized broadly into three classes, namely immunototoxicty, hemotoxicity (involving red blood cell lysis) and cytotoxicity. Recently, a tool known as ToxinPred was developed for prediction of toxic peptides/protein and identification of segments or residues leading to toxicity (Chaudhary et al., 2016). ToxinPred serve as a unique computational approach of its kind, that will be beneficial in prediction of peptides/proteins toxicity. In addition, it will be valuable for designing peptides with least toxicity as well as discovering regions in proteins that are toxic (Gupta et al., 2013).

In 2000, Seltsam et al. reported case of vaccination-related immune hemolysis in children due to production of autoantibodies against red blood cells, the mechanism underlying this process was not understood (Seltsam et al., 2000). One of the key limitations associated with HemoPI tool for hemotoxicity prediction is that, hemolytic peptides reported from literature had no clear definition. For this to be overcome, few standard were set in order to define peptides that are hemolytic and non-hemolytic. Based on that high hemolytic peptides will be discriminated from poor hemolytic from the datasets (Chaudhary et al., 2016).

CHAPTER 3: MATERIALS AND METHODS

3.1 Washing procedures

All glasswares and other washable materials were soaked in a solution containing detergent and dettol overnight. They are then rinsed thoroughly and continuously with running tapwater. After wahing, the glasswares were dried in a drying oven .

3.2 Sterilization procedure

Autoclaving also known as steam sterilization is the most reliable protocol for the killing of all the microbial life. Milli-Q water, growth media, distilled water, microfuge tube flask, preparation bottles, beakers, and pipette tips were steam-sterised using autoclave at 121^{o}C (pound-force per square inch, psi) for 15 minutes. Antibiotics and other solutions such as Isopropyl β-D-1-thiogalactopyranoside (IPTG) were sterlised using Millipore filters (Sartorius Stedim Biotech).

3.3 Growth media

3.3.1 Reagents required

1. Kanamycin 30 mg/ml stock concentration

   0.3 g Kanamycin disulfate salt was dissolved in 10 ml Mill-Q water and filter sterlised using 0.22 um Millipore filter. The solution was aliquot and stored at -20^{o}C. It was used at 1:1000 dilution (30 ug/ml working concentration) in LB or LB agar.

2. Isopropyl β-D-1-thiogalactopyranoside 1 M stock concentration

   2.38 g IPTG was dissolved in 10 ml Mill-Q water and filter sterlised using 0.22 um syringe and stored in aliquots at temperature of -20^{o}C

3.3.2 Luria-Bertani (LB) medium

The LB was obtained from Liofilchem, France. LB powder (20 g) was suspended in a liter of distilled. The solution was heated until it dissolved completely. It was then autoclaved at 15 psi (121^{o}C) for 15 minutes. The medium has typical formula in g/l as follows

Tryptone	10.0 g/l
Yeast extract	5.0 g/l
Sodium chloride	5.0 g/l

3.3.3 Luria-Bertani (LB) agar

The LB agar was obtained from Difco Laboratories, France. It is base for cultivation as well as maintenance of recombinant stains *Esterichia coli*. The LB agar powder (35 g) was suspended in 1 litre of distllied water. The solution was mixed thoughroughly, heated with frequent agitation and allowed to boiled for 1 minute until it dissolved completely. It was then autoclave for 15 minutes at 121^{o}C. The agar has typical formula in g/l as follows

Tryptone	10.0 g/l
Yeast extract	5.0 g/l
Sodium chloride	5.0 g/l
Agar	15.0 g/l

3.3.4 Luria-Bertani (LB) agar with Kanamycin (Antibiotic)

The LB agar (35 g) was suspended into 1 liter of distilled water. The solution was then mixed, boiled with agitation untit it dissolved completely. After autoclave at 121^{o}C for 15 minutes, it was then allowed to cooled to a temperature of 50^{o}C. Kanamycin (at working concentration of 30 μg/ml) was then added at that temperature. It was then poured on plates, the plates were allowed to cool at room temperature overnight

3.3.5 Luria-Bertani (LB) agar with 1mM IPTG

The LB agar (35 g) was suspended into 1 liter of distilled water. The solution was then mixed, boiled with agitation untit it dissolved completely. After autoclave at 121^{o}C for 15 minutes, it was then allowed to cooled to a temperature of 50^{o}C. IPTG (1 mM) was added. It was then poured on plates, the plates were allowed to cool at room temperature overnight.

3.3.6 Luria-Bertani (LB) agar with Kanamycin plus 1mM IPTG

After autoclave of LB agar, both Kanamycin and IPTG were added. The solution was poured on plates and allowed to cool at room temperature.

3.4 Bacterial Strains and recombinant clone Myt272-3

The bacterial strain BL21 (DE3) pLysS was obtained from Invitrogen (USA). The recombinant expressing vector (pET30a^{+}/Myt272-3) employed in this work was constructed in the Molecular Bacteriology and Toxicology laboratory, Faculty of Science, University of Malaya (unpublished). Construction of the clone involved shotgun cloning technique. The insert was derived from H37Rv strain of *Mycobacterum tuberculosis.* The insert was inserted into the recombinant plasmid at *Bam-HI* and *KpnI* endonuclease restriction digestion site. The pET vector is illustrated diagrammatically in Figure 3.1.

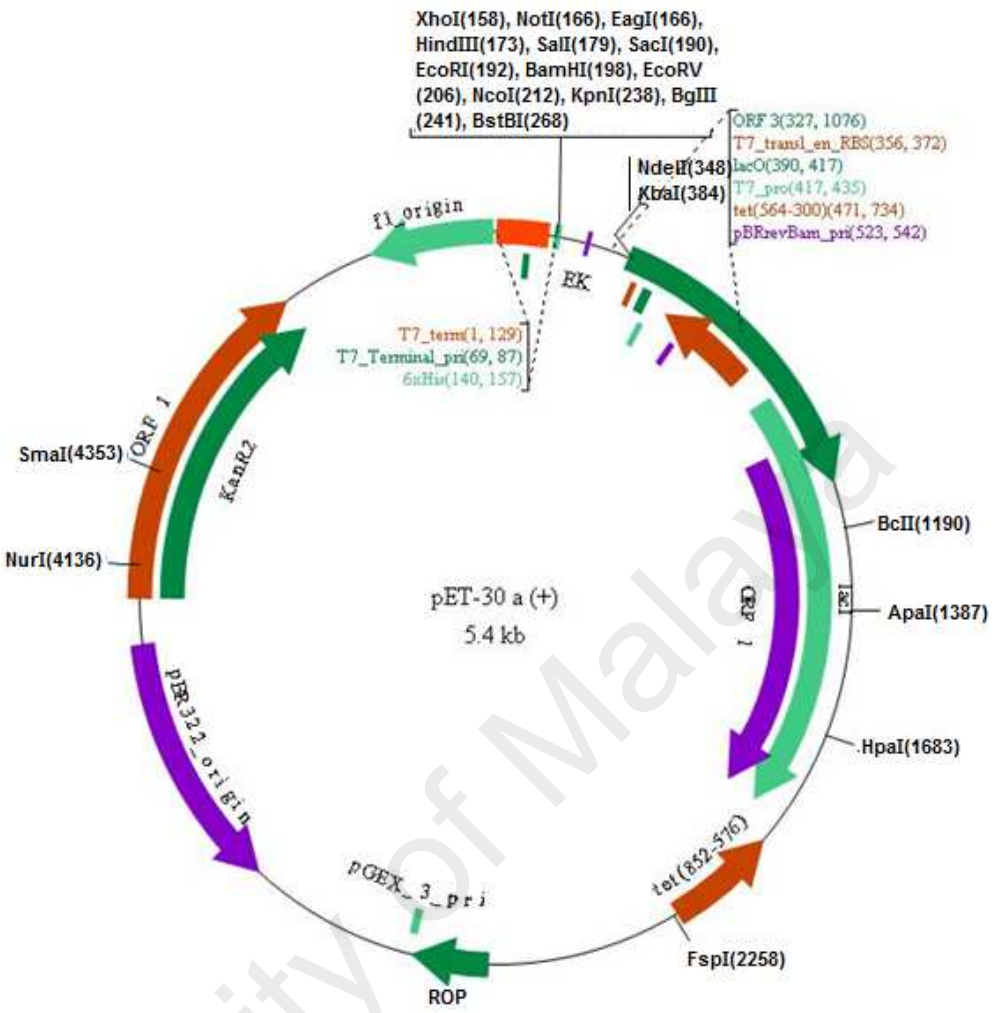

Figure 3.1: Structure of pET30a^{+} recombinant vector

The insert Myt272-3 was cloned into the *Bam-HI* and *KpnI* site of the pET vector.

The nucleotide sequence that made up the recombinant Myt272-3 protein is shown in Figure 3.2 below

atgcaccatcatcatcattcttctggtctggtgccacgcggttctggtatgaaagaa

M H H H H H H S S G L V P R G S G M K E

accgctgctgctaaattcgaacgccagcacatggacagcccagatctgggtaccctgtcg

T A A A K F E R Q H M D S P D L G T L S

cttgccgacctcggcgtcagctcccgcgacgcggtcgtactgtccggcgaactgtcagag

L A D L G V S S R D A V V L S G E L S E

ctgctgggcaggaccgtatcgccgattgacttctgggagcacccgacggatccgaattcg

L L G R T V S P I D F W E H P T D P N S

agctccgtcgacaagcttgcggccgcactcgagcaccaccaccaccaccactga

S S V D K L A A A L E H H H H H H -

Figure 3.2: Myt272-3 his-tagged protein and its corresponding nucleotide sequence

The capital letter represent the constituent amino acids of the protein derived by translating the nucleotide sequence (in small letters) using EXPASY translate bioinformatic tool (http://web.expasy.org/translate/).

3.5 Storage and maintenance of bacterial cultures

Bacterial culture isolates of recombinant *E.coli* grown overnight was mixed with sterlised 60% glycerol solution, this was achieved by mixing 500 µl of bacterial culture with the equal volume of glycerol and stored at -20^{o}C. Maintainance of the culture was also done on the agar slants and stab for a duration of 3 to 5 months.

3.6 Stability testing of the recombinant clone Myt272-3

Plasmid stability of the clone was tested by the method described by Lanka and Barth method with slight modification (Lanka & Barth, 1981). Myt272-3 cultures were grown in LB broth overnight at 37°C. The clones were sub-cultured in LB broth and LB agar and were also incubated overnight; the protocol of sub-culturing protocol was carried out repeatedly (10 times). After the tenth and the final transfer into fresh LB broth and agar

medium, the bacteria was pelleted by centrifugation then the plasmid DNA extracted using NucleoSpin® Plasmid (Macherey-Nagel, Germany) based on the manufacturer's instructions.

3.7 Plasmid DNA extraction

3.7.1 Basic principle of DNA extraction

With the NucleoSpin® Plasmid technique, the pelleted bacteria obtained was resuspended in Buffer A and plasmid DNA was liberated from the host cell (*E.coli*) using Sodium dodecyl sulfate (SDS)/alkaline lysis buffer (Buffer A2). The resulting lysate was neutralized by Buffer A3 which as result appropriate binding conditions was created for plasmid DNA to bind the silica membrane present in the NucleoSpin® plasmid column. Precipitated protein, cell debris and genomic DNA are then pelleted upon centrifugation. The supernatant obtained was then loaded on to a column. Ethanolic buffer A4 served to remove contaminants such as salts, soluble macromolecular constituents and metabolites by simple washing. Slaightly alkaline buffer (buffer AE, 5 mM Tris/HCl, pH 8.5) was used to elute the pure plasmid DNA.

3.7.2 Plasmid DNA extraction procedure

Bacterial cells were cultivated and harvested, 5 ml of a saturated *E. coli* culture was used, cells were pelleted using microcentrifuge at 11,000 × g for 30 seconds. The supernatant was discarded. The pelleted cells was resuspended in 250 µl suspension buffer (buffer A1) by vortxeing, 250 µl of lysis buffer (buffer A2) was added and mixed gently by inverting the tube 7 times. It was incubated for 5 minutes at room temperature until the lysate appeared clear. 300 µl of Buffer A3 was added and carefully mixed by inverting the tube 7 times to avoid genomic DNA shearing. To clarify the lysate, it was centrifuge at 11,000 × g for 5 minutes at room temperature. To achieved plasmid DNA binding, NucleoSpin column was placed in a collection tube and supernant was decanted

onto the column. It was then spinned at 11,000 × g for 1 minute. The flow-through was discarded and the column was taken back to the collection tube. The silica membrane of the column was then washed by 600 µl of buffer A4 and then centrifuged at 11,000 × g for 1 minute. The flow-through was discarded and the column was taken back into a new and the empty tube for collection. The silica membrane was further dried by centrifugation at 11,000 × g for 2 minutes. The plasmid DNA was eluted by placing the column on micocentrifuge tube and 100 µl elution buffer (Buffer AE) was added. It was incubated at room temperature for 1 minute, then centrifuge for a minute. The plasmid DNA was then stored at -20°C for further analysis.

3.8 Restriction digestion analysis

Restriction endonuclease digestion was carried out using two enzymes, *Bam-HI*-HF and *KpnI*-HF. Double digestion was carried out with the two enzymes in order to release the insert. Restriction endonuclease digestion was done based in compliance with conditions stipulated by the supplier (New England Biolabs, USA).

3.8.1 Agarose gel electrophoresis

3.8.1.1 Reagents/chemicals required

1. Agarose
2. Tris-acetate-EDTA (TAE) buffer

To prepare 1 × working solution, 20 ml of 50 × TAE buffer concentrate (BioRad, USA) was added to 980 ml of deionized distilled water. The solution was mixed thoroughly. The final concentration of 1 × working solution was 40 mM Tris acetate and 1mM EDTA at pH of 8.3.

3. Loading buffer (dye)
4. Gel red

3.8.1.2 Basic principle of agarose gel electrophoresis

Agarose gel electrophoresis is the most effectual method by which DNA fragments of different sizes ranging from 100 bp to 25 kb are separated. Agarose powder is obtained from the seaweed, consisting of repeated agarobiose subunits. In the course of gelation, network of bundles are formed when agarose polymers associate non-covalently. The molecular sieving properties of the gel is determine by its pore sizes (Lee et al., 2012). DNA fragments are size separated under the influence of an electric field whereby negatively charged DNA molecules moves toward positively charged pole (anode). The migration is determine entirely by molecular weight; the smaller the molecule the faster it migrate (Yılmaz et al., 2012).

3.8.1.3 Electrophoresis procedure

To prepare 1% agarose, 1g of agarose powder was measured and put into a flask, 100 ml of TAE buffer was added. The agarose was melted in a microwave until a clear solution was obtained. The solution was allowed to cool by occational swirling to about temperature of 50 °C. The casting tray was made ready with the comb placed within the tray. After cooling, the melted agarose solution was poured on the casting tray and left until it solidified which was notified with milky appearance. The comb was carefully pulled out. The gel was then placed into the electrophotetic chamber. TAE buffer was added into the electrophoretic tank with about 3 mm of buffer above the gel.

Molecular weight marker (4 µl, standard/ladder) was loaded into one lane. To 4 µl each of isolated plasmid DNA and digest, 1 µl of loading dye was added. Carefully, each sample/loading dye mixture was pipetted into separate wells (created by the comb) in the gel.

The lid was placed on the tank and the electrode connected. Electrodes were connected to the power supply. The power supply was turn on indicated by the movement of bubbles

from the electrodes. The run was carried out at 70 volt for 80 minutes. After the run, the power supply was tun off. The lid was removed. The tray and the gel were carefully removed. The gel was then inserted into a gel red and allowed to stay for 5 minutes with gentle shaking. The gel was finally viewed using image viewer.

3.9 Transformation and colony PCR

Chung and co-workers demonstrated a simple method by which a competent *E. coli* is prepared using transformation and storage solution (TSS). TSS is a LB media containing dimethyl sulfoxide, polyethylene glycol and divalent cation. In this method, ice-cold TSS are mixed with cells. The plasmid DNA is added to the cells treated with TSS, followed by incubation on ice. The transformed cells are then plated on the selective growth media (Chung et al., 1989).

3.9.1 Reagents required

1. Polyethylene glycol 10%
2. Dimethyl sulfoxide 5%
3. Magnesium sulphate 50 mM

The TSS solution was stored at -20 °C

3.9.2 Transformation procedure

Single colony of *E. coli* BL21(DE3) pLysS was cultured overnight in 5 ml of LB broth at 37°C. The following day, 500ul of the overnight culture is pipetted and subcultured into 50 ml of LB broth for 2 hours at 37 °C untl an optical density (OD) of 0.3-0.4 at 600 nm was obtained. The culture was centrifuged for 1 hour at 3500rpm, temperature of 4°C. The harvested cell was suspended in TSS solution for competent cells production. The cells were incubated for 30 minutes in ice. After incubation in ice, 5 µl of recombinant plasmid DNA was added to 100 µl of the competent cells in microtube and tapped gently before incubation again on ice for 30 minutes. To enhance uptake of DNA by the

competent cells prepared, the cells were subjected to a temperature of 42°C for 45 seconds and then taken back to ice for 2 minutes. To the cells transformed, 900 µl of prewarmed LB broth was added, followed by incubation for 1 hour at 37°C for the transformed cells to grow. After incubation, the transformed cells were centrifuged and the harvested cells resuspended in 250 µl of LB broth. The transformed cells were then lawned/plated on a LB agar plate with antibiotic (kanamycin) for selection and incubated overnight at temperature of 37°C.

3.9.3 Colony PCR

Ten colonies from the transformed cells were patched on a single petri dish and then PCR colony was carried out to confirm the presence of insert prior to expression. The PCR colony was carried out using EconoTaq PLUS GREEN 2X Master Mix from (Lucigen, USA). The master mix contains both agarose gel buffer and tracking dyes. Present in the master mix are EconoTaq DNA Polymerase, 400 µM each of four Deoxyadenosine Triphosphate (dATP), Deoxyguanosine Triphosphate (dGTP), Deoxycytidine triphosphate (dCTP), Deoxythymidine triphosphate (dTTP) and reaction buffer.

3.9.3.1 Colony PCR procedure

The reaction set up	**Reaction volume (25 µl)**
2× Master mix	12.5 µl
Forward primer	0.25 µl
Reverse primer	0.25 µl
Nuclease free water	11.0 µl
DNA template	1.0 µl

The above PCR components were carefully mixed in PCR reaction tube and briefly centrifuged in a microcentrifuge. PCR cycling conditions were set, the cycler was pre-

heated at 94 °C. The reaction was incubated at 94 °C for 2 minutes for initial denaturation process of target DNA. DNA was denatured, annelled and polymerized. After completion of PCR run, 5 µl of the PCR reaction was carefully loaded on the 1 % agarose gel for inset confirmation based on size analysis.

3.10 Plasmid stability

Immediately prior to induction, the culture was tested in order to determine the fraction of cell that are still carrying the target vector (plasmid). In this regard the cells were plated on four different plates as described in pET System Manual (Novagen, 1999). The four plates are as follows

1. LB plate. All viable cells grow on this plate
2. LB plate + Kanamycin. Cells that still carried the plasmid grow on this plate.
3. LB plate + 1mM IPTG. Cells with lost plasmid or a mutant cells that are unable to express the gene of interest grow on this plate
4. LB plate + 1mM IPTG + Kanamycin. Only mutant cells that maintain the plasmid but unable to express the target gene grow on this plate.

When the target plasmid is not stable, the fraction of cells with lost plasmid will be represented by rise in number of colonies on the LB plate + 1 mM IPTG and decline in number on the LB plate + Kanamycin. In a normal culture capable of target protein production, nearly all cells will produce colonies on both plate and on the LB plate and LB plate + kanamycin; colonies are formed by < 2% of the cells on the LB plate + 1 m M IPTG and on the LB plate + kanamycin + 1 mM IPTG < 0.01 of cells form colonies.

3.10.1 Plasmid stability procedure

Immediately prior to IPTG induction, 100 µl of aliquot from the cell culture was taken into a tube. The serial dilution of the cells were made including 10^5 and 10^6 dilutions.

Cells at a dilution of 10^5 were plated on the LB plate + 1 mM IPTG and LB plate + 1 mM IPTG + Kanamycin. The cells at a dilution of 10^6 were plated on the LB plate and LB plate + Kanamycin. The plates were incubated overnight at 37°C. The colonies were counted. The same protocol was applied for negative control (i.e for *E.coli* PLysS strain).

3.11 Protein expression

3.11.1 Basic Principle of induction of expression with IPTG

Isopropyl β-D-1-thiogalactopyranoside is a molecular analogue of allolactose capable of inducing lac operon, it is a strong inducer which is non metabolsed by *E.coli* (Wurm et al., 2016). Unlike allolactose which is hydolysable, IPTG is non hydolysable by the cell due to the presence of the Sulphur atom that provides a chemical bond. IPTG concentraction, therefore remain constant in the course of experiment. IPTG as an inducer binds to repressor. So, with an inducer bound the binding affinity of repressor to operator is greatly lowered, thereby allowing polymerase to bind its promoter and hence transcription of genes in the lac operon (Lewis, 2005). The structure of IPTG is shown in Figure 3.3 below.

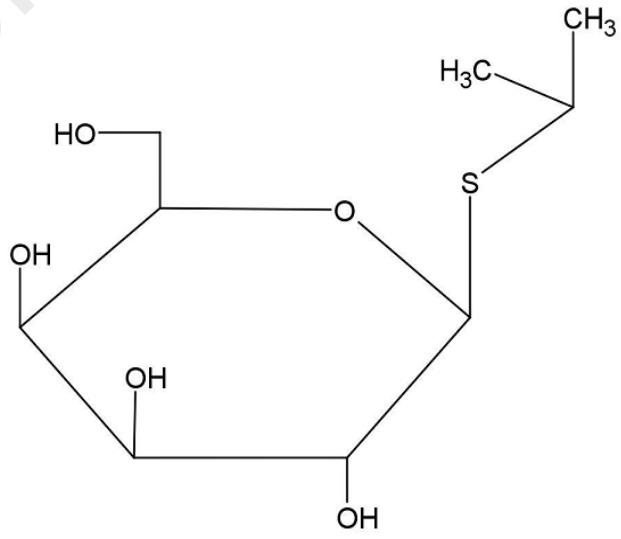

Figure 3.3: Structure of Isopropyl β-D-1-thiogalactopyranoside

The presence of Sulphur atom made IPTG non hydolysable and non metabolized by the cell. The Sulphur atom is absent in Allolactose as shown in Figure 3.4, which as a result it is hydolysed by the cell.

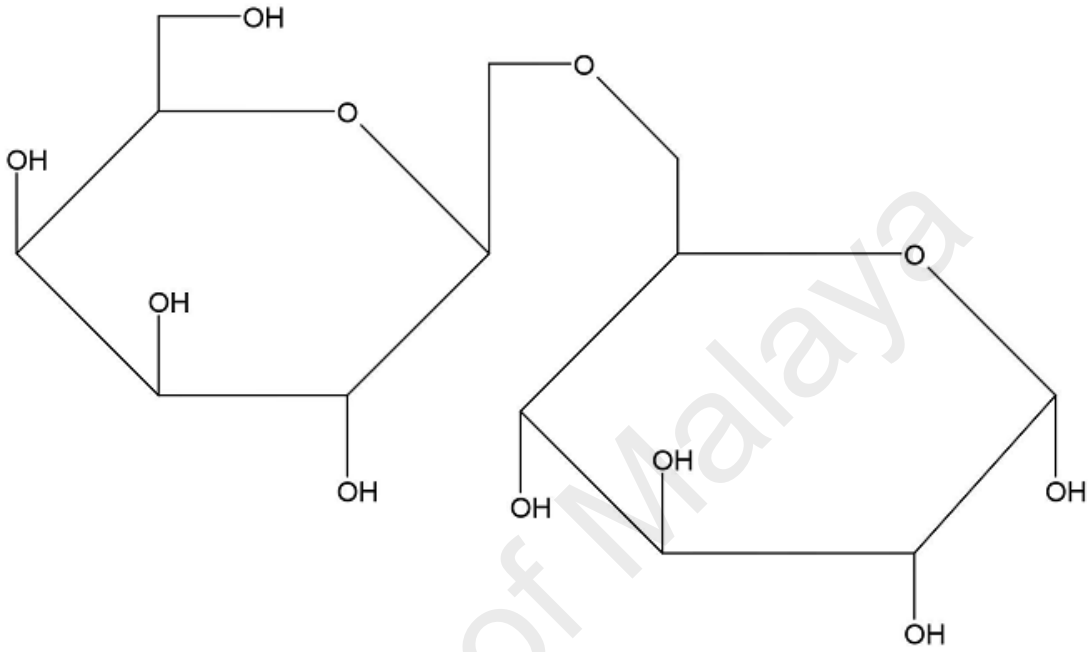

Figure 3.4: Structure of allolactose

3.11.2 Protein expression procedure

After transformation**,** the bacteria were grown in LB media containing antibiotics (Kanamycin, 30 µg/ml) at 37° C until an absorbance of 0.6-07 was observed at 600nm. Protein expression was induced by 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). After incubation for three hours cells were centrifuged (harvested) at 2500 × g for 30 min at 4°C. Pelleted cells were stored at -80°C prior to lysis. Four millilres of bacterial extraction reagent (B-PER, Thermoscientific) was added per gram of pelleted cells, suspension was pipetted to obtain homogeneous solution. The solution was incubated for 15 minute followed by centrifugation of lysate for 5 minute at 15000 × g for separation of soluble protein.

3.12 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE)

3.12.1 Reagents required

1. 30% acrylamide, bis-solution (37.5:1)

37.5g of acrylamide and 1g of N'N-methylenebisacrylamide were dissoved in distilled water and was made up to a volume of 128 ml. The solution was heated to 37°C. It was filter sterilized using 0.22 um Millipore filter The pH was then adjusted to a value less than 7. The solution was kept at in dark bottle in 4°C.

2. Ammonium persulphate 100mg/ml

Ammonium persulfate serve to provide the free radical required for the catalysis of the Acrylamide and Bis-acrylamide polymerization. Freshly prepared solution was used because ammonium persulphate decompose slowly in solution.

3. Glycerol 70%
4. Gel buffer (3 M Tri HCl / 0.3% SDS)

18.15 g of Tris HCl was dissolved in 50 ml of Milli-Q water and pH adjusted to 8.43. After pH adjustment 0.3% (0.15 g) SDS was added. The solution was stored at 4°C.

5. Tricine sample buffer

To 980 µl of Tricine sample buffer 20 µl of β-Mercaptoethanol (2% v/v) was added. Tricine sample buffer has the following constituents 200 mM Tris-HCl, 40% glycerol, 2% SDS and 0.04 Coomassie blue G-250.

6. β-Mercaptoethanol (βME)

7. Tris/Tricine/SDS buffer

To make 1× working (SDS-PAGE running) buffer, 100 ml of 10 × Tris/Tricine/ concentrate obtained from (Biorad, USA) was added to 900 ml of deionized distilled water. The solution was mixed thoroughly and yielded a final concentration, 100 mM Tris, 100 mM Tricine and 0.1% Sodium dodecyl sulfate at pH of 8.3.

8. N, N, N, N – tetramethylethylenediamine (TEMED)

Acceleration of polymerization of Acrylamide and Bis-acrylamide is achieved under the influence of TEMED.

The compositions of the resolving and stacking gel are as follows:-

Resolving gel

Components	Quantity
Dioinsed distilled water	1.1 ml
30% bis-acrlimide	7.0 ml
Gel buffer	5.0 ml
70% glycerol solution	2.0 ml
Ammonium persulphate	66 µl
TEMED	6.6 µl

Stacking gel

Components	Quantity
Dioinsed distilled water	6.7 ml
30% bis-acrlimide	1.05 ml
Gel buffer	2.5ml
Ammonium persulphate	88 µl
TEMED	8.8 µl

3.12.2 Basic principle of SDS PAGE

SDS PAGE is a separation technique by which proteins are separated due to their ability to move under the influence of electrical current, which is function of their molecular weight or the length of proteins polypeptide chains. In this technique, the proteins are coated by the SDS mostly in proportion to their molecular weight, which as a result confers the same negative charge across all the proteins present in the sample (Roy & Kumar). Protein detection was done using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with Tricine-SDS method (Schägger, 2006).

3.12.3 SDS PAGE procedure

The gels' casting frames were set on the casting strand. The resolving (separating) gel was prepared in a separate small beaker as described above. The solution was swirled gently and thoroughly. The resolving solution prepared was pipetted into the gap between the glass plates. The gap was filled with water until it overflowed so as to make the top of the separating gel horizontal. It was then allowed to gelate or solidified for 30 minutes. After gel is solidified, the water was discarded. The stacking gel solution was prepared as indicated above. The stacking gel was pipetted into the overflow. The well-forming comb was inserted into the stacking gel, it was then left for 30 minutes to gelate. The comb was taken out after solidification of staking gel. The glass plates were taken out of the casting frame and set them in the cell buffer tank. The electrophoresis (running) buffer was poured into the inner chamber until it overflow, the buffer was also poured into the lower chamber until it reaches the required level.

Tricine sample buffer was made by diluting 20 µl of βME with 980 µl of sample buffer. Protein samples were diluted with the sample buffer at ratio 1:2 and heated for 4 minutes at 95°C. The samples prepared were then loaded into the wells. The protein ladder (marker) was also loaded into the appropriate well. The top was covered and the system connected to the power supply. The volt was set to 100 V and ran for 1 hour.

After the completion of the run, the electrophoretic system was dismantelled and the gel was carefully removed followed by staining over night with shaking. The gel was destained later using deionized distilled water and finally visualized.

3.12.4 Coomassie colloidal staining of SDS PAGE gel

The colloidal coomassie staining technique was used to stain the gel after protein separation. This staining approach has detection capability of protein ranging from 50 to 500 ng (Neuhoff et al., 1985).

3.12.4.1 Reagents

1. Coomassie brilliant blue (CBB-250)
2. Ortho-phosphoric acid
3. Ammonium sulphate
4. Methanol

The CBB-250 staining solution was prepared as follows; 16 ml of phosphoric acid was mixed in 768 ml of deinised distilled water, to that solution, 80 g of ammonium sulphate was added. 5% of Coomassie brilliant blue was prepared in aqeous medium, 16 ml of this solution was added to the solution prepared in the above step. Finally, 200 ml of methanol was carefully and slowly added to the solution. The coomassie solution was shaked thoroughly for dispertion of colloid particles evently.

3.12.5 Staining procedure

After SDS PAGE separation, the gel was washed thrice using Mill-Q water with shaking. Staining was then carried out.

3.13 Purification of protein

3.13.1 Dynabeads® His-Tag Isolation & Pulldown

The protein was purified using Dynabeads® His-Tag Isolation & Pulldown (Novex life Technologies) according to the manufacture's protocol. The purification method used was designed for the isolation of histidine-tagged protein by magnetic separation; these dynerbeads were coated in a cobalt-based metal affinity chromatographic technique.

3.13.1.1 Reagents/materials required

1. 2× binding/washing buffer (50 ml). This buffer has the following constituents

100 mM Sodium phosphate, pH 8.0	0.6 g
600 mM Sodium chloride	1.75 g
0.02% Tween 20	10 µl

2. Histidine Elution buffer (50ml), with the following constitunts

300 mM Imidazole	1.02 g
50 mM Sodium phosphate, pH 8.0	0.3 g
300 mM Sodium chloride	0.88 g
0.01% Tween 20	5 µl

3. Magnet

Both binding/washing and elution buffers were kept at at 4°C. The 2× binding/washing buffer was diluted to 1× concentration before it was used.

3.13.1.2 Dynerbeads protein isolation procedure

The sample containing his-tag protein was prepared in a total volume of 700 µl 1× binding/washing buffer. The Dynabeads® in the vial was resuspended by vortexing for 30 seconds, 50 µl of suspended dynabeads which is equivalent to 2 mg was transferred

into a microcentrifuge tube. The tube was placed on a magnet to 2 minutes. The supernatant was drawn out and discarded. The sample prepared with binding/washing buffer was added to the beads and mixed well. It was incubated on a votexer for 5 minutes at room temperature. The tube was placed on the magnet for 2 minutes, then the supernatant was removed and discarded. The beads in the tube was washed 4 times with 300 µl of binding/washing buffer by putting the tube containing the beads on the magnet for 2 minutes and getting rid of the supernatant. The beads was resuspeded carefully between each washing step. After washing steps were completed, 100 µl of the elution buffer was added to the beads. The suspention was then incubated on the votexer for 5 minutes at room temperature. The tube was placed on the magnet for 2 minutes after incubation and the supernatant containing the his-tagged protein was carefully transferred into a clean tube. It was then analysed using SDS-PAGE.

3.13.2 Nickel-nitrilotriacetic acid (Ni-NTA)

The protein was also purified using Ni-NTA metal-based-affinity chromotagraphy (Qiagen) in accordance to the manufacture's procedure. Protein was isolated due to the affinity of histidine tag to the metallic nickel.

3.13.2.1 Reagents/materials required

The Ni-NTA kit has the following contents as prepared by the manufacturers

1. Lysis buffer, pH 8
2. Wash buffer, pH 8
3. Elution buffer, pH 8
4. Separation fast start column (0.5 ml)
5. Benzonase nuclease
6. Lysozyme

Before starting the purification procedure, the lysis buffer was supplemented with the benzonase nuclease and lysozyme. The lysozyme vial was dissolved in 600 µl of lysis buffer supplied. To the 10 ml aliquot solution of lysis buffer, 100 µl of lysozyme was added. The left over lysozyme solution was stored at -20°C. The vial containing nuclease was thawed and 10 µl was added to the 10 ml aliquot of lysis buffer

3.13.2.2 Ni-NTA protein isolation procedure

The harvested cells was thawed on ice for 15 minutes and the cells were resuspended in 10 ml of lysis buffer. It was incubated on ice for 30 minutes. The cell suspension was mixed 3 times gently by swirling. The lysate was centrifuged at 14,000 × g for 30 minutes at 4°C in order to pellet the cellular debris. The supernatant from the cell lysate was retained. To 5 µl of the aliquot of supernatant, 5 µl of SDS-PAGE sample buffer was added and kept at -20 °C for SDS-PAGE analysis. The resin present in the fast start column was gently resuspended by inverting it several times. The seal at the exit of the column was broken and the storage buffer was allowed to drain out. The cell lysate supernatant obtained previously was applied to the column. The flow-through fraction was collected; 5 µl of SDS-PAGE sample buffer was added to 5 µl of the flow-through and stored at the temperature of -20 °C for SDS-PAGE analysis. The column was washed twice with 4 ml of washing buffer and both wash fractions were collected. To 5 µl of each washed fraction, 5 µl of SDS-PAGE sample buffer was added and kept at -20 °C for further SDS-PAGE analysis. The bound Hitidine-tagged protein was eluted twice with 1 ml each of aliquots of elution buffer provided. Each eution was collected in a separate container. To 5 µl of the aliquot of each fraction, 5 µl of SDS-PAGE sample buffer was added and kept -20 °C for SDS-PAGE analysis. All fractions obtained were analysed by SDS-PAGE.

3.14 Quantification of protein

3.14.1 Reagent required

1. Bovine serum albumin (BSA) stock solution 10 mg/ml

100mg of BSA was dissolved in 10 ml of Milli-Q water. It was filter sterilized and stored in -20°C.

2. An alkaline solution of copper tartrate solution (Reagent A)
3. Folin reagent (Reagent B)

3.14.2 Basic principle

The protein concentration was determined in accordance of DC protein assay manual (Bio-Rad). It is a colorimetric method of protein quantification. The protein react with an alkaline solution of copper tartrate and Folin reagent. There are two steps involved for colour development. First, when the protein react with an alkaline solution of copper tartrate, then followed by reduction of Folin reagent caused by copper-treated protein. Blue colour that is measured at maximum absorbance at 750 nm and minimum at 405 nm formed.

3.14.3 Quantification procedure

Standard protein concentrations ranging from 0.2 to 1.5 mg/ml were prepared from the stock solution of serum bovine albumin protein from which standard curve was plotted. Dry microtiter plate was used, 5 µl of samples and standards were pipetted into the plate, followed by 25 µl of alkaline solution of copper tartarate. Then to each well, 200 µl of Folin reagent was added. The plate was placed on the microplate for shaking for 5 seconds. After 15 minutes, absorbance was measured at the wavelength of 650 nm. The standard curve was plotted with absorbance against protein concentration (mg/ml). Protein concentration was calculated from the graph plotted.

3.15 Physicochemical characterization of protein

The physicochemical characteristics of the protein including molecular weight, theoretical isoelectric point (pI), instability index, half-life of protein, aliphatic index as well as hydropathy (GRAVY) were determined using an *In silico tool*, Expasy's ProtParam (http://web.expasy.org/protparam/) (Gasteiger et al., 2005).

3.16 Matrx assisted laser desorption/ionization-time of flight (MALDI TOF) analysis

Trypsin digestion of the expressed protein and peptides extraction were carried out according to standard methods described by Bringans *et al*. (Bringans et al., 2008). Protein sample was dissolved in ammonium hydrogen carbonate and 5 mM tris-(2-carboxyethyl) phosphine followed by incubation of the solution at 60 °C for an hour. Prior to solvent removal 10 mM solution of methyl methanethiosulfonate was added and incubated for 10 min at room temperature. Digestive enzyme (trypsin, 0.5 µm) in ammonium bicarbonate (32 mM) was added, followed by incubation at 37 °C for 16 hours. The sample was dried and kept at -20 °C for subsequent analysis using mass spectrometry. The peptides were analysed by MALDI-TOF/TOF mass spectrometer using a 5800 Proteomics Analyzer [AB Sciex]. The sample was restored in 1 ml of 0.1 % methanoic acid (formic acid). The solution was then diluted in ratio 1 to 100 in acetonitrile/water (in 50:50 ratio). The solution obtained was spotted 1:1 with α-cyanohydoxycinamic acid (5mg/ml) which served as a matrix solution on opti-TOF steinless steel plate. The analysis of spotted samples was carried out using a first run of standard of TOF MS. The resultant spectra were identified using Mascot sequence matching software [Matrix Science] with MSPnr100 Database (MSPnr100 Taxonomy: Bacteria (Eubacteria); 45,994,506 sequences).

3.17 Allerginicity of protein

Allerginecity of protein was determined with allergen prediction software (http://www.imtech.res.in/raghava/algpred/submission.html) (Algpred) on the basis of similarity of known epitope with any region of protein (Saha et al., 2006).

3.18 Prediction of antigenicity

Antigenicity predictive value was determined using amino acid sequence of the protein with the help of Vaxijen server, which predicts whether a protein can serve as a probable antigen or not. Vaxijen prediction has a default threshold value of 0.4 and works on the basis of auto cross covariance (ACC) (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html).

3.19 Prediction of MHC class I and MHC class II binding epitopes

MHC class I binding epitopes were predicted using Propred-1 server (http://www.imtech.res.in/raghava/propred1/) containing 47 MHC class I alleles (Singh & Raghava, 2003) , while MHC class II binding epitopes were predicted using Propred server (http://www.imtech.res.in/raghava/propred/) having 51 MHC class II allele (Singh & Raghava, 2001). In both cases of servers used, protein sequence was submitted for binding to individual variant form of MHC.

The predicted were epitopes were further analyzed for antigenicity value using Vaxijen server.

3.20 Antibody epitope prediction

Kolaskar and Tongaonkar antigenicity scale from Immune Epitope Database (IEDB) was used to predict antibody epitope binding , the method utilizes physicochemical properties of amino acid residues and their frequencies of occurrence (Kolaskar & Tongaonkar, 1990) **(**http://tools.immuneepitope.org/bcell/).

3.21 Toxicity and hemotoxicity prediction of peptides

Toxicity of peptides generated were predicted by an *in silico* method via ToxinPred webserver (http://crdd.osdd.net/raghava/toxinpred/), this server was developed to predict and design toxic/non-toxic peptides. The collection of related sets of information used in this technique composed of 1805 toxic peptides (less than 35 residues). This server is characterized by the following features; Designing Peptide, a module allowing user to establish all possible single mutant analogs of their query peptides and forecast whether the analog is toxic or not. In addition, in Batch peptide submission**,** user can predict number of toxic peptides submitted through this module of ToxinPred (Gupta et al., 2013).

Hemotoxicity of the peptides was predicted computationally using HemoPI server (http://crdd.osdd.net/raghava/hemopi/design.php). This tool permit user to predict peptide's red blood cell lysing potentials. The server is aimed at facilitating scientific community taking part in therapeutic based peptides design. In HemoPI server, another dataset known as was created 'HemoPI-2', it is used to decreminate peptides showing high and low hemolytic activity. Instead of random extraction of peptides from Swiss-prot, a sum of 462 peptides that displayed low level of hemolytic potency or those which do not satisfy the cretaria above, were obtained from hemolytic database (Chaudhary et al., 2016).

CHAPTER 4: RESULTS

4.1 Restriction digestion analysis

The Myt272-3 recombinant clone was found to have an intact plasmid after it was subjected to series of stability testing up to ten generation. Restriction endonuclease digestion was carried out to determine the size of the insert. Double digestion of the clone with the two restriction enzymes, *Bam-HI*-HF and *KpnI*-HF using cut smart buffer led to generation of two fragments, an insert with approximate size of 113 bp and pET30 vector as shown in figure 4.1 below

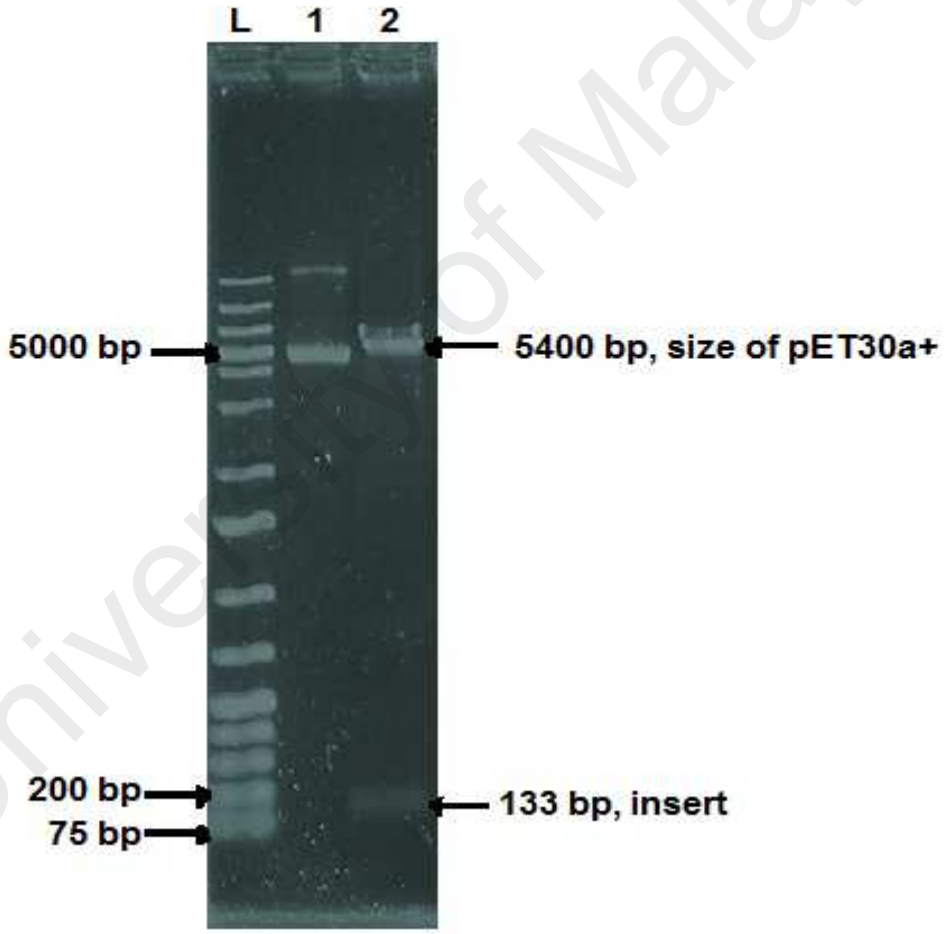

Figure 4.1: Agarose gel showing insert size

Lane L is GeneRuler^{TM} 1 kb Plus ladder; lane 1 pET30a^{+}-Myt272-3 plasmid; lane 2 indicated the restriction endonuclease restriction fragments formed by double digestion using *Bam-HI*-HF and *KpnI*-HF.

4.2 Transformation and Colony PCR

Below are pictures showing the transformed cell clones on LB agar with working concentration 30 ug/ml kanamycin.

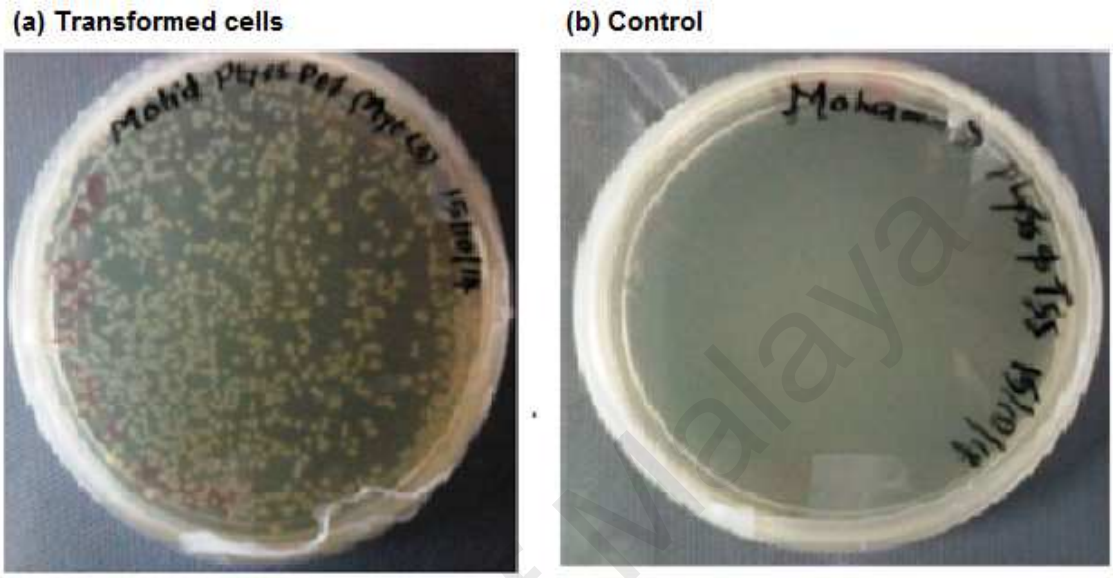

Figure 4.2: Transformed host cell

Successful transformation of *E. coli* BL21(DE3) pLysS cells with the recombinant pET30a^{+}/Myt273 plasmid DNA is indicated by colonies formed on LB plate with kanamycin as shown in Figure 4.2 (a). Figure 4.2 (b) is a control without recombinant plasmid DNA.

After transformation, 10 colonies were patched on the LB plate with antibiotics as illustrated in Figure 4.3 below.

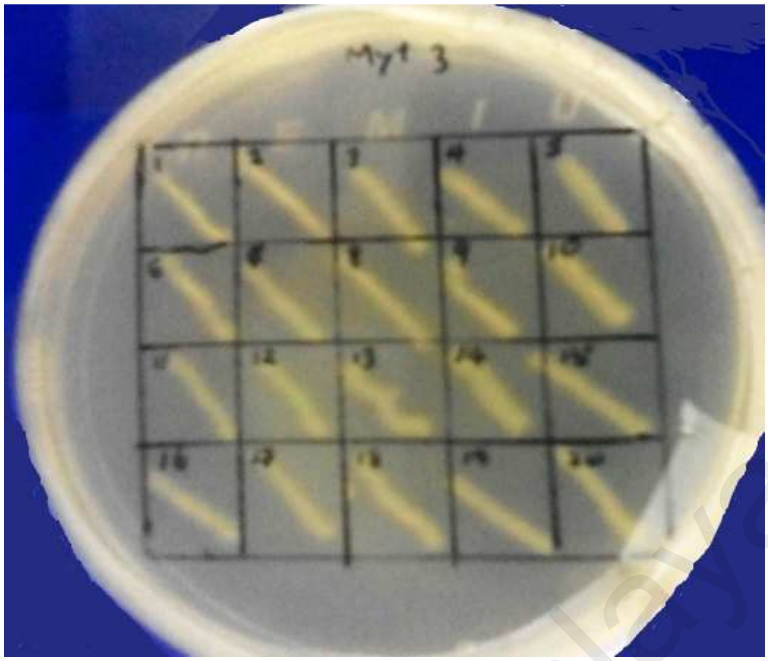

Figure 4.3: Patch culture of the transformed cell

Size analysis of an insert carried out on agarose gel was shown on Figure 4.4. The colony PCR of patch culture indicates the presence of insert of approximately 113 bp as shown in fugure below.

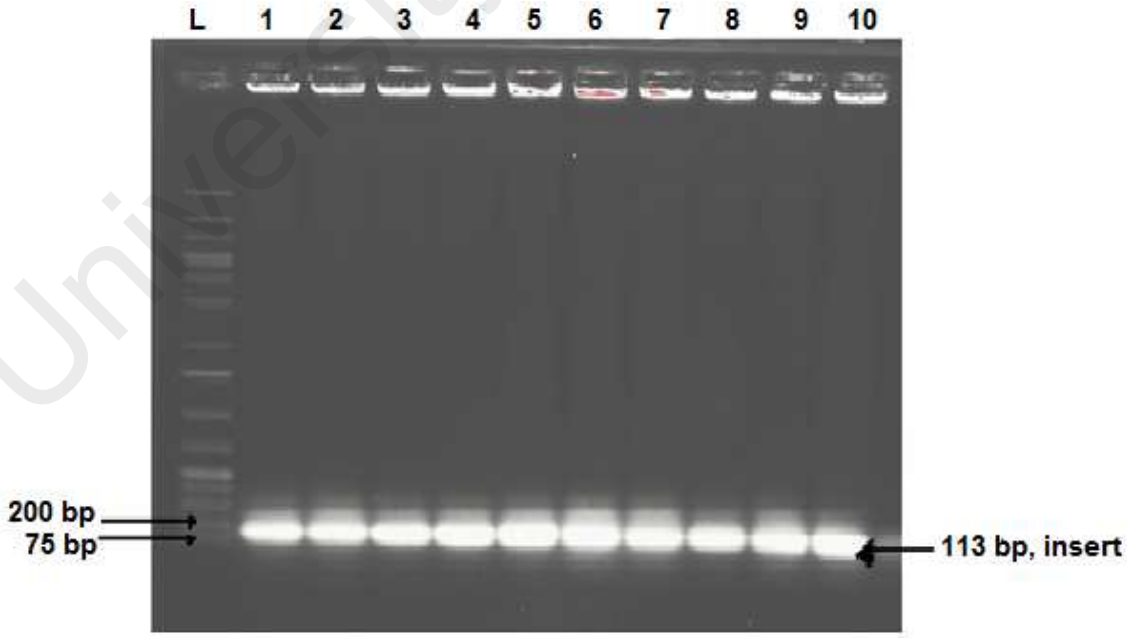

Figure 4.4: Agarose gel analysis of PCR product

Lane L is a GeneRuler^{TM} 1 kb Plus ladder; lanes 1 to 10 indicate the PCR products of the insert.

4.3 Plasmid stability

The cells grown on four different plates to acertain the plasmid stability is shown in Figure 4.5 below. The fraction of cells that take up the plasmids of interest is replected by decrease in colonies on the LB plate + 1 mM IPTG and an increase on the LB plates + kanamycin.

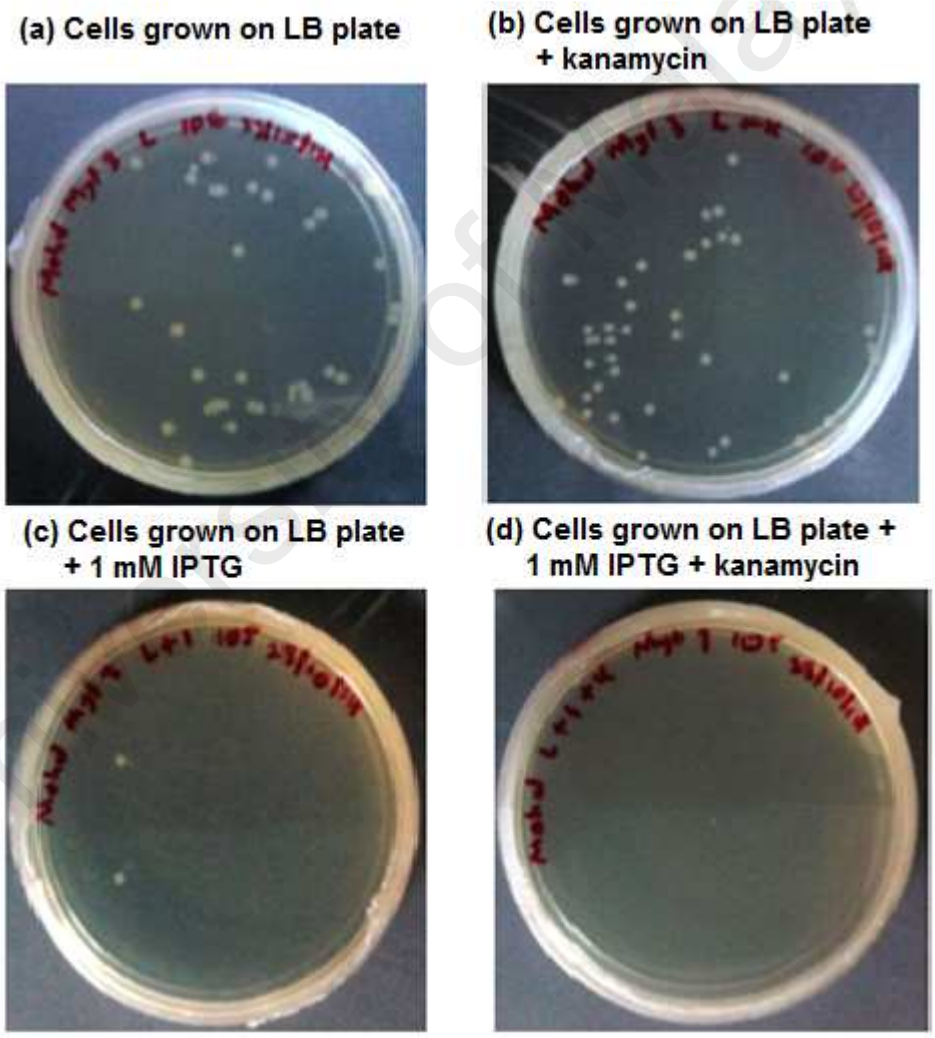

Figure 4.5: Stability of plasmid based on cells growth on four different media

4.4 Protein expression, detection and Purity

SDS-PAGE indicated the presence of Myt272-3 recombinant protein with the appearance of a band of approximately 10.58 kDa as shown with red arrow on Figure 4.6. This molecular weight matches the computed value of 10.58 kDa using the EXPASY MW bioinformatics tool.

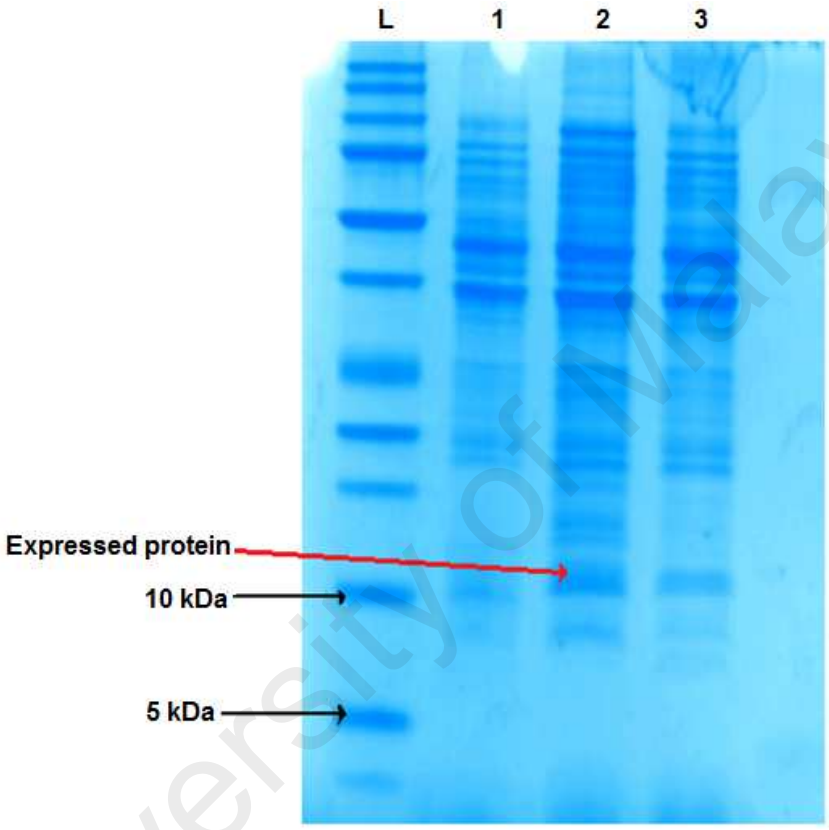

Figure 4.6: The Expression of His- tag Myt272-3 protein analyzed by SDS-PAGE Lane L is a Criterion™ 10-20% Tris-Tricine protein marker; Lane 1 indicates protein expressed without insert (control); lane 2 shows protein expression at 1 mM IPTG where red arrow indicated the expressed his-tag protein. Lane 3 is a non-induced control.

After purification protocol, the clear band of purified his- tag protein was seen with the molecular weight approximately similar to the one obtained using EXPASY MW bioinformatics tool as

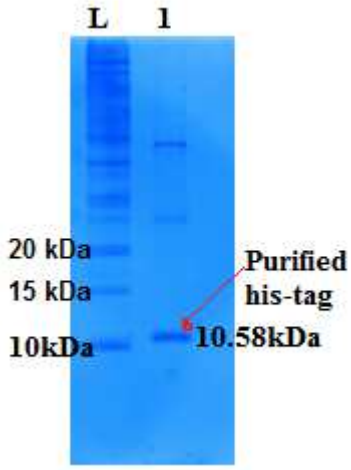

Figure 4.7: Dynerbeads Purification of His-tag protein on SDS PAGE

Lane L is a BenchMark ™ His-tagged protein standard (Novex life Technologies); Lane 1 contain Purified His-tag protein

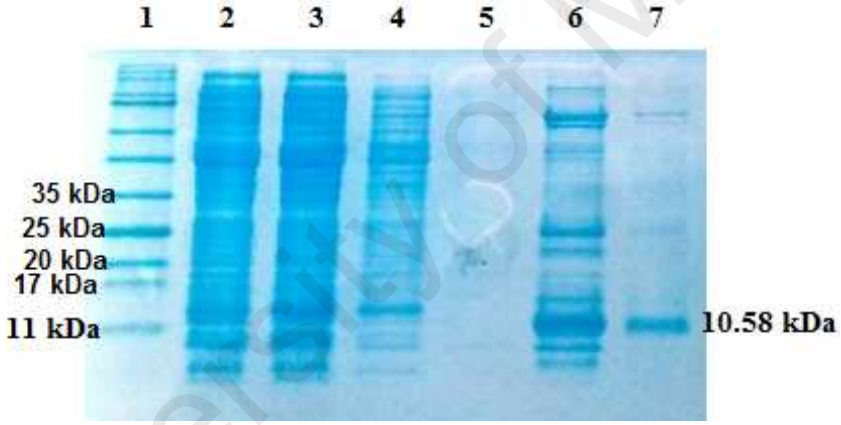

Figure 4.8: Purification of His-tag protein by Ni-NTA method

Lane L is a GangNam-STAIN™; lane 1 indicates cell lysate; lane 2 indicates flow through; lanes 3 and 4 represent wash 1 and 2 respectively, while lanes 5 and 6 indicate elutions 1 and 2 respectively.

4.4.1 Determination of protein concentration

Standard curve of Bovine serum albumin concentration was plotted as shown in Figure 4.9. The absorption of light was read at 650 nm to calculate the concentration of histidine-

tagged protein isolated. The values of optical density (absorbance) read indicate an increase with increase in concentration of Bovine serum albumin.

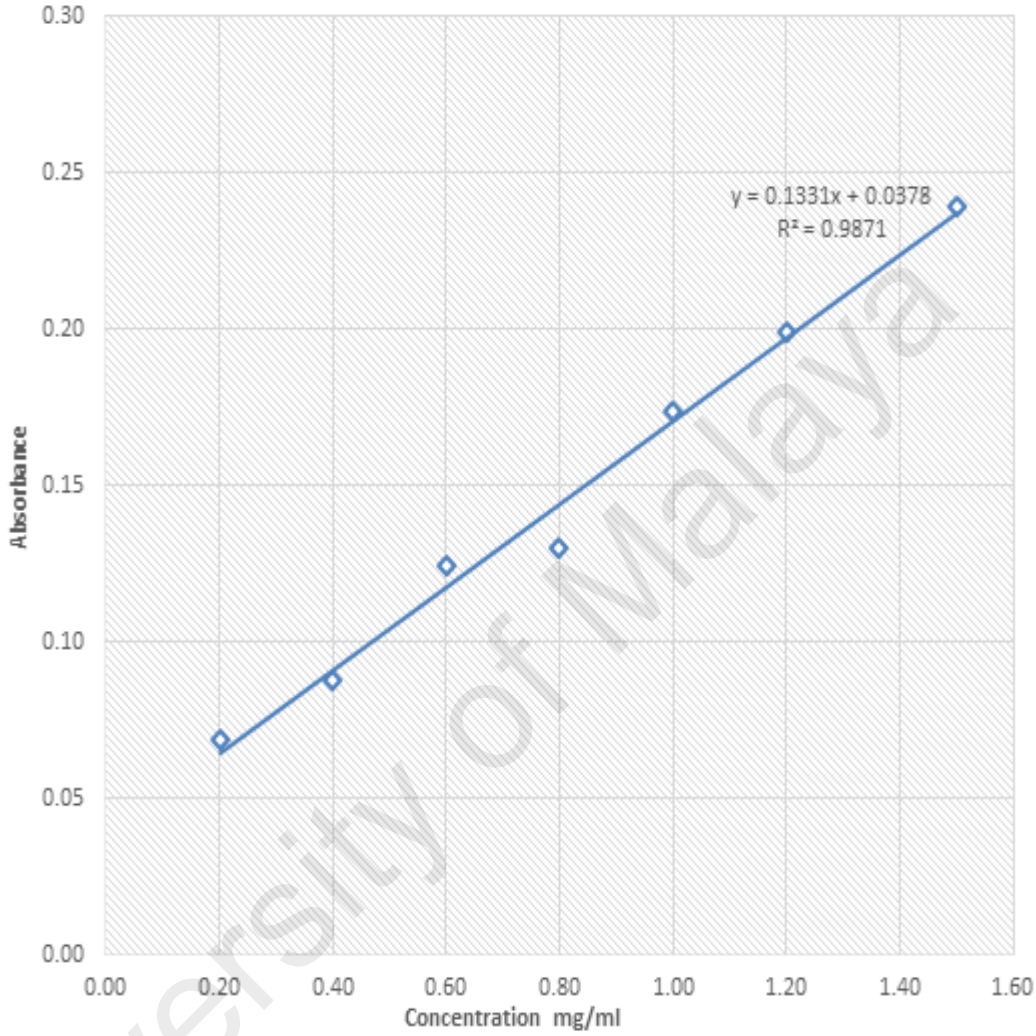

Figure 4.9: Protein concentration plot

The concentration of his-tagged protein determined using the standard curve equation was found to be 242 ug/ml.

4.5 Determination of physicochemical characteristic of protein

ProtPram values as shown in Table 4.1 indicates that the physicochemical characteristics of the protein. The total number of negatively charge residues is greater than the total number of positively charged residues. Estimated half-life of the protein was predicted to be 30 hours in mammalian reticulocytes, > 20 hours in yeast /*in vivo* and

> 20 in *E. coli*. The protein also has molecular weight of 10.58 kDa and isoelectric point of 6.1.

Table 4.1: Physicochemical characteristics of the query peptide shown by ProtParam tool

Characteristics	Parameters
1. Number of amino acids	97
2. Molecular Weight of the protein	10.85 kDa
3. Theoretical isoelectric point (pI)	6.11
4. Total number of negative residues (Asparagine + glutamine)	13
5. Total number of positive residues (Arginine + Lysine)	7
6. Estimated half-life of the protein	30 hours (mammalian reticulocytes, *in vitro*). More than 20 hours (yeast, *in vivo*). More than 10 hours (*Escherichia coli, in vivo*).
7. Aliphatic index	74.43
8. Instability index	31.85 (classified as stable protein)
9. Grand average hydropathicity (GRAVY)	- 0.564

4.6 MALDI-TOF Analysis

Analysis of spectra using Mascot sequence software indicated an identity or extensive homology to Phenolpthiocerol synthesis polyketide synthase I PpSA of *M. tuberculosis*, *M. bovis and M. africanus* with score values of 157, individual ion score > 60 is an indication of an extensive homology or identity ($p < 0.05$). The protein was also found to be identical or extensively in homology with Multispecies polyketide synthase of *M. tuberculosis* complex ($p < 0.05$) with score value of 157 which is 261.67 % greater than the reported ion score of 60.

4.7 Prediction of Allergenicity and antigenicity

Allergen prediction result from AlgPred tool indicated that the protein was non-allergen, with score value of -1.4238321[Threshold = -0.4]. In addition, the positive predictive value of the protein was 15.19% (values > 35 indicates allergenicity). The overall antigenicity value of the protein was 0.4109 (probable antigen) as determined by Vaxijen server (threshold value of 0.4).

4.8 Predicted MHC class I and MHC class II binding epitopes

Propred-I (for MHC I) and Propred (MHC II) were used for prediction of T-cell epitopes for the Myt272-3 protein. Identification of MHC-binding peptides and their subset of T-cell epitopes assist in improving our understanding of specificity of immune responses; it is also important for in discovery of vaccines (Hong H Lin et al., 2008) . MHC Class I and II binding prediction results were shown in Tables 4.2 and 4.3, respectively. All the alleles showed good match to the protein with log scores of all the peptides greater than their respective threshold values at 4 %, and Vaxijen values greater than 0.4 (Table 4.2 for MHC I). For MHC II, only alleles DRB1_1304 and DRB1_1321 matched the protein sequence, with peptide scores greater than their respective threshold values, and Vaxijen value ≥ 0.4 (Table 4.3).

Table 4.2: MHC-I binding peptides and antigenicity scores of sequences according to VaxiJen server

Allelles	Threashold	Sequence	Score achievable by any peptide on log scale value at 4%				Vaxijen value (Threashold = 0.4)
			Position	Real score	*Log* score	Highest score	
HLAA1	-1.553	LADLGVSSR	41	5	1.6094	12.324	1.2945
HLAA2	0.693	VLSGELSEL	53	27.76	3.3237	15.156	0.7474
HLAA*0201	1.143	VLSGELSEL	53	83.53	4.4252	17.857	0.7474
HLAA*0205	0.519	VLSGELSEL	53	23.80	3.1697	10.499	0.7474
		GVSSRDAVV	45	4	1.3863	10.499	0.9744
HLA-A3	-0.799	VLSGELSEL	53	1.35	0.3001	15.620	0.7474
		LLGRTVSPI	61	1.8	0.5878	15.620	0.9916
HLAA*3101	-1609	LADLGVSSR	41	0.2	-0.9163	11.408	1.2945
HLAA*3302	-0.105	LADLGVSSR	41	3	1.0986	6.109	1.2945
HLA-A68.1	1.609	GVSSRDAVV	45	6	1.7918	10.609	0.9744
HLA-A2.1	105.100	VLSGELSEL	53	119.8	119.800	145.700	0.7474
		LLGRTVSPI	61	117.9	117.900	145.700	0.9916
		GVSSRDAVV	45	108.9	108.900	145.700	0.9744
HLAB*2702	0.000	RTVSPIDFW	64	1.5	0.4055	10.309	0.5912
HLAB*2705	2.996	VLSGELSEL	53	30	3.4012	10.309	0.7474
HLAB*3501	0.693	VSSRDAVVL	46	6	17.918	8.882	0.5123
HLAB*3701	0.405	LSGELSELL	54	5	1.6094	8.006	0.4193
HLA-B40	0.000	RTVSPIDFW	64	3	1.0986	8.476	0.5192
HLAB*5101	1.649	LGVSSRDAV	44	57.2	4.0466	10.157	0.9816
HLAB*5102	2.078	LGVSSRDAV	44	120	4.7875	11.671	0.9816
HLAB*5103	0.884	LGVSSRDAV	44	40	3.6889	7.567	0.9816
HLAB*5201	1.974	LGVSSRDAV	44	7.5	2.0149	9.989	0.9816
		GVSSRDAVV	45	10	2.3026	9.989	0.9744
HLAB*5301	104.800	LGVSSRDAV	44	106.23	106.2300	150.500	0.9816
		VSSRDAVVL	46	105.84	105.8400	150.00	0.5123
HLA-B*51	104.920	LGVSSRDAV	44	115.49	115.4900	157.000	0.9816
HLAB*5801	-0.223	LSGELSELL	54	6	1.7981	9.350	0.4193
		RTVSPIDFW	64	288	5.6630	9.350	0.5912
HLA-B60	1.386	VSSRDAVVL	46	16	2.7726	8.955	0.5123
HLA-B61	0.000	LGVSSRDAV	44	2	0.6931	7.879	0.9816
HLA-B62	0.693	DLGVSSRDA	43	4	1.3863	9.199	0.7212
		LLGRTVSPI	61	4	1.3863	9.199	0.9916
HLACw*0401	1.577	VLSGELSEL	53	8	2.0794	9.342	0.7474
HLACw*0602	1.482	LSGELSELL	54	6	1.7918	4.380	0.4193
MHC-Db revised	1.276	LSGELSELL	54	22.217	3.1009	12.671	0.4193
		VSSRDAVVL	46	9.374	2.2380	12.671	0.5123
MHC-Kb	0.365	LSGELSELL	54	1.452	0.3729	6. 346	0.4193
		VSSRDAVVL	46	1.2	0.1823	6.346	0.5123
MHC-Kd	4.236	LSGELSELL	54	57.6	4.0535	8.289	0.4193
MHC-Ld	2.015	VSSRDAVVL	46	25	3.2189	6.554	0.5123

Table 4.3: MHC-II binding peptides and antigenicity scores of sequences according to vaxijen server

Allelle	Threashold for 3%	Sequence	Position	Score	Highest score achievable	Vaxijen value (Threashold = 0.4)
DRB1_0402	1.8	LLGRTVSPI	60	2.4	9.6	-0.9916
DRB1_0801	1.8	LLGRTVSPI	60	2.3	8.6	-0.9916
DRB1_0802	1.0	LLGRTVSPI	60	2.1	8.0	-0.9916
DRB1_0804	1.6	LLGRTVSPI	60	3.1	8.0	-0.9916
DRB1_0806	2.4	LLGRTVSPI	60	3.3	8.6	-0.9916
DRB1_0813	1.9	LLGRTVSPI	60	3.9	8.7	-0.9916
DRB1_1304	2.6	VVLSGELSE	51	2.8	9.0	0.8284
DRB1_1321	2.2	VVLSGELSE	51	2.8	8.9	0.8284

4.9 Antibody epitope prediction

Table 4.4: The predicted residue scores by Kolaskar and Tongaonkar antigenicity with the threshold value of 1.0

Start	End	Peptide	Score
36	42	LGTLSLA	1.087
37	43	GTLSLAD	1.032
38	44	TLSLADL	1.086
39	45	LSLADLG	1.081
40	46	SLADLGV	1.100
41	47	LADLGVS	1.100
42	48	ADLGVSS	1.066
43	49	DLGVSSR	1.039
44	50	LGVSSRD	1.039
45	51	GVSSRDA	1.012
46	52	VSSRDAV	1.085
47	53	SSRDAVV	1.085
48	54	SRDAVVL	1.119
49	55	RDAVVLS	1.119
50	56	DAVVLSG	1.119
51	57	AVVLSGE	1.117
52	58	VVLSGEL	1.143
53	59	VLSGELS	1.090
54	60	LSGELSE	1.014
55	61	SGELSEL	1.014
56	62	GELSELL	1.048
57	63	ELSELLG	1.048
58	64	LSELLGR	1.051
59	65	SELLGRT	1.003
60	66	ELLGRTV	1.056
61	67	LLGRTVS	1.079
62	68	LGRTVSP	1.052
63	69	GRTVSPI	1.038

As shown in Table 4.4, and Figure 4.10 (center yellow region). The most possible epitopes predicted for antigenic Myt272-3 protein consist of amino acids from 48-58 (SRDAVVL-VVLSGEL) with score > 1.0. Another possible epitope is from amino acids 40-47 (SLADLGV-LADLGVS).

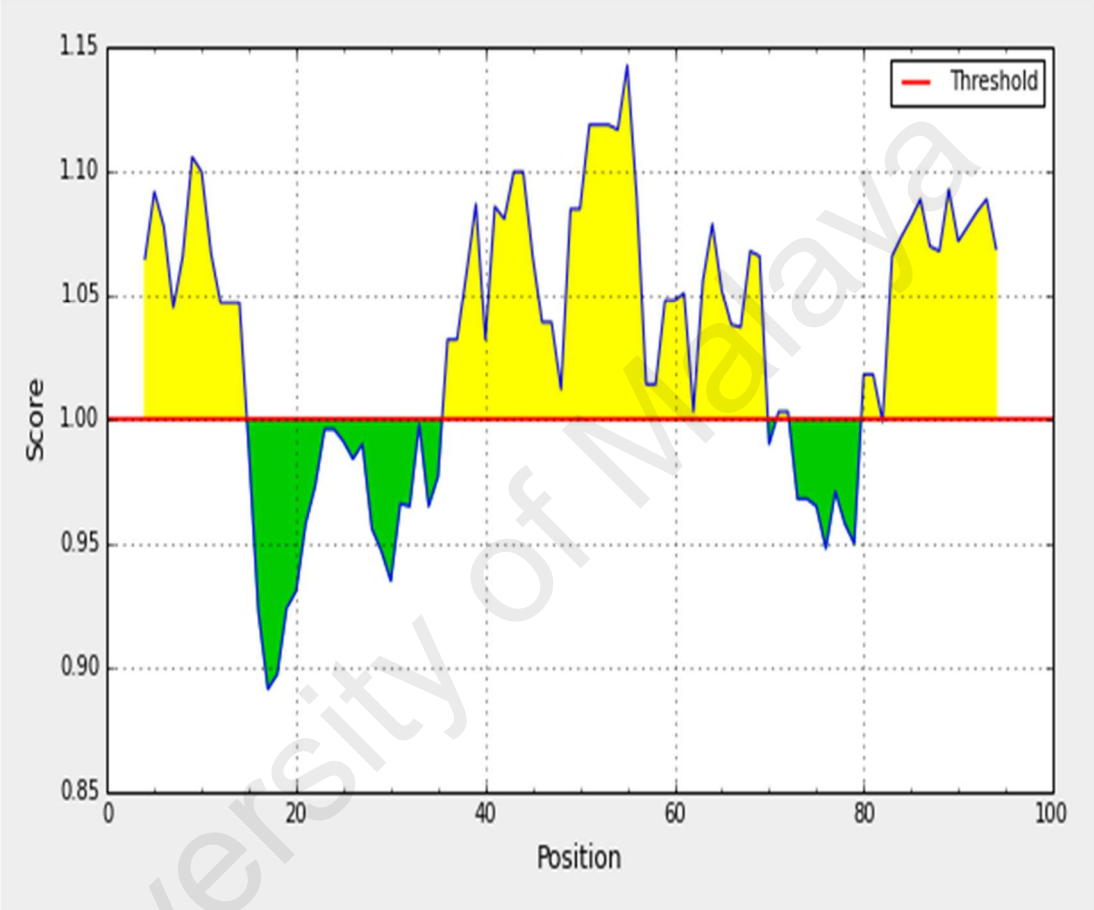

Figure 4.10: Kolaskar and Tongaonkar antigenicity plot of Myt272-3 recombinant protein

Threshold: 1.0 (horizontal redline). The yellow colors above the threshold represent higher scores, while green portions indicate unfavorable regions in relation to the region of interest.

4.10 Toxicity prediction of the epitopes

ToxinPred webserver was use to predict the toxicity of peptide epitopes generated computationally whereas hemotoxicity of the peptides was predicted computationally using HemoPI server. Toxicity and hemotoxicity prediction results of MHCs and B cell's derived sequences are shown in Tables, 4.5, 4.6 and 4.7 .The predictive results reveled non toxicity and hematotoxicy in all the sequences. The peptide toxicity threshold was 0.0, where as hemotoxicity prediction probability score ranges between 0 and 1, that is 1 very likely to be hemolytic, 0 very unlikely to be hemolytic.

Table 4.5: Toxicity of MHC-I binding peptides

	Peptide toxicity		**Peptide hemotoxicity**	
Peptide sequence	**Prob score**	**Prediction**	**Prob score**	**Mutation position**
LADLGVSSR	-1.02	Non-toxic	0.49	No mutation
VLSGELSEL	-1.21	Non-toxic	0.49	No mutation
GVSSRDAVV	-0.09	Non-toxic	0.49	No mutation
LLGRTVSPI	-1.01	Non-toxic	0.52	No mutation
RTVSPIDFW	-1.15	Non-toxic	0.49	No mutation
VSSRDAVVL	-0.93	Non-toxic	0.49	No mutation
LSGELSELL	-1.17	Non-toxic	0.49	No mutation
LGVSSRDAV	-1.04	Non-toxic	0.49	No mutation
DLGVSSRDA	-0.93	Non-toxic	0.49	No mutation

Table 4.6: Toxicity of MHC-II binding peptides

	Peptide toxicity		**Peptide hemotoxicity**	
Peptide sequence	**Prob score**	**Prediction**	**Prob score**	**Mutation position**
LLGRTVSPI	-1.01	Non-toxic	0.52	No mutation
VVLSGELSE	-1.28	Non-toxic	0.49	No mutation

Table 4.7: Toxicity of the predicted residue scores by Kolaskar and Tongaonkar

	Peptide toxicity		Peptide hemotoxicity	
Peptide sequence	**Prob score**	**Prediction**	**Prob score**	**Mutation position**
LGTLSLA	-1.14	Non-toxic	0.52	No mutation
GTLSLAD	-1.17	Non-toxic	0.49	No mutation
TLSLADL	-1.33	Non-toxic	0.49	No mutation
LSLADLG	-1.14	Non-toxic	0.49	No mutation
SLADLGV	-1.17	Non-toxic	0.50	No mutation
LADLGVS	-1.34	Non-toxic	0.50	No mutation
ADLGVSS	-1.07	Non-toxic	0.49	No mutation
DLGVSSR	-0.95	Non-toxic	0.49	No mutation
LGVSSRD	-0.99	Non-toxic	0.49	No mutation
GVSSRDA	-0.87	Non-toxic	0.49	No mutation
VSSRDAV	-0.79	Non-toxic	0.49	No mutation
SSRDAVV	-0.64	Non-toxic	0.49	No mutation
SRDAVVL	-0.91	Non-toxic	0.49	No mutation
RDAVVLS	-1.10	Non-toxic	0.49	No mutation
DAVVLSG	-1.07	Non-toxic	0.49	No mutation
AVVLSGE	-1.14	Non-toxic	0.49	No mutation
VVLSGEL	-1.32	Non-toxic	0.49	No mutation
VLSGELS	-1.06	Non-toxic	0.49	No mutation
LSGELSE	-0.99	Non-toxic	0.49	No mutation
SGELSEL	-1.07	Non-toxic	0.49	No mutation
GELSELL	-1.10	Non-toxic	0.49	No mutation
ELSELLG	-1.14	Non-toxic	0.49	No mutation
LSELLGR	-1.17	Non-toxic	0.49	No mutation
SELLGRT	-1.15	Non-toxic	0.49	No mutation
ELLGRTV	-1.00	Non-toxic	0.49	No mutation
LLGRTVS	-0.97	Non-toxic	0.49	No mutation
LGRTVSP	-0.80	Non-toxic	0.49	No mutation
GRTVSPI	-0.75	Non-toxic	0.49	No mutation

CHAPTER 5: DISCUSSION

Restriction degistion analysis revealed the presence of an insert of 113 bp on agarose gel as indicated in Figure 4.1. The approximate size of insert was also analysed from the PCR products of patch culture of the transformed cells as indicated on figure 4.4. From Figure 4.5, it was found that, the recombinant plasmid was stable for expression of the his-tagged protein (Myt272-3 protein). This could be linked to the fact that, with stable recombinant plasmids, the fraction of cells that take up the plasmids of interest is replected by decrease in colonies on the LB plate + 1 mM IPTG and an increase on the LB plates + kanamycin as reported in pET manual (Novagen, 1999).

Myt272-3 protein was expressed and purified to yield a band of approximate MW 10.58 kDa as illustrated in Figures, 4.7 and 4.8, the size of protein expressed conformed with the MW computed by EXPASY MW bioinformatics tool. The protein was found to be a non-allergen. It has been reported that a protein can be classified as an allergen when it shows > 35 % identity with a familiar allergen over a window of 80 amino acids, or presence of six 6 contiguous amino acids present in a known allergen (Dang & Lawrence, 2014; FAO, 2001). AlgPred is considered one of the web based servers used to predicts allergens through query amino acid sequences of the proteins (Sircar et al., 2014).

Protein Blast of the query protein sequence also indicated homology of 81 % with phenolpthiocerol synthesis polyketide synthase I PpSA of *M. tuberculosis,* which is consistent with the results obtained from MALDI-TOF. It has been reported that polyketide synthase is involved in the biosynthesis of unique cell surface lipids; *Mycobacterium tuberculosis* cell envelope contain the cell surface lipids that link the host and the pathogen (Passemar et al., 2014). Phthiocerol and phenolphthiocerol diesters were reported also as important virulence factors of the two main mycobacterial pathogens (*M. tuberculosis and L. leprae*) in human (Rana, 2010). The protein blast result also indicated

non-homology with human protein. It is known that vaccines are considered good when they do not show homology with human proteins, thus eliminating likelihood of their triggering an autoimmune response (Monterrubio-López, 2015).

The isoelectric point computed was less than 7 (6.11), indicating that the protein is acidic in nature. The protein was classified as stable (instability index 31.85) based on ProtParam analysis. The protein is said to be stable if its instability index < 40 (Elengoe et al., 2014). The aliphatic index determined by ProtParam was 74.43. The relative volume of protein occupied by aliphatic side chain of protein is defined as its aliphatic index, the higher the aliphatic index the higher its thermal stability (Elengoe et al., 2014), so the protein may have higher thermal stability with this relatively higher aliphatic index value. The negative grand average hydropathicity of a protein is an indication that the protein contains more hydrophilic residues (Gupta et al., 2016), which is demonstrating a hydrophilicity pattern with more effective interaction with water (Shamriz & Ofoghi, 2016). Shi et al also reported that a protein with negative GRAVY is hydrophilic in nature and with most of the residues to be available on the surface, which implies that more amino acids in the protein and to be binding residues in nature upon interaction with other protein (Shi et al., 2015).

From Table 4.2**,** it was found that thirty (30) MHC I from 30 different alleles bind peptides with binding scores higher than the threshold score of each peptide. It was also found that antigenicity values of all the peptides were above the Vaxijen server threshold (0.4). This strongly indicates that Myt272-3 protein is a probable antigenic protein for development of tuberculosis vaccine. Epitope LADLGVSSR at position 41 was found to have the highest antigenicity value of (1.2945) among all epitopes. This is an indication of maximum binding affinity.

From Table 4.3, The predicted peptides, LLGRTVSPI and VVLSGELSE at positions 60 and 51, respectively were found to bind eight different MHC II from 8 different alleles with binding scores greater than their respective threshold value at 4 %, but further antigenicity analysis using Vaxijen scale revealed only peptide, VVLSGELSE had antigenicity activity with the score of 0.8284 that is higher than the threshold score of 0.4. It has been observed that computational predictions of HLA-II binding are inferior when compared with their HLA-I counterparts, due to factors which include insufficient data used by developers of prediction methods for HLA-II binding peptides, and the fact that HLA-II molecules have relatively permissive binding sites for peptide, a property which limits their specificities (Lin et al., 2008).

The prediction of potential B-cell epitope was carried out via amino acid-based method. All epitopes with antigenic value greater than the threshold value of 1.0 were considered potential antigenic determinants. Our results show that all the epitopes from position 36 to 69 satisfied the threshold value requirement for antigenicity. The highest antigenicity scores were recorded in three peptides - SRDAVVL, RDAVVLS and DAVVLSG with score of 1.119 as shown on Table 4.4 and Figure 4.10 (Assis et al., 2014).

From Tables, 4.5, 4.6 and 4.7, the predictive analysis revealed no toxicity and hematoxicity in all the epitopes binding to MHCs as well as those predicted by by Kolaskar and Tongaonkar scale. It has been reported that an ideal epitope peptide should possess high antigenicity but less or no toxicity (Parvege et al., 2016). In addition, antigenicity carried out previously using Vaxijen server indicated that the peptides were antigenic in nature.

The best possible therapeutic peptide should have no hemolytic activity. Many prospective peptides with therapeutic effects could not reach in clinical set up because of

their high hemolytic characteristics. Therefore, considerable efforts were put in place to reduce the hemolytic capabilities of these peptides. HemoPI server was developed and will be helpful to predict more suitable therapeutic peptides (Chaudhary et al., 2016). From table, all epitope peptides were predicted to be non-hemolytic when compared to the mutant peptides from hemoPI tool, with score less than 1 unit. Therefore, all peptides generated are very unlikely to be hemolytic. This could be linked to the fact that, peptides with score value of 1 are considered very likely to be hemolytic whereas those with 0 value are very unlikely to have hemolytic activity (Chaudhary et al., 2016). A case of vaccination-related immune hemolysis was reported in children due to production of autoantibodies against red blood cells, the mechanism underlying this process was not understood (Seltsam et al., 2000). Therfore, recently developed hemolytic predictive software could be of great importance to provide guide on hemolytic activity of the query protein.

CHAPTER 6: CONCLUSION

In this study, a recombinant protein pET30a/Myt272-3 of 10.58 kDa was produced, purified and charcterised. Further more, the protein was analysed computationally toward vaccine development against tuberculosis.

Development of better and cost-effective vaccine can be achieved through immunoinformatics-based vaccine design. The prediction results obtained in the present work provide a guide for practical design of new tuberculosis vaccine. *In silico* analysis of the protein reveals that it is a non-allergen with antigenic activity. It binds both MHC I and II but with poorer to the latter, probably due to the fact that computational predictions of HLA-II binding are inferior when compared with their HLA- I counterparts. The protein possesses B-cell epitopes. The toxicity predictive analysis revealed non-toxicity. These predictive findings have implications for design and development of *Mycobacterium tuberculosis* vaccines.

Further work should include immunogenicity assessment of the his-tagged protein in evaluation of antibody responses and pathological changes in the rat model. In this regard, animals should be immunized with the his-tagged protein and compare with those to be immunized with BCG in terms of immunological response. The fact that adjuvants is needed when formulating subunits vaccine in order to trigger an adequate immune response (Liu et al., 2016). Further work should also include formulation of the Myt272-3 protein with different adjuvant in order to ascertain the effect of the adjuvant formulation.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

1. Usman, M. M., Ismail, S., & Teoh, T. C. (2016). Protein expression of Myt272-3 recombinant clone and in silico prediction of a possible vaccine candidate against *Mycobacterium tuberculosis*. *Tropical Journal of Pharmaceutical Research, 15*(7), 1393-1400. (*ISI/SCOPUS Indexed Publication*).
2. Usman, M. M., Ismail, S., & Teoh, T. C. (2017). Vaccine research and development: tuberculosis as a global health threat. *Central European Journal of Immunology, 42*(2), 196. (*ISI/SCOPUS Indexed Publication*).
3. Usman, M. M., Ismail, S., & Teoh, T.C. (2015). Expression and characterization of Myt3 recombinant protein for tuberculosis peptide vaccine development. Oral presentation at 20th Biological Sciences Graduate Congress, 9-11 December. Chulalongkorn University, Thailand. (International).
4. Usman, M. M., Ismail, S., & Teoh, T. C. (2016). *In silico* prediction analysis of Myt272-3 recombinant protein for a potential vaccine development against tuberculosis. Oral presentation at 41st annual conference of the Malaysian Society for Biochemistry & Molecular Biology, 17-18 August. Pullman Hotel, Kuala Lumpur, Malaysia. (National).