CLONING AND EXPRESSION OF prM AND IT'S USE TO DISTINGUISH HOST IMMUNE RESPONSES AGAINST DENGUE VIRUS AND JAPANESE ENCEPHALITIS VIRUS INFECTIONS

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Kota Samarahan
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HOST IMMUNE RESPONSES AGAINST
DENGUE VIRUS AND JAPANESE ENCEPHALITIS VIRUS INFECTIONS

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2001
DEDICATION

To my mum and dad. Thank you.
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First and foremost, I would like to thank my supervisor Professor Mary Jane Cardosa for her encouragement, guidance, and support throughout my studies. You will always be my inspiration of being a good scientist. Thank you Phiak Hooi, for your valuable advice and assistance through this project. To all my friends in the lab, David, Dr. Sim, Charlie, Siti, Mag, Siew Fong, Hung Ming, Yuwana, Lu, Siew Yin, Tan, Natasha, and Yee Wei, thank you for your advice and friendship. Mostly, I would like to thank my parents for their love, encouragement and understanding throughout my studies in Sarawak.
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ABBREVIATIONS

2-mer, 2-ME base pair
bp bovine BSA
C capsid
cDNA dengue
dENV deoxy
DNA deoxy
dNTPs dNTP
E envel
EDTA ethyl
ELISA enzy
FCS fetal
HRP isop
IPTG Japa
JE Japa
JEV Japa
kDa kilo
kb kilo
LD Lur
M men
M-MLV men
mRNA men
NS Not
ORF op
PAGE po
PBS ph
PCR po
PPCS po
PNR po
PPJ po
prM po
RNA po
RO po
RT po
rpm ri
S SDS
SFM s
TEMED s
TBP s
TBE s
UHQ s
WHO s
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>2-ME</td>
<td>2-mercaptoethanol</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C</td>
<td>capsid</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>DENV</td>
<td>dengue virus</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>E</td>
<td>envelope</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetraacetate</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl thiogalactosidase</td>
</tr>
<tr>
<td>JE</td>
<td>Japanese encephalitis</td>
</tr>
<tr>
<td>JEV</td>
<td>Japanese encephalitis virus</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>M</td>
<td>membrane</td>
</tr>
<tr>
<td>M-MLV</td>
<td>Moloney murine leukemia virus</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NS</td>
<td>non-structural</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffer saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PPCS</td>
<td>Perak pooled convalescent sera</td>
</tr>
<tr>
<td>PNR</td>
<td>pooled negative reference sera</td>
</tr>
<tr>
<td>PPJ</td>
<td>pooled positive JE porcine sera</td>
</tr>
<tr>
<td>prM</td>
<td>premembrane</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RO</td>
<td>reverse osmosis</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcription</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SFM</td>
<td>serum free medium</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N’-tetramethylethylenediamine</td>
</tr>
<tr>
<td>TPB</td>
<td>tryptose phosphate broth</td>
</tr>
<tr>
<td>TBE</td>
<td>tris borate EDTA</td>
</tr>
<tr>
<td>UHQ</td>
<td>ultra high quality</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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ABSTRACT

All flaviviruses share common epitopes on the envelope protein (E) that results in extensive cross-reactions in serological tests. This makes it difficult to distinguish between immune responses against dengue virus (DENV) and Japanese encephalitis virus (JEV), particularly in regions where these viruses co-circulate. We have demonstrated that DENV and JEV have unique epitopes on the prM. By detecting prM specific IgG, it is possible to distinguish between DENV and JEV infection. Current approach used in seroanalysis of DENV and JEV infections is based on antigens derived from the whole viral antigens. In this study, cloning and expression of recombinant proteins that could be used in place of the authentic antigens was investigated. Both eukaryotic and prokaryotic expression systems were used. A clonal cell line expressing DENV2 prM recombinant protein was established in insect cells. However, this recombinant cell line was unstable, which could be due to toxicity of the gene product. Complete prM and various fragments of prM (including truncated prM, M, C/pre, pre, and truncated pre) were also cloned and expressed in E. coli. Recombinant proteins encoding the pre gene were successfully expressed for both DENV2 and JEV. Specificity of these recombinant proteins was analyzed on both ELISA and western blot using a panel of patient sera (n = 129). The data observed were 15.6% (DENV) and 57.14% (JEV) in ELISA, and 62.2% (DENV) and 82.9% (JEV) on western blot. Western blot analysis suggested that specific IgG against pre could be used to distinguish humoral responses to DENV and JEV on western blot, although specificity of DENV2 pre recombinant protein needs to be improved. ELISA conditions also need to be further optimized so that the specificity is comparable to the western blot analysis.

KESAMBAHAN

Kesemua flavivirus berkon balas yang bertindihan membekakan gerak balas Jepun (JEV) yang berada menunjukkan kewujudan - JEV didapati dapat diberdasarkan kepada antitau dan pengekpresan protejai ini. Dalam kajian digunakan. Protein rekombinan yang dikenal daripada produk gen. P serpihan pre) juga telah dikaji. Kesepekatan proteJEV. Kesepekatan prote Western terhadap serum ELISA ialah 15.6% wa 62.2% serta 82.9% bo mencadangkan IgG yang imun humoral terhadap protein rekombinan p supaya kesepekatannya.

ABSTRAK
ABSTRAK

Kesemua flavivirus berkongsi epitop yang sama pada sampul protein (E) yang menyebabkan tindak balas yang bertindih berlaku dalam ujian serologi. Ini menyebabkan kerumitan dalam membezakan gerak balas imun terhadap jangkitan virus denggi (DENV) daripada virus ensefalitis Jepun (JEV) yang beredar dalam suatu kawasan yang sama. Satu kajian telah diteliti untuk menunjukkan kewujudan epitop yang unik pada prM dalam DENV dan JEV. Jangkitan DENV dan JEV didapati dapat dibezakan dengan mengesan kehadiran IgG yang spesifik terhadap prM. Pendekatan masakini dalam menjalankan analisis serum terhadap jangkitan DENV dan JEV adalah berdasarkan kepada antigen yang diperolehi daripada antigen virus lengkap. Oleh itu, pengklonan dan pengekspresan protein rekombinan yang boleh mengganakan antigen asal telah diseledik dalam kajian ini. Dalam kajian yang dilakukan, sistem-sistem pengungkapan eukariot dan prokariot telah digunakan. Protein rekombinan prM DENV2 telah diekspreskan daripada satu klon kultur sel serangga. Namun demikian, klon kultur sel ini tidak stabil, mungkin dipengaruhi oleh keadaan taksik daripada produk gen. PrM lengkap dan pelbagai fragmen prM (serpihan prM, M, C/pre, pre dan serpihan pre) juga telah dikelaskan dan diekspeskan dalam E.coli. Walaupun begitu, hanya protein rekombinan yang dikodkan oleh gen pre telah berjaya diungkapan bagi kedua-dua DENV2 dan JEV. Kespesifikan protein-protein rekombinan ini telah dikesan secara kaedah ELISA dan pemblotan Western terhadap serum daripada satu panel pesakit (n=129). Keputusan data yang diperolehi bagi ELISA ialah 15.6% untuk DENV dan 57.14% bagi JEV. Pemblotan Western pula mencatatkan 62.2% serta 82.9% bagi DENV dan JEV masing-masing. Analisis daripada pemblotan Western mencadangkan IgG yang spesifik terhadap gen pre dapat digunakan untuk membezakan gerak balas imun humoral terhadap DENV dan JEV secara pemblotan Western. Walaupun begitu, kespesifikan protein rekombinan pre DENV2 masih perlu diperbaiki. Ujian ELISA juga perlu dioptimumkan supaya kespesifikannya adalah setara dengan analisis pemblotan Western.
CHAPTER 1: GENERAL INTRODUCTION

1.1 FLAVIVIRUS

Early studies on flaviviruses began with the discovery, nearly a century ago, that yellow fever was caused by a filterable agent and transmitted to humans by mosquitoes. Further studies on yellow fever resulted in the isolation of many other arthropod-borne viruses, which in time became known as the Group A arboviruses (Alphavirus) and Group B arboviruses (Flavivirus), and was placed within the family Togaviridae (Fenner et al., 1974). In 1985, genus flavivirus was separated from Togaviridae, together with genus pestivirus and genus hepatitis C virus, they formed the family Flaviviridae (Westaway et al., 1985). The word flavus was originated from Latin flavus meaning yellow. These three genera was allocated to the same family on the basis of the similarity in terms of virion morphology and genome organization, although they have diverse biological properties and have no antigens shared between genera (Rice, 1996).

The flavivirus genus comprises at least 68 viruses separated into groups on the basis of serological relatedness (Calisher et al., 1989). Most flaviviruses are arthropod-borne, some of these cause disease in humans, ranging from febrile illness to life-threatening hemorrhagic fever and encephalitis. Dengue virus (DENV) and Japanese encephalitis virus (JEV) are members of flavivirus and rank among the most important pathogens of the developing world.

1.2 PROPERTIES OF FLAVIVIRUS

Flavivirus virions are spherical, with a diameter of 40–60 nm, and consist of an inner nucleocapsid surrounded by a lipid envelope containing peplomers glycoprotein E and small membrane protein M (Murphy, 1980). The viral genome is a single stranded positive-sense RNA of approximately 11 kb, which is 5' capped but lacks a poly-(A) tail, organized into a single long open reading frame with genes encoding the three structural proteins (the core protein C, membrane protein M, and envelope protein E), and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Figure 1.1). The premembrane (prM) protein is a glycosylated precursor protein, which is cleaved during or shortly before release from the infected cell into the non-glycosylated membrane (M) protein (7-9 kDa) with no disulphide bridges. The envelope (E) protein is usually glycosylated (51–59 kDa). Both E and M proteins are embedded in the enveloped by C-terminal hydrophobic anchor, which are derived from the host cell membrane. Replication of virus takes place in the cytoplasm of the cell in association with the rough and smooth endoplasmic reticulum.
1.3 ANTIGENIC PROPERTIES

The antigenic properties of the genus flavivirus are defined by serological tests such as the highly cross-reactive haemagglutination-inhibition (HI) test, the less cross-reactive complement fixation (CF) test and the more specific neutralization (NT) or plaque reduction neutralization (PRNT) test. Other techniques mainly used for diagnostic serology are IgG and IgM detection by enzyme-linked immunosorbent assay (ELISA) and immunofluorescent (IF) assay. The antigenic features of the virus are characterized by reactivity with antigenic domains and epitopes on the E protein of the virus. The E protein is the major component of the virion surface and it is the principal target for inducing neutralizing and protective antibodies (Gollins and Potterfield, 1986).

All members of the genus flavivirus share common antigenic sites as shown by HI test with polyclonal immune sera. The genus flavivirus is further subdivided into subgroups on the basis of the neutralization test, which is more discriminating and capable of distinguishing individual viruses in the genus and defining subgroups of closely related viruses. The member viruses within each genus are antigenetically related to each other but there is little cross-reactivity between the three genera of the family Flaviviridae.

1.4 THE prM AND M PROTEINS OF FLAVIVIRUS

The premembrane (prM) protein is the glycosylated precursor of the structural membrane (M) protein. The prM protein undergoes a delayed cleavage during or shortly before virion release to form M protein, with the N-terminal pre segment secreted into the extracellular environment. Hence, prM and M proteins are found on intracellular and extracellular virions, respectively (Friedman and Webster, 1980). Antibodies to prM function after neutralization of release.

In the current hypothesis, the virus undergoing an acid change through an acidic intracellular environment, the E-M interaction conformational change virus into a host cell infectious than the released virions.
The top and COOH ends of flavivirus envelope glycoproteins, respectively (Figure 1.2). The M protein, which is predominantly hydrophobic, is embedded in the envelope and contains a shortened ectodomain (41 amino acids). The secreted pre segment is predominantly hydrophilic. Some times, the precursor cleavage is not efficient; the released virions also contain variable amounts of the prM, thus allowing the prM to function as an additional target for neutralizing and protective antibodies. Antibodies to prM have been shown to mediate protective immunity perhaps by neutralization of released virions that contain some uncleaved prM (Rice, 1996).

In the current hypothesis, the function of prM is to stabilize the prM-E heterodimer from undergoing an acid catalyzed conformational change during export of immature virions through an acidic intracellular environment. Upon cleavage of prM and release of mature virus, the E-M interaction is destabilized. This allows E to undergo an acid-catalyzed conformational change that promotes membrane fusion in the endosome during entry of virus into a host cell. Immature virions contain exclusively unprocessed prM, are less infectious than the released virions (Rice, 1996).

![Figure 1.2. Envelope proteins of intracellular and extracellular flavivirus virions (Adapted from Rice, 1996).](image)

1.5 DENGUE VIRUSES

Dengue has been the most important mosquito-borne disease and cause of death in humans. Each year, up to 50 million people worldwide are estimated to be infected by dengue, with up to 400,000 cases of dengue haemorrhagic fever (WHO, 2000). The infection has spread widely from Southeast Asia to the Americas, the Pacific and Africa. In all major tropical areas, the incidence of dengue fever and DHF/DSS has increased dramatically over the past few years. The introduction of infection to non-endemic countries is of great concern.

There are four serotypes of dengue based on neutralization from which infection with any type gives lifelong homologous immunity, but there is no cross-protection. Dengue serotypes 1, 3, and 4 show a closer antigenic and genetic relationship to each other than dengue 2.
1.5.1 Epidemiology

The only vertebrate hosts of dengue virus in nature are humans and several species of Asian and African subhuman primates. Other vertebrates can be experimentally infected only with difficulty. The invertebrate hosts of dengue are members of the genus *Aedes*, especially the subgenus *stegomyia*. Essentially, there are three transmission cycles of dengue, involving a forest cycle in primates, a rural cycle and an urban cycle. By far the urban cycle is the most important, and the dengue viruses are maintained in an urban transmission cycle mainly by the mosquitoes *Ae. aegypti* and to a lesser extent *Ae. albopictus*.

In order to establish an infection, *Ae. aegypti* would need to feed on individuals with high levels of viremia. It usually takes interrupted blood meals by feeding on a number of individuals, thus enhancing the spread of the virus. The biting activities are maximal soon after daybreak and in the late afternoon, and are related to human activities and movements. Outbreaks are climatically influenced by heavy rainfall and high temperature.

The spread of the virus to new areas was mainly attributed to modern phenomena of human and social behavior. Uncontrolled urbanization, overcrowding and inadequate housing, greatly facilitate the spread of this vector borne disease. The spread of infection is also enhanced by modern air travel and international trade such as motor vehicle tyres, which facilitates the transmission of infected individuals as well as mosquito larvae to non-infected areas, posing the threat of introducing both the virus as well as its vector.

1.5.2 Clinical features and pathogenesis

Dengue virus infections can be subclinical whereby patients show no signs and symptoms of the disease. The clinical manifestation of dengue occurs in two forms: classical dengue fever (DF) and dengue haemorrhagic fever/dengue shock syndrome (DHF/DSS). Classical DF is an acute disease characterized by a sudden onset of fever, headache, retro orbital pain, nausea, vomiting, severe muscle and bone pain. Whereas, DHF is characterized by capillary leakage, thrombocytopenia and in some cases will lead to circulatory collapse and hypovolemic shock resulting in DSS. Numerous neurological disorders associated with dengue fever such as brain involvement, have also been described (Angibaud *et al.*, 2001).

DHF and DSS are leading causes of hospitalization and death among children in Asia. Complications of DHF/DSS are governed by two factors: prior infection and age. DHF and DSS occur in approximately 0.18% and 0.007% respectively of primary DF cases, compared with 2% and 1.1% respectively of DF due to secondary infection (Halstead, 1981). They are also rare in individuals over the age of 15 years.

The pathogenesis of dengue has yet to be established. There are two hypotheses with regards to DHF and DSS. The first hypothesis involves the immunological theory based on the phenomenon of antibody-dependent enhancement (ADE) of infection. Three elements involved in the process of ADE are antibody, virus and the receptor for the Fc portion of IgG. In the presence of subneutralizing concentrations of antibody, these antibodies, which are of the IgG class, appear to promote viral entry via Fc receptors on the membranes of monocytes/macrophages. These pre-existing antibodies may be observed in passively acquired maternal antibodies in infants less than 1 year of age, or from previous infection in children over 1 year of age. The DHF/DSS syndrome may, though it is far more completely understood, it is a prerequisite for infectivity.

The alternative hypothesis involves genetic factors. Rosen (1986) reported that the virus, in which in a particular area generally has a consistent association with virulence or heightened consistent association with virulence.

1.6 JAPANESE ENCEPHALITIS

Japanese encephalitis was first described in 1930 and was responsible for an extensive cross-reactivity with Murray Valley encephalitis. Japanese encephalitis form the mosquito-based protection.

1.6.1 Epidemiology

Humans, domestic animals particularly of the herbivorous, and second transmission to susceptible animals such as the domestic pigs play major amplifying roles. The domestic animal as the major amplifying role in the transmission of JEV in fields.

1.6.2 Clinical and pathological features

Japanese encephalitis is a febrile illness with mild febrile illness. Manifestations in children vary in degree of severity. Mild and severe in the T lymphocyte and viral antigen is also
individuals with high titering on a number of occasions are maximal subjects in an urban setting. By far the maintained in an urban setting to a lesser extent Ae.

phenomena of human behavior and inadequate housing, spread of infection is also vehicle tyres, which Vector larvae to non-
its vector.

signs and symptoms include: classical dengue (DHF/DSS). Classical signs include fever, retro orbital pain, and headache. The disease is characterized by circulatory collapse and shock associated with its vector.

Japanese encephalitis virus (JEV) is the major arboviral cause of encephalitis worldwide. It was first described in Japan in 1871 (Olitsky and Casals, 1948), and is now thought to be responsible for an estimated 50,000 cases and 15,000 deaths annually. JEV shows some cross-reactivity with St Louis encephalitis virus (SLEV), West Nile virus (WNV) and Murray Valley encephalitis virus (MVEV) (Schoub and Blackburn, 2000). Together, they form the mosquito-borne encephalitis complex. Infection with JEV will result in solid protection.

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1.6.1 Epidemiology

Humans, domestic animals and pigs are the major vertebrate hosts for JEV. Nestling birds, particularly of the heron family, play an important role in the dissemination of JEV, with a second transmission cycle involving domestic animals, especially the pigs. Other susceptible animals include cattle, horses, dogs, monkeys and bats. The high turnover of the domestic pigs resulting in a continuous supply of susceptible animals, making pigs a major amplifying host for JEV. Japanese encephalitis virus rarely causes disease in domestic animal as they are the natural reservoirs. Human however are dead-end hosts and play little role in the amplification of the virus. The major mosquito vector involved in transmission of JEV is Culex tritaeniorhynchus, which breeds in marshes and flooded rice fields.

1.6.2 Clinical and pathological features

Japanese encephalitis virus infections can be asymptomatic or show illness ranging from a mild febrile illness, headache, to aseptic meningitis or, rarely, as a variety of inflammatory manifestations in the viscera. A quarter of cases of clinical encephalitis will recover with no permanent sequelae and a quarter will die rapidly. The remaining half will recover with varying degree of permanent neuropsychiatric sequelae. JEV infections are more common and severe in the young as well as in elderly individuals. The major target cells for JEV are the T lymphocyte and the peripheral blood mononuclear cells. In fatal cases of encephalitis, viral antigen is also found in the neurons.
1.7 DIAGNOSIS

Diagnosis of DENV and JEV can be achieved by virus isolation or serological tests. For DENV, the clinical diagnosis is often unreliable, as the symptoms may clinically resemble other infections. Virus may be recovered from the blood during the early febrile phase of the illness. The conventional methods for primary virus isolation include the intrathoracic inoculation of Toxorhynchites mosquito or intracerebral inoculation of larval Toxorhynchites. However, the most commonly used system for primary dengue virus isolation is the inoculation of mosquito cell lines such as Toxorhynchites amboinenses (TRA-284), Ae. albopictus (C6/36), and Ae. pseudoscutellaris (AP-61). Virus isolation from blood and CSF for JEV is rarely successful during the acute illness because the viraemic phase is probably over by the time central nervous symptoms appear. Mammalian cell lines such as Vero and LLC-MK2 and mosquitoes cell lines can be used to isolate JEV.

Serological diagnosis depends on the demonstration of a four folds or greater rise (or fall) in antibodies by the haemagglutination inhibition (HI), complement fixation (CF), or neutralization (NT) test. The most widely used serological test is the HI test, but extensive cross-reactions occur with other flavivirus in late primary or secondary infection. The CF test is more specific than HI test, but the antibodies detected by this assay appear later and disappear earlier. The NT and PRNT tests are most specific and sensitive but are difficult to perform. Several rapid assays are now available for detection of IgM and IgG antibodies for DENV and JEV such as ELISA and dot blot. However, interpretation of the test results should be treated with caution due to extensive cross-reactivity with other flaviviruses.

1.8 CONTROL

At present, there is no licensed dengue vaccine available. Vaccine development has been hindered by the antibody dependent enhancement (ADE) properties of dengue infection. The current strategy is to develop a vaccine against all four serotypes. Successful trials of monovalent vaccines have been reported but not for experimental tetravalent vaccine. Due to the lack of availability of a vaccine, control of dengue depends on viral and serology surveillance to obtain early warning of epidemics. Routine investigation of suspected and infected individuals, collaboration with physicians, collecting blood on a regular basis and sending them to the laboratory for analysis are parts of the strategies in monitoring the disease prevalence.

Control of Japanese encephalitis infection can be achieved by widespread immunization of both human and domestic animals, especially pigs. A number of human vaccines have been developed, such as the formalin-inactivated lyophilized vaccine of mouse brain origin, derived from the Nakayama strain of JEV and the BHK prepared inactivated vaccine. Both vaccines are highly immunogenic and protection rates of over 90% are achieved (WHO, 1998b). Similarly, a number of veterinary vaccines have been used in pigs and horses.

Besides, vector control aimed at the elimination of the main mosquito vector has been carried out using ultra low-volume insecticide spraying and larvicide treatment of stagnant water. However, the effects are temporary and reinfestations often taken place within a short period. Long term based community programmes need to be implemented in endemic countries to educate people on the importance of mosquito-borne diseases.
1.9 CONCLUSION

Dengue virus (DENV) and Japanese encephalitis virus (JEV) are important arthropod-borne flaviviruses and can cause significant human diseases. As stated earlier, DENV is distributed worldwide. It now threatens up to 2.5 billion people and is still emerging throughout the world (WHO, 1998a). JEV is mainly distributed in Asia. In recent decades, epidemic incidence for JEV has increased, outbreaks have now spread to several previously non-endemic areas such as Northern India, Nepal and Sri Lanka (CDC, 2001). Other flaviviruses including yellow fever virus (YFV), tick-borne encephalitis virus (TBEV), West Nile virus (WNV), St Louis encephalitis virus (SLEV), and Murray Valley encephalitis virus (MVEV) are also important agents of regional endemic or epidemic diseases. Some of these viruses occurred or co-circulate within the same geographic regions. A notable example is DENV, which has now spread globally that it affects areas which have previously had other flavivirus such as YFV and JEV. A recent example is WNV, which has been described in Africa, Europe, the Middle East, west and central Asia, Oceania, and North America. Recent outbreaks of WNV encephalitis in humans have occurred in Europe (Romania in 1996-1997, the Czech republic in 1997, Russia in 1999), the United States (1999-2000), and Israel (2000) (CDC, 2001). Some of these outbreak areas for WNV also overlap with TBEV, which is found exclusively in Asia, eastern and central Europe (Schoub and Blackburn, 2000). Geographic distribution of the dengue and Japanese encephalitis serocomplex is shown in figure 1.3 and figure 1.4 respectively (CDC, 2000; Schoub and Blackburn, 2000).

Figure 1.3. Geographic distribution of dengue virus, yellow fever virus and tick-borne encephalitis virus (Adapted from CDC, 2000; Schoub and Blackburn, 2000).

- Areas infested with Aedes aegypti
- Areas with Aedes aegypti and dengue
- Yellow fever
- Tick-borne encephalitis
All flaviviruses are serologically related; they share common group epitopes particularly on the E protein that result in extensive cross-reactions in serological tests. The complexity of the antigenic relationships among flaviviruses has made serology diagnosis or seroepidemiology studies of flavivirus infection difficult, particularly for persons living in endemic areas where several flaviviruses co-circulate. This is a great concern especially for DENV and JEV, which co-circulate in many countries.

Current approaches used in serodiagnosis of DENV are based on antigens derived from the cocktail of the four dengue serotypes and the whole viral antigens for JEV. Serology discrimination between immune responses against DENV and JEV is complicated by antibodies which recognize shared flavivirus epitopes. The complexity of the antigenic relationship and the presence of shared epitopes have prompted the need to search for unique epitopes among flaviviruses which could serve as better antigens. Application of specific monoclonal antibodies against E protein and prM protein have demonstrated the presence of flavivirus group, serocomplex and type-specific epitopes, and uncovered antigenic relationships at the epitope level that link different flavivirus antigenic complexes (Rice, 1996). However, there is currently no information or published data which describes the use of unique epitopes in differentiating immune responses against DENV and JEV. We believe that identification of only in improving seroantigenic relationship contribute significantly.

1.10 REFERENCES

believe that identification and application of the unique epitopes is of great importance, not only in improving serology analysis but also providing a better understanding about the antigenic relationship between DENV and JEV. No doubt, this vital information will contribute significantly towards flavivirus research.

1.10 REFERENCES


CHAPTER 2: STATEMENT OF THE PROBLEM

2.1 IMPORTANCE AND CONTROL OF THE DISEASE

Dengue virus (DENV) and Japanese encephalitis virus (JEV) infections are major public health problems worldwide and number among the most important human diseases caused by mosquito-borne viruses. According to World Health Organization (WHO), each year there may be 50 million cases of dengue infection worldwide, with an estimated 2500 million people, about two fifths of the world’s population are at risk from dengue (WHO, 1998a). The related flavivirus, JEV is the major arboviral cause of encephalitis worldwide, and causes an estimated 50,000 cases and 15,000 deaths annually, mostly among children (Solomon et al., 2000).

The global prevalence of dengue has grown dramatically in recent decades. Countries that had experienced DHF epidemics had increased more than four-fold by 1995 compared to only nine countries prior 1970 (WHO, 1998a). The disease is now endemic in more than 100 countries in Africa, the Americas, the Eastern Mediterranean, particularly in Southeast Asia and Western Pacific. An estimated 500,000 cases of DHF require hospitalization each year, of which a very large population is children and roughly 5% die (WHO, 1998a). The spread of dengue is mainly attributed to the expanding geographic distribution of the four dengue serotypes and their urban mosquito vector, Aedes aegypti. Uncontrolled urbanization and rapid growth of urban population has brought more people into contact with the vector as well as provided more favorable breeding places for mosquitoes such as the household water storage system.

Japanese encephalitis (JE) is a potentially lethal infection of the central nervous system caused by JEV. Japanese encephalitis virus infection has occurred in a large number of the Asian countries, including Cambodia, China, Japan, Korea, Thailand and Vietnam. In recent decades, JE has gradually spread to previously non-affected Asian regions. The annual incidence of clinical infection in endemic areas ranges from 10 to 100 per 100 000 population (WHO, 1998b). Close to 3 billion people are now living in JE endemic regions, where more than 70 million children are born each year. Usually the infection does not cause clinical symptoms, an estimated average of one in 300 JEV infection results in symptomatic illness. The mortality in most outbreaks is less than 10% but is higher in children and can exceed 30% (WHO, 1998b). The high fatality rate and frequent residual neuropsychiatric sequelae in survivors make JE a considerable public health problem in many Asian regions.

To date, there is no licensed vaccine available for dengue (WHO, 1998a). Vaccine development for dengue and DHF is difficult due to the fact that any four different viruses could cause disease. In addition, protection against only one or two dengue viruses could actually increase the risk of more serious disease. Progress is being made in the development of a tetravalent vaccine that may protect against all four dengue viruses (WHO, 1998a). Such products could be commercially available within several years. There is also no specific treatment for DF. However, careful clinical management by experienced physician and nurses frequently save the lives of DHF patients. At present, the only available method of controlling or preventing dengue is to combat the vector mosquitoes.

Vaccination probably offers the best impact of large-scale control and vaccination campaigns. Current vaccines have been shown to be effective in preventing JE, but are not a practical alternative to the use of protective drugs. Nevertheless, there is evidence that the use of protective drugs and vaccines can be effective in reducing disease transmission and improving economic productivity. The impact of protective drug treatments in controlling or preventing dengue is the subject of current research.

2.2 OBJECTIVE OF THE LABORATORY

The laboratory director provided general information for this study. The laboratory director noted that the hemagglutination inhibition assay was adapted from the guinea pig hemagglutination inhibition test. The antigen was prepared from a virus isolate of JE. The antigen was used to capture specific antibodies in the sera of patients with known JE infection. The capture antibody bound to the epitopes of the antigen, allowing the assay to be used for early diagnosis (Chen et al., 1988). The antigens were based on the neutralizing virus strains.

All members of the research group were familiar with the diagnostic techniques used for JE. The laboratory director noted that the sensitivity and specificity of the capture antibody were essential for accurate diagnosis. The laboratory director also stated that the antigen was chosen for its ability to bind to the epitopes of the virus. The antigen was used to capture antibodies in the sera of patients with known JE infection. The capture antibody was then used to capture the epitopes of the virus, allowing the assay to be used for early diagnosis (Chen et al., 1988). The antigens were based on the neutralizing virus strains.

Numerous repeat serum samples from patients with dengue and JE were tested for the presence of antibodies against DENV and JEV using the capture antibody. The results showed that the capture antibody was able to capture the antibodies against both viruses. However, careful clinical management by experienced physician and nurses frequently save the lives of DHF patients. At present, the only available method of controlling or preventing dengue is to combat the vector mosquitoes.
Vaccination probably is currently the most effective control measure against JEV. The impact of large-scale vaccination is clearly documented in some regions of China, Korea and Japan, where the incidence of JE has decreased (WHO, 1998b). Currently, there are three types of JE vaccines available commercially; they are the mouse brain-derived inactivated vaccine, cell culture-derived inactivated vaccine and cell-culture-derived live attenuated vaccine (Tsai, 1990; WHO, 1998b). On a general basis, a large-scale mosquito control and vaccination of pigs, which serves as amplifying hosts for JEV are costly and difficult to sustain. As a consequence, large-scale human vaccination against JEV may currently be the most cost-effective measure against JE disease, although neurological side effects and allergic reactions to JE vaccines have been reported. Besides that, socioeconomic improvement and changes in agriculture practices have contributed to the reduction in disease incidence in some countries. Similar to dengue, currently there is no efficient drug treatment for JE, only supportive treatment is available.

2.2 OBJECTIVE OF THE PROJECT

The laboratory diagnosis on viral infection is important in order to provide accurate information for treatment and efficient control measures. Diagnosis is usually made serologically because virus might not be isolated from clinical specimens, due to low circulatory viral numbers and the rapid development of neutralizing antibodies. For many years, the haemagglutination-inhibition (HI) test has been employed, but this has various practical limitations, such as it requires paired serum samples, and therefore cannot give an early diagnosis (Clark and Casals, 1958). In the 1980s, the simpler ELISA and dot blot tests were developed and have been used in identifying dengue and JE viral infections by IgM capture assay or for seroanalysis by IgG capture assay (Bundo and Igarashi, 1985; Cardosa et al., 1988). These serological methods detect the presence of specific antibodies, and were based on the interaction between viral epitopes and the antiviral antibodies.

All members of Flavivirus share common epitopes on the envelope (E) protein, in addition to the epitopes that define each serotype (Rice, 1996). Serological discrimination between dengue and JE is complicated by the presence of antibodies that cross-react with the flavivirus shared epitopes. This makes it difficult to distinguish between immune responses against DENV and JEV, particularly in regions where these viruses co-circulate (Theiler and Downs, 1973). In a serological test, an antigen that is capable of recognizing the antibody produced against the infecting virus is necessary for identifying between dengue and JE infection. Usually the antigens used are prepared from cultured cells infected with flaviviruses. Since the antigens used in serodiagnosis of dengue are prepared from cocktail antigens of the four dengue serotypes and whole viral antigens for JE, cross-reaction of the antibody towards the shared epitopes on the antigens will complicate and affect the specificity and sensitivity of the diagnosis.

Numerous reports have been published which described serodiagnosis or seroanalysis of dengue and JE using ELISA and dot blot tests. Recent publications reporting serodiagnosis of JE in pigs or humans include those by Burke et al. (1982), Cardosa et al. (1991 and 1993), and Solomon et al. (1998). Cardosa and colleagues (Cardosa, et al., 1988; Cardosa and Tio, 1991; Cardosa and Zuraini, 1991; Cardosa et al., 1995), and Tio and Malasit (1995) also have various data regarding the detection of anti-dengue IgM and IgG by ELISA and dot blot tests. A commercial dengue IgM and IgG capture ELISA (PanBio Dengue Duo ELISA) for detection of specific antibodies in serum (Chew et al., 1998) or in
saliva (Cuzzubbo et al., 1998) during dengue infection have also been described. The common feature in these serological tests is the detecting antigen, which was a whole viral antigen and was prepared using infected cell culture. These serology assays can be used to diagnose flavivirus infections, provided that the individual has not previously been infected by other flavivirus. Once an individual has been infected by two different flaviviruses, the standard viral antigen preparations show very broad cross-reactivity among members of the genus.

Recently, production of viral proteins without replication of the homologous virus has been made possible by recombinant technology. The ability to produce recombinant flavivirus antigens raises the possibility that these antigens could be used in place of existing authentic antigens. Currently, information regarding the use of recombinant flavivirus antigen in serology is limited. Konishi and coworkers (1996) have expressed a JEV recombinant proteins in HeLa cells infected with a recombinant vaccinia virus that encodes the JEV prM and E genes. IgM capture ELISA has shown that the JEV antigens were antigenically equivalent to the authentic JEV antigens. A recombinant dengue domain III protein fused to the trpE protein of Escherichia coli (trpE-DEN) was also successfully expressed by Simmons and coworkers (1998). A pooled antigen consisting of four serotypes of DENV was tested for sensitivity with dengue convalescent sera and for specificity using sera from individual with no known dengue but who sequentially vaccinated with yellow fever and JE. Results indicated the trpE-DEN antigens were equally as sensitive as cell culture antigens, but the test was more specific. The vaccinated sera showed cross-reactivity with the DENV cell antigen but did not react with the trpE-DEN pooled antigens.

In studies attempting to analyze the humoral immune response against structural and non-structural proteins of DENV and JEV, we found that DENV and JEV do not share important epitopes on the prM, as compared to E and NS1. The observed specificity of prM specific IgG suggested that it may be utilized to discriminate between dengue and JE infection (Cardosa, personal communication). Based on this observation, this has led us in attempting to express a recombinant protein that could be used in place of the authentic antigens. The objective of this study is:

(1) to clone and express the prM recombinant protein for DENV and JEV, and
(2) to investigate the antigenicity of the recombinant protein expressed compared to the authentic antigen.

In these studies, both eukaryotic and prokaryotic expression systems were used to investigate the expression of recombinant protein. These options are necessary because no single strategy or condition is suitable for every target protein. The main purpose is to obtain a best possible yield of recombinant protein with properties similar to the authentic viruses that could be used in serology analysis.

2.3 REFERENCES
