CLONING AND EXPRESSION OF prM AND E GENE OF JAPANESE ENCEPHALITIS VIRUS

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ABSTRACT

The prM, complete E and fragments of E gene coding for domain III of Japanese encephalitis virus have been cloned into both pET-32a and pBAC4x-1 cloning vectors, and truncated E gene into pET-32a. The expression of the proteins in these bacterial and baculovirus expression system were studied using SDS-PAGE and Western blot methods. Expression of domain III protein in both BL21(DE3) and AD494(DE3) expression host were shown by SDS-PAGE. From these two proteins, only the E domain III protein expressed in AD494(DE3) showed antigen city by the reactivity of adsorbed pooled positive dengue patient sera, pooled positive JE patient sera and adsorbed pooled positive JE sera from swine in Western Blots. The protein, which was expressed as a C terminal fusion with S-tag was also detected by S-protein HRP conjugate (Novagen, USA), confirming that the antigenic protein band was indeed the expressed fusion protein.

Quantitation of the affinity column purified domain III protein was done and the concentration was 2.2 mg/ml. Indirect ELISA was then used to test the authenticity of the purified protein. From the results, the antigenic E domain III protein was found to be in the correct folding and specific and therefore, could be a good candidate for the development of the JE subunit vaccine.

Kuantitasi protein domain III yang ditulenkan melalui turus afiniti dijalankan dan kepekatan protein yang diperolehi adalah 2.2 mg/ml. Kaedah ELISA secara tidak langsung digunakan untuk menguji keaslian protein yang ditulenkan. Daripada keputusan yang diperolehi menunjukkan bahawa protein E domain III yang antigenik ini adalah di dalam struktur lipatan yang betul dan spesifik, dan oleh itu merupakan calon yang baik dalam penghasilan vaksin subunit terhadap JE.

CHAPTER 1: INTRODUCTION

1.1 FLAVIVIRUS

The family Flaviviridae comprises 69 viruses, 67 of which are arthropod-borne viruses or close relatives of these arboviruses. The family also includes simian hemorrhagic fever virus and hepatitis C virus. Of the 67 arboviruses, 34 (50%) are mosquito-borne, 19 (28%) are tick-borne, 12 (18%) are zoonotic agents transmitted between rodents or bats without known arthropod vectors, and 2 have unidentified transmission cycles. Thirty-eight viruses (55%) have been associated with human disease, including the most important arthropod-borne viral afflictions of humankind-dengue fever, yellow fever, and Japanese encephalitis (JE). Eight flaviviruses are pathogenic for domestic or wild animals of economic importance (Monath and Heinz, 1996).

Flavivirus virions are spherical in shape with a diameter of 40 to 60 nm (Murphy, 1980). An electron-dense spherical nucleocapsid ~30 nm in diameter is surrounded by a lipid bilayer. Particles typically have a rather smooth appearance, and regular surface projections are usually not apparent (Murphy, 1980). The genome of flaviviruses consists of a single stranded
positive sense RNA approximately 11 kb in length (Brinton, 1986, Chambers et al., 1990, Rice et al., 1986, Westaway et al., 1985) with a molecular weight of $4.2 \times 10^6$ daltons encoding for 3 structural and 7 non-structural proteins, in the sequence of NH$_2$–C–prM(M)–E–NS1–NS2A–NS2B–NS3–NS4A–NS4B–NS5–COOH (Figure 1). The genomic RNA has a type I cap at its 5' end ($^7$GpppAmp) followed by the conserved dinucleotide sequence AG. Genomic RNAs of mosquito-borne and tick-borne flaviviruses lack a 3' terminal poly (A) tract and terminate with the conserved dinucleotide CU (Chambers et al., 1990).

**Figure 1:** Flavivirus genome structure and the properties of the encoded proteins. At the top is the viral genome with the structural and nonstructural protein coding regions, the 5' cap, putative 3' secondary structure, and the 5' and 3' NTRs indicated. Boxes below the genome indicate mature proteins generated by the proteolytic processing cascade. Reproduced from Rice et al. (1986).

Translation initiation usually occurs at the first AUG in the long ORF but may also occur at a second in-frame AUG located 12 to 14 codons downstream for the mosquito-borne flavivirus (Castle et al., 1985). The primary translation product is cleaved cotranslationally and posttranslationally at specific sites by host and viral proteases to produce the virion and replicase components. The production and translocation of the structural proteins involve cellular proteases or signal peptidases, while the non-structural protein NS3 in association with NS2B constitute the viral protease responsible for the cleavages at the N-terminal ends of some of the nonstructural proteins (Preugschat et al., 1990, Falgout et al., 1991).

### 1.2 THE THREE STRUCTURAL PROTEINS

#### 1.2.1 The core protein (C)

The virion C protein is a small [predicted molecular mass (M$_r$) 11 kDa], highly basic protein that forms a structural component of the nucleocapsid. Sequence homology among C proteins from different flaviviruses is low, but regions of hydrophobic and hydrophilic amino acids are
conserved (Bartenschlager et al., 1994). C protein determinants that participate in RNA and protein interactions important for nucleocapsid assembly have not been defined. It has been detected in vitro, in the nucleus and cytoplasm of infected cells. It is believed that C is synthesized in the cytoplasm before being transported into the nucleus of infected cells (Tadano et al., 1989).

1.2.2 The membrane-associated protein (M)
There are two forms of membrane-associated protein differing with the maturity of the virus. The first form is cell-associated or associated with immature virions, and is called pre-M protein (prM). The prM protein is the glycosylated precursor (M, 19 kDa), which heterodimerizes with E protein and is essential for proper folding of E. prM prevents E from premature conformational changes in acidic transport vesicles, and undergoes a delayed cleavage to form M and the N-terminal pr segment, which is secreted into the extracellular medium (Murray et al., 1993). This cleavage occurs shortly before or coincident with virion release because prM and M are found on intracellular and extracellular virions.

The second form is extracellular or mature membrane protein (M). The cleavage of M from prM is probably by a protease present in the export vesicles of cells (Ruiz-Linares et al., 1989). The structural protein M, located in the C-terminal portion of prM is present in mature virions and contains a shortened ectodomain (41 amino acids) followed by two potential membrane-spanning domains. Antibodies to prM can mediate protective immunity (Kaufman et al., 1989) perhaps by neutralization of released virions that contain some uncleaved prM.

1.2.3 The envelope protein (E)
Among the flavivirus structural proteins, the envelope glycoprotein is the largest and has been widely studied. The glycosylated E protein (M, ~5 kDa) is located in the virion envelope and the flat, elongated dimer extends in a direction parallel to the viral surface (Rey et al., 1995). The E protein is the major envelope protein of the virion. This protein is believed to play key roles in a number of important processes including virion assembly, receptor binding and membrane fusion, and is the principal target for neutralizing antibodies (Heinz, 1986).

The E protein, which has been isolated from the virion surface, has been shown to be immunogenic in experimental animals producing neutralizing antibodies (Takegami et al., 1982a), and are protective for lethal JE virus infection in mice (Oya, 1988, Kimura-Kuroda and Yasui, 1988). Antigenicity of the E protein is analysed by monoclonal antibodies (mAb) to identify multiple epitopes with various cross-reactivity and biological properties (Kimura-Kuroda and Yasui, 1983).
1.3 JAPANESE ENCEPHALITIS VIRUS

Japanese encephalitis is a potentially lethal infection of the central nervous system (CNS) caused by Japanese encephalitis virus, a member of the mosquito-borne encephalitis complex of the family Flaviviridae. The virus causes acute encephalitis (swelling of the brain tissues) which can progress to paralysis, seizures, coma and death with fatality rates ranging from 20% to as high as 50% (Innis, 1995). The importance of this disease is its high fatality rate and its significant rate of sequelae: more than half of the survivors have neurological sequelae such as CNS impairment, emotional and behavioral disturbances, and intellectual problems (Rojanasuphot, 1994).

Epidemics of Japanese encephalitis recur in temperate areas of Asia and is found in Japan, China, Taiwan, Korea, the Philippines, the far-east provinces of the former Soviet Union, India and all of Southeast Asia. In terms of morbidity and mortality, this disease is by far the most important of the arbovirus encephalitides (Tsai, 1994, Umenai et al., 1985). Approximately 35,000 cases of JE and 10,000 deaths are reported annually in Asia, but the disease is believed to be highly underreported (Monath and Heinz, 1996).

Japanese encephalitis is endemic in Malaysia and occurs sporadically throughout the country all year round. From 1989 to 1993, a total of 172 cases of JE have been reported in Malaysia with 12 deaths. 31% of cases were in children 0-4 years; 52% in the 7-14 year age group; 9% in the 15-24 year age group and 8% in adults >25 years. Although no age is exempted, cases have occurred principally in children. Children below 14 years old have been the main target, accounting for 83% of all cases. More males (62%) are affected than females (38%) (Tan, 1994).

As a typical arbovirus, JE virus is maintained in nature by alternative growth in vertebrate hosts and arthropod vectors (Figure 2). The Culex tritaeniorhyncus mosquito is the main epidemic vector to human in Asia (Buescher and Scherer, 1959, Carey et al., 1969, Simpson et al., 1974, Sucharit et al., 1989, Ura, 1976). Classic studies in Japan by Scherer et al., (1959b) established that pigs and birds were the principal viremic amplifying hosts, serving as the source of infection of mosquito vectors and that Culex tritaeniorhyncus was responsible for transmission between these vertebrates and from them to humans.
Swine herds have been documented as the most important amplifier of JE for several reasons. Levels of viremia are high following JE virus infection, the swine population in Asia is large, high turnover rate and swine are the preferred feeding source for the vectors (Scherer et al., 1959b). Except stillbirth and/or abortion that occurs among pregnant swine infected with JE virus, swine did not exhibit any apparent diseases (Scherer, et al., 1959a).

Humans are relatively insensitive to JE virus, and no appreciable transmission of the JE virus between humans by vector mosquitoes has been documented. Therefore, humans are considered dead-end hosts in the transmission cycle of JE virus, although they can be infected by the bite of infective vector mosquitoes (Figure 2) (Igarashi, 1992b). Only one of several hundred infected humans manifests apparent encephalitis. Most of the disease remains as inapparent infection that could be detected by antibody survey (Halstead and Grosz, 1962; Grossman, et al., 1974). Experimental infection of horses, cattles and dogs induces low-level or no viremia, suggesting that they are unable to amplify virus and thus are a dead-end in the enzootic cycle (Burke and Leake, 1988; Johnsen et al., 1974).
One of the most promising approaches to reducing JEV infections in humans and domestic animals is wide-spread immunization of swine. Since 1972, both inactivated and live attenuated vaccines have been used in Japan and other Asian countries to inoculate pigs against JEV infection (Tsai, 1994, Yoshida et al., 1981). In theory, this will interrupt the transmission and amplification cycle of the JE virus and thereby reduce human infection. However, the use of live vaccines in young animals appears to stunt their growth, and inactivated vaccines are expensive to produce and require multiple inoculations to be effective. Therefore easier methods for wide-scale immunization of pigs in Southeast Asia still needs to be developed.

The WHO has proposed the development of second generation JE vaccines using recombinant DNA technology to produce inexpensive vaccines in large amounts for use against JE in developing countries in Asia (Igarashi, 1992a). Since the E protein possesses neutralizing epitopes, studies to express E gene product with authentic antigenicity have been performed using recombinant baculovirus (Matsuura et al., 1989) or vaccinia virus (Yasuda et al., 1990). Recombinant vaccinia (including the NYVAC vector, a highly attenuated strain of vaccinia suitable as a vaccine) encoding prM and E (or prM, E and NS1) of JE virus elicited neutralizing antibodies and conferred a high level of protection against challenge of mice and pigs (Konishi et al., 1992a, Konishi et al., 1992b). Infection of mammalian cell cultures with recombinant vaccinia virus expressing the prM and E genes of JEV resulted in the production of extracellular 20-nm subviral particles. These particles, composed of the prM/M and E viral proteins in a lipid bilayer, are free from nucleic acid, easily purified, and highly immunogenic, providing a possible candidate for the development of subunit vaccine (Mason et al., 1991).

CHAPTER 2: STATEMENT OF THE PROBLEM

2.1 IMPORTANCE AND CONTROL OF DISEASE

Japanese encephalitis is the leading cause of viral encephalitis in Asia (Jatanasen, 1994). It is a potentially lethal virus infection with fatality rates up to 50% (Innis, 1995). Each year, approximately 50,000 sporadic and epidemic cases of JE are reported. However, it is believe to be highly underreported (Monath and Heinz, 1996). Preliminary results based on the ongoing research at the Institute of Health & Community Medicine by Cardosa et al. (MOH data, 1998) shows that Sarawak records the highest number of JE cases in the country.

Swine herds are the most important reservoir of JEV. The virus is transmitted in a zoonotic cycle among mosquitoes and vertebrate-amplifying hosts, chiefly domestic pigs and wading
birds. It is then transferred to humans from pigs by *Culex tritaeniorhyncus*, which is the main vector in Asia. Based on the natural transmission cycle of JEV (Figure 2), three strategies have been considered for the control of human JE epidemics: vector control, swine immunization and immunization of humans.

Attempts have been made to control JE vector mosquitoes by spraying chemical insecticides, which apparently reduced vector density but their effect was relatively transient (Self *et al.*, 1973a, b, Mitchell, *et al.*, 1974). Practical application of vector control through the use of larvicides was not considered cost-effective because of the vast areas of vector breeding sites that must be treated. In general vector control alone cannot be relied upon to prevent JE. Vector control should be maintained as one of many public health components of a JE prevention program and, perhaps, has a unique place in emergency control during outbreaks (Tsai *et al.*, 1994).

Since epidemic human disease has rarely occurred in the absence of swine, it has been hypothesized that removing them from the viral transmission cycle, functionally, by immunization, should reduce the intensity of viral transmission, numbers of infected vectors and human exposure. Vaccination of pigs also prevents economic losses associated with fetal pig loss. However, difficulties arise in practice. In many parts of Asia, pigs are only semidomesticated, and wide-scale immunization is difficult. Vaccination using the available vaccines for pigs is expensive, requires high man power and this will increased the cost. Therefore, a vaccine which is economical, easily administered and suitable for wide-scale immunization needs to be develop.

### 2.2 OBJECTIVE OF THE PROJECT

The objective of this project is to determine the appropriate genes or fragments of genes of Japanese encephalitis virus for use in vaccine development. Three different constructs containing prM, complete/truncated E or fragment of E gene coding for domain III of E protein were used in this project. Since the data from passive protection studies with monoclonal antibodies have shown that the structural proteins are primarily responsible for inducing protection against the disease (Kimura-Kuroda & Yasui, 1988, Mason *et al.*, 1989, Kaufman *et al.*, 1989, Gould *et al.*, 1986), these proteins are considered as potential immunogens in the development of subunit vaccines. It is known that the major glycoprotein E plays an important role for flavivirus infection and contains antigenic determinants which elicit neutralizing antibodies and the induction of a protective immunity (Kimura-Kuroda and Yasui, 1988, Brinton, 1986). It contains 3 types of antigenic determinants: flavivirus cross-reactive, subgroup virus reactive and viral species specific (Kimura-Kuroda and Yasui, 1983). It also constitutes the major immunogen in virus infection (McAda *et al.*, 1987).
Low titer neutralizing activity and a significant degree of passive mouse protection has been observed with monoclonal antibodies against the prM protein (Kaufman et al., 1989). Mice immunized actively with prM/M also exhibit a degree of protection. The prM protein is part of the immature virions, and at the late stages of infection, its proteolytic cleavage to M protein generates mature virions. However, in certain instances this prM cleavage may not be complete, thus allowing the prM protein to be an additional target on virions for neutralizing antibodies (Bray and Lai, 1991).

Future work will put an emphasis on the use of genes or fragments of genes for use in the development of vaccine. This will involve molecular cloning of the appropriate genes or fragments of genes in a plant system for expression of proteins. The utilization of plants as expression vectors for the production of foreign proteins has recently been reported. Viral proteins (Mason et al., 1992, Mason et al., 1996, McGarvey et al., 1995, Thanvala et al., 1995), bacterial toxins (Haq et al., 1995) and antibody molecules (During et al., 1990, Hein et al., 1991, Hiatt et al., 1989, Ma et al., 1994) have been expressed successfully in transgenic plants. In most cases, the expressed proteins are fully functional as antigens (Mason et al., 1992, Mason et al., 1996, McGarvey et al., 1995, Thanvala et al., 1995, Haq et al., 1995) or in ligand recognition (During et al., 1990, Hein et al., 1991, Hiatt et al., 1989, Ma et al., 1994). More importantly, they have been shown to be effective as immunogens in eliciting specific immune responses (Mason et al., 1996, Thanvala et al., 1995, Haq et al., 1995). The use of plants as production systems for immunogens to be developed as vaccines may be an economical alternative to current methods. The technology of producing vaccines against animal and human diseases in plants is based on genetic engineering techniques which include recombinant DNA and genetic transformation of plants and plant cell and tissue culture techniques.

For the future work, sweetpotato has been chosen as the plant for expression of the prM and E protein of Japanese encephalitis virus for several reasons. Since the vaccine is targeted as a pig vaccine, it is important to make sure that the plant chosen for expressing the vaccine can and will be eaten by the animal. Another reason for choosing sweetpotato is the availability of patatin promoter. This 'tuber-specific' promoter can be used to target the expression of foreign genes in tubers (Kim et al., 1994).

Stable expression of foreign genes have been achieved in sweetpotato using Agrobacterium vector and the particle bombardment system of gene delivery. Transgenic shoots with stable expression of foreign genes have been recovered using the Agrobacterium cocultivation procedure. Plants inoculated with wild type A. tumifaciens developed crown galls confirming
that sweetpotato could be potentially transformed using the Ti plasmid vector (Prakash and Varadarajan, 1992b).

Several studies have been conducted to improve in vitro plant regeneration in sweetpotato. Regeneration using excised petioles (Gosukonda et al., 1995), leaf explants (Dessai et al., 1995) and somatic embryogenesis (Zheng et al., 1996) from leaf and petiole explants in a repetitive fashion have been reported in this crop. The availability of a reliable and reproducible somatic embryogenic system that is rapid, repetitive and that provides a choice of direct and callus-mediated embryogenesis will be useful in gene transformation research. Combining all the information above, this piece of work will ultimately lead to the design of recombinant subunit vaccine against JE.

CHAPTER 3: cDNA PREPARATION

3.1 INTRODUCTION
The genome of Japanese encephalitis virus is a single-stranded (positive sense) RNA which can act directly as messenger RNA (mRNA). RNA is chemically and biologically more labile than DNA, especially at high temperatures (>65°C) and in the presence of alkali. One of the major difficulties in working with RNA is that ribonucleases (RNase) is ubiquitous. Nevertheless, the purified mRNA molecules can serve as the template for the enzymatic conversion of these relatively unstable, single-stranded molecules into much more stable, double-stranded complementary DNA (cDNA) molecules, which can be used in cloning. This can be done by RT-PCR method. In this method, amplification of RNA is performed by annealing a primer to the RNA template and then synthesizing a cDNA copy using RNA-dependent DNA polymerase (reverse transcriptase, RT) to catalyze the reaction, followed by PCR. Two different forms of reverse transcriptase are available commercially: AMV reverse transcriptase, which is purified avian myeloblastosis virus particles, and M-MLV reverse transcriptase, which is a form of Moloney murine leukemia virus genetically altered to remove the associated ribonuclease H activity.

The polymerase chain reaction (PCR) is used to amplify the cDNA synthesized in the RT step. The cDNA, known as the first strand is used as a template for further amplification by Taq DNA polymerase. The template DNA is first denatured by heating in the presence of excess primers and dNTPs. The reaction mixture is then cooled to a temperature that allows the primers to anneal to their target sequences, after which the annealed primers are extended by DNA polymerase, in the presence of dNTPs under suitable reaction conditions. This results in the synthesis of new DNA stands. Strand synthesis is then repeated by heat
denaturation, annealing and primer extension. Each repetition of strand synthesis comprises a cycle of amplification.

This chapter describes how genes and fragments of genes of interest, i.e. prM and E genes and E domain III coding sequence from JEV were prepared.

3.2 MEDIA AND CELLS
L-15 medium (Leibovitz) with L-glutamine (Sigma, USA) was prepared in ultra high quality (UHQ) water produced in an Elgastat (England) and sterilized by negative pressure filtration through a 0.22 µm cellulose acetate filter (Milipore Corp, France). Serum-free L-15 supplemented with 10% heat-inactivated fetal bovine serum, FBS (GIBCO, BRL), 10% of tryptose-phosphate broth (TPB) and 50 U/ml benzyl penicillin and 40 U/ml streptomycin sulfate (Sigma Chem. Co., USA) was prepared as the growth medium for the cells. For maintenance medium, the concentration of FBS was reduced to 1%. Sterility tests were performed for all the media before use.

C6/36 cells (cloned Singh's Aedes albopictus cell line) were cultured in 25 cm² tissue culture flask (Costar, USA) containing growth media at 30ºC (WTC Binder incubator, Germany).

3.3 CULTIVATION OF VIRUS
JEV strain Nakayama was obtained originally from Dr. J.S. Porterfield, Sir William Dunn School of Pathology, University of Oxford.

For the propagation of virus, C6/36 cells were grown to confluence in 25 cm² tissue culture flasks. The growth media was then removed and the cells were supplemented with half volume (2.25 ml) of the maintenance media needed before virus inoculation. 100 µl of virus seed stock was added to the monolayer and the virus was allowed to adsorb to the monolayer for 4 hours on a rocker at room temperature (setting of 3 for Edmund Buhler Type WS-5 side/side rocking platform, Germany). The other half volume of maintenance medium (2.25 ml) was then added and the inoculated flasks were cultivated for 3 to 6 days until cytopathic effect was observed.

3.4 EXTRACTION OF VIRAL RNA
Extraction of viral RNA was done using TRI-Reagent (Sigma, USA), a single-step method based on the procedure by Chomczynski and Sacchi (1987) for total RNA isolation. This procedure described the isolation and purification of RNA by extraction of cells with guanidinium thiocyanate-acidic phenol-chloroform. The addition of the extraction buffer
prepared by mixing water-saturated phenol with an acidic solution of sodium acetate and chloroform would separate RNA into the aqueous phase, with DNA and proteins in the interphase and organic phase.

JEV infected cells were harvested after the cytopathic effect was seen. The media was removed before lysing the cells in TRI-Reagent. The monolayer cells were lysed directly on the culture dish by addition 2.5 ml of TRI-Reagent (1 ml of TRI-Reagent per 10 cm² of culture plate surface area). After the addition of reagent, the cell lysate was passed several times through a pipette to form a homogenous lysate.

The cell lysate was centrifuged at 12,000 x g for 10 minutes at 4°C (refrigerated centrifuge HERMLE, Z233MK, Germany) to remove the cell debris. The clear supernatant containing RNA and protein was then transferred into 3 fresh eppendorf tubes (1 ml/tube) and let stand for 5 minutes at room temperature. 0.2 ml of chloroform was added into each tube, vortexed for 15 seconds before they were allowed to stand for 15 minutes at room temperature. The resulting mixture was centrifuged at 12,000 x g for 15 minutes at 4°C to separate the mixture into 3 phases: a red organic phase (containing protein), an interphase (containing DNA) and a colorless upper aqueous phase (containing RNA).

The aqueous phase was transferred to a fresh tube and 0.5 ml of isopropanol was added. The mixture was allowed to stand at room temperature for 10 minutes, before it was centrifuged at 12,000 x g for 10 minutes at 4°C. RNA was pelleted on the side and bottom of the tube. The supernatant was removed and RNA pellet was washed using 1 ml 75% ice-cold ethanol. The RNA pellet was then dried using Savant DNA Speed Vac DNA 110 (USA) at low drying rate before the dissolution in 15 µl sterile ultra high quality water.

3.5 RT-PCR OF prM, E AND E DOMAIN III CODING SEQUENCES
All specific primers flanking the prM, full E and E domain III gene were synthesized based on the prototype strain. JEMP1 and JEMP2 primer pairs for prM gene and JEE1 and JEE2 primer pairs for E gene were obtained from University of Oxford. JEEEdIIIL and JEEEdIIIR primer pairs for E domain III gene were synthesized by Operon Technologies, USA.
Programmable Thermal Controller (PTC-100 MJ-Research, USA) was used.

Primer pairs for prM gene:
JEMP1: 5’ GAA AGA TCT ATC ATG TGG CTC GCG AGC TTG GCA 3’
JEMP2: 5’ TCC AGA TCT CTA CTA ACT GTA AGC CGG AGC GAC 3’
Expected size of product: 572 bp  
Position: nucleotides 421 to 993

Primer pairs for E gene:  
JEE1: 5’ ATA GGA TCC TGG ATG CTT GGC AGT AAC AAC GGT 3’  
JEE2: 5’ GAT GGA TCC CTA CTA TCC AGT GTC AGC ATG CAC 3’  
Expected size of product: 1.6 kb  
Position: nucleotides 895 to 2508

Primer pairs for E domain III coding sequence:  
JEEdIIIL: 5’ GCT CTG GAT ATC ACA ACC TAT GGC 3’  
JEEdIIIR: 5’ GCC CAG CGT GAT ATC TCA CTT GTG CCA 3’  
Expected size of product: 329 bp  
Position: nucleotides 1861 to 2190

3.5.1 First strand reaction (RT)  
Working concentration of primers is 10 pmol/µl. 1µl of downstream primer was added to 5 µl of extracted RNA in a 600µl reaction tube. The tube was then incubated at 70°C for 10 minutes. During the incubation, a master mix for the RT reaction was prepared in a fresh tube. The master mix contained 2 µl of 5X RT buffer and 1 µl of 10 mM dNTPs for each reaction tube. At the end of the incubation period, the reaction tube was immediately chilled on ice. The mixture of RT buffer and dNTPs was then added to the RNA mixture and the reaction tube was incubated at 37°C for 2 minutes. 1 µl (200 U) of M-MLV Reverse Transcriptase (Promega, USA) was then added to the tube and incubated at 37°C for an hour. The reaction was stopped at 65°C for 10 minutes.

3.5.2 Polymerase chain reaction (PCR)  
A master mix containing 1 nmol of downstream primer, 1 nmol upstream primer, 2.5 µl 10 mM dNTPs, 10 µl of 10X Taq buffer, 1 µl of Taq DNA polymerase (Promega, USA), 8 µl of 25 mM MgCl₂ and 48.5µl of UHQ water was prepared. The master mix was then added to the RT tube containing 10µl of first strand. The tube was then vortexed gently and spun down before starting the PCR reaction.

PCR amplification of DNA was achieved by using the following program: precycle at 94°C for 1 minute, denaturing at 94°C for 1 minute, annealing at 50°C for 1 and a half minutes and polymerization at 72°C for 1.5 minutes. Denaturing, annealing and polymerization made up 1
complete cycle. The cycle was repeated 30 times before a final extension of 4 minutes at 72°C.

3.6 CLONING OF prM AND E GENES INTO PCR-BLUNT VECTOR
All the PCR products obtained had the appropriate restriction sites engineered into the primer sequences. The 572 bp of prM PCR product and the 1.4 kb of E PCR product were cloned into the PCR-Blunt vector from the Zero Blunt PCR Cloning Kit (Invitrogen, USA) (Figure 3) before cloning into the pET-32a and the pBAC4x-1 cloning vectors. Positive transformants were selected on Luria-Bertani (LB) plates supplemented with kanamycin (50 µg/ml) and their plasmids were extracted using minipreparations of plasmid DNA by alkaline lysis method (described in 3.8).

3.6.1 Klenow reaction of PCR product
This reaction was done to blunt-end the PCR product before ligation with the Zero Blunt vector. 1µl of 10 mM dNTPs and 5 units of Klenow (Promega, USA) were added to 43 µl of the PCR product. The reaction mix was incubated at 25°C for 15 minutes before heat inactivation at 72°C for 20 minutes. The blunt-ended product was run through a low percentage (0.7%) agarose 3:1 gel (Amresco, USA). The expected band with the correct size was then excised and purified using the QIAquick gel extraction kit (Qiagen, USA) and eluted in 20 µl of UHQ water.

3.6.2 Ligation
1 µl of PCR-Blunt vector (Invitrogen, USA) was mixed with 5 µl of purified blunt-ended PCR product and 1 µl of T₄ DNA ligase (Boehringer Mannheim, Germany) in a 10 µl reaction. Ligation was done at 16°C for 1 hour.
Figure 3: Cloning of prM and E genes into PCR-Blunt (Invitrogen, USA). Blunt-ended prM and E PCR products were ligated with the PCR-Blunt vector. Two kinds of clones would be constructed, the "sense-clone and the "anti-sense clone".

3.6.3 Transformation

1 vial containing 50µl of One shot TOP 10 competent cells (Invitrogen, USA) was thawed on ice. 2 µl of 0.5 M β-mercaptoethanol was added to the vial and mixed by gentle stirring with the pipette tip. 2µl of the ligation mix was then added into the competent cells and mixed by stirring with a pipette tip before incubation on ice for 30 minutes. The vial was then transferred to a 42°C water bath for exactly 90 seconds before incubation on ice for 2 minutes. 250µl of pre-warmed (37°C) SOC medium was then added to the mixture, shaken for 1 hour at 37°C at 225 rpm in an incubator shaker (Innova 4000, USA). At the end of the incubation period, 50µl and 100 µl of the cells were then spread on LB plates containing 50 µg/ml kanamycin plates and incubated at 37°C overnight (Memmert, Germany).
3.6.4 Screening for inserts
Screening for clones that contained the correct insert was done using minipreparation of plasmid DNA as described in 3.8. The extracted DNA was digested using the restriction enzyme Eco RI. Clones with inserts were selected as a source of the gene or fragment of interest.

3.7 PREPARATION OF HIGH QUALITY PLASMID DNA
High quality DNA used for cloning or transfection was prepared using large-scale preparations described by Sambrook et al. (1989) to obtain enough DNA.

Amplification of plasmids was done by inoculation of 500 ml of LB or Terrific Broth medium (prewarmed to 37ºC) containing 50 µg/ml of ampicillin in a liter conical flask with the appropriate stock culture. The culture was then incubated overnight at 37ºC at 225 rpm in an incubator shaker (Innova 4000, USA). The next day, the bacterial cells were harvested by centrifugation at 5000 rpm for 10 minutes, 4ºC (Beckman Avanti J-25, JS 7.5 rotor, USA). The supernatant was discarded and the open centrifuge bottle was then placed in an inverted position on a pad of paper towels to allow all of the supernatant to drain away. The bacterial cells were lysed by alkali and purified by precipitation with polyethylene glycol.

The bacterial pellet was resuspended in 18 ml of ice-cold Solution I (50 mM glucose, 25 mM Tris-Cl pH 8.0, 10 mM EDTA pH 8.0) by vigorous vortexing. 40 ml of freshly prepared Solution II (0.2 N NaOH, 1% SDS) was then added. The top of the centrifuge bottle was closed and the contents mixed thoroughly by gentle inversion of the bottle several times. The bottle was then stored at room temperature for 10 minutes. 20 ml of ice-cold Solution III (5 M kalium acetate, glacial acetic acid) was then added. The contents were mixed by inverting the bottle several times until the two liquid phases were no longer distinguishable. The bottle was then placed on ice for 10 minutes and a flocculent white precipitate formed.

The bacterial lysate was centrifuged at 5000 rpm for 10 minutes at 4ºC (Beckman Avanti J-25, JS 7.5 rotor, USA). The supernatant was then transferred to a new bottle. 0.6 volume of isopropanol was then added, mixed well, and let stand for 10 minutes at room temperature. The nucleic acids was recovered by centrifugation at 5000 rpm for 15 minutes at room temperature (Beckman Avanti J-25, JS 7.5 rotor, USA). The supernatant was then carefully removed and the open bottle was then placed in an inverted position on a pad of paper towels to allow the last drops of supernatant to drain away. The pellet and the walls of the bottle were rinsed with 70% ethanol at room temperature. The ethanol was drained off and the inverted, open bottle was placed on a pad of paper towels for a few minutes at room
temperature to allow the final traces of ethanol to evaporate. The pellet of nucleic acid was dissolved in 3 ml of sterile UHQ and purified by precipitation with polyethylene glycol.

### 3.7.1 Purification of plasmid by precipitation with polyethylene glycol

The nucleic acid solution to be purified was transferred to an Oakridge tube, and 3 ml of an ice-cold solution of 5 M LiCl was added. The solution was mixed well and centrifuged at 10,000 rpm for 10 minutes at 4°C (Beckman Avanti J-25, JA 25.50 rotor, USA). The supernatant was then transferred to a fresh Oakridge tube and equal volume of isopropanol (6 ml) was added to the solution and mixed well. The precipitated nucleic acid was then recovered by centrifugation at 10,000 rpm for 10 minutes at room temperature (Beckman Avanti J-25, JA 25.50 rotor, USA).

The supernatant was then carefully removed, and the open tube was then inverted to allow the last drops of supernatant to drain away. The pellet and the walls of the tube were washed with 70% ethanol at room temperature. The open inverted tube was placed on a pad of paper towels for a few minutes at room temperature to allow the last traces of ethanol to evaporate.

The pellet was then dissolved in 500µl of sterile UHQ water containing DNAase free pancreatic RNAase (20 µg/ml). The solution was transferred to a microfuge tube and left to stand at room temperature for 30 minutes. At the end of the incubation period, 500µl of 1.6 M NaCl containing 13% (w/v) polyethylene glycol (PEG 8000) was added and mixed well. The plasmid DNA was recovered by centrifugation at 12,000 x g for 10 minutes at 4°C (refrigerated centrifuge HERMLE, Z233MK, Germany). The supernatant was removed before dissolving the pellet in 400 µl of sterile UHQ water. The solution was extracted once with phenol, once with phenol: chloroform, and once with chloroform.

The aqueous phase was then transferred to a fresh microfuge tube and 100 µl of 10 M ammonium acetate was added. The mixture was mixed well and 2 volumes (~1 ml) of absolute ethanol was added and the tube was left to stand for 10 minutes at room temperature. The precipitated plasmid DNA was recovered by centrifugation at 12,000 x g for 10 minutes at 4°C (refrigerated centrifuge HERMLE, Z233MK, Germany). The supernatant was removed, 200 µl of 70% ethanol at 4°C was used to wash the DNA pellet before dissolving it in 100 µl of sterile UHQ water.

### 3.8 MINIPREPARATION OF PLASMID DNA FOR SCREENING OF CLONES

A few colonies from the incubated plates were picked using sterile toothpicks and inoculated in 2 ml of fresh LB medium containing 50µg/ml antibiotic for each single colony. The
cultures were grown overnight at 37°C 225 rpm in an incubator shaker (Innova 4000, USA) overnight. The plasmids were extracted using minipreparations of plasmid DNA by alkaline lysis method (Sambrook et. al., 1989).

The bacterial pellet obtained from 1.5 ml culture was resuspended in 100 µl of ice-cold Solution I (50 mM glucose, 25 mM Tris-Cl pH 8.0, 10 mM EDTA pH 8.0) by vigorous vortexing. 200 µl of freshly prepared Solution II (0.2 N NaOH, 1% SDS) was added and the contents were mixed by inverting the tube a few times. The tube was then left standing at room temperature for 10 minutes. 150 µl of ice-cold Solution III (5 M kalium acetate, glacial acetic acid) was then added and the tube was left standing at room temperature for 10 minutes before centrifugation at 12,000 x g for 10 minutes (Eppendorf, 54 15D, Germany). The clear supernatant was transferred into a fresh tube. The plasmid was then recovered using MiniPreps Express Matrix (BIO 101, USA). 2 µl of the eluted plasmid was digested using appropriate restriction enzyme and the digested product was run through an appropriate percentage of agarose 3:1 gel (Amresco, USA).

3.9 RESULTS
Schematic diagram of cloning of the blunt-ended prM and E insert into PCR-Blunt vector was shown in Figure 3. Figure 4, 5 and 6 show results of the RT-PCR performed on RNA extracts using specific primer pairs. In Figure 4 a band of the expected 572 bp using primer pairs JEPM1 and JEPM2 is clearly shown.
Figure 4: Amplification of prM gene using JEPM1 and JEPM2 primers. M: λHind III/Eco RI DNA marker (Boehringer Mannheim, Germany). Lane 1: prM PCR product.

Amplification of the extracted RNA using specific primer pairs JEE1 and JEE2 produced a single approximately 1.45 kb band. This is shown in Figure 5.
Figure 5: Amplification of E gene using JEE1 and JEE2 primers. M: *Hind* III/Eco RI DNA marker (Boehringer Mannheim, Germany). Lane 1: The product of the PCR reaction; showing the expected band.

Figure 6 shows the result of the RT-PCR done on a JEV RNA extract using specific primer pairs JEEdIIIR and JEEdIIIL. Electrophoresis of the PCR product through a 2.5% agarose gel shows positive result with a band of the expected 329 bp size.
Figure 6: Amplification of E fragment coding for domain III protein using JEEdIIIR and JEEdIIIIL primers. M: 100 bp DNA ladder marker (MBI Fermentas, Lithuania). Lane 1 & 2: PCR products.

The prM and E PCR product were then treated with Klenow to blunt-end the PCR product and cloned into PCR-Blunt vector. Figure 7 shows that the prM PCR fragment was successfully cloned into the PCR-Blunt.
Figure 7: Gel electrophoresis of recombinant JEpM-ZB digested with Eco RI. M: λHind III/Eco RI DNA marker (Boehringer Mannheim, Germany). Lane 1-4: JEpM-ZB.

Digestion of the JEE-ZB transformant plasmids with the restriction enzyme Bam HI (Figure 8) yielded 2 expected bands with the size of approximately 1.45 kb (E insert fragment) and 3.5 kb (vector).
Figure 8: Gel electrophoresis of recombinant JEEM-ZB digested with Eco RI. M: 2Hind III/Eco RI DNA marker (Boehringer Mannheim, Germany). Lane 1-4: JEE-ZB.

3.10 DISCUSSION

The quality and quantity of cDNA synthesized is critically dependent on the integrity of the mRNA used as the template. Extremely stable RNAases are ubiquitous in the lab environment and therefore certain precautions should be taken to eliminate the risk of RNAase contamination whenever possible. External sources of potential RNAase contamination must be identified and neutralized from the onset of the experiment. All reagents, bottles, containers and tubes used in the process of extracting RNA must be maintained RNAase-free at all times. Gloves must be worn and changed frequently when handling RNA because the greatest source of RNAase is the oil from the fingertips. Internal RNAase activity could be avoided by the addition of RNAase inhibitor such as guanidinium thiocyanate directly to lysis and reaction buffers.

The most basic rule is to have RNA extracts of good quality that will be used as a template in RT step. Extraction of RNA using TRI Reagent (Sigma, USA) gave a high quality of RNA which is free of DNA or protein. The fact that the resuspension of the pellet can easily be done using sterile water gave the advantage of having a high concentration of RNA. The RNA extract was then used in the RT step.