INVESTIGATION INTO THE RELATIONSHIP BETWEEN JAPANESE ENCEPHALITIS VIRUS AND C6/36 CELLS

VERAWAN BOONSANAY

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Verawan Boonsanay
Miss Verawan Boonsanay
Candidate

Prof. Duncan R. Smith, Ph.D.
Major-Advisor

Assoc. Prof. Albert J. Ketterman, Ph.D.
Co-Advisor

Lect. Kanokporn Triwitayakorn, Ph.D.
Co-Advisor

Assoc. Prof. Rassmidara Hoonsawat, Ph.D.
Dean
Faculty of Graduate Studies

V. Akkarapatumwong
Asst. Prof. Varaporn Akkarapatumwong, Ph.D.
Chair
Master of Science Programme in
Molecular Genetics and Genetic Engineering
Institute of Molecular Biology and Genetics

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6 June, 2005

Verawan Boonsanay
.....
Miss Verawan Boonsanay
Candidate

.....
Prof. Duncan R. Smith, Ph.D.
Chair

.....
Assoc. Prof. Albert J. Ketterman, Ph.D.
Member

.....
Lect. Thananya Thongtan, Ph.D.
Member

.....
Lect. Kanokporn Triwitayakorn, Ph.D.
Member

.....
Assoc. Prof. Rassmidara Hoonsawat, Ph.D.
Dean
Faculty of Graduate Studies
Mahidol University

.....
Asst. Prof. Chartchai Krittanai, Ph.D.
Acting Director
Institute of molecular Biology and Genetics
Mahidol University

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Verawan Boonsanay

INVESTIGATION INTO THE RELATIONSHIP BETWEEN JAPANESE ENCEPHALITIS VIRUS AND C6/36 CELLS

VERAWAN BOONSANAY 4636852 MBMG/M

M.Sc.(MOLECULAR GENETICS AND GENETIC ENGINEERING)

THESIS ADVISORS: DUNCAN R. SMITH, Ph.D., ALBERT J. KETTERMAN, Ph.D., KANOKPORN TRIWITAYAKORN, Ph.D.

ABSTRACT

Japanese encephalitis virus (JEV) is the causative agent of Japanese encephalitis, which is the most important cause of viral encephalitis in Asia. JEV is a member of the flavivirus genus and is transmitted naturally to the vertebrate host by mosquitos, especially Culex mosquito's. This study sought to investigate the relationship between mosquito cells and JEV by using C6/36 cells, a cell line that has been derived from whole hatched larva of A. albopictus, as a model in respect of viral propagation and JEV binding proteins.

Viral growth from infected C6/36 cells was examined to determine the time needed to produce the maximum viral yield. The highest yield of JEV was produced by day 5 post infection. In case of viral production which indicated the one complete viral cycle, JEV required at least 10 hrs. after infection to complete a single viral cycle. Viral internalization profile showed an initial plateau between 1 and 2 hours and second plateau between 3 and 4 hours, possibly the recycling time of a JEV receptor protein. Similar to the dengue virus, JEV lost infectivity over the time, and showed a half-life time of 9.6 hours. The propagation and internalization pattern of JEV reflects closely the dengue virus. However, JEV internalization and propagation evidence seem to be faster than the dengue virus. For identification and characterization of JEV receptor molecules on the surface of C6/36 cell, the contribution of extra-cellular domains of plasma membrane proteins to the internalization of JEV was investigated. Pre-treatment of C6/36 cells with Heparinase and trypsin both resulted in reduction of viral production, 60% for trypsin pre-treatment and 40% for Heparinase pre-treatment. Virus overlay protein binding assay (VOPBA) identified a highly expressed JV binding band of 53kD, which was further analyzed by mass spectrometry. However limitations of information currently available in mosquito databases might be the cause of no matches with any proteins. Moreover, there is evidence of the 37/67kDa high affinity laminin receptor and heparin sulfate exhibit minor involved in the binding and entry of JEV into insect cells. This suggests that this 53 kDa binding molecule may be a possible candidate or component of the cellular receptor for JEV.

KEY WORDS: JAPANESE ENCEPHALITIS VIRUS / VIRAL PROPAGATION / VIRAL INTERNALIZATION/ PUTATIVE RECEPTORS

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การศึกษาความสัมพันธ์ระหว่างไวรัสไข้สมองอักเสบกับเซลล์ C6/36
(INVESTIGATION INTO THE RELATIONSHIP BETWEEN JAPANESE ENCEPHALITIS VIRUS AND C6/36 CELLS)

วีรวัลย์ บุญเสน่ห์ 4636852 MBMG/M

วท.ม. (อณูพันธุศาสตร์และพันธุวิศวกรรมศาสตร์)

คณะกรรมการควบคุมวิทยานิพนธ์ : DUNCAN R. SMITH, Ph.D., ALBERT J. KETTERMAN, Ph.D., กนกพร ไตรวิทยากร, Ph.D.

บทคัดย่อ

ไวรัสไข้สมองอักเสบ (Japanese Encephalitis Virus) เป็นไวรัสที่ทำให้เกิดโรคไข้สมองอักเสบซึ่งเป็นปัญหาสำคัญต่อวงการสาธารณสุขในแถบภูมิภาคเอเชีย เชื้อไวรัสไข้สมองอักเสบจัดอยู่ในกลุ่ม Flavivirus ซึ่งมียุงโดยเฉพาะในกลุ่ม Culex เป็นพาหะ งานวิจัยนี้มีจุดประสงค์เพื่อศึกษาความสัมพันธ์ระหว่างไวรัสไข้สมองอักเสบกับเซลล์ยุงโดยเฉพาะด้านการเพิ่มจำนวนของไวรัสและโมเลกุลตอบรับไวรัสไข้สมองอักเสบบนผิวเซลล์ C6/36 ซึ่งเป็นเซลล์ที่ได้มาจากตัวอ่อนของยุง C6/36 ในกลุ่ม *A.albopictus* จากการศึกษาระยะเวลาการเจริญเติบโตของไวรัสในC6/36เซลล์พบว่าไว้รัสเพิ่มจำนวนได้มากที่สุดในวันที่ 5 หลังการติดเชื้อ ส่วนช่วงเวลาที่ไวรัสใหม่ถูกผลิตออกมาซึ่งแสดงถึงภาวะการเจริญเติบโตของไวรัสครบ 1 รอบจะเกิดขึ้นในชั่วโมงที่ 10 หลังการติดเชื้อและการศึกษาการเข้าเซลล์ C6/36 ของไวรัสพบว่าการเข้าเซลล์ของไวรัสมีลักษณะคงที่อยู่ 2 ช่วงคือระหว่างชั่วโมงที่ 1 และ 2 กับชั่วโมงที่ 3 และ 4 ซึ่งอาจบงชี้ถึงการย้อนกลับของตัวตอบรับไวรัสบนผิวเซลล์ ซึ่งการลดลงของความสามารถในการติดเชื้อของไวรัสในอาหารเลี้ยงเซลล์มีลักษณะคล้ายกันกับของไวรัสเด็งกี่ จากการเปรียบเทียบพบว่าลัษณะการเข้าเซลล์และการเพิ่มจำนวนของไวรัสไข้สมองอักแกิดขึ้นได้รวดเร็วกว่าไวรัสเด็งกี่ สำหรับการพิสูจน์หาโมเลกุลตอบรับบนผิวเซลล์ C6/36 โดยการหาส่วนประกอบด้านนอกของโปรตีนบนผิวเซลล์พบว่าการใส่เอนเซมน์ Trypsin ก่อนการติดเชื้อมีผลทำให้การติดเชื้อลดลง 60%ในขณะที่การใส่เอนเซมน์ Heparinase มีผลทำให้การติดเชื้อลดลง 40% จากการใช้เทคนิค Viral overlay protein binding assay พบโปรตีนขนาด 53 kDa เกี่ยวกับการจับของไวรัส อย่างไรก็ตามจากผลการวิเคราะห์ด้วยวิธี mass spectrometry ไม่สามารถบ่งชี้ชนิดของโปรตีนดังกล่าวเนื่องจากความจำกัดของกลุ่มข้อมูลในยุง การค้นพบนี้แสดงให้เห็นถึงความเป็นไปได้ที่โปรตีนขนาด 53 kDa จะเป็นตัวตอบรับหรือเป็นส่วนประกอบของตัวตอบรับบนผิวเซลล์ยุงสำหรับไวรัสไข้สมองอักเสบ

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LIST OF ABBREVIATIONS

BJ-1	Beijing-1
C protein	nucleocapsid
CPE	cytopathic effect
cpm	count per minute
JEV	Japanese encephalitis virus
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
ER	endoplasmic recticulum
E protein	envelope protein
EBSS	Earle's balanced salts solution
EDTA	ethylene diamine tetraacetic acid
Et al	and other
FBS	fecal bovine serum
GAG	glycosaminoglycan
g	gram (s)
dH_2O	distill water
hr	hour (s)
kDa	kilodalton
M protein	membrane protein
M	molar

LIST OF ABBREVIATIONS (cont.)

MEM	Minimum Essential Medium
M-199	medium 199 (199/EBSS)
mg	milligram
min	minute (s)
ml	milliliter
mM	millimolar
moi	multiplicity of infection
NS protein	nonstructural protein
$NaHCO_3$	sodium bicarbonate
NaCl	sodium choride
ORF	open reading frame
ºC	degree Celsius
PEG 8,000	Polyethylene glycol Mw 8,000
pfu	plaque forming unit
prM protein	pre-membrane protein
RNA	ribonucleic acid
rpm	revolution per minute
SDS	sodium dodecyl sulfate

LIST OF ABBREVIATIONS (cont.)

SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TEMED	N, N, N', N' tetramethylenediamine
Tris-HCL	Tris-(hydroxymethyl)-aminomethane
UTR	untranslated region
µg	microgram
µl	microliter
µM	micromolar
WHO	World Health Organization

CHAPTER I
INTRODUCTION

The mosquito-borne flavivirus, Japanese encephalitis virus (JEV) is the leading cause of Japanese encephalitis disease which is one of the most important causes of epidemic encephalitis worldwide, with an estimated 35,000 to 50,000 cases and 10,000 deaths annually [1]. The virus is a member of the JE serogroup of the genus *Flavivirus*, family *Flaviviridae*. JEV like other flaviviruses is an enveloped, plus- sense single stranded RNA of approximately 11 Kb in length. JEV is related to St. Louis encephalitis virus, Murray Valley virus and West Nile virus. The virus is antigenic ally related to several other flaviviruses including the dengue virus. The virus contains several structural and non-structural polypeptides, wrapped in nucleocapsid and surrounded by glycoprotein-containing lipid envelope. The RNA of approximately 11 kilo base encodes three structural proteins (capsid [C], premembrane [PrM], and envelope [E]) and seven nonstructural (NS) proteins [2].

The virus was recognized as a causative agent of the disease in horses and humans as early as 1871 [3], and epidemic of Japanese encephalitis (JE) was first recognized in 1969 in the Northern part in Thailand [4]. JE is generally a disease of rural agricultural areas, where the vector mosquitoes proliferate in close association with birds, equines and swine which serve as the principal vertebrate amplifying hosts [5]. JEV is transmitted naturally in an enzootic cycle among birds, pigs and other vertebrate hosts by mosquitoes, principally by *Culex tritaeniorhynchus* and it is the principal vector in most areas of Asia [6]. JEV infection of human occurs through the bite of infected mosquitoes. The process of JEV replication is accompanied by inflammatory reaction with resultant neuronal destruction. The degree of severity of the disease in human caused by this virus is rather dependent on the degree of neural destruction. The rate of fatality averages about 10-20% and survivors are often with permanent neurological sequelae such as mental retardation, epilepsy, paralysis, deafness and blindness [7].

Nowadays, there is no efficient drug treatment for the disease, and prevention of JEV infection mainly relies on vaccination. There are three commercially available JEV vaccines that are currently used [8] which are inactivated vaccine made from virions grown in mouse brain tissue and in primary kidney cells. As well as the live attenuated vaccine produced from an attenuated strain [9]. An internationally license, fomalin inactivated, whole virion JEV vaccine prepared from virus-infected mouse brain has greatly reduced the incidence of disease in Japan, Taiwan, Thailand and The Republic of Korea. However existing products (formalin-inactivated, whole-virion vaccine produced in mouse-brain tissue) have many disadvantages, including expensive, reactogenicity, and the requirement for multiple doses to be effective [9]. For these reasons, a high priority has been placed on development of improved vaccine.

The study of vector –virus interaction will enable the characterization of important vector cell surface molecules. Moreover this knowledge could be used to develop treatment that block virus transmission and antiviral agents that target vector immunity. The objective of this study was the attempt to study the mechanism of JEV infection in mosquito cell line. C6/36 cell line has been derived from whole hatched larva of *Aedes albopitus*, as this cell line has been reported susceptible to flavivirus including Dengue virus and JEV. By testing for parental and oral susceptibility to infection with these two viruses C6/36 cell appear more susceptible to JEV than dengue virus under the same conditions [10]. Therefore it was used as a model study.

CHAPTER II
LITERATURE REVIEW

Japanese Encephalitis Virus

1. General consideration

Japanese encephalitis (JE) virus is a mosquito-borne arboviral disease that is a leading cause of major public health importance in Asia. Viral infections can be found throughout the temperate and tropical zones of Asia [1]. Approximately 3 billion people and 60% of the world's population live in endemic region and about 50,000 cases with 10,000 deaths annually. Outbreaks of encephalitis have been described in Japan from the 1870s onwards [2], and the virus was first isolated from a post-mortem human brain in Japan in 1933, and has been recognized across Asia since then (Figure 1). JE virus (JEV) is a member of the genus *Flavivirus* (family Flaviviridae) that is transmitted naturally in an enzootic cycle among birds, pigs, and other vertebrate hosts by mosquitos especially *Culex tritaeniorhynchus*. Closely related mosquito-borne flaviviruses that cause nuerological disease include St Louis encephalitis virus, Murray Valley encephalitis virus, and West Nile virus. The *Flavivirus* genus also includes the yellow fever and dengue viruses, which cause hemorrhagic fevers [1]. The origins of the virus are uncertain, but phylogenetic comparisons with other flaviviruses suggest it evolved from an African ancestral virus, perhaps as recently as a few centuries ago [3]. Humans become infected with Japanese encephalitis virus coincidentally when living or traveling in close proximity to the enzootic cycle of the virus.

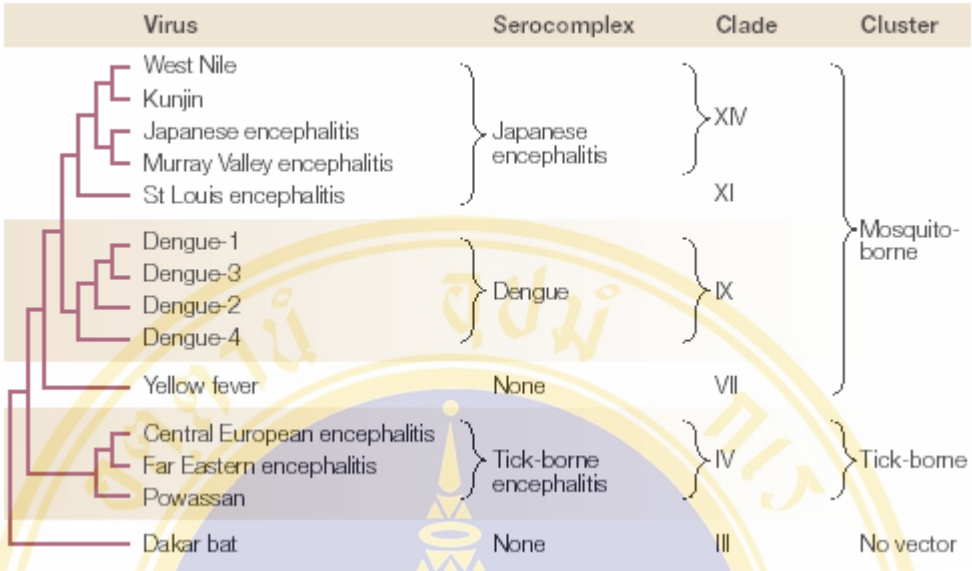


Figure 1 Flavivirus classification.

The relationships between selected flaviviruses are shown in the dendrogram on the left. Evolutionary distance is not represented in this figure. The serological (serocomplex) and phylogenetical (clade and cluster) classifications of these flaviviruses are shown on the right [4].

2. Classification

The geographic variation of JEV strains was demonstrated by studies of RNA fingerprints and nucleotide sequencing [5]. The genomic diversity of JEV has been determined by primer extension sequencing of the capsid/pre-membrane (C/prM) gene region. These analyses indicate that JEV strains could be classified into four geographically distinct genotypes based on nucleotide differences of more than 12%, and representatives of three of which have been fully sequenced. Genotype I includes isolates from northern Thailand, Cambodia, and Korea, genotype II includes isolates from southern Thailand, Malaysia, Indonesia, and Northern Australia, genotype III includes isolates from mostly temperate regions of Asia, including Japan, China, Taiwan, the Philippines, and the Asian subcontinent, and genotype IV includes isolates from Indonesia. In addition, a strain of JEV isolated in Singapore in 1952 from a

patient who originated in Muar, Malaysia (Muar strain), may represent a fifth genotype, according to cross-neutralization and limited phylogenetic evidence. Because genotypes I and III occur in epidemic regions, whereas II and IV are associated with endemic disease, it has been postulated that differences in strain virulence may explain the clinical epidemiology. However, strains are increasingly being identified that do not fit this paradigm. For example, in Vietnam epidemic disease occurs in the north and endemic disease occurs in the south, yet genotype III strains have been isolated in both areas. More recently, a genotype I strain has been found in northern Australia [3].

3. Genome structure

In common with all flaviviruses, the Japanese encephalitis virion is approximately 500 Å in diameter and is composed of an 11 kb positive-sense single-stranded RNA genome that is packaged by virus capsid protein in a host-derived lipid bilayer and surrounded by 180 copies of two glycoproteins. The most notable feature of the flavivirus genome is the presence of a single open reading frame (ORF) of ~ 11 kb which codes for 3386-3433 amino acids [6]. Untranslated regions flank the ORF at the 5' and 3' termini. The 5' untranslated region (UTR) is capped (type I) and is 95-132 nucleotides in length, starts with the conserved dinucleotide AG and contains conserved elements potentially involved in secondary structure formation. The 3' UTR lacks a poly(A) tract, terminates with the conserve dinucleotide CU and contains several conserved sequences specific for mosquitoes tick born viruses [7].

The polypeptide is subsequently cleaved by cellular and viral encoded proteinase and glycosylated by cellular glycotransferase to yield three structure proteins: capsid protein (C); precursor to the membrane M protein (PrM) consisting of 166 residues; and envelope protein (E) consisting of 495 residues and seven non-structural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Figure 2).

NS1 is a membrane-associated glycoprotein of unknown function but thought to be involved in the early stages of virus replication.

NS3 is the second largest viral protein; sequence comparisons and biochemical analyses suggest that NS3 may be trifunctional, with protease, helicase and RNA triphosphatase activities [8]. The N-terminal region of NS3 contains significant

homology with serine proteases and is thought to function in polyprotein cleavage in association with the NS2B protein; The C terminus of NS3 contains significant homology with a family of RNA helicase protein [9].

NS5 is the largest and most highly conserved flavivirus protein, is thought to function as the viral RNA polymerase and possesses a GDD motif in common with RNA–dependent RNA polymerases of other positive-strand RNA viruses. The N-terminus of NS5 is homologus with several methyltransferase proteins and it has been suggested that this domain is involved in methylation of the 5' cap [10]

NS2A, NS2B, NS4A and NS4B are poorly conserved membrane-associated proteins, whose function is currently unclear. However, the strong association of the viral polymerase complex with cell membranes suggests that these proteins may be necessaries for localization and activity of the polymerase complex.

The nucleocapsid core of the mature virion consists of the genomic RNA surrounded by multiple copies of the capsid protein C. This core in enveloped by lipid bilayer derived from the endoplasmic reticulum of host cell. Outside the membrane envelop is a layer of 180 copies of the E protein organized into a herringbone pattern [11] plus 80 copies of the M protein. Both E and M proteins are anchored in the membrane by their C terminal domains.

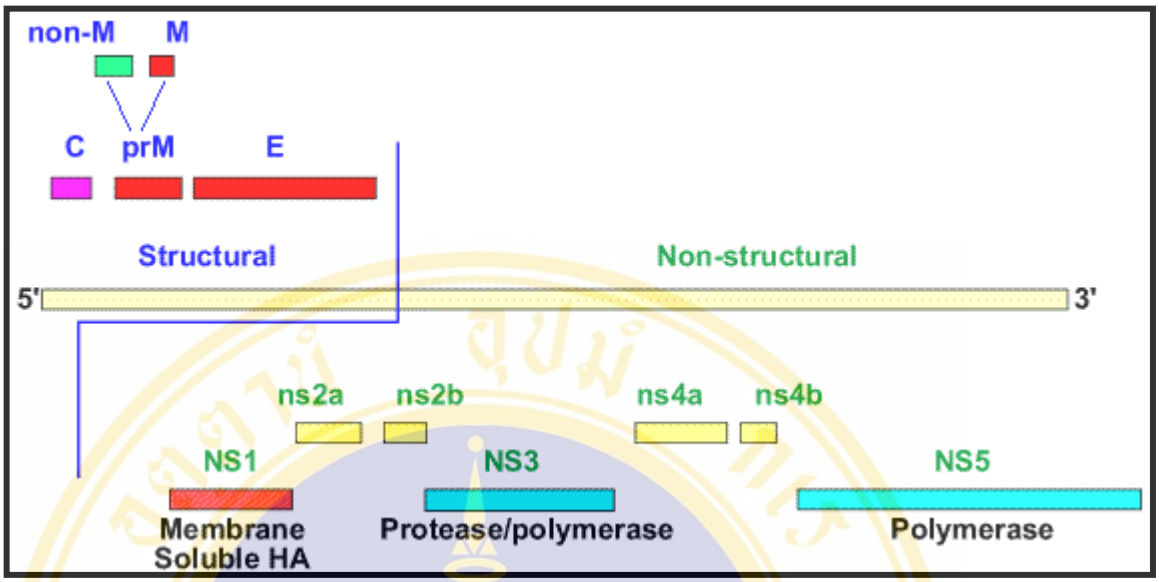


Figure 2 The flavivirus RNA genome

The single long open reading frame (ORF) spans the genome and starts at an AUG codon and terminates in a UAA codon. The ORF (yellow bar) is translated as a single polyprotein that is cleaved by viral and host protease to yield the ten viral proteins. The C protein, M protein, E glycoprotein are the structural proteins. And the NS proteins are involved in viral replication [8].

4. JEV structure proteins

a. Envelope (E) protein

The E glycoprotein, the major virion envelope glycoprotein, appears as homotrimer on the surface of mature virions. Comparison of the nucleic acid sequences of several flavivirus E genes has shown perfect conservation of 12 cysteine residues that form six disulfide bridges. The search for genetic determinants of virulence in animal models of flavivirus encephalitis has focused on the E protein. This protein is the major target for the humeral immune response, and is thought to be important in viral entry to host cells, and is thought to be the cell receptor binding protein and mediator of membrane fusion and cell entry. Studies with monoclonal antibodies suggested three antigenic domains [12], and these were confirmed more recently when the three dimensional structure of ectodomain of the flavivirus Tick-

borne encephalitis virus was determined by x-ray crystallography [13] Domain III is a putative receptor binding domain, domain II is the dimerization domain, and domain I has a central ß barrel and is the hinge domain that links the other two domains (Figure 3). A Various approaches have allowed the E gene sequences of flaviviruses to be related to virulence in animal models. These studies suggest that the E protein has a major role in determination of virulence phenotype, and that single amino acid substitutions are sufficient to cause loss of neurovirulence or neuroinvasiveness [14]. Whether such differences are important in determining the clinical presentation of Japanese encephalitis virus in humans is unknown.

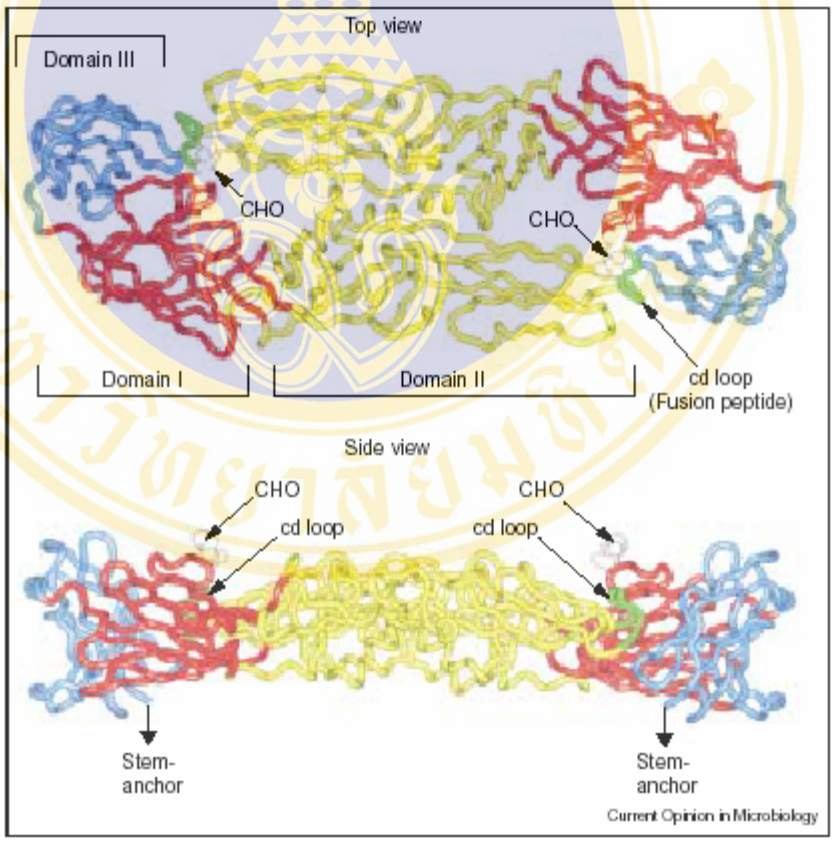


Figure 3 Ribbon diagram of E protein dimer of the TBE virus [15].

b. Membrane (PrM/M) protein

The prM protein is glycoprotein precursor of the structural protein M. Specific proteolytic cleavage of glycosylated prM precursor (22,000) during virus maturation results in the formation of an 8,000 dalton M protein. This cleavage appears to precede virus release from the cell. The formation of M from prM appears to be the crucial, terminal event in virion morphogenesis. It results in a large increase in virus infectivity and reorganization of the virus surface structure, which is composed of E-prM heterodimers in immature virions [6].

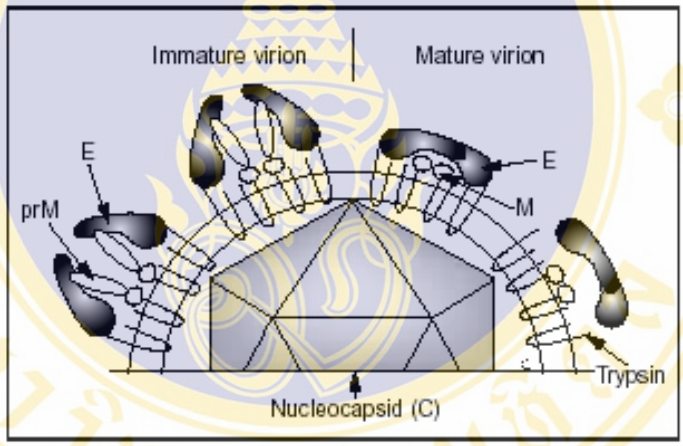


Figure 4 Schematic diagram of the composition of immature and mature flavivirus [15].

c. Capsid (C) protein

The virion C protein is the first viral polypeptide synthesized during translation, has molecular weight of about 13,500 and is rich in lysine and arginine residues (about 25%). This highly positively charged character probably enables it to interact with the virion RNA [16]. C protein lacks an N-terminal, hydrophobic signal sequence, which suggests its synthesis is on non-membrane bound ribosomes. Hydropathy plots of C protein indicate that it is conserved structurally among the flavivirus, but less so than other structural protein.

5. Processing of the structural proteins

During translation of the polyprotein, the structural proteins are translocated and anchored in the ER by various signal sequences and membrane anchor domains. The capsid protein contains a hydrophobic signal sequence at its carboxyl terminus that translocates prM into the lumen of the ER from its site of synthesis on the surface of the ER. The prM protein has two transmembrane-spanning domains, which contain a stop transfer sequence and a signal sequence. As a result, the E protein is also translocated into the lumen of the ER [4]. After the appropriate proteolytic cleavages, the capsid protein and viral RNA are localized in the cytoplasm and the capsid protein remains associated with the ER membrane. On the lumenal side of the ER, the prM and E proteins form a stable heterodimer within a few minutes of translation [15].

6. Strategies of Viral replication

6.1 Viral entry

The early events of viral infection, especially the interaction between viral attachment proteins and cellular receptors, are one of the factors determine viral tropism. JEV may attach to the target cells via a trysin-sensitive receptor. In this way, the virus will penetrate into host cells via receptor-mediated endocytosis by two methods. The virion envelope may fuse with the plasma membrane with immediate deposition of the nucleocapsid into the cytoplasm. Alternatively the plasma membrane may invaginate to form an endocytic vesicle (endosome) around the still enveloped virus. Endosomatic pH changes triggers fusion between viral envelope proteins and endosomal membrane and then delivers the nucleocapsid into cytoplasm where polyprotein translation occurs [17].

Following viral attachment to the cell surface, JEV enters cells by endocytosis and subsequence fusion of lipid membrane, with the endosome membrane allow viral RNA to penetrate into the cytoplasm of the infected cells. This fusion, which occurs as the pH of the endosome drops, is thought to be mediated by a conformational change around a putative hinge region between domain I and II of the E protein, which brings a ß barrel shaped fusion peptide at the tip of domain II to insert into the host cell membrane [4] (Figure 5).

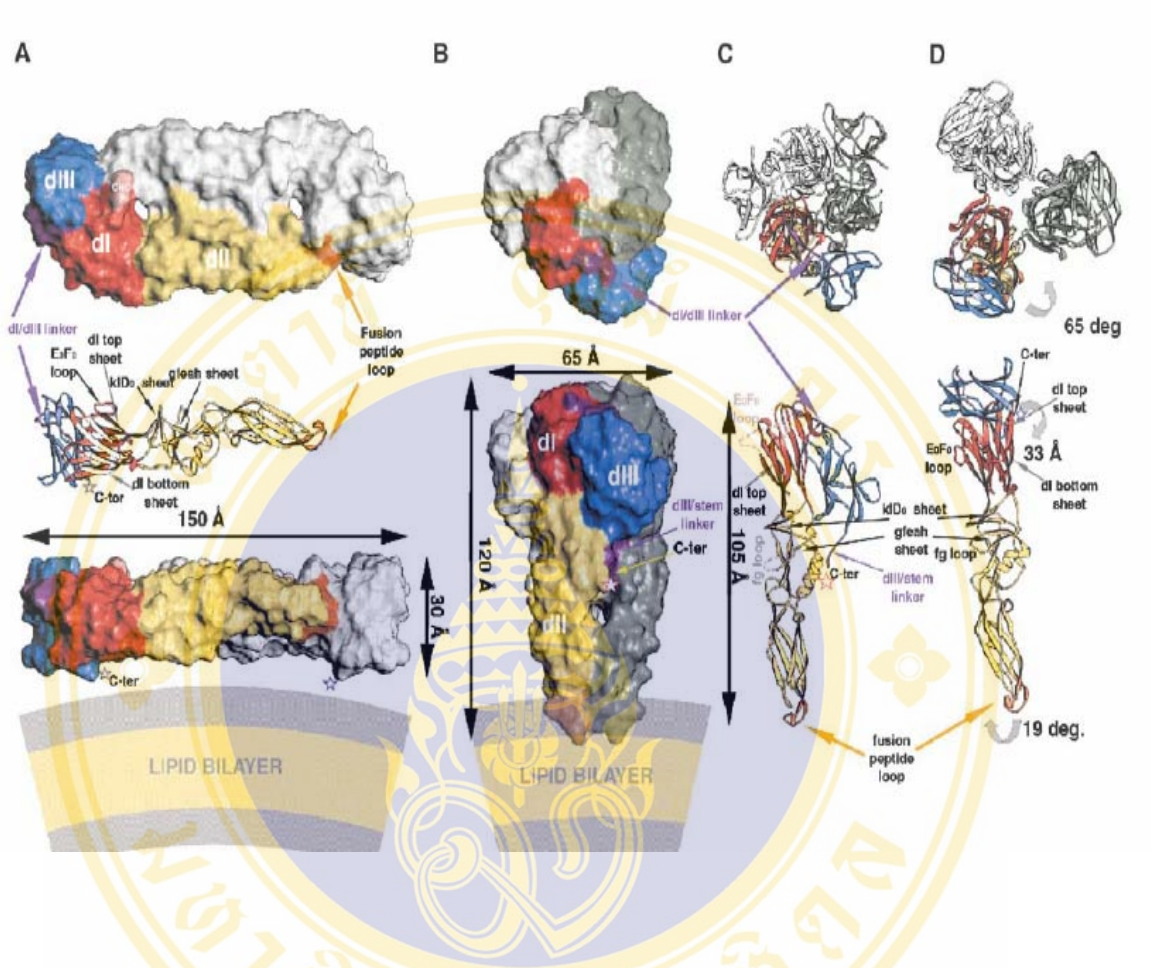


Figure 5 Conformational rearrangement of protein E. Comparison of the overall organization of the protein in the neutral- and acid-pH forms [4].

A = Neutral-pH, dimeric conformation of E protein

B = Low-pH conformation of E protein

C = Ribbon diagram of the polypeptide chain of E protein in trimeric conformation

D = Conformation rearrangement of E protein

The ‘top’ and ‘side’ views are indicated in the top and bottom rows, respectively. The three domains of E are labeled dI, dII, and dIII in red, yellow, and blue, respectively.

6.2 RNA replication

Since the JEV RNA genome has a positive sense, it must first be translated to make the RNA polymerase require for it replication. The polymerase must transcribe the positive-strand RNA to the negative-strand RNA, which then serve as a template for additional positive strands. Also, positive and negative strands should be made at approximately equal rate to allow RNA amplification to occur exponentially. However, positive-strand RNA must be diverted to virus assembly during the late eclipse. The positive strand can then be used for translation of structural and non-structural polypeptides or synthesis of negative strains or can be encapsidated into virions. The actinomycin D-resistant synthesis of flavivirus specific RNA is detectable within six hours post infection of the vertebrate cells, and a progressive increase in positive strand RNA is observed. Evident suggests that positive strand RNA molecules be synthesized from negative strand templates by a semi conservative mechanism involving replicative intermediates. The replicative form is defined as duplex RNA molecules.

It is not known how RNA replication is regulated, but it is possible that the early and the late RNA polymerase complexes have difference affinities for positive and negative strand templates, as the late replication process favors synthesis of the infectious positive strand. An increasing concentration of C protein late in infection may begin the assembly of nucleocapsids, removing positive strands as a substrate for replication. It has been proposed that binding of C protein to a site at the 3’ end of positive strand RNA prevents it from being recognized by RNA polymerase, but not by ribosomes which bind at the 5’ end. This would allow for its continued translation and explain the predominance of positive strand RNA later in infection [17].

6.3 Virus assembly and Release

Flavivirus nucleocapsids assemble from C protein which has two forms. One form of C protein contains a hydrophobic stretch of amino acids at carboxy terminus which may anchor it to the membrane of rough endoplasmic reticulum and is removed by proteolytic cleavage during the maturation of the virions. This evidence led to the speculation that membrane-bound core protein assembles into nucleocapsids which then simultaneously budding so that unenveloped nucleocapsid is not seen.

Release of virus from the infected cell presumable occurs via secertory excytosis as virus-containing secretory vesicle fuse with the plasma membrane and is mediated by a host protease or possible viral protease. The cleavage site sequence, Arg-X-Arg/Lys-Arg (where X is variable), is similar to those recognized in maturation cleavages of numerous viral glycoproteins and cellular pro-proteins. The inability to detect intracellular M-containing virions suggests that this cleavage occurs just prior or during release of virions. Cleavage of prM is accompanied by reorganization of virion envelop from one containing prM-E heterodimers to one containing E protein trimers. The biological role of prM cleavage is still unclear, but like similar glycoprotein cleavages for other envelop viruses, it may trigger changes in the virion envelop that promote infectivity [17].

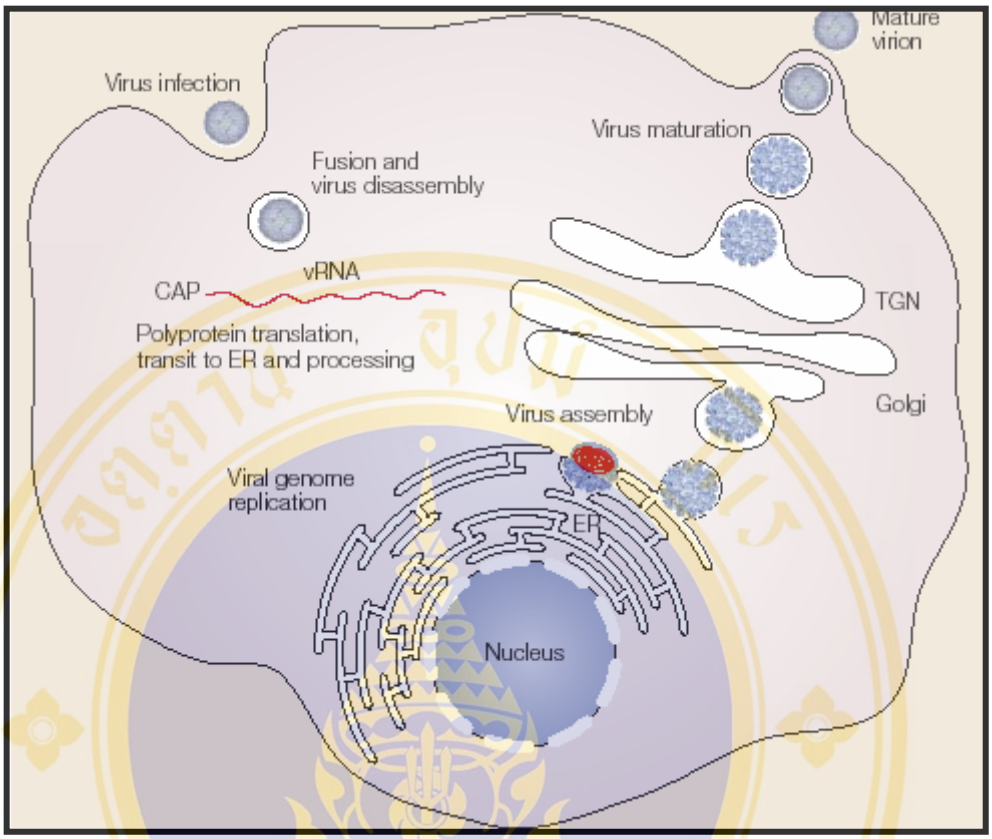


Figure 6 The flavivirus life cycle [4]

Assembly of JEV has four continues phases;

1. Assembly of nucleocapsids from C protein and RNA
2. Budding of nucleocapsids through membrane containing integral E and prM proteins to acquired an envelope
3. Exit from the cells, either as a result of the budding process or afterwards, in exocytic vesicles
4. Cleavage of the prM protein, resulting in the reorganization of the virion surface and virion maturation

7. Clinical features of Japanese encephalitis infection

Japanese encephalitis is a serious disease caused by the virus transmitted by the kind of mosquito, *Culex tritaeniorhyncus*. The majority of the population in rural Asia has been infected with the virus by early adulthood. Humans become infected during the bite of an infected mosquito. In humans, JEV infections range from a febrile headache syndrome to an acute and possibly fatal encephalitis. The main symptoms are fever, meningeal irritation and encephalitis. There is an incubation period of about two weeks after infection to onset of the illness. Most patients show sudden fever and headache. The temperature rises to 39-40 ºC or higher within a few days. Other early symptoms are anorexia, feeling of disorientation of the whole body, vomiting, diarrhea, pains at different parts of the body, etc. In people who develop a more severe disease, JE usually begins with fever, chills, tiredness, headache, nausea and vomiting and the illness can progress to encephalitis and can be fatal in 30% of cases. Normally neurologic disease typically develops in patients after an incubation period of 5 to 15 days or recently described polio-like acute flaccid paralysis [18]. Severe cases may progress to confusion and coma. Confusion and agitation can also occur in the early stage. Illness usually begins with high fever, change in mental status and headache, followed gradually disturbances in speech, gait or other motor dysfunction. The initial presentation in children usually begins with gastrointestinal symptoms of anorexia, nausea, or abdominal pain. Irritability, vomiting and diarrhea or an acute convulsion may be the earliest objective signs of illness in an infant or child. Seizures occur in over three-fourths of pediatric patients but are observed less frequently in adults. Conversely, headache and meningimus are more common in adults.

8. Pathogenesis

The pathogenesis of infection of Japanese encephalitis is not completely known yet. After the viruses have invaded the human body by a mosquito's bite, it primarily proliferates in the local lymph node, and subsequently enters into the blood sweam causing viremia. The virus comes to the lymphatic tissue of the whole body and the bone marrow. The viruses can able to proliferate there, causing secondary viremia, and come to the tissue of the cranial nervous system. It is thought that in most

cases, the infection is unapparent because the virus does not enter the brain because of the blood-brain barrier. The morbidity of the disease is low, being one morbid case per 100-1,000 infected cases. In a small scale epidemic however, the morbidity was estimated at one morbid case per 25 infected cases. The pathogenesis of flavivirus encephalitis is consisting of direct, viral mediated damage and the host inflammatory response. The host immune response comprises antibody-mediated immunity, particularly against the E and NS1 proteins, and cell-mediated immunity, including T lymphocyte. It has also been shown the role of apoptosis in the pathogenesis of JEV [19]. Its replication triggers apoptosis in various cell lines. In vitro apoptosis was associated with rise in the expression of nuclear factor kappa B [20]. Cytolytic viruses are known to cause their host cells to disintegrate by increasing plasma membrane permeability, causing a loss of cellular ion gradients and leakage of essential compounds from the cell, which leads to necrosis [21].

When a person has been exposed to one flavivirus, cross-reacting antibodies may affect the outcome of infection with a second flavivirus. Thus, for patients with Japanese encephalitis, prior infection with dengue virus, which circulates through much of Asia, appears to protect against severe of disease [22]. These provide the reduction in secondary flavivirus infections.

9. Epidemiology

JEV is the leading cause of viral encephalitis in Asia. Approximately 50,000 sporadic and epidemic cases of encephalitis are estimated to occur annually. A growing number of cases have been seen in China, India, Nepal, Philippines, Sri Lanka, northern Thailand, Vietnam and Myanmar [2]. JEV is the most common documented cause of viral encephalitis in Cambodia. The main characteristics of JE epidemiology in Taiwan in the past three decades is the transmission mode remain unchanged; that is the amplification of virus is followed by a human epidemic each year. Rise in population density, deforestation and increasing irrigation of agricultural areas may contribute to the rise in JE incidence in South East Asia. JEV infections were occasionally found in Indonesia and northern Australia and never found in United States. In recent years, JE is rare in Japan due to JE-virus vaccination, use of agricultural pesticides and controlled pig farming. The occurrence of JE has also been

reported to be less in Korea, China and Taiwan due to JE-virus vaccination of children[18].

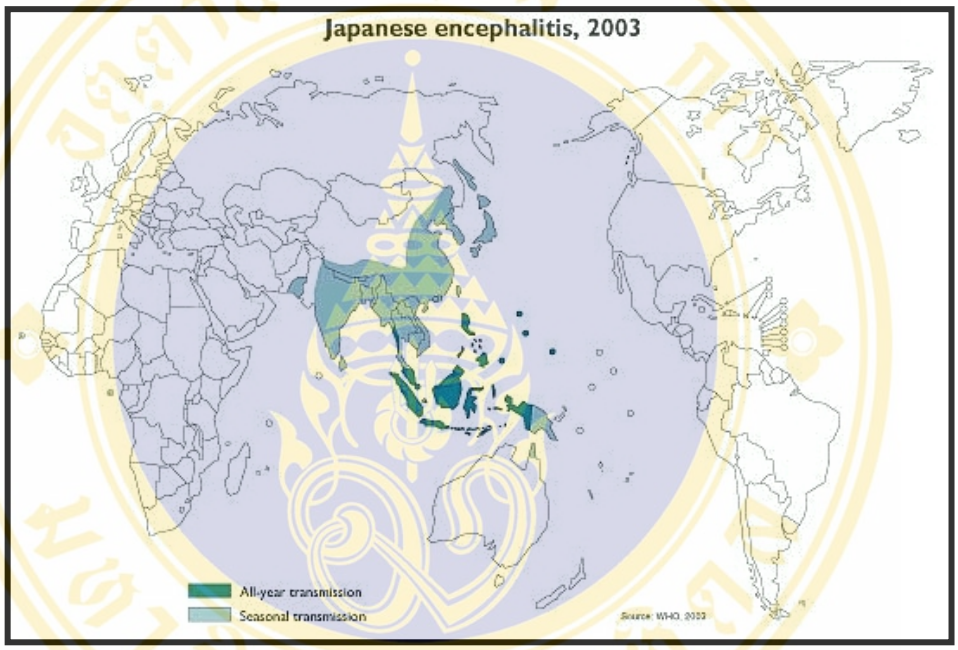


Figure 7 Current distribution of Japanese encephalitis, 2003
(www.info.gov.hk)

The dark green represents the all-year transmission.
The light green represents the season transmission.

Two epidemiological patterns of JE are recognized [3]. In northern temperate areas JE occurs in summer epidemics, whereas in southern tropical areas the disease is endemic and occurs year-round. Risk for acquiring JE among most travelers to Asia is extremely low; however, the risk for an individual traveler is highly variable and depends on factors such as the season, locations and duration of travel, and activities of the person. Travel during the transmission season and exposure in rural areas, especially for extended periods of time, are the principal factors contributing to risk. The nature of outdoor activity, use of protective clothing, bed nets and repellents, and lodging in air-conditioned or well-screened rooms are additional factors that affect exposure. Residents of developed countries usually have no natural immunity to JE virus and travelers of all ages are equally susceptible to infection with JE virus; however, the elderly may be more susceptible to developing neuroinvasive disease.

Although the probability of exposure to JE viral infection and illness increases with the duration of stay in rural endemic areas, one case occurred in a traveler who made only a few excursions into rural areas while on a 2-week vacation [23].

Thailand

In 1969 the first epidemics of JEV was reported in Chiang Mai valley and nearby areas in Northern Thailand during the rainy season. The total number of patients was 655 with 152 deaths (23%). Approximately 70% of them were children under 15 years old. Since then epidemics have occurred regularly every year in June, July and August with the highest peak in 1980 (2,387 cases with 439 deaths). The disease attaches the population in the Northern Thailand most heavily followed by the Northeast. The age-specific rate is highest among 5 and 9 years old. *C. tritaeniorhynchus* is the main vector. Pigs are important amplifying hosts. Vaccine trails (purified mouse brain) were carried out in an epidemic province with very satisfactory results [24].

10. Transmission

Japanese encephalitis is not spread from person to person [25] but it transmitted via infected mosquitoes. It transmitted in an enzootic cycle among mosquitoes and vertebrate-amplifying hosts, chiefly domestic pigs and Ardeid

(wading) birds. Culex mosquitoes, primarily Cx. tritaeniorhynchus, are the principal vectors. Pigs are the principle host for viral amplification. Large aquatic birds may the second principle host in areas where pigs are absent. Other domesticated animals, such as dogs, sheep, cows, and chickens, and rodents may become infected, however fail to develop a sufficient viremia to support further viral amplification [25]. Viral infection rates in the mosquitoes range from less than 1% to 3%. These species are prolific in rural areas where their larvae breed in ground pools and especially in flooded rice fields. All elements of the transmission cycle are prevalent in rural areas of Asia, and human infections occur principally in this setting. Because vertebrate-amplifying hosts and agricultural activities may be situated within and at the periphery of cities, JE cases occasionally are reported from urban locations.

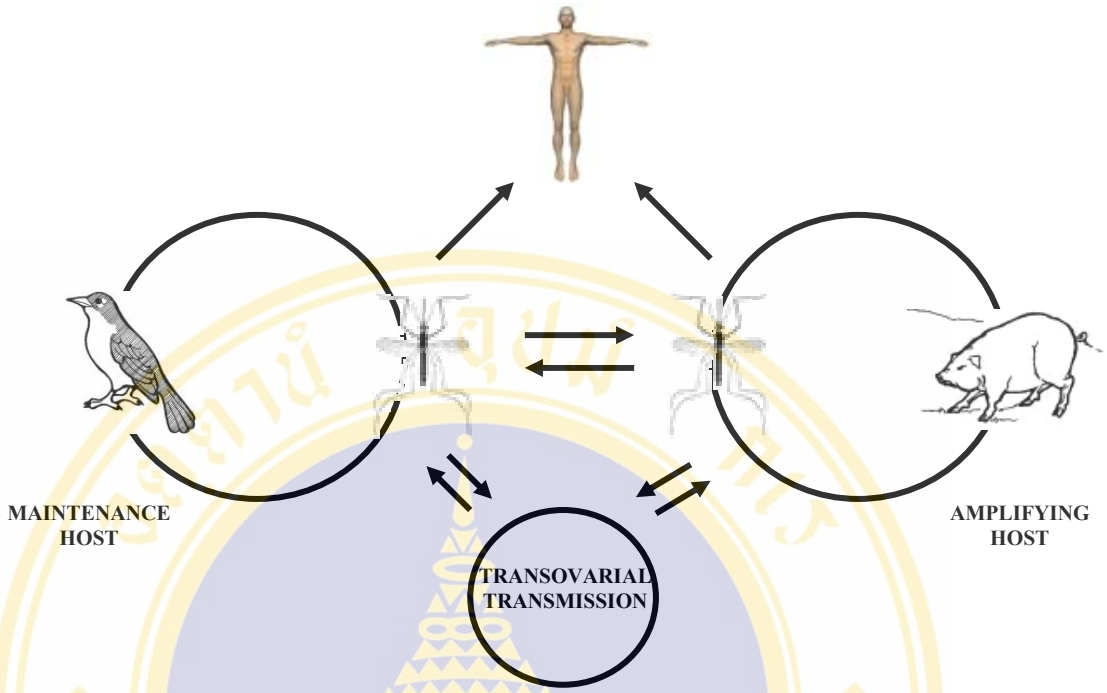


Figure 8 Transmission cycle of Japanese encephalitis virus

JE virus is transmitted seasonally in most areas of Asia. In temperate regions, JE virus is transmitted during the summer and early fall, approximately from May to September [26]. In subtropical and tropical areas, seasonal patterns of viral transmission are correlated with the abundance of vector mosquitoes and of vertebrate-amplifying hosts. These, in turn, fluctuate with rainfall, with the rainy season, and with migratory patterns of avian-amplifying hosts. In some tropical locations, however, irrigation associated with agricultural practices is a more important factor affecting vector abundance, and transmission may occur year-round. Patterns of JE viral transmission vary regionally, within individual countries, and from year to year.

11. Vaccine

There is no drug treatment for JE. Although improvements in agricultural practices have contributed to the reduction in disease incidence in some countries, JE vaccination is the single most important control measure. There is only one WHO approved JEV vaccine that is currently available in the international market. The vaccine is developed by formalin-inactivation of JEV cultured in mouse brain. The major problems associated with the mouse brain–derived inactivated vaccine are the lack of long-term immunity and practical difficulties in production of large amounts of vaccine needed for mass immunization programs [27]. To obtain effective protection, multiple boosts of the inactivated vaccine are routinely required, making the vaccination program costly. In addition, repeated immunizations with killed vaccine prepared from mouse brain may cause hypersensitivity reactions in vaccines [28].

A live, attenuated JEV vaccine based on the SA14- 14-2 strain is licensed for use in China. More than 200 million doses of this vaccine have been delivered since 1988 with excellent record of safety and efficacy [29]. However, this vaccine is produced in primary hamster kidney cells that are not approved by WHO for human vaccine production.

In the past few years, there has been a rapid growth in the knowledge of molecular information on JEV, and on the basis of this information, several potential recombinant vaccines have been developed using different approaches.

One new vaccine in development is a chimeric vaccine in which the prM and E genes of attenuated JEV stain SA14-14-2 were inserted into infection clone of the 17D yellow fever vaccine strain. The chimeric virus replicated efficiently in vitro, and was shown to be safe in mice. The vaccine has recently been given to human, and was also shown to be safe [30].

12. Cellular targets of virus

In human, the primary sites for JEV multiplication are likely either in myeloid cells or in vascular endothelial cells. How JEV penetrates the blood-brain barrier to infect the central nervous system (CNS) remain unclear. Several lines of evidence suggest that the principle target cells for JEV in the CNS are neurons and as a result of infection, massive destruction of neurons should be responsible for the disease manifestation of encephalitis. In fact, in a fetal human case, JEV antigens have been localized mainly in neurons but not in the neighboring glia cells and the major pathological changes were primarily observed in the brain's gray matter where the neurons in thalamus and brain stem appear to be particularly vulnerable to JEV infection. Additionally, a wide variety of primary and continuous cell culture from different origin, e.g. monkey, hamster, porcine, chicken and mosquito can support the productive growth of JEV. Among them, due to apparent cytopathic effects (CPE) induce by JEV infection, Vero, LLC-MK2 (monkey kidney cells) and BHK-21 (baby hamster kidney cells) are frequency used for virus titration by plaque assay [31].

13. Putative receptor(s) for JEV

Virus infection requires entry of the virus into the host cell. The interaction of a virus with its cellular receptor is multistep process in itself, or in a cell-type specific manner, and co-receptor may be involved. The viral genome can enter the cytoplasm by penetrating the plasma membrane at the cell surface, or at the endosome after endocytosis. The process of the early phase of flavivirus infection is not clearly understood; however, there are reports that flaviviruses enter the cell by receptor-mediated endocytosis [15]. Clathrin-dependent and caveola-dependent pathways were both reported to be the virus entry mechanism. Endocytic activity of cells can be analysed by using pharmacological agents demonstrated that chlorpromazine induced the assembly of clathrin lattices on endosomal membranes and at the same time prevented coated pit assembly on the cell surface of human fibroblasts [32]. Nystatin is a sterol-binding agent and removes membrane cholesterol, which is required to maintain caveolae in the plasma membrane. Cytochalasin D was used to inhibit macropinocytosis of the cell.

Virus attachment to the host cell is the first stage of the virus replication cycle. It requires the molecular interaction between the virion surface and a host cell receptor and is often the basis for viral species and tissue tropisms as well as virulence properties. The cellular receptors for some viruses have been defined and reveal diverse strategies for virus attachment, ranging from binding to specific cell surface proteins to attachment via widely distributed carbohydrate moieties, such as sialic acid and heparan sulfate [33]. For a large number of viruses, however, specific host cell receptors have not been identified. The use of multiple receptors on individual or different cells could be one reason for this lack of knowledge. Nowadays there are a little known about cellular receptor for Japanese encephalitis virus.

Many lines of evidences about receptor usage by some viruses suggest that viruses can recognize more than one receptor. For instant, at least two receptors have been proposed to mediate entry of human hepatitis C virus (HCV) in to hepatocytes: CD81, a member of the tetraspanin superfamily of proteins, and the low-density lipoprotein receptor (LDLR) [4, 34].

Numerous candidate receptor proteins with have been found to associate with JEV in binding assays but they have been not successfully characterized. Kimura [35] studies found that in early interactions between viruses and cells appear to determine the susceptibility of target cells to JEV infection. Moreover he found that JEV bound to a 74 KD molecule present in membrane fraction of Vero cells and this binding was inhibited by MAb 301 which is known to have anti-hemagglutinin activity. Importantly, this antibody also inhibited binding of JEV to Vero cells and this raises the possibility that the 74 kD molecule may be a possible candidate or component of the cellular receptor for JEV on Vero cell.

In addition, in an *in vitro* binding assay of Chen, shown that the envelope (E) protein of JEV bound specifically to a cellular protein of 57-kDa derived from BHK-21 cells. However, the nature of cellular 57 kDa in JEV infection remains to be determined, although this protein is not likely to be the viral receptor on BHK-21 cells, because the attenuated variants of JEV can readily infect BHK-21 cells despite its failure to bind to this protein. It is possible that this 57 kDa protein is accessory molecule on the surface of BHK-21 cells, which facilitates the interaction between the putative receptor on the JEV E protein during receptor-mediated endocytosis [36].

These evidences suggest that JEV also employs more than one receptor for attachment to the cells, which depend on the type of host cells.

Furthermore, an important role of heparin sulfate has also been demonstrated for the attachment of Japanese encephalitis virus to target cells. Interestingly, many enveloped and nonenveloped viruses utilize cell surface glycosaminoglycans (GAGs) as cellular receptors for attachment in a process thought to concentrate virus particles at the cell surface for subsequent binding to high-affinity receptors including vaccinia viruses [37], herpesviruses [38], Human immunodeficiency virus C [39],and Sinbis viruses [40]. In the present study, found that highly sulfated glycosaminoglycans (GAGs) are involved in infection of JEV in BHK-21 cells. Competition experiments using highly sulfated GAGs, heparin and dextran sulfate, demonstrated an inhibition of JEV's attachment and subsequent infection of BHK-21 cells, the addition of heparin suppressed the cytopathic effects induced by JEV infection in cultured cells suggesting that GAG play a critical role on the JEV infection [41].

No experimental evidence on the use or nature of the high-affinity receptor for the Japanese encephalitis virus on mosquito cells exists at present. However the mosquito tissue's studies have documented that the two glycoproteins of 40 and 45 kDa present on the surface of C6/36 cells bound dengue virus serotype 4 (DEN 4) specifically. DEN 4 binding to C6/36 can be blocked antibodies raised against the 40 and 45 kDa molecules or by the partially purified 40 and 45 kDa proteins, suggesting that their participation in the virus-receptor complex [42].

CHAPTER III
OBJECTIVES

1. To investigate the relationship between Japanese encephalitis virus and C6/36 mosquito cells.

2. To identify Japanese encephalitis virus binding proteins in *Adese albopictus* mosquito cell using C6/36 cells.

CHAPTER IV
MATERIALS AND METHODS

1. Source of materials

1.1 Chemical

Polyethylene glycol Mw 8,000 (PEG 8,000)	Bio Basic Inc
Bovine serum albumin fraction V	Sigma
Penicillin/Streptomycin	Hyclone, USA
Gentamycin	TP. Drug Laboratory
Fungizone	Bristol-Myer Squibb
Lactalbumin hydrolysate	Sigma
Crytal violet	Bio Basic Inc
Dimethyl sulfoxide (DMSO)	Amresco®
Ethelynediamine trichloroacetic acid (EDTA)	Bio Basic Inc
Magnisium chloride	Fluka, Chemical, Germany

1.2 Culture media and reagents

Calf serum	Gibco BRL
Fetal bovine serum	Gibco BRL
Medium 199 (199/EBSS)	Hyclone
Dulbecco's modified Eagle's medium (DMEM)	Hyclone
Minimum Essential Medium (MEM/EBSS NEAA Modified)	Hyclone
L-glutamine	Gibco BRL
Trypsin (1:250)	Gibco BRL

1.3 Miscellaneous materials

Standard protein marker	BIORAD
Prestain protein marker	BIORAD
Nitrocellulose membrane	Schleicher & Schuell

1.4 Cell lines

1.4.1 C6/36: Whole hatch larva of mosquito cell line from *Aedes albopictus*

1.4.2 Vero: kidney cell line from *Cercopithecus aethiops* (African green monkey)

1.5 Japanese encephalitis virus

JEV strain Beijing-1 (BJ-1)

Source: Human from China

1.6 Culture medium

C6/36: Minimum Essential Medium (MEM), 10% fetal bovine serum (FBS), 100 unit/ml penicillin and 100 ug/ml streptomycin and 1% L-glutamine

Vero: Dulbecco's modified Eagle's medium (DMEM), 5% FBS,100unit/ml penicillin and 100 ug/ml streptomycin.

1.7 Medium for virus infection in solution

MEM supplement with 100 unit/ml penicillin and 100 ug/ml streptomycin exception in medium mixture is the lack of heat-inactivated FBS.

2. Freezing cells for liquid nitrogen storage

Healthy cells were trypsinized and cell suspensions were centrifuged at 1500 rpm for 5 min at room temperature. The supernatant was removed to get rid of both dead cells and cell debris. Cell pellet was resuspended in MEM supplemented with 40% FBS and 10% DMSO to final concentration 2 x 10^7 cell/ml. Vials containing 1 ml of cell suspension were wrapped with several layers of cotton in foamed box and placed at -80ºC. Following freezing, the vials were transferred to liquid nitrogen for long-term storage.

3. Recovering cells from liquid nitrogen storage

Frozen vials were removed from liquid nitrogen and cells were quickly thawed in a 37ºC water bath. When the cells were almost thawed, the vials were cleaned outside with 70% ethanol. The cell suspensions were transferred to centrifuge tubes containing 10 ml of medium and centrifuge at 1500 rpm for 5 min. The medium was discarded and cells were resuspended with MEM supplemented with 10% FBS. The

cells were transferred to 75 cm^2 tissue culture flask and incubated at 28 ºC with no CO_2.

4. Counting cells

The number of the cells was determined by using a hemocytometer. By counting the number of cells within the 0.1 mm^3 chamber formed by the 1x1 mm square and the height of the coverslip, an accurate quantitation of cells per milliliter can be calculated. A drop of cell suspension was transferred to the edge formed between the coverslip and slide following by examining under the microscope. The number of cells was counted in several of the 1x1 mm squares.

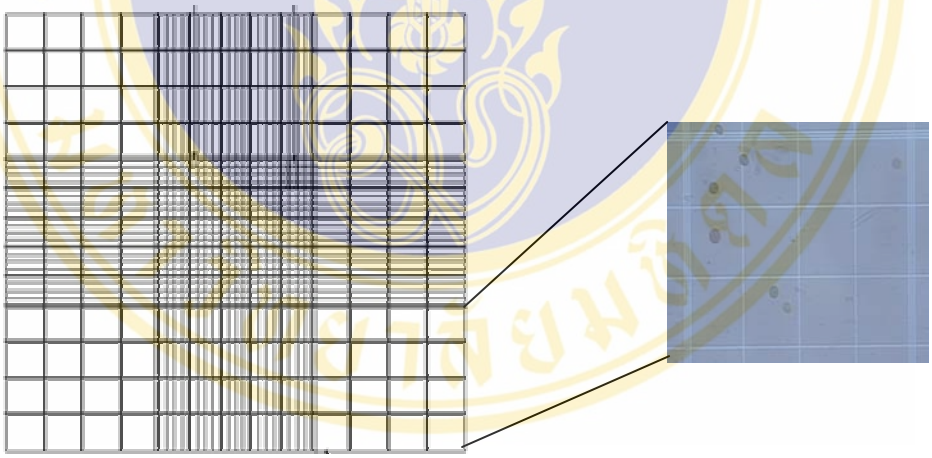


Figure 9 Hemocytometer for cell counting

One entire grid on standard hemacytometers can be seen at 40x (4x objective). The main divisions separate the grid into 9 large squares. Each square has a surface area of one square mm, and the depth of the chamber is 0.1 mm. Thus the entire counting grid lies under a volume of 0.9 mm-cubed.

5. Culturing of C6/36 cells and Vero cells

The whole hatch larva of mosquito cell line C6/36 was cultured at 28 ºC with no CO_2 in Minimum Essential Medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 unit/ml penicillin, 100 ug/ml streptomycin and 1% L-glutamine. The African green monkey kidney cell line Vero was cultured at 37 °C/5% CO_2 in Dulbecco's modified Eagle's medium (DMEM), 5% FBS, 100unit/ml penicillin and 100 ug/ml streptomycin. This cell line was allowed to titer Japanese encephalitis virus (JEV).

6. Viral growth curve

C6/36 cells were grown in 75 cm^2 tissue culture flasks containing 20 ml of growth medium. Subconfluently (3 x 10^6 cells/flask), the culture media was discarded and replaced with 3 ml of MEM without FBS containing with the JEV at multiplicity of infection (MOI) of 1 pfu/cell. Viral absorption was allowed to proceed for 120 minutes at 28 ^oC with constant agitation. After this fresh culture media was added to the cells and the cells incubated further for 6 days at 28 ºC with no CO_2 which is the standard conditions. Aliquots of the growth medium were taken every days for or 6 days. Each sample was assayed in triplicate by standard plaque assay on Vero cells, and each experiment was undertaken in triplicate. Data was analyzed using the GraphPad Prism program (GraphPad Software Inc, San Diego, CA, USA).

7. Virus titeration and optimization of JEV plaque assay on Vero cell

Vero cells were seeded in 6-well plates in DMEM supplemented with 5% heat –inactivated FBS, 100 U/ml penicillin, and 100 ug/ml streptomycin. Cells were incubated at 37 ^oC in 5% CO_2 for 2 days before the medium was removed and then infected with 200 ul serially diluted virus solution. Incubation of cells and virus at 37 ^oC with aligation every 10 minutes has been proposed to allow virus infection. Subsequently, 2% (w/v) Seakem Le agaloseMD) mixed with nutrient overlay (Earle's Balancedsalts supplemented with 0.5% (w/v) yeast extract, 2.5% lactalbumin hydrolysate, 3% FBS) was added to each well. The plates were further incubated at 5 sampling plate, the agarose plugs were removed and cells fixed with 3.7% formaldehyde for 1 hour at room temperature. Plaques were visualized by staining

with 1% crystal violet in 10% ethanol. After drying, the diameter of each plaque on plates was measured to optimize the appropriated size. In addition the number of plaques were counted and calculated in plaque forming unit per virus sample per 1 ml (pfu/ml).

Viral titer (pfu/ml) = number of plaques x sDF x iDF

sDF = serial dilution factor (e.g.10^5)

iDF = injection dilution factor e.g. if the injection volume is 0.2 ml; the iDF currently equals to 1ml/0.2 ml which is 5.

In this experiment, all titration experiments were undertaken independently in triplicate, with duplicate assay of each point.

8. Viral stocks

C6/36 cells were grown in tissue culture flasks until reach to subconfiuent (3 x 10^6 cells/flask). At that time the culture media was discarded and replaced with 3 mls of MEM without FBS containing the JEV at a MOI of 1. Viral infection was allowed to proceed for 120 minutes at 28 ^oC with constant agitation. At the day which gave the highest viral yield determined by viral growth curve experiment, medium containing progeny virus was collected and kept at -80 ºC. The viral titer was determined by standard plaque assay on Vero cells.

9. JEV viral production

C6/36 cells were grown in 75 cm^2 tissue culture flasks containing 20 ml of growth medium. Prior to confluence, when the cell number reached 7.5 x 10^6 the culture media was discarded and replaced with 3 mls of MEM containing the JEV at a MOI of 1. Viral absorption was allowed to proceed for 120 minutes at 28^oC with constant agitation. After this period cells were washed three times with PBS to remove unabsorbed viruses. After that cells were treated with acid glycine (pH 3.0) for 1 minute to inactivate any un-internalized extra cellular viruses followed by washing in PBS. Fresh culture media was added to the cells and the cells incubated under standard conditions. Aliquots of the growth medium were taken every hour for the first 24 hours post infection. Each sample was assayed in triplicate by standard plaque assay

on Vero cells, and each experiment was undertaken in triplicate. Data was analyzed using the GraphPad Prism program (GraphPad Software Inc, San Diego, CA, USA).

10. Viral Internalization

C6/36 cells were grown in small culture plate (23.75 cm^2 in diameter) until the number of cell reach to 3 x 10^6 cells/plate. After that the culture media was discarded and cells were then incubated with JEV at a MOI of 1 in MEM with no FCS added at 4 ^oC with gentle agitation for 2 hours to allow attachment of the virus to the cells surface but not internalized. After this time, cells and virus were temperature shifted to 37 ^oC to allow internalization to proceed. After incubation, the mixture of cell and virus were then shifted to 28 ^oC. At time points of 0, 10, 20, 30, 60, 90, 120, 180, 240 and 300 minutes, the infected cells were washed once with PBS and then treated for 1 minute with acid glycine buffer (pH 3.0) at room temperature to inactivate any uninternalized viruses. Following acid glycine treatment cells were again washed with PBS. After washing normal growth media was added and cells incubated under standard conditions for one and a half virus replication cycles (10 hours). After 10 hours the media was collected to analyze by standard plaque assay for viral titer in triplicate. Experiment was undertaken in triplicate.

11. Detection of Mature and Immature virions

C6/36 cells were grown in 23.75 cm^2 plate until the number of cells reach to 3 x 10^6 cells. Cells were then incubated with JEV at a MOI of 1 in MEM with no FCS added at 28 ^oC for 2 hours following by washing with PBS. Cells were then treated for 1 minute with acid glycine buffer (pH 3.0) at room temperature to inactivate any uninternalized viruses and washed again with PBS. After washing normal growth media was added and cells incubated under standard conditions. At each time point, 0 hour to 12 hours, medium was collected following by treated with 0.06 % trypsin to detach cells. After that cells were transferred to eppendoft a tube following by centrifugation at 3,000 x rpm for 5 minutes. BA-1 was added to resuspend prior collected at -80 °C until use. Finally, levels of intracellular viruses were determined by release from infected cells by one freeze-thaw cycle followed by 5 minutes of sonication at 4 °C and assay by standard plaque assay. In addition the levels of

produced viruses were also determined by standard plaque assay. Experiment was undertaken in triplicate.

12. Viral Stability

A known titer of infectious JEV (as determined by plaque titer assay) were added to MEM supplemented with 10% FBS, a medium normally employed for the propagation of C6/36 cells, to give a final viral titer of 3 x 10^7 pfu/ml. The virus/medium mixture was incubated at 28 °C. At various time-points (0, 3, 6, 9, 12, 24, 36, and 48 hours.) aliquots were taken and the level of infectious JEV per milliliter assayed by standard plaque titration assay on Vero cells. Experiment was undertaken in triplicate.

13. Pre-treatment of cell with trypsin

C6/36 cells were washed with PBS and trypsinized with 0.06% trypsin-EDTA at 37^{o}C for 5 minutes followed by the addition of fresh culture medium to inactivate trypsin. The cell suspension was distributed to eppendoft tubes (1.1x10^6 cells per tube) and cells infected with JEV at a MOI of 1 at 28 ^{o}C for 1 hour. After infection, uninternalized extracellular viruses were removed by washing infected cells with PBS and treatment with acid glycine pH 3 followed by a further wash with PBS. After each washing step the infected cells were collected by centrifugation at 1,500 x g for 5 minutes. The infected cells were finally plated into 60 mm tissue culture dishes. Levels of infectious virus in the medium were assayed at one and a half virus a replication cycle, which is sufficient time for viral production but not re-infection, by standard plaque assay. Experiment was undertaken in triplicates. Control infections were undertaken identically, with the exception that cells were grown to subconfluence directly and infected which non-trypsin treated.

14. Pre-treatment of cells with Heparinase III

C6/36 cells (2.5 x 10^5) were seeded into 24 well culture plates and grown at 28 °C for 2 days after which time cells were washed once with MEM medium. Cells were then treated with 1 unit/ml Heparinase III (Sigma Chemical Co., St Louis, MO, USA) in MEM medium without FBS 28 ^{o}C for 1 hour. Treated cells were then washed

twice with MEM medium without FBS before incubating with the JEV at a MOI of 1 for 2 hours, following which cells were washed with PBS and extra cellular viruses inactivated with acid glycine for 1 minute at room temperature. Cells were again washed once with PBS followed by once with MEM. Subsequently MEM supplemented with 10% FBS was added and cells incubated at 28 ^{o}C for one and a half virus a replication cycle at which point levels of virus in the media were assayed by standard plaque assay in triplicate. Control samples were run in parallel and incubated with only MEM instead of heparinase III. Experiment was undertaken independently in triplicate.

15. C6/36 cell membrane protein preparation

Confluently grown C6/36 cells were detached by scraping with TBS buffer and harvested by centrifugation. Cell pellets were pelleted by centrifugation at 1200 x *g* for 3 minutes at 4 ^{o}C in an Eppendorf bench-top centrifuge and subsequently resuspended in ice-cold modified buffer M (100 mM NaCl, 20 mM Tris-HCl [pH8], 2 mM $MgCl_2$, 1 mM EDTA, 0.2% Triton X-100, 1 mM PMSF) and lysed by vigorous vortexing. Nuclei and debris were removed by centrifugation at 600 x *g* for 3 minutes at 4 ^{o}C , and membranous organelles were pelleted from the supernatant by further centrifugation at 6,000 x *g* for 5 minutes. Membrane proteins were pelleted by centrifugation at 20,800 x *g* for 10 min at 4 ^{o}C and resuspended in modified buffer M containing 0.3% Triton X-100. The membrane protein sample was kept at -80 ºC. In addition, the concentration of protein was quantified by the Bradford method.

16. Viral purification

Japanese encephalitis virus strain Beijing-1 (BJ-1), (accession no. L48961) was propagated in C6/36 cells. The virus was partially purified through sucrose step gradient and stored at -80 °C. In methodology, subconfluent C6/36 cells were infected with JEV at MOI of 1. On the day that give the highest viral yield, the growth medium that contain progeny virus was clarify by centrifugation at 1,000 x g at 4 °C for 5 minutes. The supernatant was then transfer to a flask and all proteins were precipitated with 10% (w/v) PEG 8000 and 1.5M NaCl at 4 °C overnight. The precipitated proteins were pelleted by centrifugation at 15,000 rpm (55,566 x g) at 4 °C for 1 hour. The

pellet was then resuspended with TNE buffer (10mM Tris-Cl [pH 7.5], 140mM NaCl, 1 mM EDTA) containing 10% (w/v). The protein/sucrose mixtured was laid on to a 30/60% (w/v) discontinuous sucrose gradient. Viral protein was harvested by high speed centrifugation at 30,000 rpm (111,132 x g) for 3.5 hours at 4 ºC following by punching at the bottom of tube to collect the viral protein band. After that viral protein was pelleted by high speed centrifugation at 30,000 rpm (111,132 x g) for 2 hours at 4 ºC. Finally pellets were solved.

17. Virus Overlay Protein-Binding Assays (VOPBA)

17.1 Protein sample preparation

Prior loading, the protein samples were mixed with 5x sample buffer (200 mM Tris-HCL pH 7.5, 4 mM EDTA pH 8.0, 4% (w/v) SDS, 40% glycerol and 0.1% (w/v) bromophenol blue) in the ratio 4:1. The sample mixture was then heated at 95 ºC for 5 minutes following by short centrifugation to spin all samples down.

17.2 SDS Polyacrylamide gel electrophoresis

Electrophoresis of approximately 150 µg of C6/36 membrane protein was performed according to the protocols for Mini-protein II electrophoresis (Bio-Rad) and Sambrook. Protein was loaded into adjacent wells of a single 10% SDS polyacrylamide gels which consist of a resolving or separating gel and a stacking gel. The sample was electrophoreses in Tris-glycine buffer (25 mM Tris-Hcl (pH 8.3), 192 mM glycine, 0.1% SDS) with constant voltage of 100 at room temperature.

17.3 Electrotransfer of proteins

After protein samples were separated, SDS-PAGE gel was soaked with transfer buffer (15.6 mM Tris-Base, 120 mM glycine) for 10 minutes. One nitrocellulose membrane and six pieces of filter paper were cut as same as size with gel and soaked with transfer buffer for 10 minutes. Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) was applied to electrophoretically transfer protein from SDS-PAGE gel to a nitrocellulose membrane. A pre-soaked fiber pad was placed onto the side of cassette flowing by three pieces of filter paper, SDS gel, nitrocellulose membrane, three additional pieces of filter paper and finally fiber pad on another side of cassette. Following this, entire cassette was assemblies into electrophoretic tank. Pre-cooled

transfer buffer was then added. Finally electroblotting was carried out at constant voltage of 30 at 4 ºC for 16 hours.

17.4 Detection of virus binding protein

After the electro transfer process was completed, the nitrocellulose membrane was stained with 1x Ponceau S (0.1% (w/v) Ponceau S in 5% (v/v) acetic acid) to examine the quality of transferation. After staining, membrane was washed and blocked with blocking solution (5% skim milk in TBS [20mM Tris-HCL (pH 7.5), 140 mM NaCl]) for 2 hours, following by incubated with 5 x 10^{11} pfu/ml purified JEV in 1% skim milk in TBS. Hybridization was undertaken at room temperature for 5 hours followed by hybridizing overnight at 4 °C, washing three times with TBS at room temperature. Immunoblotting was undertaken with a pan specific anti-flavivirus monoclonal antibody produced by hybridoma cell line HB-112, (a kind gift of Siritorn Butrapet) at a dilution of 1:50 in 5% skim milk in TBS. Incubation was undertaken at room temperature for 2 hours followed by washing three times with TBS. Following this, membranes were incubated at room temperature for 1 h with HRP-conjugated rabbit anti-mouse IgG (Sigma Chemical Co., St Louis, MO, U.S.A.) at a dilution of 1:3000 in 5% skim milk in TBS. Finally, signal was developed using the ECL Western blotting detection reagent kit (Amersham Phamacia Biotech, Buckinghamshire, England) followed by autoradiography.

18. Westernblotting

150 μg of C6/36 cell membrane proteins were subjected to 10% SDS-PAGE and prior transferred to nitrocellulose membranes. The membrane was incubated with a polyclonal antibody against the human 37/67kDa high affinity laminin receptor (sc-21534, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:200 in 5% skim milk in TBS for 2 hours at room temperature. Following this, membranes were incubated at room temperature for 1 hour with HRP-conjugated goat anti-mouse IgG (Sigma Chemical Co., St Louis, MO, U.S.A.) at a dilution of 1:3000 in 5% skim milk in TBS. Finally, signal was developed using the ECL Western blotting detection reagent kit (Amersham Phamacia Biotech, Buckinghamshire, England) followed by autoradiography.

19. VOPBA on Non-denaturing gel

The 150 μg of C6/36 membrane protein was separated through polyacrylamide gel which consists of a resolving or 10% separating gel and 3% stacking gel. Protein samples were mixed with 5X loading dye without DDT and then were loaded on to gel without prior boil. The sample was electrophoreses in Tris-glycine buffer (25 mM Tris-Hcl (pH 8.3), 192 mM glycine, 0.1% SDS) with constant current between 4 to 7 A at 4 °C in order to eliminate heat production. After proteins were completely separated, protein on gel were transferred on to nitrocellulose membrane prior to be performed VOPBA with the same procedure as shown.

20. Infection inhibition assays

C6/36 cells were grown on 6-well plates until the cells number reached 3 x 10^6, following which the culture medium was discarded and the cells were incubated with a soluble laminin (basement membrane laminin, Engelbreth-Holm-Swarm murine sarcoma; L2020, Sigma Chemical Co.) at various concentration as indicated at 28 ^oC for 1 hour with aligation every 10 minutes. After incubation, the cells were washed twice with PBS and then infected with JEV at MOI of 1 at 28 ^oC for 2 hours. After viral adsorption, the extracellular viruses were removed by washing with PBS, followed by acid glycine treatment for 1 minute. The infected cells were washed again with PBS and fresh growth medium was added. The infected cells were then incubated for a further one and a half virus a replication cycle at which point levels of infectious virus in the media were assayed by standard plaque assay. Each sample was titered in triplicate, and the experiment undertaken three times independently. In this experiment the nonpre-incubated cells with soluble laminin was used as the control.

21. Immunoflourescence

21.1 Poly-L-lysine coating glass slides

Place slides in wash glass rack which containing detergent and wash for 30 minutes at room temperature on an orbital shaker. And then rinse slides by plunging racks up and down in fresh water with several times. After washing slides were placed in 1M HCL in wash glass rack for 1 hour at room temperature on an orbital shaker and

washed with fresh water. Following this slides were dipped in 95% ethanol for 3 minutes and following by acetone dip for 3 minutes. Slides were then incubated in 30 ^{o}C incubator until dry. Each slide was coated with 0.01% poly-L-lysine (PLL) solution and leaved at room temperature for drying. Rinse slides in ddH2O by plunging rack up and down 10 times. Coated slides were autoclaved, wrapped with foil and stored at room temperature.

21.2 Seeding cells onto coated slide and Immunofluorescence

Prior cells were seeded onto slide; slides were framed with nail enamel to locate the seeding area on slide. The 3 x 10^5 of C6/36 cells were then grown on glass slides for 2 days. Cells were fixed with 1% formaldehyde for 10 minutes at room temperature. After fixing, cells were pre-incubated with 40 ug of soluble laminin for 1 hour at 4 °C following by washed twice with PBS. JEV was then added at MOI of 10 and incubated for 1 hour at 4 °C. After washing twice with PBS, cells were further incubated with a pan specific anti-flavivirus monoclonal antibody produced by hybridoma cell line HB-112, which was kindly provided by Dr. Siritorn Butrapet at a dilution of 1:100 for 1.5 hours at room temperature. Cells were subsequently incubated with a 1:10 dilution of a polyclonal goat anti-mouse IgG conjugated with FITC (Molecular Probes, Eugene, Oregon, U.S.A.) for 30 minutes at room temperature in the dark. After washing twice with PBS the slides were air dried, and observing signal under a fluorescent microscope.

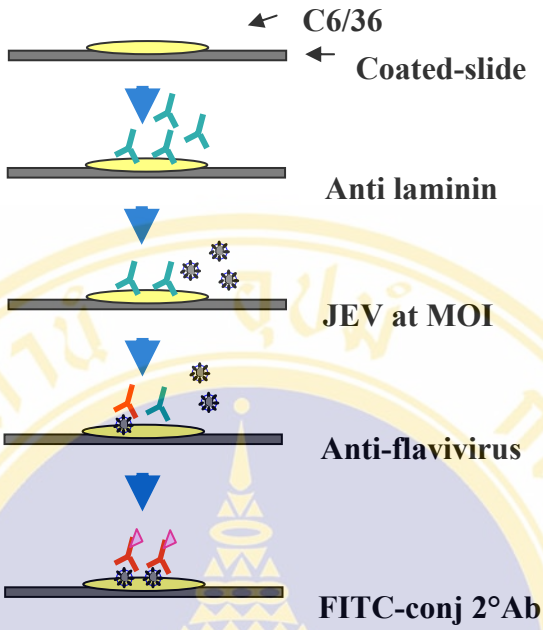


Figure 10 Binding Inhibition Assay

Binding inhibition assay was done following these steps. Prior cells were seeded onto coated-slide, and then grown on glass slides for 2 days. Cells were fixed with 1% formaldehyde for 10 minutes at room temperature. After fixing, cells were pre-incubated with soluble laminin for 1 hour at 4 °C following by washed twice with PBS. JEV was then added at MOI of 10 and incubated for 1 hour at 4 °C. After washing twice with PBS, cells were further incubated with a pan specific anti-flavivirus monoclonal antibody produced by hybridoma cell line HB-112 at a dilution of 1:100 for 1.5 hours at room temperature. Cells were subsequently incubated with a 1:10 dilution of a polyclonal goat anti-mouse IgG conjugated with FITC for 30 minutes at room temperature in the dark. After washing twice with PBS the slides were air dried, and observing signal under a fluorescent microscope.

22. Protein fingerprint

Peptide mass fingerprinting is an analytical method use to identify proteins based on their mass and charge. The fingerprinting for each protein was generated by protease digestion. The masses of each peptide fragments from a protein fingerprint can be accurately determined by mass spectrometry. Firstly, the binding of JEV on C6/36 cell membrane proteins were carried out by VOPBA. The protein bands corresponded with VOPBA signal was excised from the gel. Then the excised gel pieces were submitted for commercial mass spectrometer analysis [21]. Briefly, protein in excised gel was digested by protease enzyme. After digestion, the peptides were extracted from the gel and mass analyzed. The resultant peptides were analyzed by the MS/MS spectra that were produced for each sample was searched against the MSDB non-redundant protein database using the MASCOT search engine for a match and the identity of the protein was determined.

CHAPTER V
RESULTS

1. Optimization of JEV plaque assay procedure

Vero cells were infected with serially diluted virus solution. The plates were incubated for 2 hours at 37 ^{o}C with shaking every 10 minutes. Subsequently, 2% (w/v) Seakem Le agarose MD mixed with nutrient overlay was added to each well. The plates were further incubated at 37 ºC in 5% CO_2 and were sampled 3 to 10 days post incubation. In each sampling plate, the agarose plugs were removed and cells fixed with 3.7% formaldehyde for 1 hour at room temperature. Plaques were visualized by staining with 1% crystal violet in 10% ethanol. After drying, the diameter of each plaque on plates was measured to optimize for the appropriate size. The plaque size data is shown in Table 1. The result informed that plaque incubation for 7 days given rise the most appropriate plaque size for easy counting.

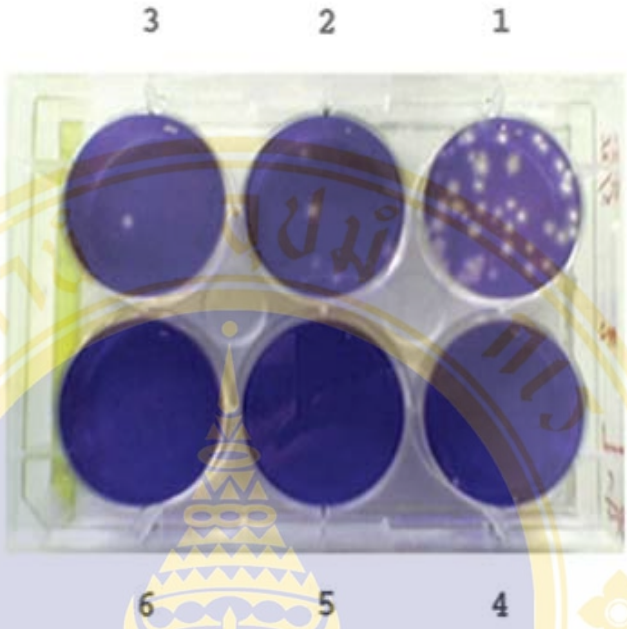


Figure 11 The appropriated plaque formation (Day 7)

This figure shows the result of plaque incubation at day 7 of 6-well plate. This assay is the most accurate method for determining the concentration of infectious viral particles in a suspension. A cell monolayer is infected with a serial dilution of the virus suspension to be titrated, and covered with agar. Plaques are counted easily at seven days after infection.

Well 1 represents 10^{-5} dilution of the original virus stock.

Well 2 represents 10^{-6} dilution of the original virus stock.

Well 3 represents 10^{-7} dilution of the original virus stock.

Well 4 represents 10^{-8} dilution of the original virus stock.

Well 5 represents 10^{-9} dilution of the original virus stock.

Well 6 represents 10^{-10} dilution of the original virus stock.

Table 1 Plaque size of each day after plaque incubation

Day after plaque incubation	Diameter of each plaque (randomly for 8 plaques, cm)	Average plaque size (cm)
3	0	0
4	0	0
5	0.08, 0.1, 0.08, 0.07, 0.1, 0.08, 0.08, 0.08	0.084
6	0.4, 0.4, 0.6, 0.5, 0.4, 0.6, 0.5, 0.5	0.487
7	0.7, 0.6, 0.9, 1.0, 0.9, 0.9, 0.8, 0.8	0.825
8	1.1, 1.0, 1.2, 1.0, 1.2, 1.3, 0.9, 1.1	1.100
9	1.4, 1.4, 1.3, 1.3, 1.4, 1.4, 1.2, 1.2	1.325
10	1.6, 1.8, 1.4, 1.7, 1.6, 1.8, 1.8, 1.7	1.675

2. Propagation of JEV in C6/36 cells

The propagation profile of JEV on C6/36 cells was investigated over two time periods, the kinetics of early viral production (hourly over the first 24 hours and long term (daily for 7 days).

2.1 Growth curve of JEV in C6/36 cells

In long term production profile, to determine the production profile of JEV over a long term period of time, whole hatch larva of mosquito cell line from *Aedes albopictus*, C6/36 cells was examined. As the maximum viral yields are required in order to apply for other experiments, the growth curve of JEV in C6/36 cells was first determined to obtain primary information of which day had the highest viral yield. C6/36 cells were infected with JEV at MOI of 1 and future incubated under standard condition for 6 days. Culture medium containing progeny virus was sampled daily. The level of infectious virus was determined by standard plaque assay on Vero cells. Data was analyzed using the GraphPad Prism program (GraphPad Software Inc, San Diego, CA, USA).

Result (Figure 12) showed that JEV gave maximum titers at day 5 post infection. Growth curve result of JEV on C6/36 cells benefits the information for virus stock production, which the highest yield of virus will be collected at that day.

2.2 Viral production of JEV in C6/36 cells

In early kinetics, whole hatch larva of mosquito cell line from *Aedes albopictus*, C6/36 cells were required to assess the first virus that are released out of host cells. Cells were grown in 75 cm^2 tissue culture flasks containing 20 ml of growth medium. Prior to confluence, when the cell number reached 7.5 x 10^6 the culture media was discarded and cells were infected with the JEV at a MOI of 1 in MEM with no FBS. Viral absorption was allowed to proceed for 120 minutes at 28 ^oC with constant agitation. After this period cells were washed three times with PBS to remove unabsorbed viruses. After that cells were treated with acid glycine (pH 3.0) for 1 minute to inactivate any un-internalized extra cellular viruses followed by washing in PBS. Fresh culture media was added to the cells and the cells incubated under standard conditions. Aliquots of the growth medium were taken every hour for the first 24 hours post infection. Each sample was assayed in triplicate by standard plaque assay on Vero cells to determine viral titer, and each experiment was undertaken in

triplicate. Data was analyzed using the GraphPad Prism program (GraphPad Software Inc, San Diego, CA, USA).

Result (Figure 13) shows that JEV was produced as early as 10 hours post infection. This result benefits to serve as primary information which indicate the appropriate time that virus was produced for one viral production cycle.

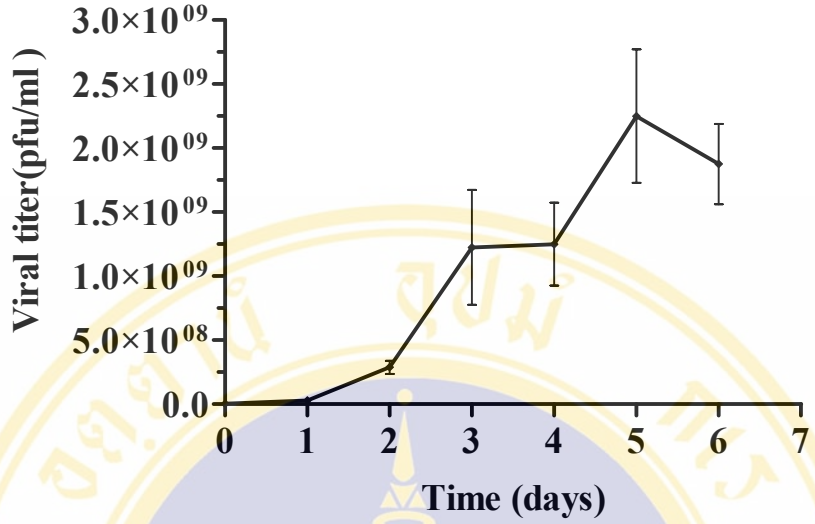


Figure 12 JEV Growth curve in C6/36 cells

This figure shows viral growth curve in C6/36 cell line. Cells were infected with JEV at an MOI of 1. After 2 hours of viral absorption, the growth medium was sampled daily for 6 day and determined for level of infectious viruses by standard plaque assay on Vero cells. Experiments were undertaken independently in triplicate with triplicate titer of samples. Each point represents the triplicate count. Error bars represent SEM.

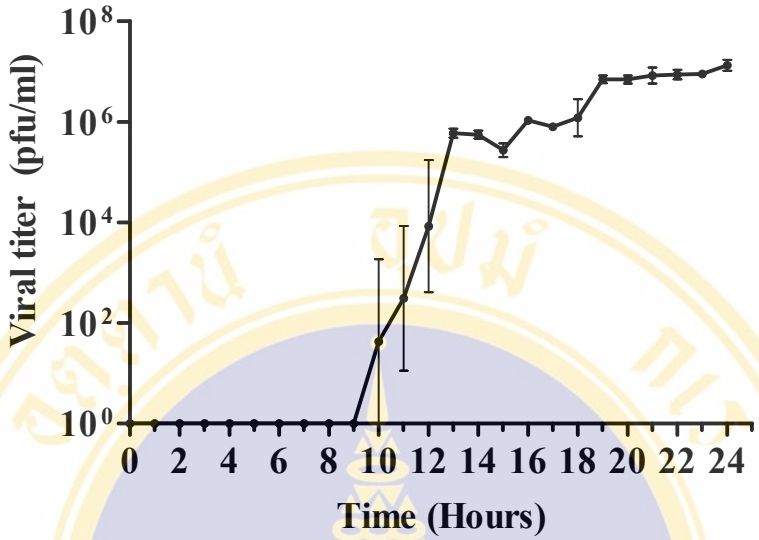


Figure 13 JEV viral production in C6/36 cells

This figure shows viral growth curve in C6/36 cell line. Cells were infected with JEV at an MOI of 1. After 2 hours of viral absorption, the growth medium was aliquot every hour for the first 24 hours post infection and determined for level of infectious viruses by standard plaque assay on Vero cells. Experiments were undertaken independently in triplicate with triplicate titer of samples. Each point represents the triplicate count. Error bars represent SEM.

3. Viral Internalization

C6/36 cells were assayed to determine the time course of JEV internalization, using a similar methodology to previous studies in the laboratory. The methodology, the level of internalization is determined by using a combination of acid glycine treatment and plaque assay, as only viruses that have internalized will give rise to plaques. Given that only viruses that are internalized can produce progeny viruses, the greater the degree of internalization, the greater the number of viruses produced. Incubation of cells and virus at 4 ^{o}C has been proposed to allow virus attachment but not internalization, and so initially cells were incubated with virus at 4 ^{o}C for 2 hours. After this time, cells and virus were temperature shifted to 37 ^{o}C to allow internalization to proceed. At times ranging from 0 minutes to 300 minutes, cell/virus mixtures were treated with acid glycine to inactivate any un-internalized viruses. Cells were then plated and allowed to grow for on and half cycle of viral replication (15 hours), following which the levels of infectious viruses in the media were established by plaque titer. Result (Figure 4) shows that initial results indicated that even at 0 minutes at the higher temperature, a degree of internalization had occurred. This suggested either that acid glycine did not inactivate extra cellular viruses completely or that a degree of internalization occurred at 4 ^{o}C. Results were normalized as a percentage of maximum virus titer (at 240 minutes) and show that internalization of the JE virus is quite rapid. At 2 hours, more than 20% of the 5 hours value has been internalized, and the levels internalized increase sharply between 2 hours and 3 hours which increasing from 35% to 80% within 1 hour. Moreover internalization profile of JEV appears twice of plateau phases, initial plateau occur between 1 and 2 hours and the second between 3 and 4 hours which indicate the saturation of viral entry. This result suggesting that there is the recycling of receptors back onto cell surface after viral usage in order internalized within 1 hour.

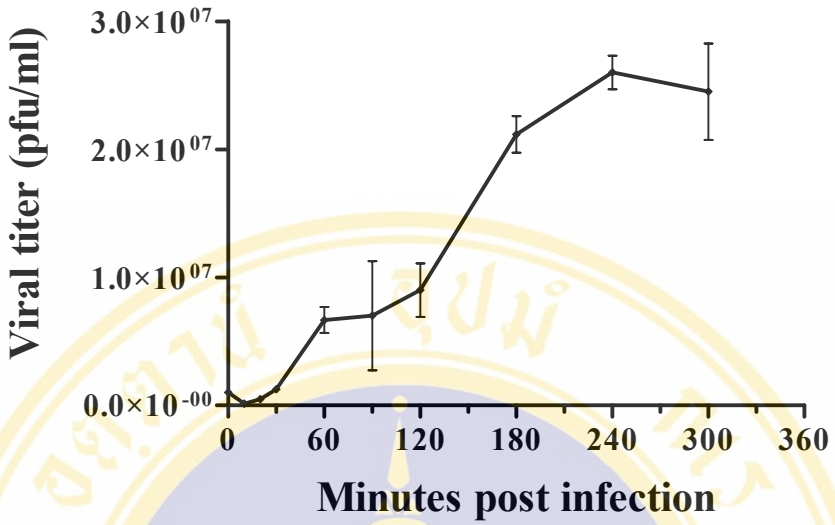


Figure 14 Internalization profile of JEV in C6/36 cells

Internalization profile: C6/36 cells were incubated at 4 ^{o}C with JEV for 2 hours at MOI of 1 and the temperature subsequently shifted to 28 ^{o}C. At the time points shown the cell/virus mixtures were treated with acid glycine pH 3.0 to inactivate un-internalized viruses and the cells allowed to grow in growth medium for one and a half virus replication cycles at which point the growth medium was sampled and the level of infectious viruses present assayed by standard plaque assay on Vero cells. Experiments were undertaken independently in triplicate with triplicate assay of virus titers. Each point represents the triplicate count. Error bars represent SEM.

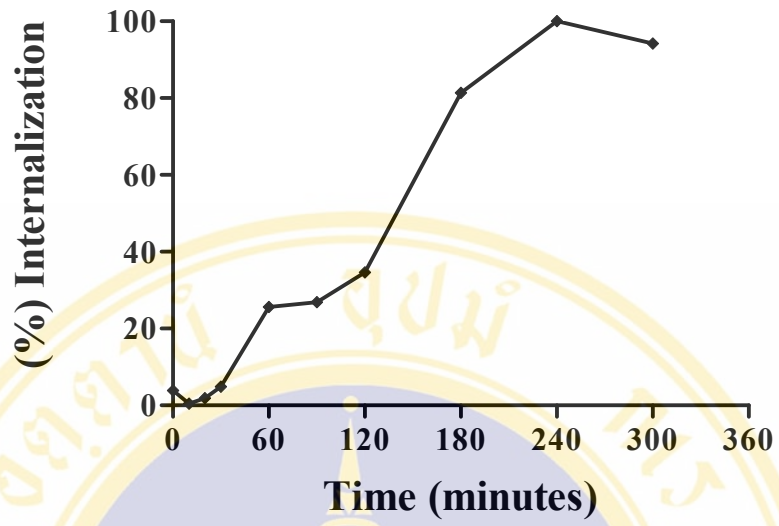
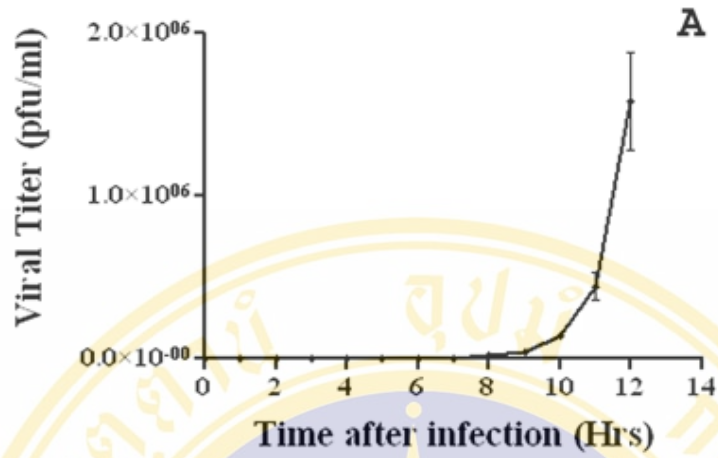


Figure 15 Percent Viral Internalization of JEV in C6/36 cells

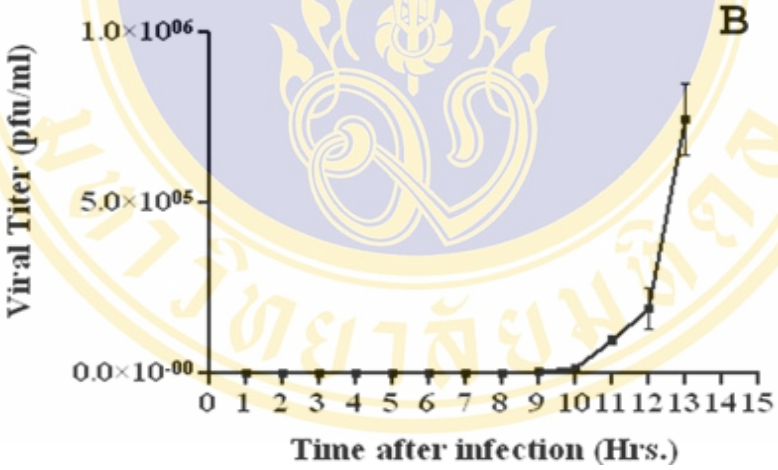
Internalization of JEV into C6/36 cells: Virus was incubated with C6/36 cells on ice for two hours then temperature shifted to 28 ^{o}C for the times indicated from 0 to 300 minutes at which point acid glycine was added to inactivate extracellular viruses. After one cycle of viral replication the level of virus in the medium was assayed by plaque titer on Vero cells. Results are shown as a percentage of the maximum viral titer at the 240 time point.

4. Detection of Mature-Immature virions

Upon virus infection, the progeny viruses are produced inside the cell before releasing to the culture medium. However, these un-releasing viruses of intracellular viruses are also infectious allowed the detection via plaque assay. By using both cell separation technique and breakage of cells, the intracellular viruses can be detected in cytoplasm fraction. C6/36 cells were used to determine the time that the complete intracellular viruses were produced. Cells were infected with JEV at a MOI of 1 for 2 hours following by treated for 1 minute with acid glycine buffer (pH 3.0) to inactivate any uninternalized viruses. After washing normal growth media was added and cells incubated under standard conditions. At each time point, medium was collected following by treated with 0.06% trypsin to detach cells. After those cells were transferred to an eppendoft a tube followed by centrifugation at 3,000 x rpm for 5 minutes. BA-1 was added to resuspend prior collected at -80 °C. Finally, levels of intracellular viruses were determined by release from infected cells by one freeze-thaw cycle followed by 5 minutes of sonication at 4 °C and assay by standard plaque assay. The level of intracellular viruses shows that JEV was detected inside the cell after 8 hours post infection (Figure 16, Panel A). Moreover the levels of produced viruses were detected as early as 10 hours post infection (Figure 16, Panel B).



Panel A. The level of intracellular viruses



Panel B. The levels of produced viruses

Figure 16 Detection of Mature-Immature virions

This figure shows cell lysate experimente in C6/36 cell line that represented in both level of infectious virus, intracellular (Panel A) and extracellular (Panel B). Experiments were undertaken independently in triplicate with triplicate titer of samples. Each point represents the triplicate count. Error bars represent SEM.

5. Viral Stability in growth media

JEV stability profile was determined to investigate the time that JEV is still infectious in absence of target cells. JEV was added to a medium normally employed for the propagation of C6/36 cells. The virus/medium mixture was incubated at 28 °C. At various time-points aliquots were taken and the level of infectious dengue viruses per milliliter assayed by standard plaque titration assay on Vero cells (Figure 17). The loss of viral viability fitted an exponential decay equation, and calculated half-lifetimes of 9.6 hours.

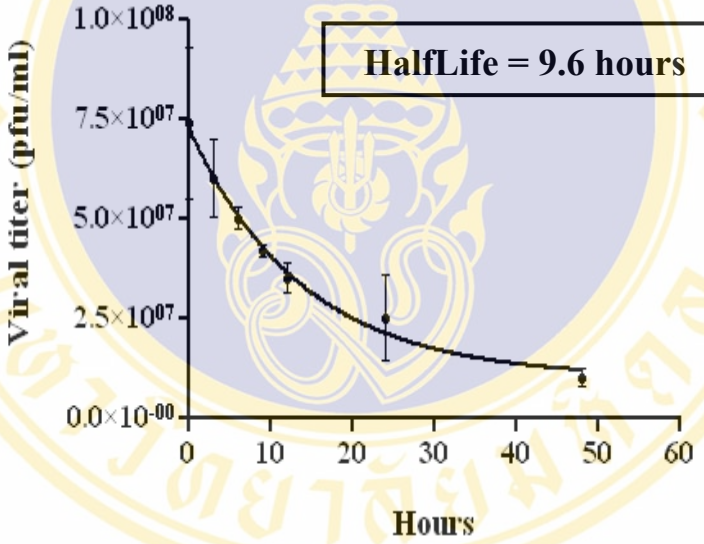


Figure 17 Time course profiles of loss of JEV infectivity in culture medium

A known titer of infectious JEV were added to MEM supplemented with 10% FCS, a medium normally employed for the propagation of C6/36 cells, to give a final viral titer of $1x10^7$ pfu/ml. The virus/medium mixture was incubated at 28 ^oC. At various time aliquots were taken and the level of infectious JEV per milliliter assayed by standard plaque assay on Vero cells. Experiments were undertaken independently in triplicate with triplicate titer of samples. Each point represents the triplicate count. Error bars represent SEM.

6. JEV receptor molecules on C6/36 cell surface

6.1 Effect of pre-trypsinization on viral entry

To assess the relative contribution of protein and non-protein moieties of molecules involved in JEV infection. Thus the ability of JEV infection that lack of extracellular protein was assessed. C6/36 cells were pre-treated with 0.06% trypsin prior to infection. Confluent of C6/36 cells were trypsinized and trypsinized C6/36 cells were incubated with JEV at 28 °C for 2 hours. The cell/virus mixtures were then treated with acid glycine to inactivate uninternalized viruses. Cells were then plated and allowed to grow further under appropriate conditions for one and a half virus a replication cycle. Level of infectious virus in the medium was determined by plaque assay titer on Vero cells. Experiment was undertaken in triplicate, with triplicate assay of virus levels. Experiment was undertaken in parallel with untreated control infection. Results (Figure 18) show that a very significantly reduction of viral titer of infectious virus for trypsinizing compare to normal cells with approximately 60% reduction of control levels.

6.2. Effect of heparinase pre-treatment on viral entry

While trypsinization significantly reduced the level of productive virus for JEV it did not completely eliminate the ability of the C6/36 cells to be infected completely. As glycosaminoglycans have been implicated in the process of viral binding [12, 15], we also sought to investigate the contribution of this class of molecules to virus internalization. Cells were pre-treated with heparinase III for 1 hour, and then incubated for 2 hours with JEV, followed by acid glycine treatment to inactivate any un-internalized viruses. Cells were then plated and allowed to grow under optimal conditions for one and a half virus a replication cycle which levels of infectious virus were determined by plaque titration on Vero cells. Experiments were undertaken in triplicate with triplicate titer assay. Experiment was undertaken in parallel with untreated control infection. Results (Figure 19) showed the reductions of approximately 40% of control levels.

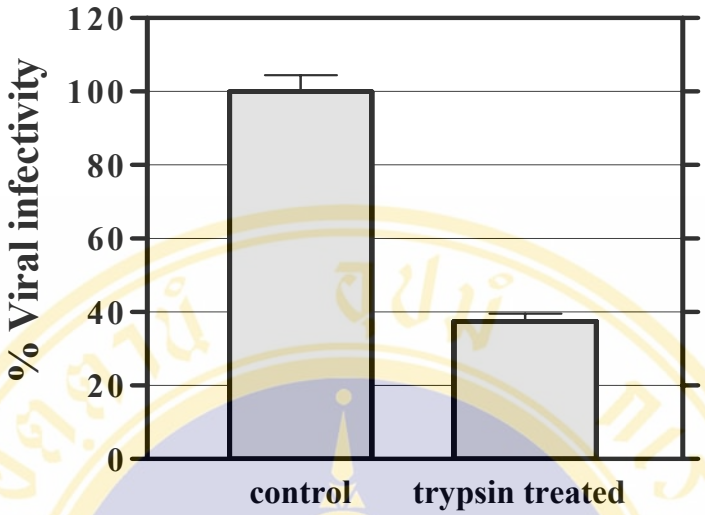


Figure 18 Trypsin treated Inhibition Infection Experiment

Trypsin treated inhibition: Confluent C6/36 cells were pre-treated with 0.06% trypsin for 5 minutes at 37 ^{o}C in parallel with untreated control reactions. After treatment, cells were infected with JEV at a MOI 1 for 2 hours at 28 ^{o}C. Infected cells were treated with acid glycine pH3.0 for inactivating uninternalized virus prior to the addition of normal growth medium. Medium was assayed at one and a half viral replication cycle and titered by plaque assay. Experiments were undertaken independently in triplicate with triplicate assay of virus titer. Error bars represent SEM.

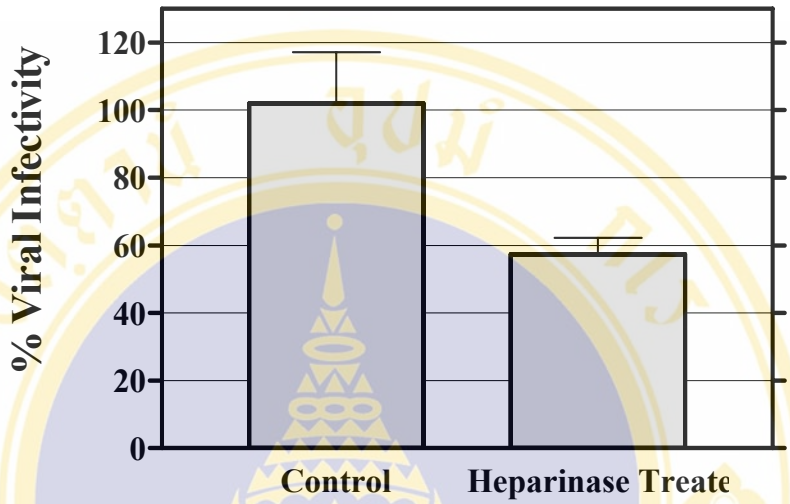


Figure 19 Heparinase III treated Inhibition Infection Experiment

Heparinase III treated inhibition: C6/36 cells were pre-incubated with HeparinaseIII at 1 unit/ml for 1 hour at 28 ^{o}C followed by incubation with JEV at MOI of 1. After 2 hours of virus adsorption period completed, virus/cell mixtures were treated with acid glycine pH 3.0. The infected C6/36 cells were grown further for one and half viral replication cycles, after which the levels of extracellular virus were assayed by plaque assay. Results are normalized against the level of virus produced with no Heparinase III preincubation. Each experiment is the sum of triplicate experiments with triplicate assay of titer. The non-treated cells were used as a control. Error bars represent SEM.

7. JEV receptor molecules on C6/36 cell surface

To further assess the contribution of extracellular elements towards the internalization of the JEV into C6/36 cells, we undertook to further define protein elements by using the viral overlay protein binding assay (VOPBA), an assay my colleagues have used to successfully identify HepG2 dengue virus receptor proteins. They have identified one protein associated with the internalization of dengue virus serotype 2 (DEN-2), namely GRP78 (BiP) [71]. As well as the 37/67 kDa high affinity laminin receptor has been identified as a dengue virus serotype 1 binding protein [72]. In methodology, membrane proteins were prepared from C6/36 cells and separated by electrophoresis through a 10% denaturing polyacrylamide gel prior to transfer to nitrocellulose membrane by wet electroblotting. The membrane was then incubated with purified JEV and virus binding visualized by incubation with a pan-specific anti-flavivirus monoclonal antibody and a secondary anti-mouse IgG conjugated with horseradish peroxidase. Five C6/36 membrane protein bands of 150, 90, 53, 51 and 35 kDa were found to be able to bind to purified JEV as several VOPBA (Figure 20). One band were cut at the positions corresponding to the 53 kDa bands which highly expressed and appear highest band intensity from VOPBA and subjected to mass spectroscopy fingerprint analysis (MALDI-TOF) (Figure 20. lane C).

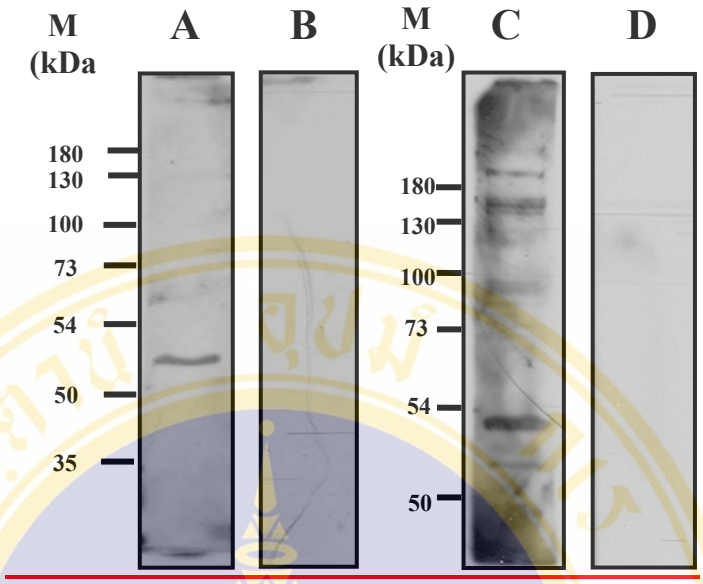


Figure 20 Japanese encephalitis binding proteins were identified by viral overlay protein binding assay (VOPBA) with C6/36 cell membrane proteins. Binding was carried out in 10% SDS-PAGE (lane A, C). No virus in the binding step was added in parallel with the negative control (lane B, D). The C6/36 membrane proteins that used in this experiment derived from lot 1. Molecular size markers are indicated on the left. The series of protein bands as well as four C6/36 membrane protein bands of 150, 90, 53 and 51 kDa were found to be able to bind to purified JEV. A 53 kDa band which appears on both gels was cut at the positions corresponding to the bands from VOPBA and subjected to mass spectroscopy fingerprint analysis (MALDI-TOF).

8. Peptide Mass Fingerprinting Analysis

Peptide mass fingerprinting analysis was used in this study to futher identidy JEV binding proteins revealed by VOPBA. The protein bands corresponded with VOPBA signal will be excised from the gel. Then the excised gel piece was submitted to Australian Proteome Analysis Facility (APAF, Macquarie University, Australia) for analysis. Briefly, proteins in excised gel were digested by protease enzyme. After digestion, the peptides were extracted from the gel and mass analyzed. The result analysis was used to search in the databases for a match and the identity of the protein can be determined.

The best matching protein of JEV binding protein analyzed by MALDI-TOF mass spectrometer is shown (Figure 21). After that the protein name (ENSANGP00000024137) was searched via Expasy site in Swiss-Pro/TrEMBL. The JEV binding protein (53 kDa) significant matched to ATPase. Its function is the production of ATP from ADP in the presence of a proton gradient across the membrane (Figure 22).

Match to: gi|30174085; Score: 319
ENSANGP00000024137 [Anopheles gambiae str. PEST]
Found in search of C:\Documents and Settings\rwitherdin\My Documents\Mass Spec\MAR3105_ROXANNE\Peak List\ppw_A1_111222785400.txt

Nominal mass (M_r): 51357; Calculated pI value: 4.96
NCBI BLAST search of gi|30174085 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Anopheles gambiae str. PEST
Links to retrieve other entries containing this sequence from NCBI Entrez:
gi|31240065 from Anopheles gambiae str. PEST

Variable modifications: Oxidation (M),Propionamide (C)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: 27%

Matched peptides shown in Bold Red

```
    1 LARSYAAKAA VNAAAGAQGK VVAVIGAVVD VQFDEQLPPI LNALEVQGRS
   51 ARLVLEVAQH LGENTVRTIA MDGTEGLVRG QRVLDTGSPI RIPVGAETLG
  101 RIINVIGEPI DERGPIDTNL SAPIHAEAPE FIEMSVEQEI LVTGIKVVDL
  151 LAPYAKGGKI GLFGGAGVGK TVLIMELINN VAKAHGGYSV FAGVGERTRE
  201 GNDLYNEMIE GGVISLKDKS SKVALVYGQM NEPPGARARV ALTGLTVAEY
  251 FRDQEGQDVL LFIDNIFRFT QAGSEVSALL GRIPSAVGYQ PTLATDMGSM
  301 QERITTTKKG SITSVQAIYV PADDLTDPAP ATTFAHLDAT TVLSRAIAEL
  351 GIYPAVDPLD STSRIMDPNI IGAEHYNIAR GVQKILQDYK SLQDIIAILG
  401 MDELSEEDKL TVARARKIQR FLSQPFQVAE VFTGHAGKLV PLEETIKGFT
  451 KILNGELDHL PEVAFYMVGP IEEVVEKAER
```

Figure 21 Mass spectrometry reports

Protein Name:	**ENSANGP00000024137**
Organism:	**Anopheles gambiae str. PEST;**
Sequence length:	**480** amino acid residues
SPROT/TrEMBL accession code:	**Q7PKD7 (ID: Q7PKD7)**
Secondary accession code:	
Gene name:	**Name=ENSANGG00000014374;;**
Protein description:	
Taxonomy:	**Eukaryota; Metazoa; Arthropoda; Hexapoda; Insecta; Pterygota; Neoptera; Endopterygota; Diptera; Nematocera; Culicoidea; Anopheles;**
TaxId:	**180454**
InterPro:	**Domain structure**
FUNCTION:	Produces ATP from ADP in the presence of proton gradient across the membrane

Figure 22 Matching protein search via Expasy

9. Native gel

To further confirm the possibility of 53 kDa protein binding band which analyzed in denaturing condition the non-denaturing gel was utilized. Native VOPBA appeared two virus binding band at the bottom of gel (Figure 23). Then both bands were subjected to analyze by commercial mass spectrometry. The result shows no match with any protein in mosquito database on both proteins.

Figure 23 VOPBA analysis of JEV on C6/36 cell membrane proteins in non-denaturing condition. C6/36 cell membrane proteins were separated on non-denaturing gel which no SDS including and no heat samples (lane A). Control reaction was identical, except that no virus was incubated (lane B).

As we have recently identified the 37/67 kDa high affinity laminin receptor as a dengue virus serotype 1 binding protein, we assessed this as a possible candidate binding protein and one strip from the filter was incubated with an antibody against the human 37/67 kDa high affinity laminin receptor in a Western analysis in parallel with the VOPBA analysis. Surprisingly, a major band of detected by this antibody was present at a closely similar or identical position to the 51 kDa VOPBA band (Figure 24).

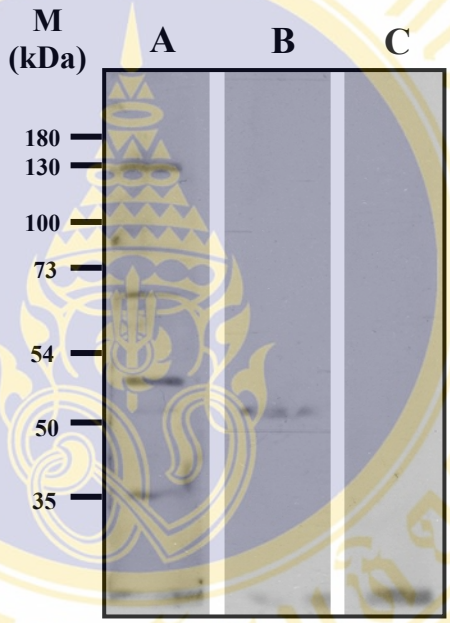


Figure 24 Membrane proteins of C6/36 cells were separated on 10% SDS-PAGE gels and transferred to a nitrocellulose membrane and subjected to either western blotting with an anti-37/67 kDa high affinity laminin receptor polyclonal antibody (lane B) and to virus overlay protein binding assay with purified JEV (laneA) in parallel with a negative control, no virus in the binding step (lane C). Size markers derived from Prestained protein ladder was indicated on the left. The series of virus bind bands were observed as well as with the 53 kDa highly expressed.

10. Inhibition of Infection

To further evaluate the possibility that the 37/67kDa high affinity laminin receptor was served as a component of JEV receptor protein expressed on the surface of the C6/36 cells, inhibition experiments were undertaken with a ligand for this receptor, soluble laminin. C6/36 cells were pre-incubated with 10 μg and 20 μg of soluble laminin, prior to infection with JEV. As in previous experiments, the cells were treated with acid glycine pH3 to inactivate un-internalized viruses prior to incubation in growth medium for one and a half virus replication cycles at which point the medium was assayed by standard plaque assay to determine the levels of infectious viruses in the medium. Experiments were undertaken independently in triplicate, with triplicate assay of virus titer. Results (Figure 25) show JEV appears some levels of inhibition in the presence of the soluble laminin. In the presence of 10 μg soluble laminin, internalization is reduced to about 20% of control levels. While at 20 μg soluble laminin, approximately 50% inhibition was observed.

11. Inhibition of binding

To verify that incubation of the C6/36 cells with soluble laminin results in a reduction of JEV binding, the binding of the JEV to C6/36 cells was visualized by immunoflourescence. C6/36 cells were incubated directly with the JEV at an MOI of 10, or pre-incubated with 10 μg soluble laminin, prior to incubation with JEV at the same MOI. JEV binding was visualized by incubations with a pan-specific anti-flavivirus monoclonal antibody and a FITC labeled goat anti-mouse IgG (Molecular Probes, Eugene, Oregon, U.S.A.). Results (Figure 26. Panel A, B, C, and D) showed that soluble laminin resulted in slight inhibition of binding of JEV to the C6/36 cells.

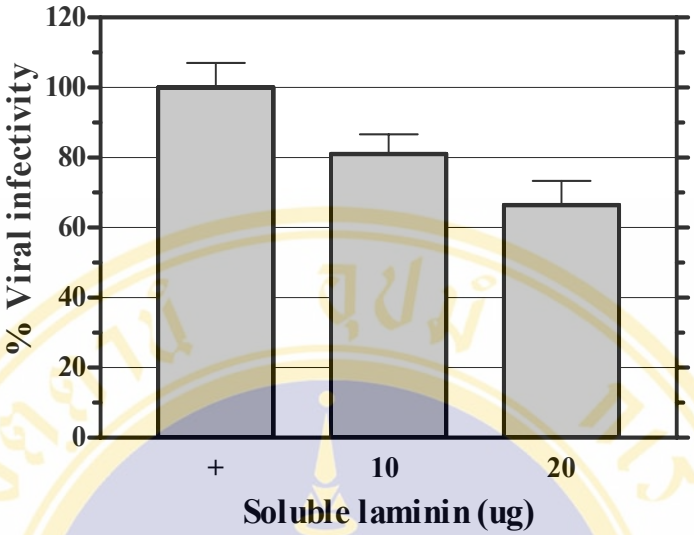


Figure 25 Infection Inhibition assay

Infection inhibition assay: C6/36 cells were pre-incubated with 10 or 20 µg of a soluble laminin prior to infection with JEV at MOI of 1. Levels of viral production were assayed after one and half viral replication cycles by plaque assay. Results are normalized against the level of virus produced with no antibody pre-incubation. Each experimental point is the sum of triplicate experiments with triple assay of titer. Error bars represent SEM.

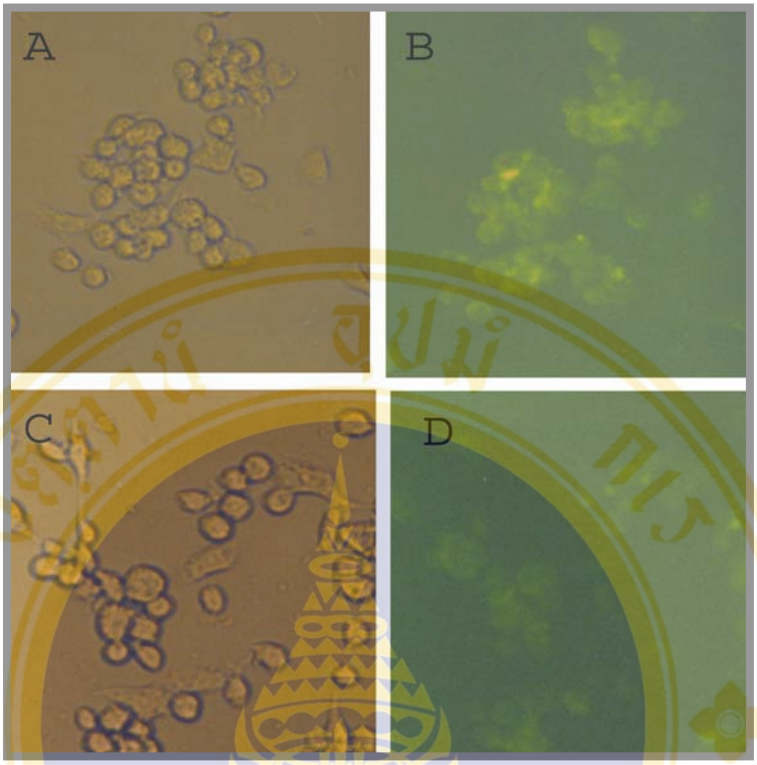


Figure 26 Binding Inhibition assay

Binding inhibition assay: The immunofluorescent staining was used to investigate the soluble laminin inhibition of JE binding in C6/36 cells. The C6/36 cells were cultured on glass slides and fixed with 1% formaldehyde. C and D show the inhibitory effect of JEV binding to C6/36 cells by soluble laminin. Cells were pre-incubated with soluble laminin and then incubated with JEV at MOI of 10. Virus-binding was detected with a pan-specific anti flavivirus monoclonal antibody and a FITC labeled goat anti-mouse IgG. The slides were investigated under fluorescent microscope and the positive cells were estimated in whole slide. Unpre-incubated with soluble laminin (A and B) were used as control.

CHAPTER VI
DISCUSSION

During the previous years, several groups have been studying JEV infection in mosquitoes [48-51] however, little known about the interaction between virus and insect cell. Although the initial steps of viral infection are a critical determinant of tissue tropism and therefore of pathogenesis, little is known about the process of early phase of JEV infection. However the studies of entry modes of Japanese encephalitis virus (JEV) into C6/36 mosquito cells by the electron microscopy revealed in direct penetration of the virions into the cytoplasm at the cell surface in three stages [48]. At stage one, virions attach to the plasma membrane of the host by their envelope spikes; at the second stage, the virions envelopes approximated to and eventually overlapped the host plasma membrane, and in the process the plasma membrane at the attachment sites dissolved. The last stage, virion penetrated into the cytoplasm through the plasma membrane disruptions created at the absorption sites. Coated pits did not form at the virion attachment sites, and virion attachment sites, and the virion-containing vesicles were not found in the cytoplasm.

However the present study on vertebrate cell indicates that JEV entry cells through the clathrin-dependent endocytic pathway. They showed the inhibition of productive JEV infection after treated cells with chlorpromazine, inhibiting the assembly of clathrin lattices on endosomal membranes [52]. In step, initial attachment of single viral particles to clathrin-coated pits on the cell surface and then the uptake of virions into coated vesicles followed. Uncoating of virions occurred at acidic pH where the envelope of the virus fused with the membrane of the prelysosomal endocytic vacuoles before degradation in lysosomes. The viral genome was eventually released into the cytoplasm for replication. However no cellular molecule has thus far been identified as receptor for JEV. This study sought to investigate the interaction between mosquito cells and the Japanese encephalitis virus with respect to internalization, viral production, viral stability, and comparison the level of un-

releasing infectious and releasing one. Pretreated cell with trypsin and heparinase have been carried out base on the sensitivity of the viral binding site on the cell surface. Furthermore the possible candidate or components of cellular receptor for JEV were determined. The identification of the interaction and number of cellular receptor molecules would be of the future importance in understanding viral replication, pathogenesis, and tissue tropism in vector organism.

1. Propagation of Japanese Encephalitis Virus in C6/36 cells

In these studies, a precise understanding of the replication cycle, as well as, a better understanding of the mechanisms of viral internalization in insect cells may yield strategies to eliminate the viral infectious state. The viral production profile of JEV was analyzed in both early event and long term profile. The kinetics of early virus production shown the production of infectious virus particles was detected as early as 10 hours post infection. These suggest the time for one complete infectious viral cycle for JEV. While the study of early viral profile of all 4 serotype of dengue viruses in the laboratory have been shown that dengue serotype 4 was produced earliest at 14 hours post infection, while serotypes 1 and 2 were first produced at 17 hours and serotype 3 by 19 hours (Figure 27). By 20 hours, JEV will have undergone 2 rounds of viral production and reinfection while all four still present in the first round. It does suggest that JEV is produced from the mosquito cells much higher in the level of infectious virus than dengue virus over the same time interval.

The long term profile of JEV production shows the highest yield of virus was produced by day 5 post infection. In a parallel experiment C6/36 cells were infected with the four dengue serotypes, the result showed that serotypes 1 and 2 gave maximum titers at day 6 post infection, whereas serotypes 3 and 4 gave maximum titers at days 10 and 7 respectively. Moreover the maximum titer of JEV appeared higher level than that of all four serotype (Figure 28). At this point in time it is unclear why JEV has such a shorter mature virus production time. However, it is interesting to note that JEV also has a more rapid early internalization profile than dengue viruses leading to the short viral process inside the host cells. It is corresponding to the past evidence, remarked that JEV has a more gradual onset but greater case-fatality rate (20% vs. less than 5%), than the dengue virus [53].

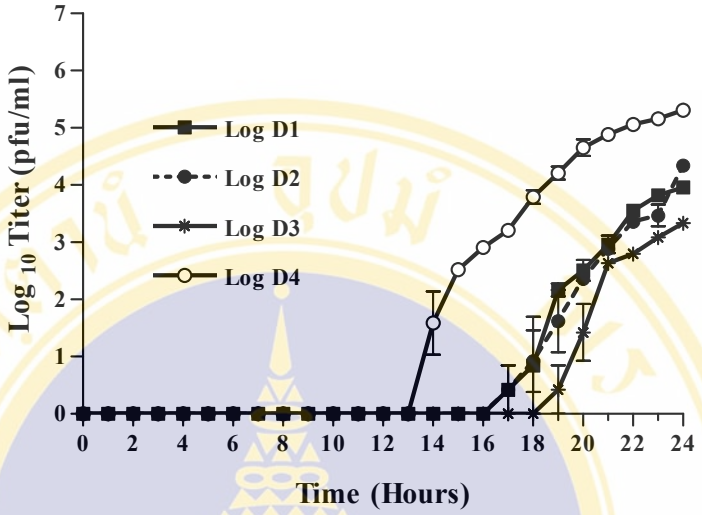


Figure 27 Dengue viral production on C6/36 cells
(PRIRAYAPAK SAKOONWATANYOO)

This figure shows all four serotype of dengue virus growth curve in C6/36 cell line. Cells were infected with dengue virus at an MOI of 1. After 2 hours of viral absorption, cells were treated with acid glycine pH 3.0 the growth medium was sampled hourly for 24 hours post infection and determined for level of infectious viruses by standard plaque assay on Vero cells. Experiments were undertaken independently in triplicate with triplicate titer of samples. Each point represents the triplicate count. Error bars represent SEM.

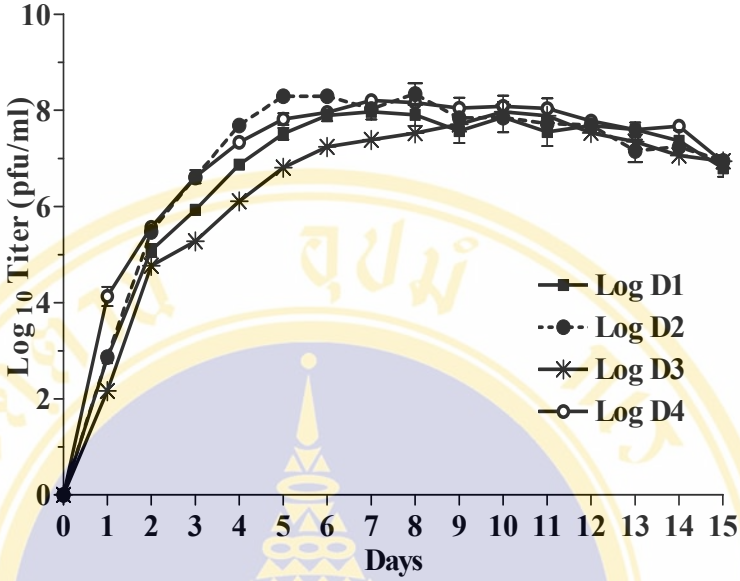


Figure 28 Dengue viral growth curve on C6/36 cells

(PRIRAYAPAK SAKOONWATANYOO)

This figure shows viral growth curve in C6/36 cell line of all four dengue serotypes. Cells were infected with dengue virus at an MOI of 1. After 2 hours of viral absorption, the growth medium was sampled daily for 15 days and determined for level of infectious viruses by standard plaque assay on Vero cells. Experiments were undertaken independently in triplicate with triplicate titer of samples. Each point represents the triplicate count. Error bars represent SEM.

2. Internalization of Japanese Encephalitis Virus into C6/36 cells

In the profile of internalization, however direct analysis of the media by plaque titration on samples withdrawn immediately after acid glycine treatment showed no infectious virus present in other experiments, suggesting that acid glycine can completely inactivate the virus. Because of at low temperature membrane becomes rigid. Even at low temperatures a degree of JEV internalization can occur in C6/36 cells might due to incompletely membrane rigid at that temperature point. JEV internalization profile appears twice plateau phases between 2 and 3 hours and also 3 and 4 hours, it does suggest that the internalization of JEV is not a smoothly continuous process, proceed by periods of rapid internalization followed by plateaus of little or no internalization. It is interesting to speculate that the internalization plateau of JEV may be related to the cycling time of a JEV receptor protein. Although JEV has been proposed that the receptor-independent direct fusion can be occurred in the JEV entry process. When compare the speed of internalization between JEV and dengue virus, the data indicated that JEV is quite rapidly entry into the mosquito cells than dengue virus as at third hours JEV internalized reach to 80% while dengue virus is less than 25% comparing to the maximum titer (Figure 29). It is able to suggest that JEV exhibited a higher absorption rate than dengue virus. In addition, there is no evidence of a plateau in the internalization of any of the dengue serotypes, shown an almost linear increase between 1 and 5 hours.

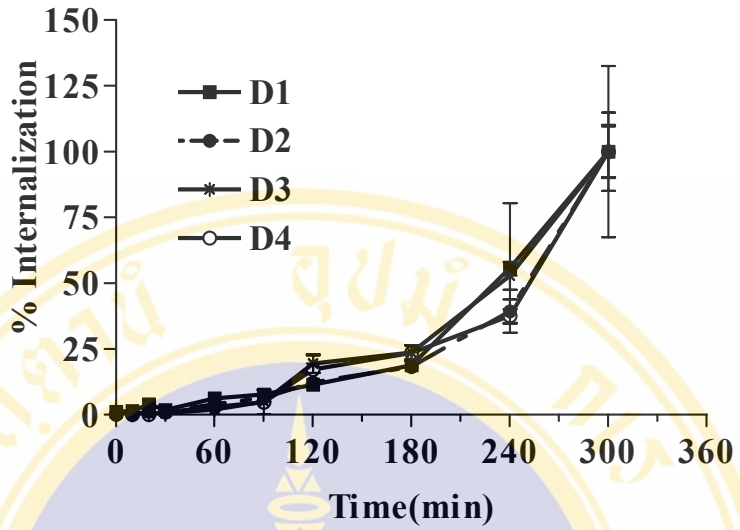


Figure 29 Dengue internalization in C6/36 cells

(PRIRAYAPAK SAKOONWATANYOO)

This figure shows the internalization profile of all four dengue serotypes. The C6/36 cells were incubated on ice with all four serotypes of the dengue virus individually for 2 hours at an MOI of 1 and the temperature subsequently shifted to 28 ^{o}C. At the time points shown the cell/virus mixtures were treated with acid glycine pH 3.0 to inactivate un-internalized viruses and the cells allowed to grow in growth medium for one and a half virus replication cycles at which point the growth medium was sampled and the level of infectious viruses present assayed by standard plaque assay on Vero cells. Experiments were undertaken independently in triplicate with duplicate assay of virus titers. Error bars represent SEM.

3. Detection of extra and intracellular infectious virus

Upon virus infection, the progeny viruses are produced inside the cell before releasing to the culture medium. However, these un-releasing viruses of intracellular viruses are also infectious allowed the detection via plaque assay. The mature JEV particles can be first detected inside the mosquito cells at 8 hours after infection in addition the extracellular mature viral particle were detected as early as 10 hours post infection. This result is corresponding to the viral production curve of JEV, which appear JEV was produced as early as 10 post-infection. The combination of both results would suggest that JEV spend at least 8 hours for processing, include uncloating, viral genome translation, and assembly. The exocytosis and subsequently releasing out from the cells require about 2 hours. Hase [54] demonstrated the maturation process of JEV in C6/36 cells *in vitro* by electron microscopy. They proposed that the mode of JEV maturation, termed trans-type maturation, the viral structure proteins that are synthesized on host ribosomes are presumed to appear within the cisternae of RER for viral assembly, and the assembed virions the pass through the host secretory channel including the Golgi apparatus, and are eventually carried within secretory vesicles to the cell surface for secretory-type exocytosis. JEV virion at the maturation stage appeared within cisternae of RER of infected cells at 24 hours post infection. From this evidence, JEV might spend shorter time in order to acquire the maturation process as coincident with my study due to the observation times, 24, 48, and 72 hours post infection, of Hase experiment.

4. Japanese Encephalitis Stability in normal growth medium

During studies on the interaction between the JEV and mosquito cells, the ability of the JEV to retain it infectivity in normal growth medium became interesting. The result shown the JEV in normal growth medium loses infectivity over time. It is evident that the decline in virus titer over time in the standard plaque assay. Preliminary result in the laboratory have determined that dengue virus produced in both Vero cells and HepG2 cells showed a faster rate of loss of stability as compared to JEV made in C6/36 cells for all four serotypes [55]. As the rate of loss of infectivity is primarily determined by temperature and pH of the solution (Figure 30) [55]. EM data of dengue virus in the laboratory shown that dengue virus tends to

aggregated overtime and it is evidence that the aggregated virus is no longer able to be internalized into cells, as evidenced by the decline in virus titer overtime the aggregation is non-reversible. However, in the previous studies of Tick Borne Encephalitis (TBE) virus, it is known that low pH trigger a conformation change in E protein leading to fusion with endosome membrane [56]. As such it is interesting to speculate that the aggregation of virus in solution is related to this mechanism. In addition, previous data showing that JEV is cleared more severity in fatality rate that dengue virus [53], indicate that the issue of lost of infectivity of virus may also have clinical significant. However, the issue of loss of infectivity in human samples is vastly more complex, given the complicated relationship between the JEV and the immune system [57].

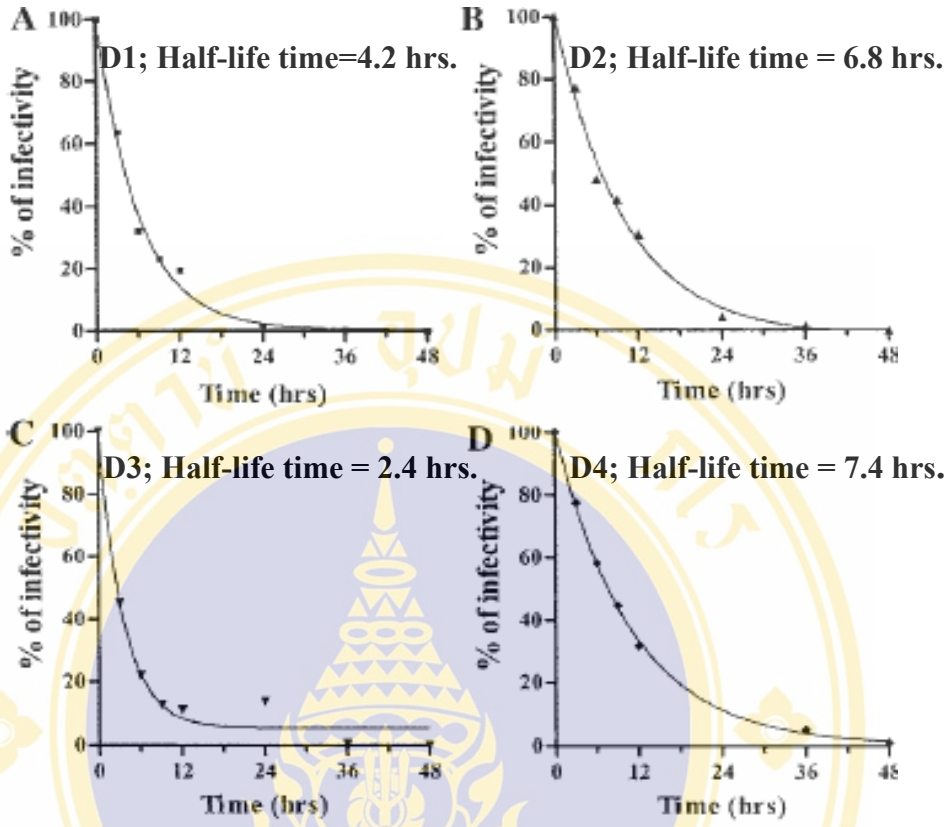


Figure 30 Time course profile of loss of infectivity of dengue virus in normal culture medium [55]

A known titer of infectious all four dengue serotypes were added to DMEM supplemented with 10% FCS, a medium normally employed for the propagation of Vero cells, to give a final viral titer of $1x10^7$ pfu/ml. The virus/medium mixture was incubated at 37 ^oC. At various time aliquots were taken and the level of infectious virus per milliliter assayed by standard plaque assay on Vero cells. Experiments were undertaken independently in triplicate with triplicate titer of samples. Each point represents the triplicate count. Error bars represent SEM. For dengue serotype 1 (Panel A), 2 (Panel b), 3 (Panel C), and 4 (Panel D) Points represent the mean of three independent experiments.

5. Isolation and characterization of Japanese Encephalitis Virus binding receptor molecules on C6/36 cells

JEV is believed to utilize receptor-mediated endocytosis for the initiation of infection [52]. No cellular molecule has thus far been identified as a receptor for JEV, whereas its counterpart, a surface protein on virion, has been suggest to be glycoprotein E, because E but not other viral structure proteins, is the major target for neutralizing antibodies [58]. Many previous studies have reported different binding proteins for JEV and in different cell types [59, 60]. However, no other research group has identified JEV receptor in C6/36 cells.

Many microbial infections have been postulated to be related to heparan sulfate (HS) [43, 44, 61-63] which is a member of the highly sulfated glycosaminoglycans (GAGs) and is mostly localized on cell surfaces [64]. Tissue tropism and pathogenesis of various viruses in an infected host can also be consequence of the efficient attachment of an envelope protein with HS proteoglycans. Some viruses such as herpes simplex virus gain entry into target cells through a multistep process [43] that includes an initial attachment to the surface mediated by heparan sulfate, followed by the stage that require the cooperation of different cell surface molecules that act as coreceptor. Binding appears to involve electrostatic interactions between ionizable groups on the virus and the cell membrane. It is highly dependent on the pH of the medium, and binding is reduced in medium of elevated ionic strength [64]. The contribution of glycosaminoglycans to the entry of the JEV into cells is somewhat controversial. Heparan sulfate has been shown to play a role in the entry of the JEV into CHO-K1 [65, 65, 65], however no reports suggest that they play a significant role in mosquito cells. In this study preincubation of C6/36 cells with heparinase before JE virus infection has caused a significant reduction to almost 40% of control level. It is noted that the effect of heparinase on JEV infection probably mediated through its interference with the virus binding to highly sulfated molecules on the cell surface. As the preincubation experiments with heparin clearly localized the GAG binding domain to the viral surface showed most likely as a part of the E-protein. Proposing a homologous 3-dimensional structure of DEN-2 E-protein to the crystallographically partially determined structure of another flavivirus E-protein (tick-borne encephalitis virus) [66] they localized two potential GAG-binding motifs on the assumed external

domains of the E-protein, which possess binding activity for GAGs. The binding activity depends largely on electrostatic interaction among positively charged Arg and Lys in side chains with negatively charged sulfate moieties. So the highly sulfate from GAGs are critically involved in JEV infection *in vitro* in mosquito cells, preliminary at the early stage of the virus life cycle. Nevertheless it did not completely eliminate the ability of C6/36 cell to be infected.

In addition the identification of the extra-cellular proteins utilized by the JEV to gain entry into cells was conducted. Pre-trypsinization of C6/36 cells prior to be infected with JEV produced significant effects for JEV which 60% reduction as compare to the non-treated cells. It does suggest that the entry of JEV was relatively affected by removal of extra cellular protein domains. From this result, it is possible that major elements used by JEV are trypsin insensitive protein. However viral binding have great effectively suppressed by cell pretreatment with trypsin, presumably because of cleavage of the proteoglycan core protein ectodomains, leading to almost complete release of cell surface heparin sulfate as shown in other cell lines [67].

To investigate the additional trypsin-sensitive molecules that involved in JEV entry, viral overlay protein binding assay (VOPBA) were performed. It has been widely used to characterize putative receptors for a number of viruses, including visna virus [68], human cytomegalovirus [69], and dengue virus [70, 71]. Through several VOPBA performing, JEV was observed to bind to the series of several protein bands and the four major virus binding proteins, which are 150, 90, 53 and 51 kDa membrane protein band. From various patterns of VOPBA bands that appeared in each independent experiment, the 53 kDa protein band was observed consistently while others series of binding bands might be the non-specific binding. Hence this viral binding band has been subjected to further identified. The various pattern of VOPBA might be derived from the effect of freeze-thaw of sample proteins in each sample protein that will break protein molecule as non-specific breaking. Although the boiling protein and SDS condition on SDS-PAGE cause the specifically break protein structure. As high temperature will break H-bond and the SDS is usually necessary to reduce disulphide bridges in proteins before they adopt the random-coil configuration necessary for separation by size. So the non-specific breaking might create many

various protein fragments which were separated on SDS gel via their size. Some fragment still express the viral binding part that why the series of protein bands were found. In addition the difference in lot of membrane protein could provide difference in protein component. As well as the different in cell passage could be involved in different virus binding proteins [72]. Hence, it is difficult to compare between different experiments. Through the VOPBA technique is the method which used to specify the possibility of viral binding protein. Because of in a standard VOPBA, complex protein preparations are separated according to molecular mass and transferred to membranes to be probed with purified virus. When complex mixtures are used there are often many comigrating proteins that cannot be adequately resolved for accurate interpretation of mass spectrometric data. Moreover the binding of virus and membrane via VOPBA technique is not conferring the real native conformation of proteins. Hence the non-denaturing condition is used. In the absence of denaturing agents, gel electrophoresis can be used to study the native conformation, physico-chemical properties and biological activities of proteins. Native PAGE has been used to analyze biological systems involving protein–protein interactions. And the further analyzing methods are necessary such as inhibition of virus binding and infection by antibody that specific to those proteins.

For mass spectrometry result, the strategies to identify protein by mass spectrometry require more than one analyzing process in order to confirm the identify protein. Since the first analysis matched only one protein, ATPase, but without any confirm due to the native gel does not provide the positive result. The reason for this may be due to insufficient protein that cut from the gel or the limited information in the databases for this species. Therefore, further examination to confirm this viral protein binding of 53 kDa was required prior to be establishing as candidate receptor for JEV on C6/36 cells.

As our laboratory have recently identified the 37/67 kDa high affinity laminin receptor as a dengue virus serotype 1 binding protein [71], one strip from the filter was incubated with an antibody against the human 37/67 kDa high affinity laminin in a Western analysis in parallel with the VOPBA analysis. The evidence of the 37/67 kDa high affinity laminin receptor protein was found at a closely similar or identical position to the 50kDa VOPBA band (Figure 24). Although the 37/67 kDa high affinity

laminin receptor protein probably not the major element is utilized by JEV however both inhibition assays by soluble laminin revealed the minor reduction in both viral binding and virus infection. Hence it is possible that the 37/67 kDa high affinity laminin receptor protein might serve as an accessory molecule on the surface of C6/36 cells, which facilitate the interaction between the putative receptor and the JEV E protein during receptor-mediate endocytosis. This speculates the specificity in flavivirus group, given the degree of homology between the flavivirus members. However, although the envelope protein (E protein), which plays role in receptor recognition, is highly conserved in this genus, domain III, the host cell binding domain [73] shows high variation in amino acid sequences. Such variation might lead to the specificity in host cells receptor recognition noted here.

The role of cell surface heparin sulfate in the internalization of the JEV appears to have a significant role. Interestingly, both the 53 kDa identified protein high affinity receptor and heparin sulfate have been shown to be involved in the internalization of the JEV into mosquito (C6/36) cells that corresponding to the previous studies in many other flavivirus [65, 74]. This provide one possible evidence, heparin sulfate might sever as a primary receptor probable concentrating viral particles on the cell surface, and that subsequently viral penetration could require other high affinity molecule such as a 53 kDa identified protein.

Based upon the studies of relationship between Japanese encephalitis virus and mosquito vector cells, it make a determination of the nature of the early interactions of the infecting JEV with the host cells especially with the molecules on the surface of vector cells. Understanding the interaction of viruses and vector cells provides the possibility to the development of therapeutic agents that can be used to inhibit virus transmission.

CHAPTER VII
CONCLUSIONS

1. The propagation scheme of Japanese encephalitis virus in insect cells could be directly compared to the closely related flavivirus, dengue virus. The present study examined the infection of C6/36 mosquitoes cells with JEV, under conditions similar to those used previously for dengue virus study. The propagation pattern of JEV appears in parallel with the dengue virus including viral propagation profile, viral internalization, and its stability in the normal growth medium. However JEV evidence seems to be more rapidly than the dengue virus in every profile. The reason of this might due to the higher severity of JEV than that of dengue virus.

2. Heparan sulfate and the 37/67 kDa high affinity laminin receptor which has been identified as dengue virus serotype 1 receptor (Thepparit and Smith 2004) might have a minor role in the internalization of JEV into C6/36 mosquito cells. They might serve as an accessory molecule on the surface of C6/36 cells, which facilitate the interaction between the putative receptor and the JEV E protein during receptor-mediate endocytosis.

3. Analysis of the newly identified viral binding protein (53 kDa) from VOPBA via mass spectrometry revealed no significant match with any protein in the mosquito protein databases, possibly owing to their limited information currently available. Thereby, further characterization of the putative receptor needs to be conducted.

REFERENCES

1. Solomon, T. 2003. Recent advance in Japanese Encephalitis. *Journal of Neurology* 9:274-283
2. Sumiyoshi, H., Mori C., Fuke, I., Morita, K., Kuhara, S., Kondon., J., Kikuchi, Y., Nagamatu, H., and Igarashi, A. 1987. Complete nucleotide sequence of the Japanese Encephalitis Virus genome RNA. *Virology* 161:497-510
3. Jacobson, J. and Sivalenka, S. 2004. Japanese encephalitis globally and in India. *Indian J.Public Health* 48:49-56
4. Thongcharoen, P. 1985. Japanese encephalitis in Thailand. *J.Med.Assoc.Thai.* 68:534-545.
5. Takegami, T. 2003. [Japanese encephalitis]. *Uirusu* 53:25-30.
6. Solomon, T., Ni, H., Beasley, D. W., Ekkelenkamp, M., Cardosa, M. J., and Barrett, A. D. 2003. Origin and evolution of Japanese encephalitis virus in southeast Asia. *J.Virol* 77:3091-3098.
7. Solomon, T. and Winter, P. M. 2004. Neurovirulence and host factors in flavivirus encephalitis evidence from clinical epidemiology. *Arch Virol Supp*l161-170.
8. Chang, G. J., Kuno, G., Purdy, D. E., and Davis, B. S. 2004. Recent advancement in flavivirus vaccine development. *Expert Rev.Vaccines*. 3:199-220.
9. Jones, T. 2004. A chimeric live attenuated vaccine against Japanese encephalitis. *Expert Rev.Vaccines*. 3:243-248.
10. Rosen, L., Roseboom, L. E., Gubler, D. J., Lien, J. C., and Chaniotis, B. N. 1985. Comparative susceptibility of mosquito species and strains to oral and parenteral infection with dengue and Japanese encephalitis viruses. *Am.J.Trop.Med.Hyg*. 34:603-615.

11. Kabilan, L., Rajendran, R., Arunachalam, N., Ramesh, S., Srinivasan, S., Samuel, P. P., and Dash, A. P. 2004. Japanese encephalitis in India: an overview. *Indian J.Pediatr.* 71:609-615.

12. Bressanelli, S., Stiasny, K., Allison, S. L., Stura, E. A., Duquerroy, S., Lescar, J., Heinz, F. X., and Rey, F. A. 2004. Structure of a flavivirus envelope glycoprotein in its low-pH-induced membrane fusion conformation. *EMBO J.* 23:728-738.

13. Chen, W. R., Tesh, R. B., and Rico-Hesse, R. 1990. Genetic variation of Japanese encephalitis virus in nature. *J.Gen.Virol 71* (Pt 12):2915-2922.

14. Westaway, E. G., Brinton, M. A., Gaidamovich, S. Y., Horzinek, M. C., Igarashi, A., Kaariainen, L., Lvov, D. K., Porterfield, J. S., Russell, P. K., and Trent, D. W. 1985. *Flaviviridae. Intervirology* 24:183-192.

15. Rice, C. M., Aebersold, R., Teplow, D. B., Pata, J., Bell, J. R., Vorndam, A. V., Trent, D. W., Brandriss, M. W., Schlesinger, J. J., and Strauss, J. H. 1986. Partial N-terminal amino acid sequences of three nonstructural proteins of two flaviviruses. *Virology* 151:1-9.

16. Gorbalenya, A. E., Blinov, V. M., Donchenko, A. P., and Koonin, E. V. 1989. An NTP-binding motif is the most conserved sequence in a highly diverged monophyletic group of proteins involved in positive strand RNA viral replication. *J.Mol.Evol*. 28:256-268.

17. Koonin, E. V. 1993. Computer-assisted identification of a putative methyltransferase domain in NS5 protein of flaviviruses and lambda 2 protein of reovirus. *J.Gen.Virol 74* (Pt 4):733-740.

18. Kuhn, R. J., Zhang, W., Rossmann, M. G., Pletnev, S. V., Corver, J., Lenches, E., Jones, C. T., Mukhopadhyay, S., Chipman, P. R., Strauss, E. G., Baker, T. S., and Strauss, J. H. 2002. Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. *Cell* 108:717-725.

19. Roehrig, J. T., Hunt, A. R., Johnson, A. J., and Hawkes, R. A. 1989. Synthetic peptides derived from the deduced amino acid sequence of the E-glycoprotein of Murray Valley encephalitis virus elicit antiviral antibody. *Virology* 171:49-60.

20. Rey, F. A., Heinz, F. X., Mandl, C., Kunz, C., and Harrison, S. C. 1995. The envelope glycoprotein from tick-borne encephalitis virus at 2 A resolution. *Nature* 375:291-298.

21. Shevchenko,A., Jensen,O.N., Podtelejnikov,A.V., Sagliocco,F., Wilm,M., Vorm,O., Mortensen,P., Shevchenko,A., Boucherie,H., Mann,M. 1996. Linking genome and proteome by mass spectrometry: large-scale identification of yeast proteins from two dimensional gels. *Proc.Natl.Acad.Sci.U.S.A* 93:14440-14445.

22. Monath, T. P., Arroyo, J., Levenbook, I., Zhang, Z. X., Catalan, J., Draper, K., and Guirakhoo, F. 2002. Single mutation in the flavivirus envelope protein hinge region increases neurovirulence for mice and monkeys but decreases viscerotropism for monkeys: relevance to development and safety testing of live, attenuated vaccines. *J.Virol* 76:1932-1943.

23. Heinz, F. X. and Allison, S. L. 2001. The machinery for flavivirus fusion with host cell membranes. *Curr.Opin.Microbiol*. 4:450-455.

24. Hahn, C. S., Hahn, Y. S., Rice, C. M., Lee, E., Dalgarno, L., Strauss, E. G., and Strauss, J. H. 1987. Conserved elements in the 3' untranslated region of flavivirus RNAs and potential cyclization sequences. *J.Mol.Biol*. 198:33-41.

25. Leyssen, P., De Clercq, E., and Neyts, J. 2000. Perspectives for the treatment of infections with Flaviviridae. *Clin.Microbiol.Rev*. 13:67-82, table.

26. Jan, L. R., Yueh, Y. Y., Wu, Y. C., Horng, C. B., and Wang, G. R. 2000. Genetic variation of Japanese encephalitis virus in Taiwan. *Am.J.Trop.Med.Hyg*. 62:446-452.

27. Liao, C. L., Lin, Y. L., Wu, B. C., Tsao, C. H., Wang, M. C., Liu, C. I., Huang, Y. L., Chen, J. H., Wang, J. P., and Chen, L. K. 2001. Salicylates inhibit flavivirus replication independently of blocking nuclear factor kappa B activation. *J.Virol* 75:7828-7839.

28. Liao, C. L., Lin, Y. L., Wang, J. J., Huang, Y. L., Yeh, C. T., Ma, S. H., and Chen, L. K. 1997. Effect of enforced expression of human bcl-2 on Japanese encephalitis virus-induced apoptosis in cultured cells. *J.Virol* 71:5963-5971.

29. Libraty, D. H., Nisalak, A., Endy, T. P., Suntayakorn, S., Vaughn, D. W., and Innis, B. L. 2002. Clinical and immunological risk factors for severe disease in Japanese encephalitis. *Trans.R.Soc.Trop.Med.Hyg.* 96:173-178.

30. Macdonald, W. B., Tink, A. R., Ouvrier, R. A., Menser, M. A., de Silva, L. M., Naim, H., and Hawkes, R. A. 1989. Japanese encephalitis after a two-week holiday in Bali. *Med.J.Aust*. 150:334-6, 339.

31. Thongcharoen, P. 1989. Japanese encephalitis virus encephalitis: an overview. *Southeast Asian J.Trop.Med.Public Health* 20:559-573.

32. Halstead, S. B. and Jacobson, J. 2003. Japanese encephalitis. *Adv.Virus Res.* 61:103-138.

33. Ku, C. C., King, C. C., Lin, C. Y., Hsu, H. C., Chen, L. Y., Yueh, Y. Y., and Chang, G. J. 1994. Homologous and heterologous neutralization antibody responses after immunization with Japanese encephalitis vaccine among Taiwan children. *J.Med.Virol* 44:122-131.

34. Nothdurft, H. D., Jelinek, T., Marschang, A., Maiwald, H., Kapaun, A., and Loscher, T. 1996. Adverse reactions to Japanese encephalitis vaccine in travellers. *J.Infect*. 32:119-122.

35. Liu, Z. L., Hennessy, S., Strom, B. L., Tsai, T. F., Wan, C. M., Tang, S. C., Xiang, C. F., Bilker, W. B., Pan, X. P., Yao, Y. J., Xu, Z. W., and Halstead, S. B. 1997. Short-term safety of live attenuated Japanese encephalitis vaccine (SA14-14-2): results of a randomized trial with 26,239 subjects. *J.Infect.Dis.* 176:1366-1369.

36. Monath, T. P. 2002. Japanese encephalitis vaccines: current vaccines and future prospects. *Curr.Top.Microbiol.Immunol.* 267:105-138.

37. Solomon, T. 2004. Flavivirus encephalitis. *N.Engl.J.Med.* 351:370-378.

38. Wang, L. H., Rothberg, K. G., and Anderson, R. G. 1993. Mis-assembly of clathrin lattices on endosomes reveals a regulatory switch for coated pit formation. *J.Cell Biol*. 123:1107-1117.

39. Bergstrom, T., Trybala, E., and Spillmann, D. 1997. Heparan sulfate and viral tropism. *Nat.Med*. 3:1177.

40. Andre, P., Komurian-Pradel, F., Deforges, S., Perret, M., Berland, J. L., Sodoyer, M., Pol, S., Brechot, C., Paranhos-Baccala, G., and Lotteau, V. 2002. Characterization of low- and very-low-density hepatitis C virus RNA-containing particles. *J.Virol* 76:6919-6928.

41. Kimura, T. 1994. [Characterization of binding and entry process of Japanese encephalitis virus infection to high-susceptibility and low-susceptibility cell lines]. *Hokkaido Igaku Zasshi* 69:857-865.

42. Chen, L. K., Lin, Y. L., Liao, C. L., Lin, C. G., Huang, Y. L., Yeh, C. T., Lai, S. C., Jan, J. T., and Chin, C. 1996. Generation and characterization of organ-tropism mutants of Japanese encephalitis virus in vivo and in vitro. *Virology* 223:79-88.

43. Chung, C. S., Hsiao, J. C., Chang, Y. S., and Chang, W. 1998. A27L protein mediates vaccinia virus interaction with cell surface heparan sulfate. *J.Virol* 72:1577-1585.

44. Laquerre, S., Argnani, R., Anderson, D. B., Zucchini, S., Manservigi, R., and Glorioso, J. C. 1998. Heparan sulfate proteoglycan binding by herpes simplex virus type 1 glycoproteins B and C, which differ in their contributions to virus attachment, penetration, and cell-to-cell spread. *J.Virol* 72:6119-6130.

45. Patel, M., Yanagishita, M., Roderiquez, G., Bou-Habib, D. C., Oravecz, T., Hascall, V. C., and Norcross, M. A. 1993. Cell-surface heparan sulfate proteoglycan mediates HIV-1 infection of T-cell lines. *AIDS Res.Hum.Retroviruses* 9:167-174.

46. Byrnes, A. P. and Griffin, D. E. 1998. Binding of Sindbis virus to cell surface heparan sulfate. *J.Virol* 72:7349-7356.

47. Su, C. M., Liao, C. L., Lee, Y. L., and Lin, Y. L. 2001. Highly sulfated forms of heparin sulfate are involved in japanese encephalitis virus infection. *Virology* 286:206-215.

48. Yazi, M. M., Salas-Benito, J. S., Lanz-Mendoza, H., Hernandez-Martinez, S., and del Angel, R. M. 2002. A putative receptor for dengue virus in mosquito tissues: localization of a 45-kDa glycoprotein. *Am.J.Trop.Med.Hyg*. 67:76-84.

49. Hase, T., Summers, P. L., and Eckels, K. H. 1989. Flavivirus entry into cultured mosquito cells and human peripheral blood monocytes. *Arch Virol* 104:129-143.

50. Hsu, S. H., Huang, W. C., and Cross, J. H. 1978. The isolation of Japanese encephalitis virus from Taiwan mosquitoes by mosquito cell cultures and mouse inoculation. *J.Med.Entomol*. 14:698-701.

51. Mason, P. W. 1989. Maturation of Japanese encephalitis virus glycoproteins produced by infected mammalian and mosquito cells. *Virology* 169:354-364.

52. Rosen, L., Tesh, R. B., Lien, J. C., and Cross, J. H. 1978. Transovarial transmission of Japanese encephalitis virus by mosquitoes. *Science* 199:909-911.

53. Nawa, M., Takasaki, T., Yamada, K., Kurane, I., and Akatsuka, T. 2003. Interference in Japanese encephalitis virus infection of Vero cells by a cationic amphiphilic drug, chlorpromazine. *J.Gen.Virol* 84:1737-1741.

54. Nathin, M. A., Harun, S. R., and Sumarmo 1988. Dengue haemorrhagic fever and Japanese B encephalitis in Indonesia. Southeast Asian *J.Trop.Med.Public Health* 19:475-481.

55. Hase, T., Summers, P. L., Eckels, K. H., and Baze, W. B. 1987. Maturation process of Japanese encephalitis virus in cultured mosquito cells in vitro and mouse brain cells in vivo. *Arch Virol* 96:135-151.

56. Sithisarn, P., Suksanpaisan, L., Thepparit, C., and Smith, D. R. 2003. Behavior of the dengue virus in solution. *J.Med.Virol* 71:532-539.

57. Allison, S. L., Schalich, J., Stiasny, K., Mandl, C. W., Kunz, C., and Heinz, F. X. 1995. Oligomeric rearrangement of tick-borne encephalitis virus envelope proteins induced by an acidic pH. *J.Virol* 69:695-700.

58. Sierra, B., Garcia, G., Perez, A. B., Morier, L., Rodriguez, R., Alvarez, M., and Guzman, M. G. 2002. Long-term memory cellular immune response to dengue virus after a natural primary infection. *Int.J.Infect.Dis*. 6:125-128.

59. Heinz, F. X. 1986. Epitope mapping of flavivirus glycoproteins. *Adv.Virus Res.* 31:103-168.

60. Chen, L. K., Lin, Y. L., Liao, C. L., Lin, C. G., Huang, Y. L., Yeh, C. T., Lai, S. C., Jan, J. T., and Chin, C. 1996. Generation and characterization of organ-tropism mutants of Japanese encephalitis virus in vivo and in vitro. *Virology* 223:79-88.

61. Kimura, T., Kimura-Kuroda, J., Nagashima, K., and Yasui, K. 1994. Analysis of virus-cell binding characteristics on the determination of Japanese encephalitis virus susceptibility. *Arch Virol* 139:239-251.

62. Byrnes, A. P. and Griffin, D. E. 1998. Binding of Sindbis virus to cell surface heparan sulfate. *J.Virol* 72:7349-7356.

63. Feyzi, E., Trybala, E., Bergstrom, T., Lindahl, U., and Spillmann, D. 1997. Structural requirement of heparan sulfate for interaction with herpes simplex virus type 1 virions and isolated glycoprotein C. *J.Biol.Chem.* 272:24850-24857.

64. Song, B. H., Lee, G. C., Moon, M. S., Cho, Y. H., and Lee, C. H. 2001. Human cytomegalovirus binding to heparan sulfate proteoglycans on the cell surface and/or entry stimulates the expression of human leukocyte antigen class I. *J.Gen.Virol* 82:2405-2413.

65. Sawitzky, D. 1996. Protein-glycosaminoglycan interactions: infectiological aspects. *Med.Microbiol.Immunol.(Berl)* 184:155-161.

66. Liu, H., Chiou, S. S., and Chen, W. J. 2004. Differential binding efficiency between the envelope protein of Japanese encephalitis virus variants and heparan sulfate on the cell surface. *J.Med.Virol* 72:618-624.

67. Allison, S. L., Schalich, J., Stiasny, K., Mandl, C. W., and Heinz, F. X. 2001. Mutational evidence for an internal fusion peptide in flavivirus envelope protein E. *J.Virol* 75:4268-4275.

68. Wang, A., Miralem, T., and Templeton, D. M. 1999. Heparan sulfate chains with antimitogenic properties arise from mesangial cell-surface proteoglycans. *Metabolism* 48:1220-1229.

69. Crane, S. E., Buzy, J., and Clements, J. E. 1991. Identification of cell membrane proteins that bind visna virus. *J.Virol* 65:6137-6143.

70. Adlish, J. D., Lahijani, R. S., and St Jeor, S. C. 1990. Identification of a putative cell receptor for human cytomegalovirus. *Virology* 176:337-345.

71. Jindadamrongwech, S., Thepparit, C., and Smith, D. R. 2004. Identification of GRP 78 (BiP) as a liver cell expressed receptor element for dengue virus serotype 2. *Arch Virol* 149:915-927.

72. Thepparit, C. and Smith, D. R. 2004. Serotype-specific entry of dengue virus into liver cells: identification of the 37-kilodalton/67-kilodalton high-affinity laminin receptor as a dengue virus serotype 1 receptor. *J.Virol* 78:12647-12656.

73. Cresci, B., Giannini, S., Pala, L., Mavilia, C., Manuelli, C., Cappugi, P., Maggi, E., and Rotella, C. M. 2003. AT1 and AT2 receptors in human glomerular endothelial cells at different passages. *Microvasc.Res.* 66:22-29.

74. Crill, W. D. and Roehrig, J. T. 2001. Monoclonal antibodies that bind to domain III of dengue virus E glycoprotein are the most efficient blockers of virus adsorption to Vero cells. *J.Virol* 75:7769-7773.

75. Lin, Y. L., Lei, H. Y., Lin, Y. S., Yeh, T. M., Chen, S. H., and Liu, H. S. 2002. Heparin inhibits dengue-2 virus infection of five human liver cell lines. *Antiviral Res*. 56:93-96.

APPENDIX

APPENDIX
REAGENT PREPARATION

1. Culture medium and reagent preparation

1.1 C6/36 cells Growth medium: MEM with 10% FBS

5X MEM		100	ml
FBS		50	ml
100x Penicillin/Streptomycin		5	ml
Tissue culture graded dH_2O to		485	ml
7.5% $NaHCO_3$		15	ml
Distilled water	up to	500	ml

1.2 Vero cell Growth medium: DMEM with 5% FBS

5x DMEM		100	ml
FBS		25	ml
100x Penicillin/Streptomycin		5	ml
Tissue culture graded dH_2O to		485	ml
7.5% $NaHCO_3$		15	ml
Distilled water	up to	500	ml

Note: Heat inactivated fetal bovine serum (FBS) at 56 ºC 30 min before used. The medium was stored at 4 ºC

2. Reagents for Plaque assay

2.1 YE-LAH medium

Yeast extract	1 g
Lactalbumin hydrolysate	5 g
Distilled water	100 g

2.2 20x Earl's Balance Salt Solution (EBSS)

$CaCl_2.H_2O$	2.65	g
KCL	4	g
$Mg_2SO_4.7.H_2O$	2	g
NaCl	68	g
$NaH_2PO_4.H_2O$	1.25	g
Glucose	10	g
Tissue culture graded dH_2O to	500	g

2.3 2x Nutrient Solution: for 100 ml

20x EBSS	9.8	g
YE-LAH	6.6	g
FBS	6	g
Gentamycin (80 mg/ml)	0.5	ml
Fungizone (2.5 mg/ml)	100	ul
7.5% $NaHCO_3$	6	ml

2.4 BA-I virus diluent

10X M-199E		10 ml
1M Tris-HCL, pH 7.6		5 ml
2% BSA fraction V (final 1 g)		50 ml
7.5% $NaHCO_3$		6 ml
100X P/S		1 ml
Distilled water	up to	100 ml

2.5 0.15 M PBS (pH 7.4) for 1 liter

NaCl	8	g
KCL	0.2	g
Na_2HPO_4	1.15	g
KH_2HPO_4	0.2	g

3. Reagents for JEV Purification

3.1 TNE buffer

10 mM	Tris-HCL, pH 7.5
140 mM	NaCl
1 mM	EDTA

3.2 Sucrose gradients

Prepare three concentrations of sucrose solutions:

60% Sucrose in TNE buffer

30% Sucrose in TNE buffer

10% Sucrose in TNE buffer

BIOGRAPHY

NAME	Ms. Verawan Boonsanay
DATE OF BIRTH	January 13, 1981
PLACE OF BIRTH	Bangkok, Thailand
INSTITUTE ATTENED	Mahidol University, Thailand: 1999-2003; Bachelor of Science (Biology) Mahidol University, Thailand: 2004-2005; Master of Science (Molecular Genetics and Genetic Engineering)
SCHOLARSHIP	The Development and Promotion of Science and Technology Talents Project (DPST)
PRESENTATION	1. Oral presentation at Congress on Science and Technology of Thailand, 10-12 March, 2005, Bangkok, Thailand 2. Poster presentation at Joint tropical disease International Meeting, 21-23 March, 2002