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# "Cloning of VacII into pNMN013: Development of a Recombinant BCG vaccine against Tuberculosis"

Dissertation submitted in partial fulfillment for the
**Degree of Bachelor of Science (Health) in Biomedicine**


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# CERTIFICATE

This is to certify that the dissertation entitled

**"Cloning of VacII into pNMN013: Development of recombinant BCG vaccine against Tuberculosis"**

is the bona fide record of research work done by

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# ABSTRACT

In previous study, the VacII gene has been constructed through the technique of assembly PCR and has been cloned into pKK to produce surface display vaccine candidate (pTMSInakVacII). VacII gene is a synthetic gene consists of selected T cell epitopes of *Mycobacterium tuberculosis* (MTB) genes namely ESAT6, MTP40, 38kD and MPT64.

In this study, VacII gene was cloned into pNMN013 for development of a recombinant BCG vaccine. PCR was used for the amplification of the gene DNA from pTMSInakVacII. The PCR products (VacII gene) were visualized directly on agarose gel. The PCR product was first cloned into pTOPO vector. The PCR product were also digested with *Nhe*I and *Nde*I and cloned into *Nhe*I and *Nde*I digested pNMN013. The plasmids were transformed into *E.coli*. There were some colonies grown on the agar plate.

Finally, the colonies were screened using PCR technique and restriction enzyme analysis. But after the screening procedures were done no expected recombinant plasmid were obtained.

# INTRODUCTION

The acid–fast bacillus *Mycobacterium tuberculosis* is the causative agent of tuberculosis (TB). Humans are the natural host for this pathogen, which is transmitted via the respiratory route. Tuberculosis is easily spread from one person to another person through the air by coughing, sneezing, laughing and even talking. The group that at high risk are health workers who work in long term care of the infected persons, intravenous drug users, people in close contact with infected active tuberculosis and in HIV/AIDS patients(Ramaswamy and Musser,1998).

This bacteria can affect several organs includes lung, brain, kidney, bone and other organs. Strains of *M.tuberculosis* resistant to antimycobacterial agents are recovered from immunocompetent and immunocompromised patients' worldwide. This infection is the leading cause of mortality in adults due to an infectious agents and account for 26 percent of all preventable adult deaths globally (Ramaswamy and Musser,1998).There are approximately 8 million new cases of tuberculosis worldwide and at least 1.5 million deaths per year.

Increasing trends can also be seen in Malaysia. In the early 1940s and 1950s, tuberculosis (TB) was the number one cause of death in Malaysia. Patients with TB were admitted to the many sanatoria in various parts of the country and were often managed by surgical means. TB chemotherapy became available only in the late 1950s. At this time, TB was already a major cause of morbidity and mortality.

Realizing its seriousness, the Malaysian government launched its National TB Control Programme (NTP) in 1961. At that time, the recommended treatment for TB was a combination of three drugs, namely, streptomycin, isoniazid and paraaminosalicylic acid (PAS) given for 2 months followed by isoniazid and PAS given for 12 months. Generally the treatment used to last 1–2 years (Kupusamy, 2004).

Over the past 10 years there has been a steady increase in the number of TB notifications. In the year 2000, a total of 15,057 cases of all forms of TB were notified. The incidence rate per 100,000 populations is 64.7. Of these 8154 were smear positive. 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 (Kuppusamy, 2004).

TB will undoubtedly increase in prevalence in most countries due to the human immunodeficiency virus (HIV) pandemic. In alarming statistics and trends, the World Health Organization declared TB to be a global public health emergency. The US centers for Disease Control and Prevention national surveillance system recorded 93 449 TB cases from 1993 through 1996, including 1457 cases of multidrug resistant TB (MDR-TB) (Ramaswamy and Muasser, 1998).

Vaccines against tuberculosis (TB) provide one of the earliest examples of human immunoprophylaxis, since tuberculin was used by Koch over 100 years ago, for treating tuberculosis in humans infected with *Mycobacterium tuberculosis*.

The introduction of BCG, the attenuated strain of *Mycobacterium bovis*, by Calmette and Guerin in 1921, represents the earliest use of a live attenuated vaccine in human medicine (Griffin *et al.*, 2001).

BCG is the most widely used and safest attenuated human vaccine, with more than 1 billion doses used worldwide in the past 80 years. Whereas BCG vaccination has been widely accepted and used in many countries for more than 50 years, controversy concerning its efficacy has emerged, since a systematic evaluation of BCG was carried out by WHO in Chingleput, South India, in the 1970s (Griffin *et al.*, 2001).

Results from this study showed that children vaccinated with BCG produced no discernable protection against TB, compared with non-vaccinated controls. Overall results from individual trials of human BCG vaccination carried out worldwide have shown highly variable levels of protection. The only consistent observation has been that BCG provides significant protection against bacteremic spread of tuberculosis and TB meningitis in children (Griffin *et al.*, 2001).

A number of reasons have been advanced to explain the variations seen in the levels of protection obtained with BCG. These include prior TB infection or exposure to host immunocompetence, associated with malnutrition and environmental stressors and differing levels of ability and immunogenicity of strains of BCG might also have contributed to the inconsistent results. Vaccine may failure also have been due to the way in which the vaccine has been used, rather than due to any deficiency

in the vaccine (Griffin *et al.*, 2001).

Therefore, development of an effective vaccine against tuberculosis is a big task. There are a many approaches in development TB vaccines. The current vaccine, BCG, is live attenuated mycobacteria. Attenuated vaccine is a live microbe but is weakened so that is mimicry an actual infection without causing actual disease. Live vaccines may give long term effectiveness because attenuated microbe replicate in body. But this vaccine is not recommended for people whose immune response is compromised because it will cause actual disease.

One type of new vaccine being developed is the subunit vaccine consisting of proteins or peptides. Subunit vaccines have the ability to enhance immune responses and increases the duration of protection. However, these non-living subunit proteins have still failed to provide better protection than BCG. Furthermore, the subunit proteins are very expensive to produce and require multiple boosters (Norazmi and Mustaffa, 2002).

Another approach in development of vaccine is recombinant BCG. BCG is re-engineered to enhance its efficacy through inserting genes encoding immunodominant antigens or immunostimulatory genes. Some genes in BCG which has been deleted during the attenuation process may re-introduce and re-expressed in the recombinant BCG. The vaccine also would share the safety and low side effects of BCG. Furthermore, it should be inexpensive and easy to mass produce because costly purification and synthesis are not required. It is also noteworthy that

BCG vaccination could protect against leprosy. So, this approach needs to explore for the development of an effective vaccine against TB (Norazmi and Mustaffa, 2002).

# REVIEW OF LITERATURE

The doubtful efficacy of bacille Calmette-Guerin (BCG) immunization against tuberculosis and the spread of the dual epidemic of human immunodeficiency virus (HIV) and tuberculosis has created a renewed global interest in developing improved preventive therapeutic reagent and diagnostic assays against tuberculosis. Furthermore, progressive innovations of molecular biology techniques and quite comprehensive information of *Mycobacterium tuberculosis* also encourage development of new vaccine (Prabhakar *et al.*, 1998).

There are a current trends are seen in developing of vaccination, the first approach involves modifying BCG itself. BCG was developed through passaging a virulent strain of *M.bovis* over a period of almost 11 years. The second approach involves the use of subunit proteins and the more recent format of DNA vaccines. DNA vaccines involve cloning of antigen encoding genes into plasmid constructs that can be expressed in mammalian cells. The DNA vaccines generally induce effective Th1 cellular responses, which are important for protection in tuberculosis (Zodpey *et al.*, 1999).

Morris *et al.*, (2000) documented that a single DNA vaccine encoding both the MPT-63 and MPT-83 tuberculosis antigens evoked partial protection against an aerogenic challenge with *M.tuberculosis* Erdman in the mouse model of pulmonary tuberculosis. Further he showed that immunization with a multivalent combination DNA vaccine containing the ESAT-6, MPT-64, MPT-63 and KatG constructs elicited

a strong protective response relative to the protection evoked by conventional BCG vaccine.

DNA vaccine involves direct injection of plasmid DNA encoding specific antigens that can led to protective immunity (Davis *et al.*, 1993; Donnelly *et al.*, 1994;Cohen and Steinman, 1997).

According to Murray *et al.*, (1996) and Wangoo *et al.*, (2000), recombinant BCG expressing various cytokines such as interleukin-2, interferon gamma, or granulocyte-macrophage colony-stimulating factor have been shown to improve the response against *M. tuberculosis* antigens

Recombinant BCG expressing listeriolysin of *Listeria monocytogenes* showed an enhanced capacity to stimulate CD8+ T cells according to Hess *et al.*, (1998). Horwitz *et al.*, (2000) reported that better protective efficacy of a recombinant BCG expressing an epitope of *M.tuberculosis* compared to the parent stain. Recombinant BCG that over expresses the 30 kDa Ag85 protein has been reported to provide better protection than BCG (Horwitz *et al.*, 2000). Hetzel *et al.*, (2000) have constructed recombinant mycobacteria by inserting specific foreign epitopes into the superoxide dismutase gene of the host.

## Lacuna in literature

Since BCG vaccine is not reliable, new vaccine against tuberculosis is urgently needed. Recombinant BCG approach for the development TB vaccine could be explored.

# OBJECTIVE OF THE STUDY

The aim of this research project is to clone VacII, a synthetic multi-epitope, multi-gene of *Mycobacterium tuberculosis* into pNMN013 for development of a recombinant BCG vaccine against tuberculosis.

# MATERIALS AND METHODS

## MATERIALS

### Plasmids

Plasmid pTMSInakvacII as shown in Figure 1, containing the VacII gene was obtained from Mohamed Sarhan, a PhD student of Associate Professor Dr. Mustaffa Musa, Department of Immunology, School of Medical Sciences, USM. Plasmid pNMN013 was obtained from research group Professor Dr. Norazmi Mohd. Nor. The map of pNMN013 is shown in Figure 2.

### Primers

Primers were obtained from MWG Biotech (Germany). The sequences of primers used in this research project are:

VacIINhe1 (Forward) CTCTGCTAGCATGACAGAGCAGCAG

VacIINDE1 (Reverse) CTATCATATGTTAATGATGGTGGTG

### Bacterial strain

*E.coli* host strain TOP10 was obtained from the culture collection of the Molecular biology research laboratory, School of Medical Sciences, USM.

**Ampicillin stock solution 50mg/ml**

500mg of Ampicillin powder was dissolved in 10ml sterile $dH_2O$ and sterilized by filtration. The solution was aliquoted into sterile tubes and kept in 4°C.

**Kanamycin stock solution 100mg/ml**

1000mg of Kanamycin powder was dissolved in 10ml sterile $dH_2O$ and sterilized by filtration. The solution was aliquoted into sterile tubes and kept in 4°C.

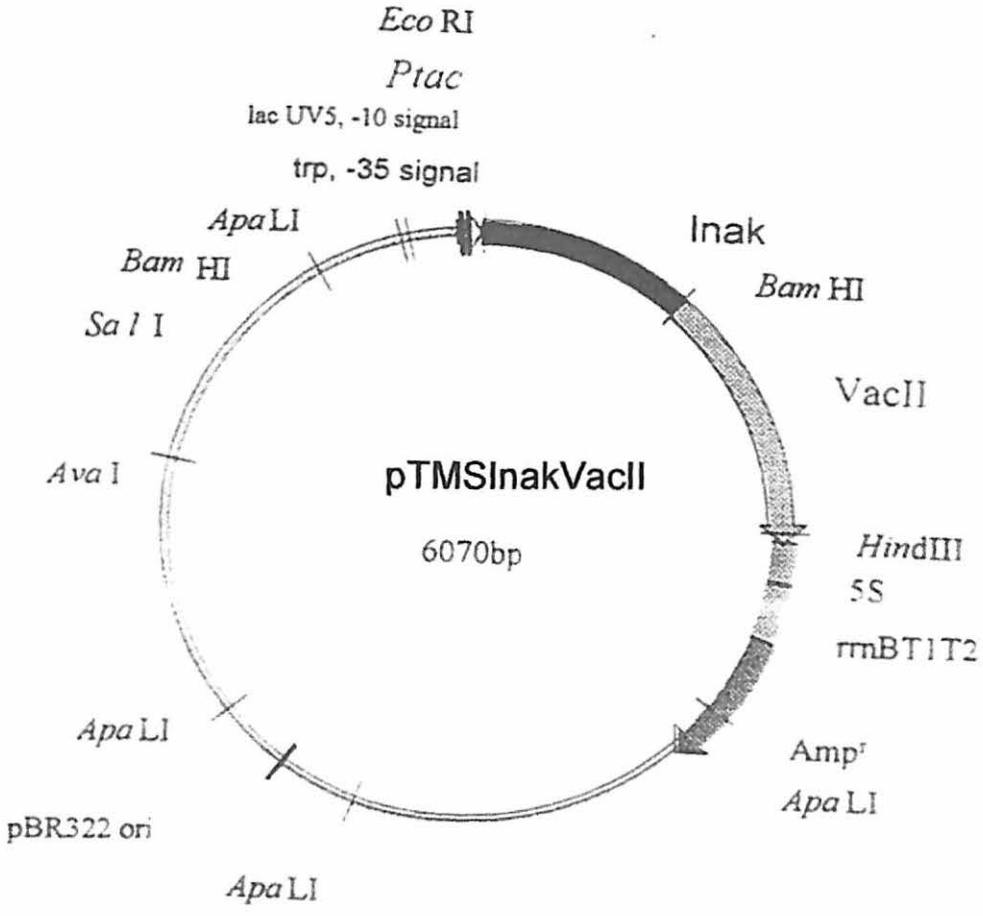


**Figure 1: pTMSInakVacII**

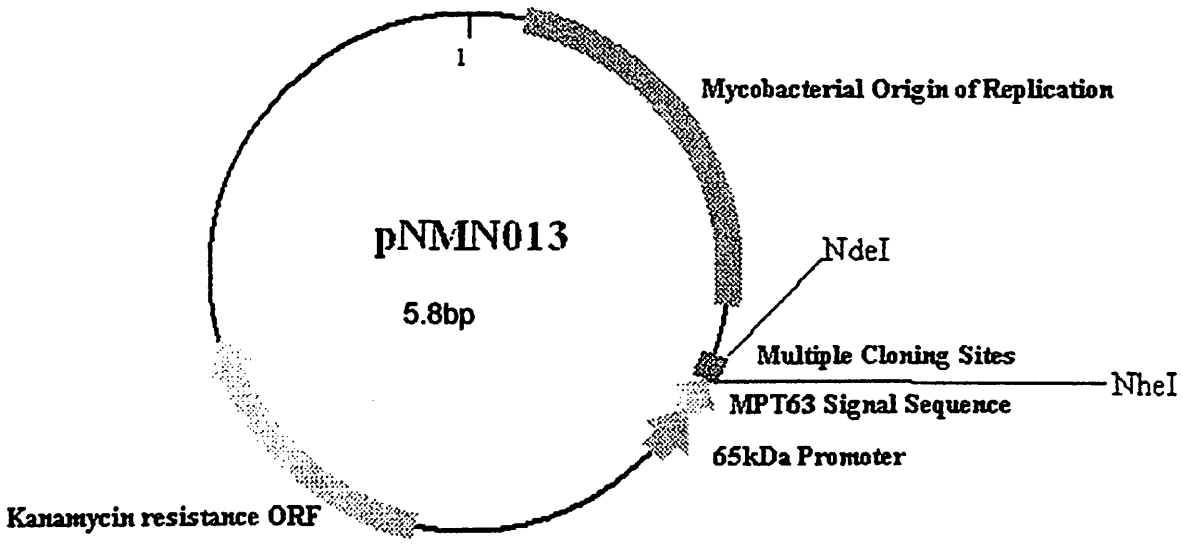


**Figure 2: pNMN013 Vector**

## Media

### Luria- Betani Broth and Agar

| | |
|---|---|
| Tryptone | 5.0g |
| Yeast extracts | 2.5g |
| $NaCl_2$ | 5.0g |
| $dH_2O$ | 500ml |

All ingredients were dissolved in 500ml $dH_2O$ and mixed thoroughly using a magnetic bar. The mixture then adjusted to increase pH 7.2 using NaOH. After that, the mixture was autoclaved at 121°C in 15 minutes and aliquoted into sterile universal tubes. To make LB broth Ampicillin (50µg/ml), the broth is cooled to 50°C before adding 500µl of the stock Ampicillin solution (50mg/ml).To make broth contain Kanamycin (50µg/ml), the broth is adding with 250µl of the stock Kanamycin solution (100mg/ml).

To make LB agar, all ingredients are the same as the LB broth but 7.5g bacteriological agar was added to a final concentration. The mixture was autoclaved at 121°C in 15 minutes and cooled to 50°C.After that, antibiotic whether Ampicillin or Kanamycin was added to the mixture before pouring out into sterile petri dishes.

**10X stock solution of Tris-Borate (TBE) buffer**

| | |
|---|---|
| Tris- base | 108g |
| Boric acid | 55g |
| 0.5M EDTA pH 8.0 | 40ml |
| Or disodium EDTA.$H_2O$ | 9.3g |

All ingredients were mixed together and autoclaved at 121°C for 15 minutes. To prepare 1000ml of 0.5X TBE buffer, 50ml 10X stock solution will be diluted with 950ml $dH_2O$.

**Loading buffer**

| | |
|---|---|
| Glycerol (BDH chemicals) | 3ml |
| Bromophenol Blue (Sigma, USA) | 0.03g |

The ingredients were mixed together with deionized distilled water ($ddH_2O$) to the final volume of 10ml. The mixture was kept in a bottle covered with aluminium and was kept at 4°C.

**Ethidium bromide ($EtBr_2$) solution (10mg/ml)**

1g ethidium Bromide (Sigma, USA) was diluted with 10 ml $ddH_2O$ and mixed until completely dissolved. Then, the mixture was kept in dark bottle.

**λ HindIII marker (50µg/µl)**

| | |
|---|---|
| λ HindIII 0.5mg/µl | 10µl |
| Loading Buffer | 80µl |
| 1X Tris-EDTA | 10µl |

All ingredients were mixed and kept at 4°C.

**100bp Ladder (100µg/µl)**

| | |
|---|---|
| 100bp DNA ladder (0.5mg/ml) | 20µl |
| Loading buffer | 20µl |
| 1X Tris-EDTA | 60µl |

All ingredients were mixed together and kept at 4°C.

**10mM $MgCl_2.6H_2O$**

0.203g 0f $MgCl_2.6H_2O$ was dissolved in 100ml of sterile distilled water. The $MgCl_2.6H_2O$ solution was poured in one bottle and then autoclaved at 121°C for 15 minutes. After that, the bottle was allowed to cool and finally was kept at -20°C.

**100mM $CaCl_2.2H_2O$**

1.4702g 0f $CaCl_2.2H_2O$ was dissolved in 100ml of sterile distilled water. The $CaCl_2.2H_2O$ solution was poured in one bottle and then autoclaved at 121°C for 15 minutes. After that, the bottle was allowed to cool and finally was kept at -20°C

# METHODS

## Agarose Gel Electrophoresis

### Preparation of 0.8% Agarose Gel

0.24g of LE agarose (Promega) was mixed with 30ml of 0.5X TBE buffer and was heated in a microwave oven for approximately 2 minutes or until all ingredients were completely dissolved. The mixtures were then cooled using running tap water. After that, 0.5 µl of 10mg/ml ethidium bromide was added. The solution was poured into a casting tray that assembled with comb. The gel was left about 15 minutes to let gel cold and firm.

### Running Agarose Gel Electrophoresis

The solidified gel was placed into the running tank. 0.5X TBE was placed into the tank until the gel was flooded. Then, 10 µl of each samples are mixed with 2 µl of loading buffer and then placed into each well. After that, 1 µl of 100bp marker and λ HindIII marker were loaded into another well. Electrophoresis was run at 50V for one hour. The DNA in gel was visualized using an image analyzer.

**Calculation of Annealing Temperature**

Annealing temperature was calculated using the formula below:

Melting temperature (Tm) = 2(A + T) + 4 (C + G)

Annealing Temperature =Tm - 5°C (range 2-5°C)

VacIINhe1 (Forward primer) 5' CTCTGCTAGCATGACAGAGCAGCAG 3'

A=6, T=1, C=3, G=5 Tm= 2(6+1) + 4(3+5)

=46°C

Annealing Temperature =46°C - 5°C

=41°C

VacIINDE1 (Reverse primer) 5' CTATCATATGTTAATGATGGTGGTG 3'

A=3, T=6, C=0, G=6 Tm= 2(3+6) + 4(0+6)

=42°C

Annealing Temperature =42°C - 5°C

=37°C

Nhe1 and Nde1 sites are not include for annealing temperature calculation because these region do not bind to the template DNA.

**PCR Reaction**

Reagents:

| | |
|---|---|
| $ddH_2O$ | 25.0 µl |
| MPOR Buffer 1-Buffer 6 | 5.0 µl |
| Forward primer(10 µm) | 1.0 µl |
| Reverse primer(10 µm) | 1.0 µl |
| GC Normalizer | 10.0µl |
| DMSO | 2.5µl |
| dNTPs (3.125mM) | 4.0µl |
| Taq Pfu polymerase | 1.0µl |
| DNA | 1.0µl |
| Total | 50.0µl |

Polymerase chain reaction (PCR) was performed with Taq deoxyribonucleic acid (DNA) polymerase according to the manufacturer's instructions (MBI Fermentas).

The PCR reactions was subjected to initial denaturation at 95°C for 1 minute, 30 cycles each of 30 seconds at 95°C, 30 seconds at 59°C, and 30 seconds at 72°C followed by final extension at 72°C for 30 minutes. The PCR products were analyzed on a 0.8% agarose gel and stained with ethidium bromide.

**Gel Purification**

Impure PCR products are caused by smearing and multiple banding. Therefore, purification was needed in order to obtain pure product. The purification of gel was done using QIAquick Gel Extraction Kit. Up to 400mg agarose can be processed per spin column.

The DNA fragment visualized under UV and the band excised from the agarose gel with a clean and sharp scalpel. The size of each gel slice was minimized by removing extra agarose because gel purification will result in a dilution of PCR product.

The gel slices were weighed in colorless tubes. 3 volumes of Buffer QG were added to 1 volume of gel (100mg~100 µl).Tubes were incubated at 50°C for 10 minutes or until the gel slice were completely dissolved. After the gel slices had dissolved completely, the color of the mixture was checked yellow. The adsorption of DNA to the QIAquick membrane is efficient only at pH $\leq$ 7.5.Buffer QG contains a pH indicator which is yellow at pH$\leq$ 7.5, allowing easy determination of the optimal pH for DNA binding.

The dissolved gel is applied to the QIAquick column and centrifuged for 1 minute at 24°C at 13 000 rpm. Flow-through discarded and the QIAquick column was placed back in the same collection tube. 500µl of QG buffer was added into column then spun again. This step will remove all traces of agarose.

750µl of PE buffer was added to QIAquick column and centrifuged for 1 minute. The Flow-through was discarded and QIAquick column was centrifuged for an additional minute at 13 000 rpm. The residual ethanol from PE buffer will not be completely removed unless the flow through is discarded before this additional centrifugation.

QIAquick column was placed into a clean 1.5ml microcentrifuge tube. To elute DNA, 50µl of EB buffer (10mM Tris-Cl, pH8.5) added to the center of the QIAquick membrane and centrifuged for 1 minute. Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5.

**Cloning into TOPO**

To ensure that most of the PCR product has an A overhang, an A-Tailing procedure was carried out as follows:

| | |
|---|---|
| DNA (purified product) | 10 .0µl |
| 10X PCR buffer with $MgSO_4$ | 1.0 µl |
| *pfu* DNA polymerase | 0.75 µl |
| dATP (10mM) | 0.50 µl |
| Total | 12.25 µl |

All the solutions were mixed together and briefly spun then, incubated at 72°C for 30 minutes. Cloning into TOPO was performed according to the manufacturer's instructions (Invitrogen).