'Construction of plasmids expressing specific genes of *Mycobacterium tuberculosis*: towards the development of vaccine against tuberculosis'

Mustaffa Musa, Salwana Ahmad, Jamaruddin M. Asan, Zainul F. Zainuddin,

Project number: 06-02-05-8012
IRPA 1999-2001

End Of Project Report

December 2001

End Of Project report

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Project Title: 'Construction of plasmids expressing specific genes of *Mycobacterium tuberculosis*: towards the development of vaccine against tuberculosis'

Project leader: Prof. Madya Dr. Mustaffa Musa

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Summary for the MPKSN Report (For publication in the Annual MPKSN Report, please summarise the project objectives, significant results achieve, research approach and team structure)

The objective of the project was to construct plasmid(s) expressing specific genes of *Mycobacterium tuberculosis (MTB)*: towards the development of potential **DNA vaccine candidates** against tuberculosis. Several T-cell epitopes from various specific genes of MTB were selected which include ESAT-6, MTP40, MPT64 and 38kDa. In order to construct the synthetic gene consists of those selected epitopes, the innovative PCR technology known as `Assembly PCR` was employed. In this project, the synthetic gene (consist of multiple epitopes of *M. tuberculosis*) was successfully constructed and the gene is designated as vacII. The vacII was then cloned into plasmid DNA vector, pJW4303. The recombinant plasmid containing the vacII designated as **pJWvacII** was obtained. The DNA sequence of the cloned gene (vacII) or the insert was confirmed by DNA sequencing method. So, in this project, we have constructed one **DNA vaccine candidate** for tuberculosis. Further studies that to be done to evaluate this vaccine candidate include immunogenecity and protection efficacy in animal model.

In this project, the immunogenicity of pJWvacII was studied in mice in order to determine its ability to induce various immune responses. Mice were immunised with the pJWvacII and control plasmid (pJW blank vector). At the end of the immunisation protocol, the spleenic lymhocytes were prepared from the two groups of mice. Lymphocyte responses in vitro were determined by Flow Cytometric analyses, Lymphocyte Transformation Test and IFN gamma production (by ELISA) after culturing them with several peptides derived from the vacII construct.

Results of Flow Cytometry which measure lymphocyte activation status did not show any significant different between the control and test group. But, results of LTT and IFN gamma production showed that some mice vaccinated with pJWvacII were positive when compared to the control group. These results indicated that the DNA vaccine candidate (pJWvacII) is immunogenic in mice *ie.* able to induce immune response. The ability of pJWvacII to induce IFN gamma production suggested that the DNA vaccine construct stimulated the Th1 type of immune response which is essential for immunity to TB. However, more studies need to be done to confirm these prilimanary findings. Such studies include protection or challenge studies in mice but this part of the study can be done here due to lack of facility required.

Objectives achievement

- **Original project objectives** (Please state the specific project objectives as described in Section II of the Application Form)

  - To clone genes/epitopes of *M.tuberculosis* into a plasmid (pCMV).
  - To prepare DNA constructs/recombinant protein for vaccination.
  - To study antibody and T-cell responses of the vaccinated animals.

- **Objectives Achieved** (Please state the extent to which the project objectives were achieved)

We have succesfully constructed a plasmid expressing multiple T-cell epitopes of *M. tuberculosis* as DNA vaccine candidate for tuberculosis.

- **Objectives not achieved** (Please identify the objectives that were not achieved and give reasons)

Technology Transfer/commercialisation Approach *(Please describe the approach planned to transfer/commercialise the results of the project)*

This project has established recombinant DNA technology/approach especially assembly PCR technique for DNA vaccine development. This technology is not only usefull for TB but also for other diseases in future. This technology can be transferred to other research institutions/universities in this country.

Benefits of the Project *(Please identify the actual benefits arising from the project as defined in Section III of the Application Form. For examples of outputs, organisational outcomes and sectoral/national impacts, please refer to Section III of the Guidelines for the Application of R & D Funding under IRPA)*

- **Outputs of the project and potential beneficiaries** (Please describe as specically as possible the outputs achieved and provide an assessment of their significance to users)

- Laboratory set up/technology for DNA vaccines development can be a trainning center for other reseachers in this country.

- The plasmid DNA construct (DNA vaccine candidate) obtained from this project has potential for further vaccine development as an alternative vaccine for the prevention and control of TB.

- **Organisational Outcomes** (Please describe as specifically as possible the organisational benefits arising from the project and provide as assessment of their significance)

- establised training center for other reseachers/postgraduate students in this country with regards to recombinant DNA/PCR technology.

- **National Impacts** (If known at this point in time, please describe as specifically as possible the potential sectoral/national benefits arising from the project and provide an assessment of their significance)

- Established linkage with Mycobacteriology Group, University of Sydney.

- The clone product (DNA construct) obtained from this project has potential for further vaccine development as an alternative vaccine for the prevention and control of TB.

Assessment of project structure

• Project Team (Please provide an assessment of how the project team performed and highlight any significant departure from plan in either structure or actual man-days utilised)

- Assoc. Prof. Dr. Mustaffa: overall management of the project, supervising immunogenicitiy studies (10 man-months)

- Assoc. Prof. Dr. Zainul: organising PCR analyses and DNA cloning (4 man-months)

- RO (Pn. Salwana Ahmad): performing all laboratory works (36 man-months)

• Collaborations (Please describe the nature of collaborations with other research organisations and/or industry)

Colaboration with Mycobacteriology laboratory (Assoc. Prof. W. Britton), Centenary Institute for Cancer Medicine & Cell Biology, University of Sydney with regards to the information on the latest technology in DNA vaccine development.

Assessment of Research Approach *(Please highlight the main steps actually performed and indicate any major departure from the planned approach or any major difficulty encountered)*

Main steps:

1. Designing synthetic gene of *M. tuberculosis* consist of multipe T-cell epitopes of *M. tuberculosis* using a computer programme (Mac DNAsis & Oligo programme) and generation of oligonucleotides based of the gene construct (vacII). Performing Assembly PCR to construct the vacII.
2. Cloning of the vacII gene into an expression vector (a DNA vaccine vector - pJW4303) and analyses of the recombinant clones by PCR, restriction digest and DNA sequencing.
3. Vaccination mice with pJWvacII and pJW4303 (control) for immunogenicity studies.
6. Lymphocyte Transformation Test and IFN gamma production assay.

Assessment of the Project Schedule *(Please make any relevant comment regarding the actual duration of the project and highlight any significant variation from plan)*

The project was running according to project schedule except at the beginning of the project due to time consumed for purchasing of reagents and training of the research officer.

Appendix A

UNIVERSITI SAINS MALAYSIA
KAMPUS CAWANGAN KELANTAN

GERAN PENYELIDIKAN (R&D) JANGKA PANJANG
PUSAT PENGAJIAN SAINS PERUBATAN
(305/PPSP/6110243.....)

NAMA PENYELIDIK : DR MUSTAFFA MUSA

NAMA PROJEK : CONSTRUCTION OF RECOMBINANT PLASMIDS EXPRESSING SPECIFIC OF MYCOBACTERIUM TUBERCULOSIS TOWARDS THE DEVELOPMENT OF VACCINE TUBERCULOSIS.

PENYATA PERBELANJAAN BAGI TEMPOH BERAKHIR PADA : 30 NOVEMBER 2001

PECAHAN KEPALA (VOT)	BAKI TAHUN LALU	PERUNTUKAN 2000	BELANJA 2000	PINDAAN PERUNTUKAN	BAKI 2000	PERUNTUKAN 2001	JUMLAH PERUNTUKAN	BAYARAN 2001	TANGGUNGAN	PERBELANJAAN 2001	BAKI KESELURUHAN
11000 GAJI	18,118.73	37,078.50	37,654.76	0.00	17,542.47	0.00	17,542.47	35,135.10	0.00	35,135.10	(17,592.63)
14000 ELAUN	950.00	950.00	0.00	0.00	1,900.00	0.00	1,900.00	0.00	0.00	0.00	1,900.00
15000 BONUS	558.50	1,871.50	0.00	0.00	2,430.00	0.00	2,430.00	1,397.80	0.00	1,397.80	1,032.20
21000 PERJALANAN	1,473.00	7,600.00	1,046.00	0.00	8,027.00	0.00	8,027.00	0.00	0.00	0.00	8,027.00
22000 PENGANGKUTAN	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
23000 PERHUBUNGAN	421.02	475.00	57.40	0.00	838.62	0.00	838.62	9.00	0.00	9.00	829.62
24000 SEWAAN	0.00	3,800.00	0.00	0.00	3,800.00	0.00	3,800.00	0.00	0.00	0.00	3,800.00
25000 MAKAN & MINUM	0.00	1,900.00	0.00	0.00	1,900.00	0.00	1,900.00	0.00	0.00	0.00	1,900.00
26000 BAHAN MENTAH	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
27000 BAHAN LAIN	15,148.74	26,600.00	21,196.21	0.00	20,550.53	0.00	20,550.53	16,048.10	6,672.50	22,718.60	(2,169.07)
28000 PEMBAIKAN KEC	1,900.00	1,900.00	0.00	0.00	3,800.00	0.00	3,800.00	0.00	0.00	0.00	3,800.00
29000 HOSPITILITY	413.75	475.00	282.45	0.00	606.30	0.00	606.30	205.50	0.00	205.50	400.80
35000 HARTA MODAL	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
JUMLAH BESAR	38,981.74	82,650.00	60,236.82	0.00	61,394.92	0.00	61,394.92	52,793.50	6,672.50	59,466.00	1,928.92

Technology Transfer/commercialisation Approach *(*Please describe the approach planned to transfer/commercialise the results of the project*)*

- Patent (*Please state full title of the patent by giving the patent number or application number*)

  Nil

- Publication pertaining to the research finding

  (a) Report/Conference Paper

  1. "Construction of synthetic genes of *Mycobacterium tuberculosis* using assembly PCR technique. 10th MSMBB Scientific Meeting;14-16th May 2000, Hyatt Regency Saujana, Subang. ***(see Appendix B)***

  2. `Construction of synthetic gene of *Mycobacterium tuberculosis*: towards the development of DNA vaccine against tuberculosis`. Global alliance for TB drug Development Conference on R & D coalition for TB drug development in Asia. May 2nd – 4th, 2001. Penang Parkroyal Resort Batu Feringghi Beach, 11100 Batu Feringghi, Penang, Malaysia.

  3.Norazmi Mohd Nor and Mustaffa Musa (2001). `Potential approaches towards the development of a vaccine against tuberculosis: Recombinant BCG and DNA vaccine`. Monograph. Biotechnology Symphosia I. UMS.

  (b) Journal Publication (*Use only the standard accepted abbreviations for journal titles. If there are none, give the full journal title)*

  In preparation

  (c) Others:

1. *The Sun:* 10th June 1999. `New vaccine from USM`.
2. *Utusan Malaysia*: 9th June 1999. `USM researchers construct vaccine prevent TB`
3. Segment `Good Morning Malaysia`, TV1 RTM 5/10/99 on `TB vaccine research activities in PPSP`.

- Post Graduates (*Who graduated or who are still participating the project)*

Student Name &Year of Registration/Nationality	Thesis Title	PhD/ MSc	Year of completion
Nil			

- No. of Research Assistants or Officers funded by the project:

(a) Research Officers: 1 (b) Research Assistants: 1

Collaboration (*Please describe the nature of collaborations with other research organizations and/or industry)*

Institutions:

(a) Local Institutions: Hospital Kota Bharu- provide blood samples of TB patients.

(b) International Institutions: Mycobacterial Research Group, Centenary Institute of Cancer Medicine and Cell Biology (Professor Warwick Britton & Dr Adrew Bean). Performing protective/challenge studies in mice to compare the protective efficacy of the DNA vaccine with BCG-immunised mice. Experiments need to be carried out in the P3 level laboratory which involve aerosol tuberculosis.

Details of experimental results of the project

Objectives of the project

To construct plasmid DNA expressing mutiple T-cell epitopes of *Mycobacterium tuberculosis*: towards the development of potential **DNA vaccine candidates** against tuberculosis.

Methodology

1. Designing of a synthetic gene (vacII) consist of multiple T-cell spitopes of *M. tuberculosis* using MacDNAsis and OLIGO software (Figure A).
2. Construction of vacII by assembly PCR technique (Figure B).
3. Cloning of vacII into plasmid expression vector, pJW4303.
4. Immunization protocol for DNA vaccine candidate (Figure C).
5. Immunogenicity studies: LTT, ELISA IFNγ (Figure D)

Results

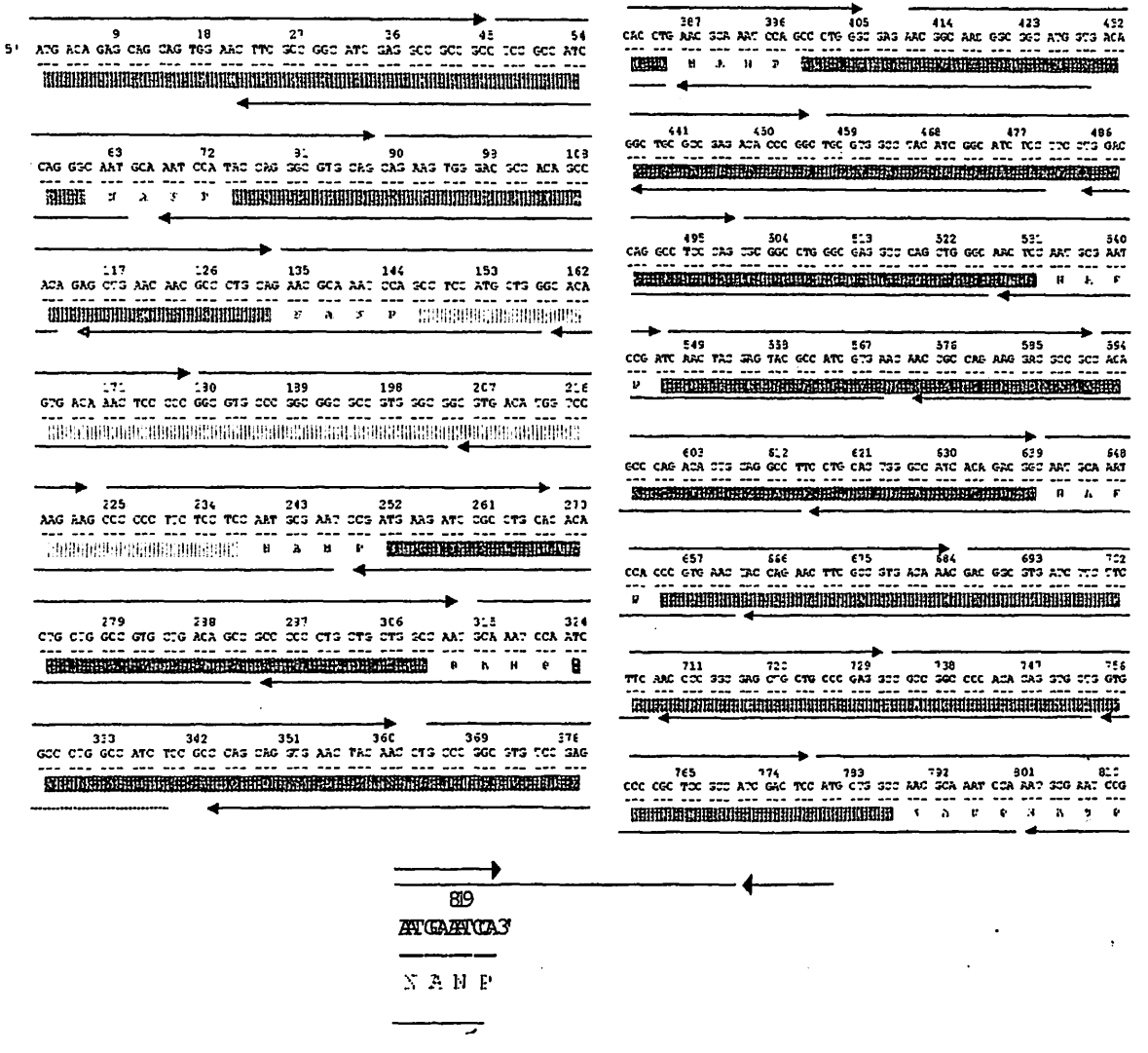
1. Construction of synthetic gene(designated as vacII) consist of multiple T-cell spitopes of *M. tuberculosis* and cloning of vacII into plasmid expression vector, pJW4303. The recombinant clone (designated as pJWvacII) was secreened by double enzyme digestion (Figure E) and confirmed by DNA sequencing (Figure F).

2. Immunogenicity studies: LTT, ELISA IFNγ (Table A & B)

Conclusion

We have constructed one plasmid DNA expressing multiple T-cell epitopes of *Mycobacterium tuberculosis* (designated as pJWvacII). Preliminary studies on the immunogenicity of this plasmid has been done in mice and the results suggested that this plasmid is potentially useful as **DNA vaccine candidate** for tuberculosis. Further studies that to be done to evaluate this vaccine candidate include protection efficacy in animal model.

Figure 4 DNA and protein sequence of vac II (consist of selected T-cell epitopes of ESAT6, mtp40, 38kD and MPT64 genes of *M. tuberculosis*).



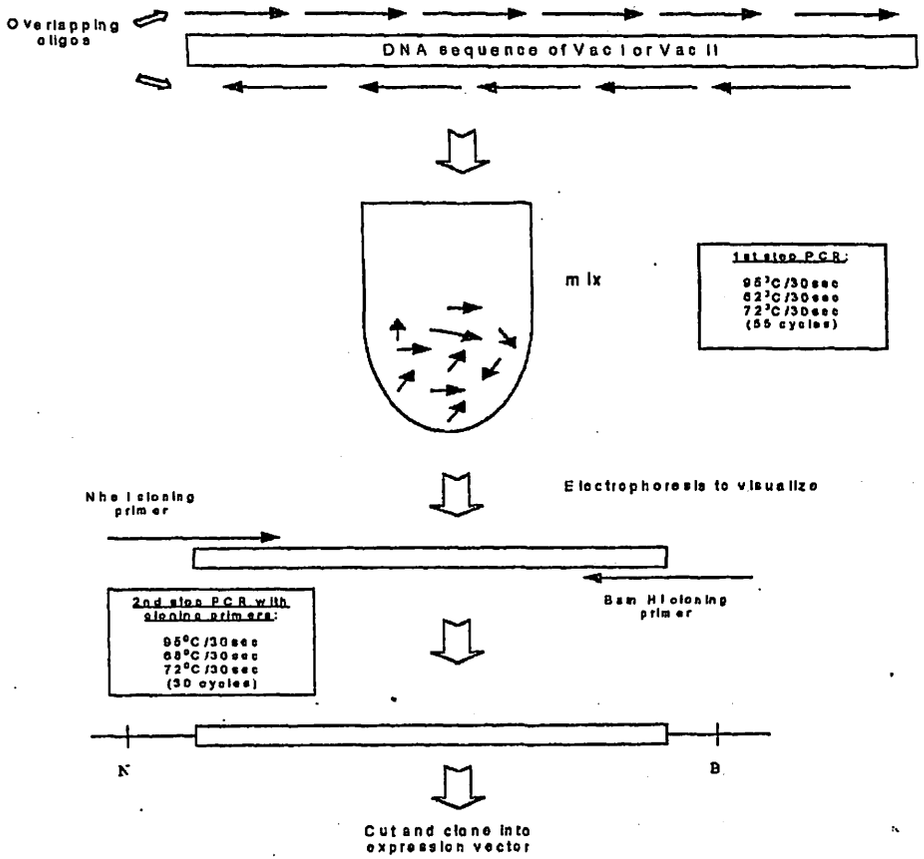


Figure 1: Assembly PCR to generate Vac I & Vac II genes

Figure C

Immunization Protocol

Plasmid DNA injected i.m. into C57BL/6J

[5 mice for pJWvacII

5 mice for the plasmid vector pJW]

⇩ **2 weeks**

2nd injection

⇩ **2 weeks**

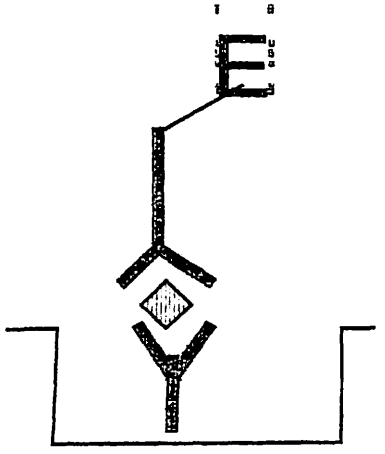
3rd injection

⇩ **2 weeks**

Collect spleen (lymphocytes)

Figure D

ELISA IFNγ (using commercial kit)



Culture S/N is added into wells coated with Ab to IFN γ

⇓

Washing

⇓

Enzyme Ab conjugate is the added

⇓

washing

⇓

Enzyme substrate is added and colour reaction is measured by spectrophotometer (the value of IFN γ is calculated based on the standard curve)

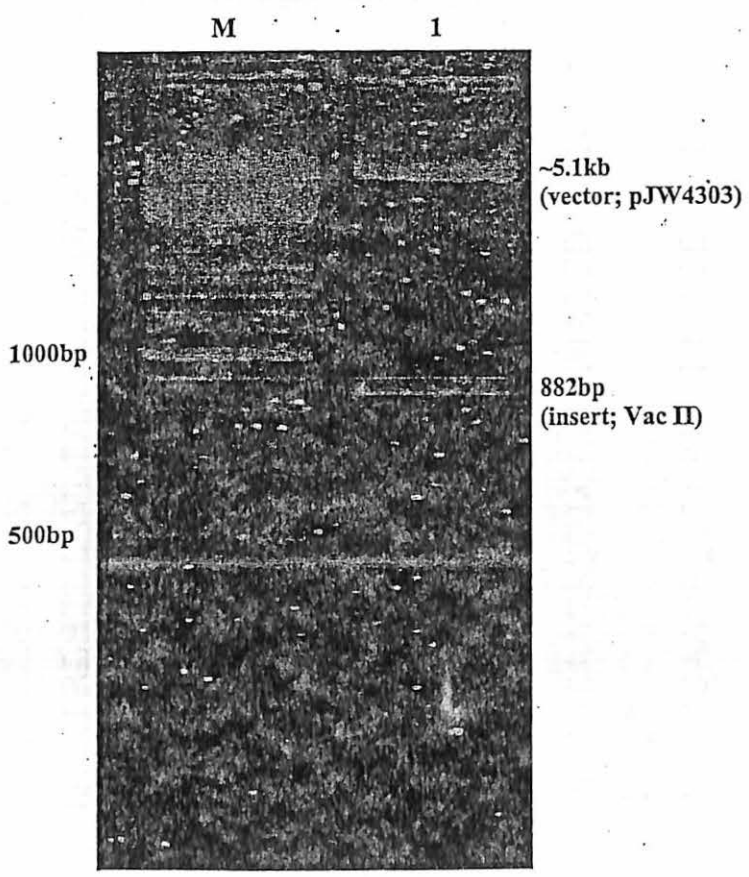


Figure E. Electrophoresis of recombinant clone (pJWVac II) after double enzyme digestion with *Nhe*I and *Bam*HI (Lane M: 100bp DNA ladder, lane 1: clone pJWVacII digested with *Bam*HI and *Nhe*I)

Figure F

Confirmation by DNA sequencing

ESAT mtp40

MTEQQWNFAGIEAAASAIQGNANPYQGVQQKWDATATELNNALQNANPASMLGT

MTEQQWNFAGIEAAASAIQGNANPYQGVQQKWDATATELNNALQNANPASMLGT

38kD

VTNSPGVPGGAVGGVTWSKKPPFSSNANPMKIRLHTLLAVLTAAPLLLANANPI

VTNSPGVPGGAVGGVTWSKKPPFSSNANPMKIRLHTLLAVLTAAPLLLANANPI

AVGGVTWSKKPPFSSNANPMKIRLHTLLAVLTAAPLLLANANPI

ALAISAQQVNYNLPGVSEHLNANPALGENGNGGMVTGCAETPGCVAYIGISFLD

ALAISAQQVNYNLPGVSEHLNANPALGENGNGGMVTGCAETPGCVAYIGISFLD

ALAISAQQVNYNLPGVSEHLNANPALGENGNGGMVTGCAETPGCVAYIGISFLD

QASQRGLGEAQLGNSNANPINYEYAIVNNRQKDAATAQTLQAFLHWAITDGNAN

QASQRGLGEAQLGNSNANPINYEYAIVNNRQK

QASQRGLGEAQLGNSNANPINYEYAIVNNRQKDAATAQTLQAFLHWAITDGNAN

MPT64

PPVNYQNFAVTNDGVIFFFNPGELLPEAAGPTQVLVPRSAIDSMLANANPNANP

PPVNYQNFAVTNDGVIFFFNPGELLPEAAGPTQVLVPRSAIDSMLANANPNANP

LYMPHOCYTE TRANSFORMATION TEST (LTT)

Table A: Stmulation Index (SI) of lymphocyte cultures from mice vaccinated with pJWvacII (U) and control mice (K) vaccinated with pJW4303 (blank plasmid) after stimulation with various vacII peptides (5ug/ml).

Experiment 1

	K1	K2	K3	U1	U2	U3
POOLED 1.25ug	0.8	0.21	2.2	3.4	0.6	0.6
POOLED 2.5ug	0.7	0.6	1.0	0.9	0.5	1.1
POOLED 5.0ug	1.0	1.0	0.7	0.7	0.2	3.8
POOLED 10ug	1.0	0.3	0.5	2.8	1.0	2.3
ESAT6 5ug	0.7	0.4	1.1	2.5	0.3	1.2
ESAT 10ug	0.11	0.6	0.9	2.5	0.8	1.0
38A 5ug	0.3	1.0	2.0	1.6	0.3	3.6
38A 10ug	0.04	1.02	3.3	4.1	0.8	2.8
38B 5ug	0.1	0.4	1.7	4.3	0.6	1.2
38B 10ug	0.14	1.5	0.9	3.4	0.4	0.7
64A 5ug	0.11	1.1	1.8	3.0	0.4	5.5
64A 10ug	0.13	0.5	0.9	4.3	0.4	0.4

Experiment 2

	K4	K5	U4	U5	U6
POOLED 5.0ug/ml	1.8	1.1	5.8	27.6	3.2
POOLED 10ug/ml	2.5	3.1	1.0	44.1	1.5
ESAT 5.0ug/ml	1.5	0.5	0.8	1.4	7.3
ESAT 10.0ug/ml	5.2	1.4	1.7	4.3	4.2
38A 5ug/ml	0.8	0.5	8.2	4.7	2.6
38A 10ug/ml	1.1	1.0	1.9	0.8	2.8

38B 5ug	0.7	0.3	6.0	3.8	2.5
38B 10ug	1.4	0.8	8.1	14.0	2.4
64A 5ug	0.4	0.6	4.1	4.3	0.6

ELISA IFN

Table B: Production of IFNγ (ug/ml) by lymphocyte cultures from mice vaccinated with pJWvacII (U) and control mice (K) vaccinated with pJW4303 (blank plasmid) after stimulation with various vacII peptides.

Experiment 1

	K1	K2	K3	U1	U2	U3
Pooled peptides (5ug/ml)	500	1750	1100	500	>2,000	>2,000
ESAT6 (5ug/ml)	65	365	35	800	>1,000	>1,000
38kD –A (5ug/ml)	100	>1,000	100	>1,000	>1,000	>1,000
38kD- B (5ug/ml)	900	150	550	>1,000	>1,000	950

Experiment 2

	K4	K5	U4	U5	U6
Pooled 1.25ug	457	204	72	626	168
ESAT6 5.0ug	216	361	108	819	1361
38A 5.0ug	493	746	301	3060	349
38B 5.0ug	168	409	409	301	277
64A 5.0ug	554	-	192	951	327

Appendix B: Paper presented at10th MSMBB Scientific Meeting;14-16th May 2000, Hyatt Regency Saujana, Subang (Poster P8).

"Construction of synthetic genes of *Mycobacterium tuberculosis* using assembly PCR technique"

Mustaffa Musa , Salwana Ahmad & Zainul F. Zainuddin*Department of Immunology, School of Medical Sciences, School of Health Sciences*, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan.

Abstract

The development of improved subunit vaccines is considered a high priority in the effort to control TB worldwide. Naked DNA or genetic vaccination is the current concept in vaccinology with the use of plasmid DNA expressing certain gene. Single or multiple genes of an organism can be cloned into the expression plasmids. In our study, assembly PCR technique was used to construct a synthetic genes namely, Vac Vac II. (0.82 kb). This synthetic gene were based on ESAT6, mtp40, mtp64 and 38kD genes of *M. tuberculosis.* Vac II gene consist of selected T-cell epitopes of those four MTB genes. In assembly PCR technique, mixture of Vac II derived-oligos was amplified for 55 cycles (95^{0}C/30sec, 62^{0}C/30sec, 72^{0}C/30sec). The PCR product with the expected DNA band size was reamplified using vector cloning primers (*Nhe* 1 and *Bam* HI primers). The final PCR products of Vac II are ready to be cloned into the expression vector.

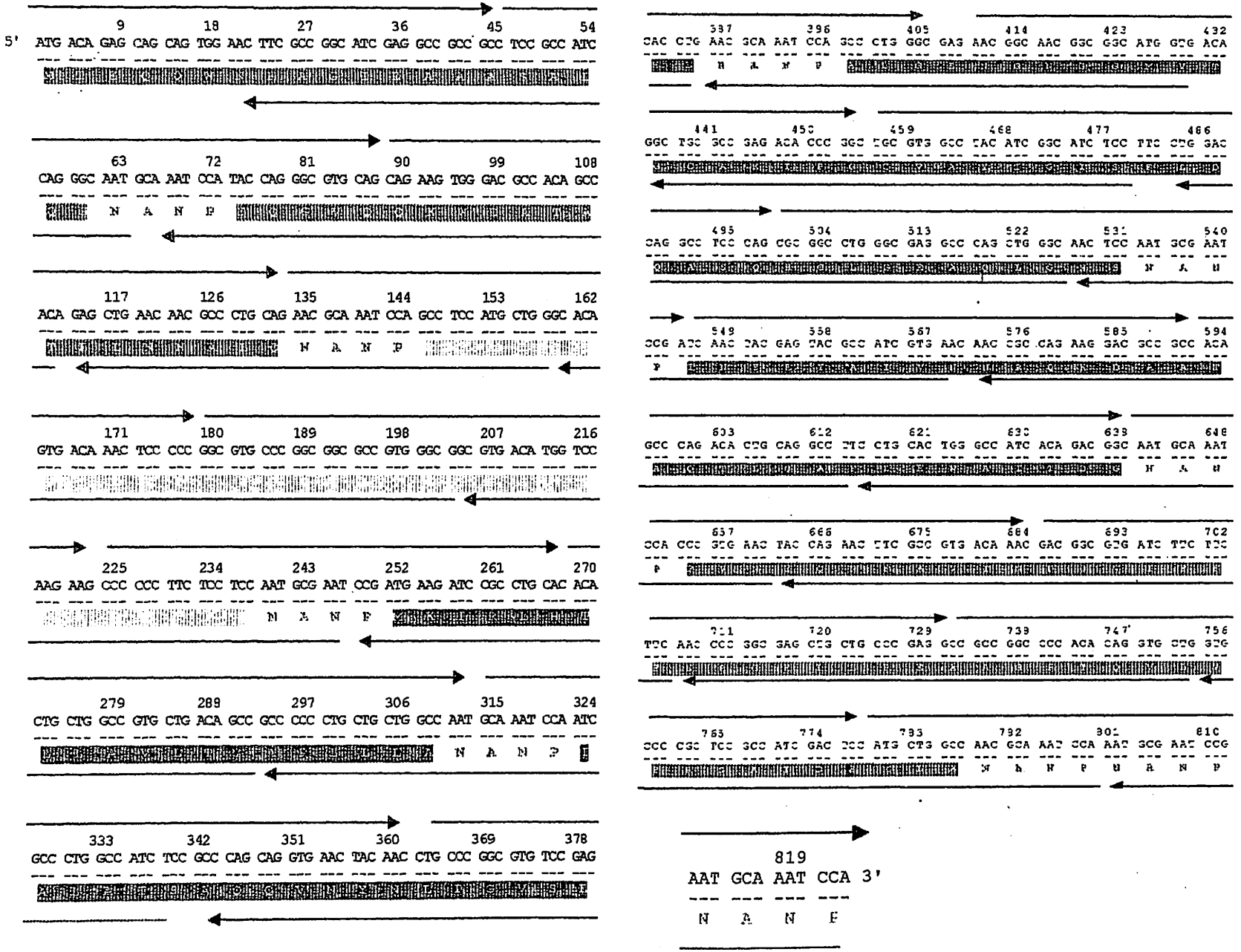
Introduction

Tuberculosis is a major bacterial disease of humans accounting for 8 million cases of clinical disease and 3 million deaths annually. The development of improved subunit vaccines is considered a high priority in the effort to control TB worldwide. DNA vaccines are a new and powerful approach to the generation of the needed vaccines (1-4). Dubbed as the "third revolution" in vaccine development, this novel method of immunization relies on plasmid expression vectors to produce the immunizing protein(s) in the vaccinated host. It involves direct injection of plasmids, loops of DNA that contains genes for proteins produced by the organism targeted for immunity. Once injected into the host muscle tissue, the DNA is taken up by host cells, which then start expressing the foreign protein. The protein serves as an antigen that stimulates immune responses.

Materials and Methods

Overlapping oligos (19 upper strand + 19 lower strand) based on the DNA sequence of the synthetic gene (vac II - see Figure 1) were designed using a computer programme (Oligo 5 & McDNAsis). Oligos were commercially made by Operon Technologies Inc. and BioBasic Inc. through Bionsyntech Sdn. Bhd. Assembly PCR was

DNA and protein sequence of vac II



performed with slight modification according to the method described by Stemmer *et al.* (5). This technique involves 2 steps (Figure 2); 1st step: ovalapping oligos were mixed in PCR reaction buffer, amplified for 55 cycles (95^{0}C/30sec, 62^{0}C/30sec, 72^{0}C/30sec). 2nd Step: PCR product of the first step (with the expected band size) was re-PCR (95^{0}C/30sec, 62^{0}C/30sec, 72^{0}C/30sec) using vector cloning primers (*Nhe*1/*Bam*H1 primer) for 55 cycles. The PCR products were analysed by agarose gel electrophoresis.

Results and Discussion

Assembly PCR (1st step PCR) using the Vac II oligos showed smear band when analysed with agarose gel electrophoresis (Figure 3A). From the first step product as a template, we did the 2nd step PCR using the cloning primers (*Nhe* I dan Bam HI). We managed to get the correct size of the product which was 0.82kb (Vac II) (Figure 3B). The synthetic gene is being cloned into plasmid expression vector (pJW4303) for further analysis. Such analysis included DNA sequencing to determine the gene sequences and whether the gene is in frame or not. The results showed that assembly PCR technique employed in this study were able to generate selected genes of *M. tuberculosis*. It shows that assembly PCR technique is a useful tool to generate synthetic gene or multiple genes for the development of DNA vaccine.

References

1. Robinson HL, Hune LA, Webster RG (1993). Protection against a lethal influenza virus challenge by immunization with a haemaglutinin-expressing plasmid DNA. Vaccine 11:957-960.

2. Ulmer JB, Donnelly JJ, Parker SE, Rhodes GH et al (1993). Heterologous protection against influenza by injection of DNA encoding a viral protein. Science 259:1745-1749.

3. Wang B, Ugen KE, Srikantan V, et al (1993) . Gene inoculation generates immune responses against human immunodeficiency virus type I.Immunology 90: 4146-4160.

4. Fynan EF, Webster RG, Fuller DH, haynes JR,Santoro JC,Robinson HL (1993) DNA vaccines: Protective immunizations by parental, mucosal, and gene-gun inoculations. Immunology 90:11478-11482.

5. Stemmer *et al.* (1995) Single-step assembly of a gene and entire plasmid from large numbers of oligodeoxyribonucleotides. Gene.164:(49-53).

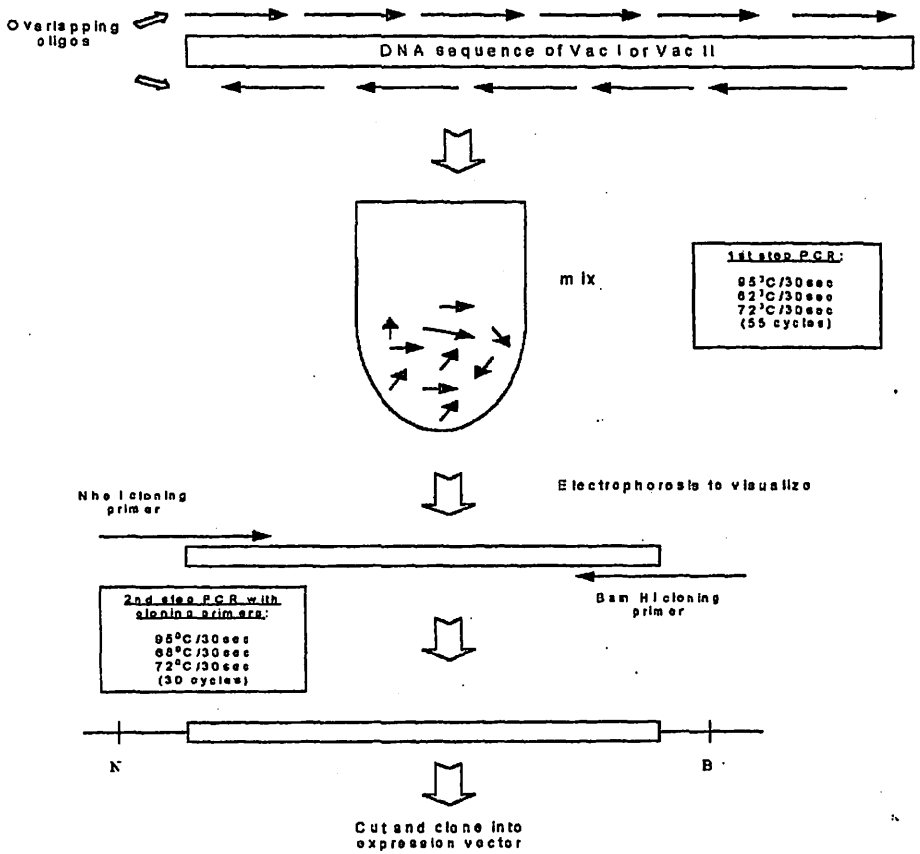
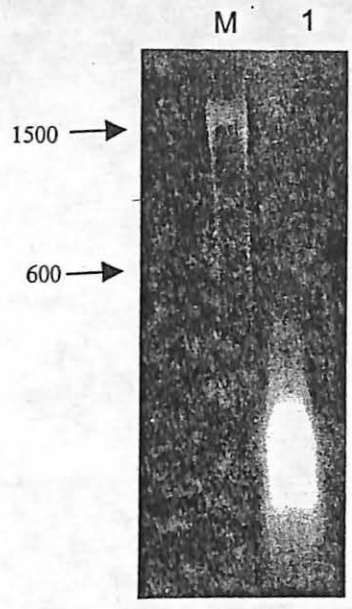


Figure 2: Assembly PCR to generate Vac I & Vac II genes

A. 1st step PCR



Lane M – 100bp marker
Lane 1 – PCR product for Vac II

B. 2nd step PCR (cloning primers)

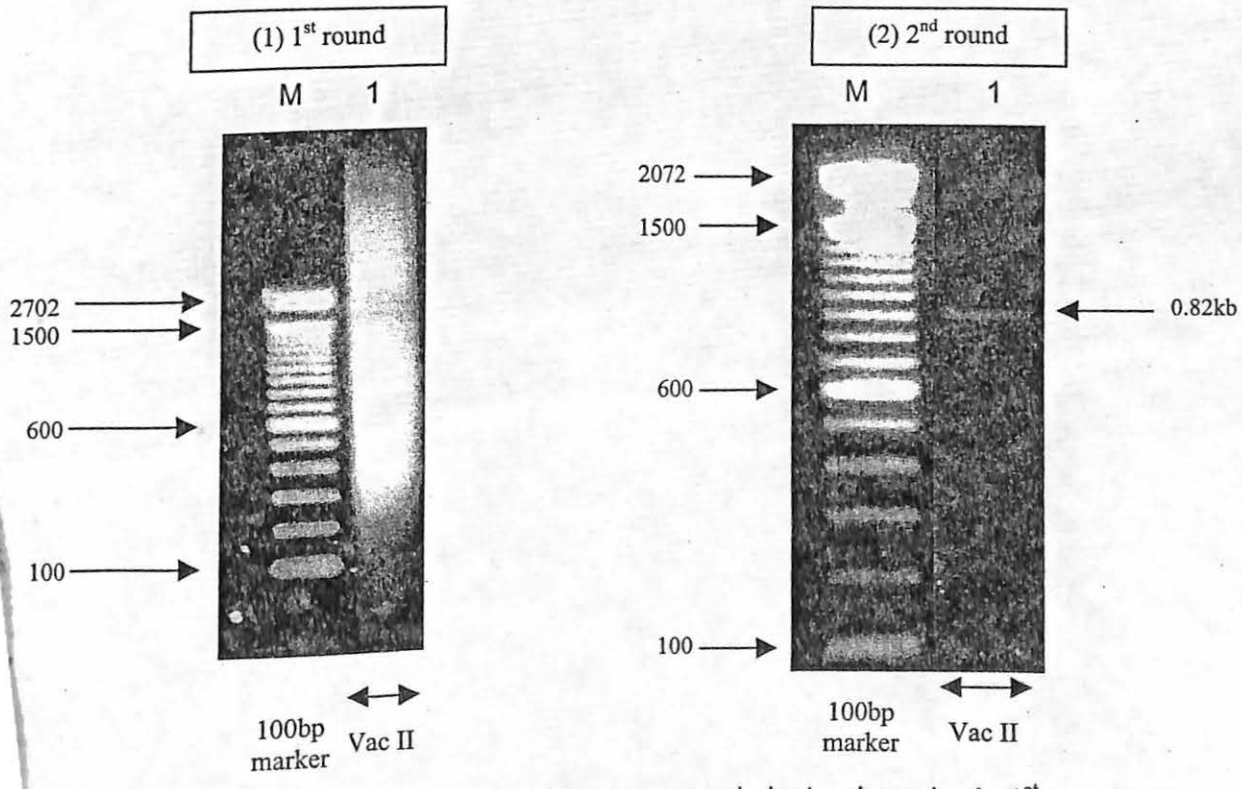


Figure 3: Analysis of the PCR product by agarose gel electrophoresis. A. 1st step PCR B(I),(2). 2nd step PCR