CONSTRUCTION OF VAC IV: TOWARDS THE DEVELOPMENT OF DNA VACCINE CANDIDATE AGAINST TUBERCULOSIS

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DEDICATIONS

This thesis is specially dedicated to:

My beloved husband, Badrul Syam b. Mat Zainuddin
My daughter, Amni Batrisyia
My parents, Dr. Hj. Fauzi Yaakub and Hjh. Che Patimah

Thank you for your love, support and patience.

May Allah bless you all..

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LIST OF ABBREVIATION

α/ß	Alpha beta
Ag85	Antigen 85
AIDS	Acquired Immunodeficiency Syndrome
APC	Antigen presenting cell
B.C	Before Century
BCG	Bacille Calmette Guerin
bp	Base pair
Con A	Concanavalin A
CO_2	Carbon dioxide
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DNA	Deoxyribonucleic acid
ddH2O	Deionized distilled water
HF	High Fidelity
HIV	Human Immunodeficiency Virus
IFN	Interferon
IL	Interleukin
IULTD	International Union against TB and Lung Disease
Kb	kilo base
KDa	kilo Dalton
LB	Luria-bertani
LTT	Lymphocyte Transformation Test
MHC	Major Histocompatibility complex
OD	Optical density
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PPD	Purified protein derivatives
RE	Restriction enzyme
SI	Stimulation index
TB	Tuberculosis
Th	T helper

TNF	Tumour Necrosis Factor
UV	Ultra violet
WHO	World Health Organization

CONSTRUCTION OF VAC IV: TOWARDS THE DEVELOPMENT OF DNA VACCINE CANDIDATE AGAINST TUBERCULOSIS

ABSTRACT

Tuberculosis (TB) is the one of leading cause of death in the world, caused by a bacterium, *Mycobacterium tuberculosis*. The disease affects 1.7 billion people every year which is equivalent to one-third of the entire world population. The recent increase in the incidence of TB, particularly antibiotic-resistant TB underscores the need for an effective vaccine against this important disease. The only vaccine currently in use is the live, attenuated strain of *Mycobacterium bovis*, bacille Calmette-Guérin (BCG) that was produced in the early 1920s. In this study, a plasmid DNA encoding Mtb8.4, 30kDa (Ag85B) and 32kDa (Ag85A) genes of *M. tuberculosis* was constructed as an alternative vaccine candidate against TB. Using assembly polymerase chain reaction (PCR) method, the synthetic gene, designated as VacIV gene was constructed from overlapping oligonucleotides of the desired genes. The VacIV gene was successfully cloned into an eukaryotic expression vector, pVAX1© to produce a DNA vaccine candidate namely pVaxVacIV. The VacIV gene also was successfully expressed in *E. coli* expression system. The immunogenicity of this vaccine candidate was then tested in mice. Mice were immunized intramuscularly with the vaccine candidate. Control mice were immunized with the blank vector (pVAX1©). The splenocytes were cultured with antigens such as purified protein derivatives (PPD), rVacIV protein and Mtb8.4 synthetic peptide for lymphocytes transformation test (LTT) and cytokines assay. Sera were also collected to determine the level of serum IgG subclasses. Our results showed that lymphocytes from mice immunized with the pVaxVacIV secreted a higher gamma interferon (IFN-γ) but not the interleukin-4 (IL-4) levels compared to the lymphocytes from the control mice. Mice immunized with

pVaxVacIV also showed high lymphocytes stimulation index and high ratio of IgG2a:IgG1 as compared to control group. These results showed that the newly constructed DNA vaccine candidate, pVaxVacIV was immunogenic in mice and can be further developed as a potential vaccine candidate for TB.

PEMBINAAN VAC IV: KE ARAH PEMBANGUNAN CALON VAKSIN DNA TERHADAP PENYAKIT TUBERKULOSIS

ABSTRAK

Tuberkulosis (TB) merupakan salah satu punca kematian di dunia ini, disebabkan oleh bakteria *Mycobacterium tuberculosis*. Penyakit ini telah menyerang 1.7 billion penduduk dunia setiap tahun, bersamaan dengan satu per tiga daripada keseluruhan jumlah popoulasi di dunia. Disebabkan peningkatan jumlah kes TB pada masa kini, terutamanya TB rintang antibiotik, maka vaksin yang lebih efektif adalah diperlukan bagi melawan penyakit ini. Satu-satunya vaksin yang digunakan sekarang adalah vaksin strain teratenuat, *Mycobacterium bovis*, bacille Calmette-Guerin (BCG) yang telah dibangunkan sejak tahun 1920-an. Dalam penyelidikan ini, plasmid DNA yang mengekodkan gen Mtb8.4, 30kDa (Ag85B) dan 32kDa (Ag85A) dari *M. tuberculosis* telah dibangunkan sebagai vaksin alternatif terhadap TB. Dengan menggunakan teknik tindakbalas polymerase berantai (PCR), gen sintetik yang dikenali sebagai VacIV telah dibina daripada oligonukleotida-oligonukleotida yang mengekodkan gen yang dikehendaki. Gen VacIV juga telah berjaya diklonkan ke dalam vektor pengekspresian eukariotik iaitu pVAX1, bagi menghasilkan calon vaksin yang diberi nama pVaxVacIV. Gene VacIV telah berjaya diekspreskan dalam sistem pengekspresan *E. coli*. Kajian imunogenisiti terhadap calon vaksin ini telah dilakukan ke atas mencit. Mencit telah diimunisasikan melalui kaedah intramuskular (intra-otot) dengan calon vaksin tersebut manakala mencit kawalan diimunisasikan dengan vektor tanpa gen VacIV (pVAX1). Sel limfosit telah dikulturkan dengan antigen-antigen iaitu 'purified protein derivatives' (PPD), protein rekombinan VacIV dan peptida sintetik Mtb8.4 untuk ujian transformasi limfosit (LTT) dan penentuan asai sitokin. Serum mencit telah dikumpul untuk menentukan aras sub-kelas IgG. Hasil kajian

kami telah menunjukkan bahawa mencit yang diimunisasikan dengan pVaxVacIV merembeskan interferon gamma (IFN-γ) lebih tinggi tetapi tidak interleukin-4 (IL-4) berbanding mencit kawalan. Mencit yang diimunisasikan dengan pVaxVacIV juga menunjukkan indeks stimulasi limfosit dan nisbah IgG2a terhadap IgG1 yang tinggi berbanding mencit kawalan. Keputusan ini menunjukkan bahawa calon vaksin DNA yang baru ini adalah imunogenik terhadap mencit boleh dibangunkan sebagai calon vaksin yang berpotensi untuk mencegah TB.

CHAPTER ONE

INTRODUCTION

1.1 Background of Tuberculosis

1.1.1 Overview of TB

Tuberculosis (TB) is among the most frequent infectious disease nowadays. In 1882, Robert Koch had identified the responsible causative agent of this disease which is the tubercle bacillus, *Mycobacterium tuberculosis* (Kanai, 1990). *M. tuberculosis* infects about one-third of the world's population, causing approximately 3 million deaths annually (Dye *et al.*, 1999). In 1993, WHO declared the current TB epidemic 'a global emergency' as it became a major cause of death in the developing world (WHO, 1994). Until now, TB remains a major global health threat and almost 200 millions people are currently infected with *M. tuberculosis*, resulting in 50,000 new cases of disease every year (WHO, 2005, Kaufmann, 2005).

TB is accountable for the deaths of an additional 900,000 people with Acquired Immunodeficiency Syndrome (AIDS) annually (Dye *et al*., 1999; Kochi, 1991). Co-infection with human immunodeficiency virus (HIV) is the most important risk for the development of latent *M. tuberculosis* infection (Ibanga *et al.*, 2006). In addition, the problem is compounded by the global emergence of *M. tuberculosis* strains which are resistant to the major antibiotics used to treat TB (Lauzardo & Ashkin, 2000).

1.1.2 History of TB

The existent of *M. tuberculosis* was realized since 2400 B.C through the fragments of the spinal column from Egyptian mummies, which showed definite signs of TB. The term “phthisis”, consumption, appears first in Greek literature. Around 460 B.C, Hippocrates identified phthisis as the most common disease of the times, and notable that it was almost fatal. Due to common phthisis-related fatalities, he warned his colleagues to beware of TB patients in late stages of the disease, because their expected deaths might damage the reputations of the attending physicians (Kanai, 1990).

TB was present many centuries before Robert Koch first isolated the tubercle bacillus in 1882 (Kanai, 1990). Koch’s discovery has lead to the detection of the tubercle bacilli in variety methods such as microscopy, animal inoculation and by an *in vitro* culture. Koch’s finding was the starting point for the development of scientific research of TB, both practical and theoretical.

Clinically, there are variety of types of TB such as bone TB, lymph node TB, pleural TB, genitourinary TB, central nervous system (tuberculomeningitis) tuberculosis, abdominal TB, pericardial TB and pulmonary TB (Kumar *et al.,* 2003). Among all of these, pulmonary TB is the most common and is responsible for the most death per year (Butler, 2000). The disease remained a bane for many years as there is no effective treatment available (Goodfellow & Magee, 1998).

In 1908, a bacteriologist from France Pasteur Institute named Calmette, together with his friend Guerin had succeeded to produce a vaccine against TB from a live-attenuated *M. bovis*. This vaccine is known as bacille Calmette-Guerin (BCG) and practically used in

human since 1921. The development of this vaccine also led to the production of antituberculous drugs such as isoniazid (INH), kanamycin (KM), pyrazinamide (PZA), ethambutol (EB) and rifampicin (RFP) (Kanai, 1990).

1.1.3 Epidemiology of TB in Malaysia

The number of TB notification had increased for over 10 years. In Malaysia, the incidence rate per 100,000 populations is 64.7. The incidence of smear positive cases is 34.7 per 100,000 populations. The state with the highest disease burden is Sabah, followed by Wilayah Persekutuan, Sarawak and Pulau Pinang respectively. In the meantime, the majority of cases are in the 15 to 54 years age group (67.7 %) followed by above 55 years age group (29.5 %) and children below 14 years age group (2.8 %) respectively (Kuppusamy, 2004)

The increase number of TB notification is associated with co-infection with HIV infection. Since most patients with TB/HIV co-infection are seen with advanced TB in Malaysia, the number of deaths due to TB/HIV has also increased. Moreover, the existence of immigrant population contributes to increase the number of TB cases. About 10 % of TB cases notified in Malaysia are discovered among the immigrant population, particularly the immigrant workers from high TB burden neighbouring countries such as Philippines, Indonesia, Thailand, Burmese and Bangladesh. Among the variety types of TB, pulmonary TB represents 91 % of the total cases. The most common type of TB in Malaysia are TB lymphadenitis, bone and joint TB and miliary TB (Kuppusamy, 2004).

1.1.4 *M. tuberculosis*

Mycobacteria is a gram-positive (no outer cell membrane), belongs to the family of Actinomyces. Most mycobacteria are found in habitats such as water or soil. However, a few are intracellular pathogens which infect animals and humans. *M. tuberculosis*, along with *M. bovis*, *M. africanum* and *M. microti* are the causative agents of TB and are members of the *M. tuberculosis* complex species. Each member of the TB complexes are pathogenic, but *M. tuberculosis* is the most pathogenic for humans while *M. bovis* usually infects animals (online at http://www-micro.msb.le.ac.uk/Video/Mtuberculosis.html)

M. tuberculosis is a non-motile rod-shaped bacterium. The rods are 2-4 um in length and 0.2-0.5 um in width. *M. tuberculosis* is an obligate aerobe, so it can grow most successfully in tissues with high oxygen content, such as the lungs. A *Mycobacterium* is a facultative intracellular parasite, usually of macrophages, and has a slow generation time, 15-20 hours, a physiological features that may supply to its virulence. *Mycobacterium* species are classified as acid-fast bacteria due to their impermeability by certain dyes and stains. Once stained, acid-fast bacteria will retain dyes when heated and treated with acidified organic compounds. One acid-fast staining method for *M. tuberculosis* is the Ziehl-Neelsen staining. When this method is used, the *M. tuberculosis* smear is fixed, stained with carbol-fuchsin (a pink dye) and decolorized with acid-alcohol. The smear is counterstained with methylene-blue or certain other dyes. Acid-fast bacilli appear pink in a contrasting background (Todar, 2005). The genome of *M. tuberculosis* consists of a single circular chromosome with 4.4 mega base pair and G+C contents approximately 65.6 % (Cole *et al.,* 1998).

The cell wall structure of *M. tuberculosis* earns special consideration because it is unique among prokaryotes and is a major determinant of virulence for the bacterium. The cell wall complex contains peptidoglycan and complex lipids (Bloom, 1994). Over 60% of the mycobacterial cell wall is lipid. The lipid fraction of *M. tuberculosis* cell wall consists of three major components. They are mycolic acid, cord factor and wax-D (Kanai, 1990).

Mycolic acids are unique alpha-branched lipids found in the cell walls of *Mycobacterium* and *Corynebacterium.* The composition is 50% of the dry weight of the mycobacterial cell envelope. Mycolic acids are strong hydrophobic molecules that form a lipid shell around the organism and affect the permeability properties at the cell surface. Mycolic acids are considered to be a significant determinant of virulence in *M. tuberculosis*. Most likely, they prevent attack of the mycobacteria by cationic proteins, lysozyme and oxygen radicals in the phagocytic granule. They also protect extracellular mycobacteria from complement deposition in serum (Todar, 2005).

The high concentration of lipid in the cell wall of *M. tuberculosis* has been associated with these properties of the bacterium including impermeability to stains and dyes, resistance to many antibiotics, resistance to kill by acidic and alkaline compounds, resistance to osmotic lysis via complement deposition and resistance to lethal oxidations and survival inside the macrophages.

Meanwhile, cord factor is toxic to mammalian cells and most abundantly produced in virulent strains of *M. tuberculosis*. Wax-D in the cell envelope is the major component of Freund's complete adjuvant (CFA), which is responsible in the impermeability of the mycobacterial cell wall (Todar, 2005).

1.1.5 Pathogenesis of TB

Tuberculosis is commonly acquired by inhalation of the tubercle bacilli through talking, coughing and sneezing. Most of the healthy people will beat a potential infection with activated microphages, especially when the dose is low. Disease progression depends on strain of *M. tuberculosis*, prior exposure, infectious dose and the immune status of the host (Tortora *et al*. 1997; Todar, 2005).

The pathogenesis of TB start once after droplet nuclei are inhaled, the bacteria are nonspecifically taken up by alveolar macrophages. However, the macrophages are not activated and are unable to destroy the intracellular organisms. TB begins when droplet nuclei reach the alveoli. The smaller droplet nuclei may reach the small air sacs of the lung (the alveoli), where infection begins. The second stage begins 7-21 days after initial infection. *M. tuberculosis* multiplies within normal macrophages until the macrophages burst. Other macrophages are released from peripheral blood. These macrophages also phagocytose *M. tuberculosis* but they are also unactivated and cannot destroy *M. tuberculosis*. Macrophages and other defensive cells accumulate at the infection site, forming a surrounding layer, walled-off lesion called a tubercle (means lump or knob) (Tortora *et al*. 1997; Todar, 2005).

The third stage begins after several weeks. Many of the macrophages die, releasing tubercle bacilli. The interior of tubercle becomes caseous (cheeselike in consistency). *M. tuberculosis* cannot multiply within these tubercles because of the low pH and anoxic environment. The disease may become dormant after this stage and *M. tuberculosis* persist within these tubercles for extended periods (Tortora *et al*. 1997; Todar, 2005).

At the fourth stage, a caseous lesion is slowly enlarged and become less cheeselike and more liquid, which called liquefaction. Although many activated macrophages can be found

surrounding the tubercles, many other macrophages present remain unactivated or poorly activated. *M. tuberculosis* uses these macrophages to replicate and hence the tubercle grows. The growing tubercle may attack a bronchus. Thus, *M. tuberculosis* infection can spread to other parts of the lung. The tubercle may invade an artery or other blood supply line. The hematogenous spread of *M. tuberculosis* may result in an extrapulmonary TB otherwise known as milliary tuberculosis (Tortora *et al*. 1997; Todar, 2005).

At the fifth stage, the disease now becomes very infectious. The liquid makes for an efficient aerosol droplets when coughed up and bacteria begin to escape the tubercle. These microorganisms begin to enter the airways of the lung, and then the circulatory and lymphatic systems (Tortora *et al*. 1997; Todar, 2005).

The symptoms that often occur in TB patient are fever, night-time sweating, loss of weight, persistent cough (often bringing up blood), constant tiredness and loss of appetite (Tortora *et al*., 1997; Todar, 2005).

1.1.6 Diagnosis of TB

In order to identify and diagnose *M. tuberculosis* infection, several ways can be used such as bacteriological or by immunological methods (Kanai, 1990). *M. tuberculosis* can be detected in sputum sample from patient by microscopic examination using Ziehl-Neelsen staining method. This method is performed to detect acid-fast bacilli in sputum. Ziehl-Neelsen staining method is less expensive and can detect the mycobacteria easily and quickly (Cambiaso *et al.,* 1990; Kanai, 1990). Ziehl-Neelsen staining method is recommended by the International Union against TB and Lung Disease (IUATLD) and WHO (Todar, 2005).

Other method used to make a diagnosis of TB is sample culturing. The culturing method commonly used is the BACTEC System. The media used in the BACTEC system contains radio-labeled palmitate as the sole carbon source. As *M. tuberculosis* multiplies, it breaks down the palmitate and liberates radio-labeled CO_2. *M. tuberculosis* growth can be detected in 9-16 days through the BACTEC system, in contrast of using conventional media which takes 4-6 weeks (Chien *et al*., 2000; Todar, 2005).

More recently, a polymerase chain reaction (PCR) assay was used for the rapid detection of *M. tuberculosis* in sputum samples. The most commonly target DNA is a 123 bp segment of IS6110, which is repeated in the *M. tuberculosis* chromosome and is specific for the *M. tuberculosis* complex. The PCR assay involves several steps which include lyse the mycobacteria, extraction the DNA, and amplification the 123-bp target DNA. This assay provides a sensitive and specific means for the laboratory diagnosis of TB within 48 hour that is relatively simple to perform (Eisenach *et al*., 1991). One of the TB detection kits currently available in this country is EZ TB Amp PCR Detection Kit (MBDr, Malaysia). Due to the high sensitivity and short time needed to detect the presence of the bacteria in a clinical sample, this kit can help rapidly identify TB patients and thus help to slow down the spread of the disease (online at: http://www.informm.usm.my/index.php?option=com_content&task=view&id=88&Itemid=126).

TB infection also can be detected using immunological approach such as tuberculin skin test. This particular *in vivo* test is commonly used for identifying latent infection of *M. tuberculosis*. Using protein purified derivatives (PPD) as a tuberculin, PPD is administered intracutaneously to the subject. If the injected person has been infected with TB in the past, sensitisized T cells react against this protein and produce a delay hypersensitivity

reaction within 48 hours. This reaction appears as a reddening of the area around the injected site (Tortora *et al.,* 1997; Lee & Holzman, 2002; Frieden *et al.,* 2003).

1.2 Immunity to TB

Immunity against *M. tuberculosis* infection is closely related with innate and adaptive immunity. Primary cell such as dendritic cell and macrophages are involved in the innate immunity and play an essential role in the initiation of the adaptive immunity. Both humoral and cell-mediated immune responses are elicited by *M. tuberculosis* infection (Laal *et al*., 1997).

Humoral responses in TB have been studied for several decades, primarily for the purpose of developing serodiagnostic assays (Laal *et al*., 1997). It is acknowledged that TB patients produce antibodies to more than one proteinaceous antigen. The humoral responses exist in TB patients, depending upon the disease stage, the patient's immunological background, the antituberculous therapy, and/or the differential gene expression of different strains of *M. tuberculosis* (Julian *et al*., 2004).

Cellular-mediated immune response is the major protective immune response more eagerly than humoral responses (Kaufmann, 1995; Feng *et al.,* 1999). Macrophages and T-lymphocytes are important properties in cellular mediated immunity. Exclusion of *M. tuberculosis* infection mainly depends on the success of the interaction between infected macrophages and T lymphocytes. Acquired immunodeficiencies, especially HIV infection, have significantly shown the importance of cellular immunity in TB. $CD4^+$ T-cell affect their protective effect by the production of cytokines, mainly gamma interferon (IFN-γ) after stimulation with mycobacterial antigens. $CD8^+$ T cells are other T cell subset, which

contribute as well, by secreting cytokines and lysing infected cells (Stenger & Modlin, 1999; Geluk *et al.,* 2000).

Macrophages are the major component in immune system and play a multiplicity of roles in *M. tuberculosis* infection. The organism can grow inside resting macrophages but can be inhibited or killed when the macrophage is activated (Flynn, 2004). The role of macrophages including antigen processing and presentation and effector cell functions (Adams & Hamilton, 1992). The interaction between infected macrophages or dendritic cells and T lymphocytes is a main key in elimination of *M. tuberculosis* infection. Mycobacterial antigens presentation by macrophages and dendritic cells elicit T-cells response in the context of major histocompatibility complex (MHC). MHC molecules include class I and class II for the presentation of mycobacterial antigens (Flynn, 2004). After processing mycobacterial antigen in phagolysosomal compartments of professional antigen-presenting cells (APC), MHC class II molecules present these mycobacterial proteins to antigen specific $CD4^+$T-cells. In contrast with MHC class II, MHC class I molecules present mycobacterial proteins to antigen-specific $CD8^+$T-cells, which then lead to the terminating of infected cells upon peptide-MHC recognition through cytolytic function of cytotoxic T-lymphocytes (CTL) by releasing cytotoxic molecules, perforin which induce apoptosis in infected cells (Esser *et al*., 2003).

The major cell population in cell-mediated immunity in TB is the $CD4^+$ T-lymphocytes. $CD4^+$ T-cell express the α/ß T-cell receptor, and they involved in recognition of antigens that has been processed in phagosomes and presented as small fragment in the context of MHC class II molecules on the surface of antigen presenting cell such as macrophages or dendritic cells. $CD4^+$ T-cells help to amplify the host immune response by activating effector cells and taking on other immune cells of the site of disease (Berman *et al.*, 1996;

Boom, 1996). Many studies of animal models of TB have suggested that both $CD4^+$ and $CD8^+$ T-cells have a protective role in *M. tuberculosis* infection (Flynn *et al.*, 1995; Coler *et al*., 2001). Within one week of infection with virulent *M. tuberculosis*, the number of activated $CD4^+$ and $CD8^+$T-cells in the lung-draining lymph nodes had increased. This finding indicated that activated T-cells had migrated to the site of infection and had interacted with APC (Serbina *et al*., 2000).

$CD4^+$ T-cells can be divided into two subsets of effector $CD4^+$ T-lymphocytes, called T helper type 1 (Th1) and type 2 (Th2) respectively, which were characterized by their cytokines production. Th1 cells were characterized by the production of interferon gamma (IFN-γ), tumor necrosis factor-α (TNF-α) and IL-2, whereas Th2 cells produce cytokines such as IL-4, IL-5 and IL-10 (Barnes, 1993). Immunity to *M. tuberculosis* is dependent on the generation of Th1 type cellular immune response. Th1 type cytokine seem to be essential for protective immunity against TB. IFN-γ is the key activating agents that triggers the antimycobacterial effects (Rook *et al.,* 1986; Flesch & Kaufmann, 1990). IFN-γ play an important role to activate macrophages, followed by killing the mycobacterial during phagocytosis. Bacteria that are not killed by this process have their multiplication inhibited inside IFN-γ activated macrophages. An additional function is then handed out by cytotoxic $CD8^+$ T cells which release intracellular bacteria from infected cell, so that they can be killed during phagocytosis by activated macrophages (De Lebiro *et al.,* 1988).

IL-12 production may be an important regulator of T-cell phenotypes in TB and as a crucial cytokines in controlling *M. tuberculosis* infection. Flynn and collegues (1995) showed that the exogenous administration of IL-12 into BALB/c mice can improve survival from *M. tuberculosis* infection. Zhang *et al.,* 1994 has demonstrated that cytokines production in pleural fluid from patients with tuberculosis pleurisy and found high level of IL-12 after

stimulation of pleural fluid cells with *M. tuberculosis*. Human with recessive mutation in IL-12p40 are more susceptible to mycobacterial infection (Ottenhof *et al*., 1998). IL-12 is known to induce a Th1-type response in undifferentiated $CD4^+$ cells (Lin *et al.,* 1996). IL-12 is induced subsequent phagocytosis of *M. tuberculosis* bacilli by macrophages and dendritic cells which drives enlargement of a Th1 response with the production of IFN-γ.

IFN-γ, which is the major macrophages activating cytokines and other Th1 cytokines are essential as stated previously. It is already demonstrated that T-cells function in direct killing of mycobacterial and lyse infected macrophages. Macrophage lysis appears to be the qualification for killing by T-cells of microbes living inside macrophages. Furthermore, lysis of infected macrophages could promote release of mycobacteria from inability macrophages to other competent monocytes (Silva *et a*l., 2001).

1.3 TB vaccine development

1.3.1 Failure in BCG vaccination

Bacille Calmette-Guerin (BCG) vaccine is an attenuated strain of *M. bovis* applied in 1921 in France by Albert Calmette and Camille Guerin as a vaccine against TB. The mechanism of protection from BCG vaccination involves a reduction of the haematogenous spread of bacilli from the site of primary infection. It also protects against acute symptom of the disease and reduces a lifelong risk of endogenous reactivation and propagation from prior infection (Luelmo, 1982; Arbelaez *et al.,* 2000).

The efficacy of BCG vaccination, however, has been strongly issued. Multiple studies have been conducted to evaluate BCG vaccine efficacy as a preventive measure against TB.

Although vaccination with BCG is widely practiced worldwide, the efficacy is ranging from 0 % to 85 %, among different clinical trials and geographically distinct populations. Baily (1980) has demonstrated through his study that BCG vaccine was found to offer no protection to individual over the age of 12-years among Tamil Nadu population in South India. A current review of all previous controlled clinical trials concluded that vaccination with BCG reduced the overall risk of tuberculosis by approximately 50 % (Colditz *et al.,* 1994).

The reasons for the different effect of BCG vaccination are subjected to differences of substrains of the vaccine used in different trials and also due to differences dose and vaccination schedules. The difference substrains used including Glaxo, Danish, Paris, Montreal, Phipps, Tice, Birkhaug and Madras (Chan & Kaufmann, 1994). Furthermore, genetics differences in human population also contribute to the efficacy of BCG vaccination. Genetic factors are important in determining the host susceptibility to infection against mycobacteria (Bellamy & Hill, 1998).

In addition, BCG is less effective at preventing late reactivation and pulmonary TB, thus, BCG vaccination has not contributed to control the spread of TB (Kristensen *et al.*, 2000). Furthermore, the effectiveness of early BCG vaccination in preventing TB among adult could be modified by the presence of HIV infection. The immunosuppression produced by HIV infection could alter the response to vaccine and increase the risk of mycobacterial infection (Arbelaez *et al.,* 2000).

Due to the less effectiveness of BCG vaccination, there is an urgent need to develop a better or improved TB vaccine as an alternative to BCG.

1.3.2 Features of an ideal vaccine

In order to develop an ideal vaccine candidate in preventing TB infection, there are some characteristics that should be considered. The vaccine candidate should be safe to be used in newborns, adults and immunosuppressed person (including the HIV-infected person). An ideal vaccines should have an ability to induce both humoral and cellular immune response in newborns and adults, provide long lasting immunity, immunogenic in HIV infection, protective against primary disease including pulmonary and meningeal TB, effective against reactivation disease, useful in person with previous BCG immunization and definitely low cost, easy to manufacture and administer (von Reyn & Vuola, 2002; McShane, 2004).

1.3.3 New approaches in TB vaccine development

Several approaches have been used in the development of TB vaccine, including DNA vaccination, subunit vaccine, recombinant BCG and recombinant microbial vaccine.

1.3.3.1 Subunit vaccine

Since the current whole cell vaccine for TB did not show hopeful result, researchers are now targeting individual protein fraction of the BCG or *M. tuberculosis* either alone or in combination to boost the immune system (Kumar *et al.,* 2003). The advantages of subunit vaccine delivered as protein or DNA or non-replicating viral vector include their safety profile and the lack of tissue damage or scarring (Britton & Palendira, 2003). Subunit vaccine is considered as a booster on top of a BCG vaccination. These subunit vaccine

candidates therefore need to perform better only when given as a booster after BCG priming (Baumann *et al.,* 2006).

1.3.3.2 DNA vaccine

DNA vaccination is an effortless method to generate both humoral and cellular immune responses. DNA vaccine can stimulate both exogenous (MHC-class II restricted) and endogenous (MHC-class I restricted) antigens presentation pathways (Huygen, 2003). Both antibodies and cell-mediated immune responses have been induced by direct intramuscular inoculation with plasmid DNA encoding viral proteins. These responses have been found to be protective in various animal models of viral diseases. Immunization by direct transfection *in vivo* with plasmid DNA will elicit $CD8^+$ cytotoxic T lymphocytes (CTL). These CTL recognize peptides 9-11 amino acids in length bound to Class I molecules of the MHC. Therefore, protein antigens produced by DNA vaccination put on access to pathways of antigen presentation via Class I MHC molecules, a system of antigen presentation that is most frequently utilize by live attenuated virus vaccines, recombinant viral vectors, and intracellular bacteria such as *Listeria* or *Shigella* (Donnelly *et al.,* 1997).

The mechanism of action of plasmid DNA vaccine is illustrated in Figure 1.1. DNA vaccine is constructed by inserting the most promising antigens under strong promoters in an expression vector. Then, the DNA vaccine is administered via intramuscularly route. This technique involves the preparation of pure DNA which is less expensive than pure protein, make it very cost effective and appealing.

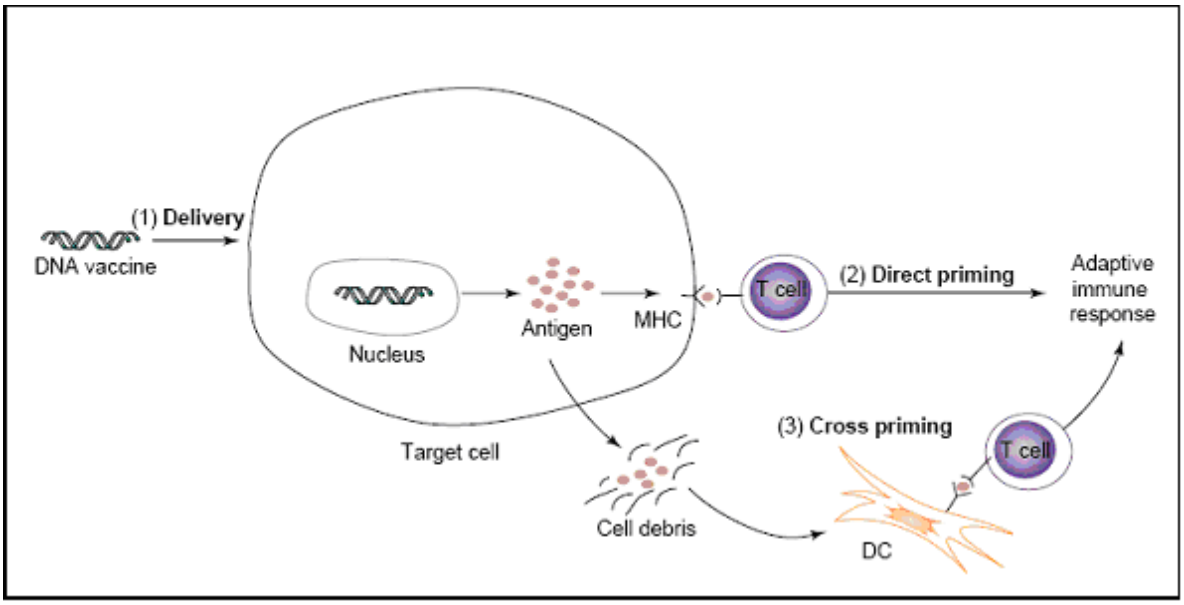


Figure 1.1: Mechanism of action of plasmid DNA vaccines. (1) DNA uptake by cells can be facile (for naked DNA) or facilitated (using delivery vehicles). (2) When expressed by antigen presenting cells (APCs), the antigens can be processed and presented by major histocompatibility complex (MHC) molecules directly to naive T cells (direct priming). (3)Alternatively, the antigens that are released from transfected cells (e.g. by apoptosis) can be internalized by 'bystander' APCs such as dendritic cells (DCs) for presentation by MHC molecules (cross priming)(adapted from Ulmer *et al.*, 2006).

DNA vaccine candidates expressing selected *M. tuberculosis* epitopes have been tested in animal models and have shown to be immunogenic and also provide some protection against infection (Huygen *et al.,* 1996; Kamath *et al.,* 1999; Lowrie *et al*.,1999).

1.3.3.3 Recombinant BCG

Recombinant BCG (rBCG) is one of approaches in vaccine development strategies. The advantages of using BCG as a platform for new TB vaccine are the low cost, safe and widespreadly used (Huebner, 1996). The genes encoding immunodominant antigens have been inserted into BCG to increase its efficacy. Recent studies have demonstrated that rBCG expressing protective viral or bacterial antigens may induce strong cellular immune response and under some condition good antibody responses (Dhar *et al*., 2000). Recent study by Rapeah and Norazmi (2006) had shown recombinant BCG expressing the malarial epitopes F2R(II)EBA and $(NANP)_3$ as well as two T cell epitopes of the *M. tuberculosis* ESAT-6 antigen, elicited specific immune response against these epitopes. Flow cytometric analyses showed elevated numbers of $CD4^+$ lymphocytes expressing IFN-γ and IL-2 against the ESAT-6 peptides, suggesting a specific Th1-mediated response. Currently, another advanced candidate has been developed, so called rBCGΔureC:hly, which express the listeriolysin molecule (Hly) of *Listeria monocytogenes* has increased the immunogenecity of this vaccine (Grode *et al.*, 2005).

1.3.3.4 Recombinant microbial vaccine

Recombinant modified vaccinia virus Ankara (MVA) is an example of recombinant microbial vaccine. MVA is an attenuated strain of vaccinia virus which has an excellent safety record, as it was used in the final stages of the smallpox eradication. MVA

expressing a major secreted antigens from *M. tuberculosis*, antigen 85A, was the first new tuberculosis vaccine to enter into clinical trials in September 2002. This vaccine is known as MVA85A. In a series of phase I clinical trials in the United Kingdom, MVA85A had an excellent safety profile and was highly immunogenic (Ibanga *et al.*, 2006). MVA85A is a new-generation vaccine against TB which produced higher levels of long-lasting cellular immunity as it used as a booster in BCG priming (McShane *et al.,* 2004)

1.3.4 Mycobacterial epitopes as an antigen and its potential

The genome of *M. tuberculosis* has been sequenced earlier (Cole *et al.,* 1998). The availability of the genome sequence has enabled further characteristics of the mycobacterial protein to identify the suitable gene that can stimulate immune response against TB. A number of immunodominant antigens (e.g ESAT-6, Ag85B and Mtb8.4) are recognized by infected subject from different ethnic background. Both $CD4^+$ and $CD8^+$ T cell epitopes have been identified on these proteins and may be used to evaluate host response to the vaccine incorporating the antigens (Britton & Palendira, 2003).

1.3.4.1 6 kDa Early Secretory antigenic target (ESAT-6)

ESAT-6 is a low mass culture filtrate with highly immunoreactive properties (Brandt *et al.,* 1996; Andersen, 2001). The major T-cell epitope on this molecule is recognized by a high frequency of T-cell during the recall of immunity to TB. The ESAT-6 gene is present in *M. tuberculosis* and virulent *M. bovis* but not in *M. bovis* BCG vaccine strain (Harboe *et al.*, 1996; Behr *et al.*, 1999; Bao *et al.*, 2003). ESAT-6 antigen is considered to play a protective role as a target of protective immune response (Kamath *et al.*, 1999; Brandt *et al.*, 2000, Andersen, 2001).

1.3.4.2 Antigen 85 complex

The proteins of the antigen 85 (Ag85) complexes are the major secretion product of *M. tuberculosis* and *M. bovis* (Wiker & Harboe, 1992). The Ag85 complex (A, B and C) consists of a family of 30-32 kDa protein that acts as mycocyl transferases (Belisle *et al.,* 1997). Ag85 complex induces strong T-cell proliferation and IFN-γ production in most healthy individuals infected with *M. tuberculosis* or *M. leprae* and in BCG-vaccinated mice and human. Tanghe *et al.* (2000) had demonstrated that vaccination with plasmid DNA encoding Ag85A resulted in a strong humoral and cellular immune response and gave important protection in C57BL/6 mice challenged by aerosol with live *M. tuberculosis* H37Rv. The three immunodominant epitopes of the 30, 32 and 16 kDa major extracellular proteins of *M. tuberculosis* had been identified by Lee & Horwitz in 1999. Proliferative response assays from outbred guinea pig which was immunized with each of the three proteins showed that 30 kDa (known as Ag85B) is the most immunogenic of the three proteins tested.

1.3.4.3 Mtb8.4

Mtb8.4 is a low molecular weight of *M. tuberculosis* culture filtrate protein (estimated molecular weight was 8.4 kDa) which is an immunodominant T cell antigen. Immunogenecity study in mice has demonstrated that Mtb8.4 elicits Th1 cytokines profile, which leads to IFN-γ production which is important for protective immunity against TB. In addition, Mtb8.4 was reported to elicit proliferation and production of IFN-γ from human peripheral blood mononuclear cell (PBMC) of PPD^{+} healthy individuals (Coler *et al.,* 1998, Coler *et al*., 2001).

1.4 Aim of the study

As described previously, BCG is the only vaccine available against TB, which is routinely practiced in many countries. However, due to the differentiation and limitation of BCG vaccination, there is an urgent need for another potential vaccine candidate. Therefore, we aimed to construct a new multi-epitopes synthetic DNA vaccine candidate expressing the most promising mycobacterial antigens of *M. tuberculosis* as an alternative vaccine against TB. The specific objective of this study as follows:

1. To synthesis a multi synthetic gene (VacIV) of *M. tuberculosis*
2. To study the expression of the cloned gene (VacIV)
3. To construct a DNA vaccine candidate (pVaxVacIV).
4. To determine the immunogenicity of DNA vaccine candidate in C57BL/6 mice

Firstly, we aim to synthesis a VacIV gene encoding Mtb8.4, Ag85A (32 kDa) and Ag85B (30 kDa) gene of *M. tuberculosis* by assembly polymerase chain reaction (PCR). Then, the gene will be cloned into pCR®2.1 TOPO cloning vector to construct a recombinant plasmid namely pTOPOVacIV for DNA sequencing analysis, restriction enzyme analysis and screening by PCR. As we get the positive clone, the VacIV gene will be removed from pTOPOVacIV and cloned into pVAX1© eukaryotic expression vector to produce a DNA vaccine candidate namely pVaxVacIV. In addition, the VacIV gene will be cloned into pPROEX™ HTb prokaryotic expression vector for expression study in *E. coli* and further purified of recombinant VacIV protein. The DNA vaccine candidate will be tested in C57BL/6 mice for immunogenicity study which involved measurement of IFN-γ and IL-4 cytokines, lymphocytes proliferation assay and determination of IgG subclasses. The flowchart of the overall study is illustrated in Figure 1.2.

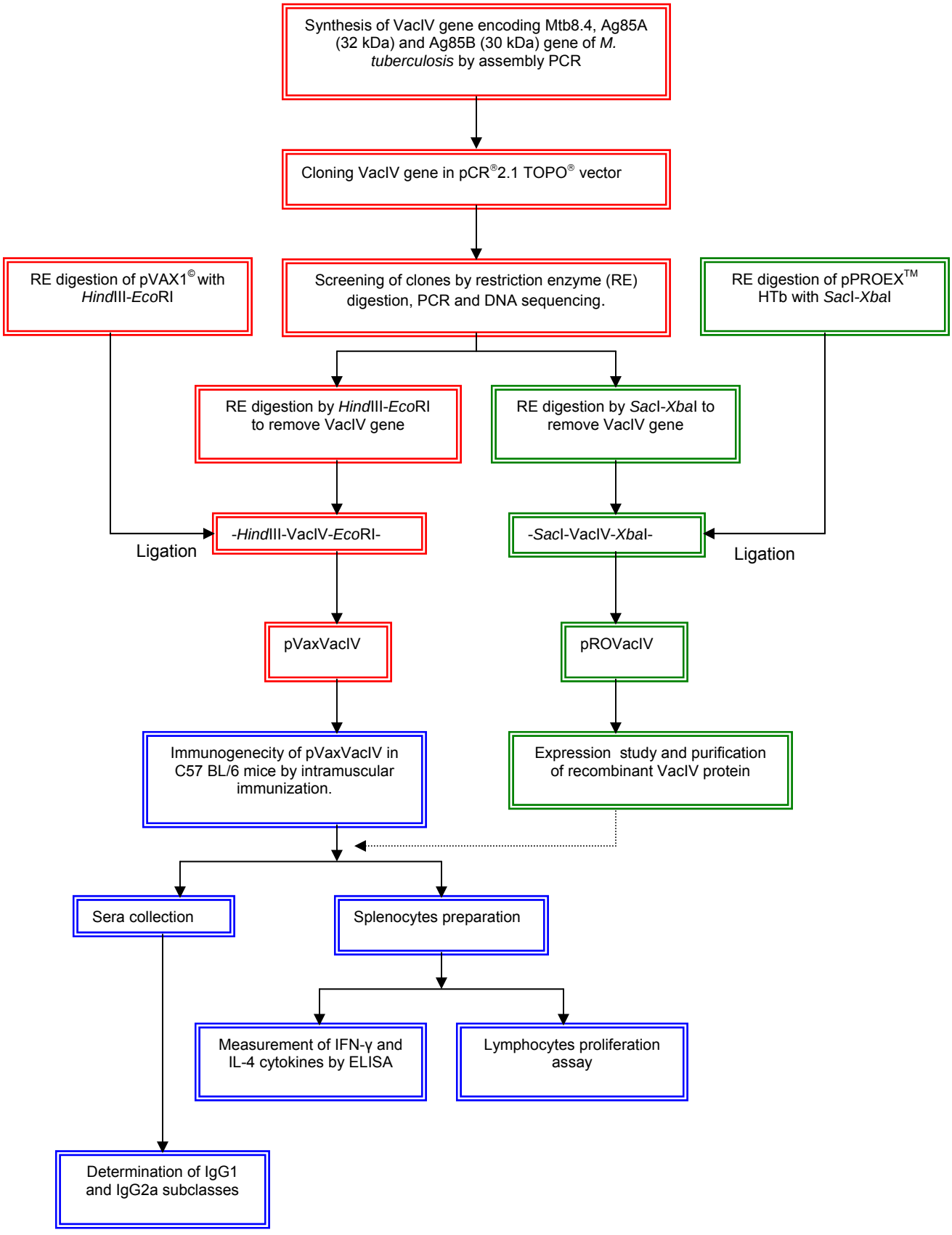


Figure 1.2: The flowchart of the overall of this study

CHAPTER TWO

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Mice

Female mice strain C57BL/6 at 6-8 week-old were used in this study were obtained from Animal House of USM Health Campus, Kelantan. Animal care and experimentation procedures have been approved by the Animal Ethics Committee, USM.

2.1.2 Bacterial strains

Two different strains of *Escherichia coli* (*E. coli*) which are TOP 10 and DH5α from Invitrogen (USA) were used in this study. The bacterial cells were grown in Luria-bertani (LB) broth and LB-agar. The bacteria were stored in glycerol stock at –80^{o}C.

2.1.3 Oligonucleotides and primers

Thirty five overlapping nucleotides and primers for assembly PCR used in this study were manufactured by MWG (Germany) which purchased through Research Instruments Sdn. Bhd. Malaysia. All oligonucleotides and primers were resuspended overnight at 4^{o}C by adding an appropriate amount of distilled deionized water to make a stock of 250 μM. The primers were diluted to make a 10 μM of working stock solution. All oligonucleotides and primers were stored at –20^{o}C.

2.1.4 Plasmids

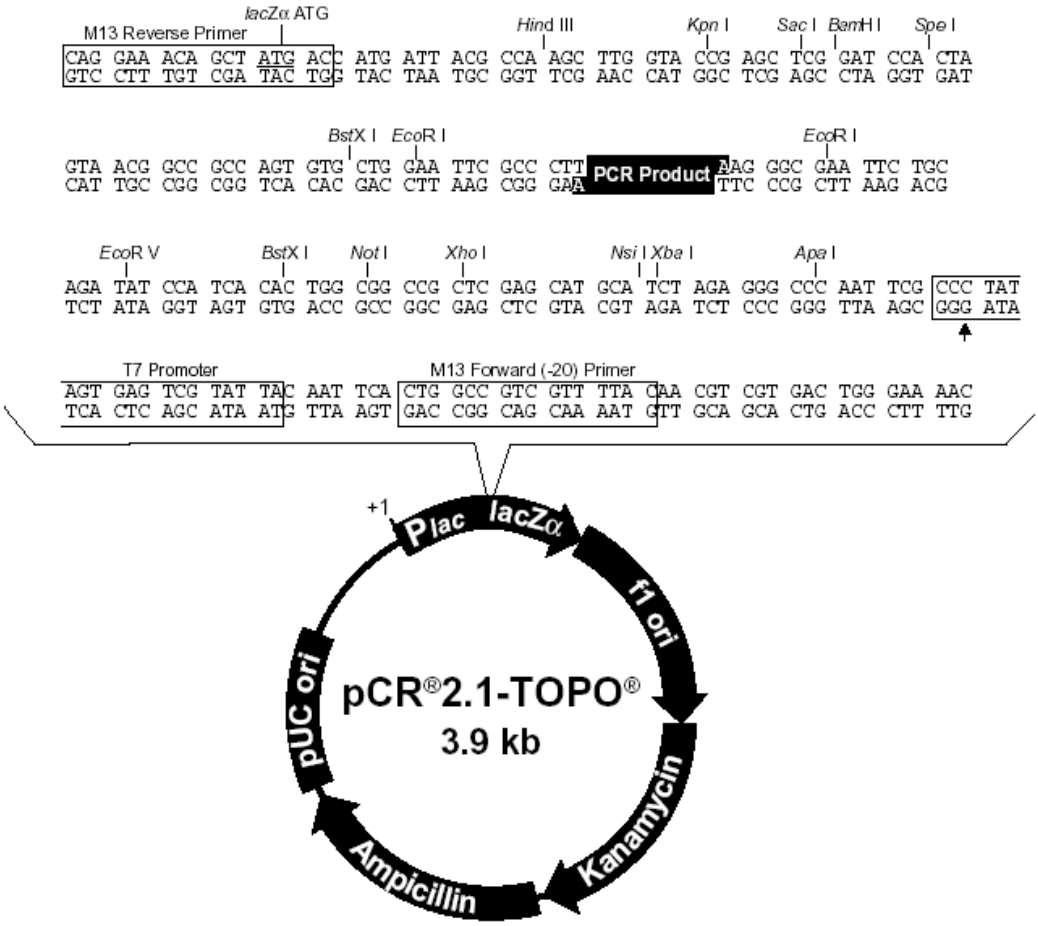
The plasmids used in this study were pCR®2.1 TOPO cloning vector (Figure 2.1a), pVAX1© eukaryotic expression vector (Figure 2.1b) and pPROEX™HTb prokaryotic expression vector (Figure 2.1c). All plasmids were purchased from Invitrogen (USA).

2.1.5 Chemicals and reagents

All chemicals, reagents and enzymes (restriction enzymes, DNA polymerase and DNA ligase) used in this study are listed in Table 2.1.

2.1.6 Kits and laboratory equipments

All kits and equipment used in this study are listed in Table 2.2 and 2.3.



Comments for pCR®2.1-TOPO®
3931 nucleotides

*Lac*Zα fragment: bases 1-547
M13 reverse priming site: bases 205-221
Multiple cloning site: bases 234-357
T7 promoter/priming site: bases 364-383
M13 Forward (-20) priming site: bases 391-406
f1 origin: bases 548-985
Kanamycin resistance ORF: bases 1319-2113
Ampicillin resistance ORF: bases 2131-2991
pUC origin: bases 3136-3809

Figure 2.1a: The map of pCR®2.1 TOPO® cloning vector (adapted from http://www.invitrogen.com/content/sfs/vector/pcr2_1topo_map.pdf.)