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The Hong Kong Polytechnic University
Department of Health Technology and Informatics

MOLECULAR AND EPIDEMIOLOGICAL ASPECTS OF DENGUE VIRUS INFECTION IN HONG KONG

LO Lek Hang Constance

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

June 2010

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LO Lek Hang Constance

Abstract

Dengue has emerged as a global public health concern and the incidence has increased dramatically in recent decades. The disease is caused by the dengue virus (DV), and currently has no specific treatment. In the present study, we investigated the molecular and epidemiological aspects of dengue virus infection. For the molecular aspects, we focussed on the development of diagnostic tools and investigation of three recombinant proteins for their potential as vaccine candidates. For the epidemiological aspects, the seroprevalence of DV among the population of Hong Kong and abundance of DV vectors among mosquitoes collected in Hong Kong were studied.

Two diagnostic tools, a molecular and a serological assay for DV, have been developed. For the molecular assay, a one-step reverse transcription-polymerase chain reaction (RT-PCR) LightCycler assay was developed for rapid and simultaneous detection and typing of DV. The assay's strategy for detection was based on colour and melting temperature (T_m) multiplexing. The serotypes of DEN-1 and DEN-3 amplicons were detected by their characteristic emission generated from induced fluorescence resonance energy transfer (iFRET). The presence of DEN-2 and DEN-4 amplicons was indicated by SYBR Green I (SGI) signals at the specific amplicon T_m. The assay had a dynamic range of 10^3–10^8 plaque-forming units/L and could be performed in 2 hours. By using in-house RT-PCR cocktail replacement for the commercial reagent kit, an economical in-house assay was modified based on the same detection strategy. The running cost per reaction of the in-house assay was about

one-third of that of the kit-based assay. T_m profiles and detection limits of the in-house assay were comparable to the original kit-based assay. This in-house assay was validated with clinical sera collected from Hong Kong, Mainland China and Brazil.

Three recombinant fusion proteins, prM (24 kiloDalton, kDa), ED3 (32 kDa) and prM-ED3 (43 kDa), of DEN-2 were produced from the Champion™ pET SUMO protein expression system in *Escherichia coli* for the development of a serological assay and investigation of their potential as subunit vaccine candidates. A serological assay based on the three recombinant proteins as detection antigens was set up. This assay was evaluated with sera collected from the community in Hong Kong and clinical sera collected from Brazil. Performance of the assay was compared with the commercially-available Panbio dengue IgG indirect ELISA kit. We confirmed that this test had high specificity (>90%), but low sensitivity (<30%). Interestingly, prM as capture antigen was able to detect dengue IgG-positive sera with the highest frequency. To investigate their potential as vaccine candidates, purified recombinant proteins were administrated into rabbits for polyclonal antibody production. Four rabbits were respectively injected with prM, ED3 or prM-ED3 fusion proteins or a mixture of prM plus ED3 fusion proteins. Antisera were analysed and characterised by immunoblot and ELISA. The neutralisation potential of antisera were investigated by means of an inhibition assay to determine DEN-2 recombinant subviral particle (RSP) binding to Vero E6 cells using flow cytometry. The inhibition of RSP binding to Vero E6 cells was indicated by decreased FITC fluorescence. Individual antisera showed varied ability to inhibit RSP binding to Vero E6 cells from 5.0-24.7%. Antiserum against

prM-ED3 chimeric protein showed the strongest inhibition of RSP binding among the four tested.

Two epidemiological aspects were investigated in the present study. For vector epidemiology study, a total of 1888 mosquitoes, of which 31.4% (593) were *Aedes albopictus,* were collected and screened for DV by our in-house RT-PCR assay and a conventional nested RT-PCR; however, DV was not detected. No PCR inhibitor was detected from the pooled mosquito materials spiked with DV RNA except mosquito pool Jan 2008. For the seroepidemiology study, a total of 685 subjects were recruited. The overall prevalence of DV was 1.61%, which was much lower than that reported by other nearby Asian countries. It was observed that seropositivity was significantly associated with increased risk for subjects who were not born and did not grown up locally in Hong Kong (P<0.01, OR 18.00, CI 2.18-148.36). Individuals with an active travel history and who had visited some areas of Eastern Asia (P=0.07, OR 5.60, CI 0.68-46.03) or Southeast Asia (P=0.04, OR 4.27, CI 0.984-18.55) in the past 12 months were more likely to be dengue seropositive.

In conclusion, a rapid and economical one-step RT-PCR LightCycler assay for the detection and typing DV (DEN-1 to-4) was developed and validated. Three recombinant proteins of DEN-2 were generated. A serological assay based on the three recombinant proteins as detection antigens was set up and validated with serum samples. Polyclonal antisera were raised from rabbits and their neutralising potentials against DEN-2 RSPs were demonstrated. No DV was detected from

mosquitoes collected in the present study and the seroprevalence of DV was very low among subjects recruited in Hong Kong.

Publications

Paper in refereed journal:

Lo CL, Yip SP, Cheng PK, To TS, Lim WW, Leung PH. (2007) One-step rapid reverse transcription-PCR assay for detecting and typing dengue viruses with GC tail and induced fluorescence resonance energy transfer techniques for melting temperature and color multiplexing. *Clin Chem* **53**, 594-9.

Conference papers

C. Lo, P Leung, S.P. Yip, T. To, W. Lim. (2006) Development of a one-step real-time RT-PCR assay for detection and serotyping of dengue viruses.. *Int J Infect Dis* **10**, S65. 12th International Congress of Infectious Diseases. Lisbon, Portugal, 15-21 June 2006

C.L.H. Lo, P.H.M. Leung, S.P. Yip. Seroprevalence of dengue infection in Hong Kong. 2nd International Conference on Dengue and Dengue Haemorrhagic Fever. Phuket, Thailand, 15-17 Oct 2008

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List of Abbreviations

%	percent
A	Absorbance
Ae. albopictus	*Aedes albopictus*
ACN	acetonitrile
ADE	antibody dependent enhancement
AFCD	Agriculture Fisheries and Conservation Department
AOI	area ovitrap index
APC	antigen presenting cell
BLAST	Basic logical alignment search tool
BSA	Bovin serum albumin
bp	base pair
C	capsid
cDNA	complimentary DNA
CI	confidence interval
CO_2	carbon dioxide
CPE	cytopathic effect
CS	cyclization sequences
Ct	threshold cycle
CTL	cytotoxic T lymphocytes
CV	coefficient of variations
DC	dendritic cell
DC-SIGN	DC express specific ICAM3-grabbing non-integrin receptors
DF	dengue fever

DHF	dengue haemorrhagic fever
DNA	deoxyribonucleic acid
DMEM	Dulbecco′s modified Eagle′s medium
dNTPs	deoxyribonucleotide triphosphates
	[N= adenosine (A), guanosine (G), cytidine (C), thymine (T)]
DSS	dengue shock syndrome
DV	dengue virus
	[serotype 1(DEN-1), serotype 2 (DEN-2), serotype 3 (DEN-3), serotype 4 (DEN-4)]
E	envelope
E. coli	*Escherichia coli*
ED	envelope domain
	[domain 1 (ED1), domain 2 (ED2), domain 3 (ED3)]
EDTA	ethylene-diamine-tetra acetic acid
ELISA	enzyme-linked immunosorbent assay
EM	electron micrographs
ER	endoplastic reticulum
FBS	fetal bovine serum
FEHD	Food and Environment Hygiene Department
FITC	fluorescein isothiocyanate
FMEL	Florida Medical Entomological Laboratory
FRET	fluorescence resonance energy transfer
FRhL	fetal rhesus lung
GSK	GlaxoSmithKline
GST	glutathione S-transferase

HAI	haemagglutination inhibition (HAI)
hr	hour
HKSAR	Hong Kong Special Administrative Region
HRP	horseradish peroxidase
IC	immunochromatography
IFAS	Institute of Food and Agricultural Sciences
IFN	interferon
iFRET	induced fluorescence resonance energy transfer
IL	interlukin
IMAC	immobilized metal affinity chromatography
IPTG	Isopropyl-β-D-thiogalactopyranoside
KC	Central Kwai Chung Park
kb	kilo-base
kDa	kilo-dalton
L	litre
LAMP	liposome-associated membrane protein
LB	Luria-Bertani
LCSD	Leisure and Cultural Services Department
LM	Lady MacLehose Holiday Village
LN	Lions Nature Education Centre
mAb	monocloncal antibody
MAC-ELISA	cellulose membrane-based IgM capture-ELISA
MBP	Maltose binding protein
MCS	multiple cloning site
$MgCl_2$	magnesium chloride

$MgSO_4$	magnesium phosphate
MHC	histocompatibility complex
min	minute
MIR	minimum infection rate
mL	milli-litre
mM	millimolar
MOI	monthly ovitrap index
MS	mass spectrometry
MW	molecular weight
NCBI	National Center for Biotechnology Information
ND	North District Park
ng	nanogram
nm	nanometer
NPV	negative predictive value
NS	non-structural [NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5]
OR	odd ratio
P	prevalence
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PDK	primary dog kidney
PF	Pok Fu Lam Country Park
PFU	plaque forming unit
PGMK	primary green monkey kidney
PHLC	Public Health Laboratory Centre
PPV	positive predictive value

prM	membrane precursor
PU	The Hong Kong Polytechnic University
PVDF	polyvinylidene fluoride
QB	Quarry Bay Park
r	correlation
RNA	ribonucleic acid
RSPs	recombinant subviral particles
RT	room temperature
RT-PCR	reverse transcription-polymerase chain reaction
s	second
S2	*Drosphilia melanogaster* Schneider 2
SB	Silvermine Bay Outdoor Recreation Camp
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SGI	SYBR Green I
SL	stem-loop
SM	Shing Mun Country Park
ST	Sha Tin Park
SUMO	small ubiquitin-like modifier
SV	So Kwun Wat Valley
T	time point
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween-20
TC	Tung Chung Outdoor Recreation Camp
T_m	melting temperature
TM	Tuen Mun town centre

TMB	tetramethylbenzidine
TNF	tumor necrosis factor
TT	Tai Tam Country Park
U	unit
UTR	untranslated region
WHO	World Health Organization
WRAIR	Walter Reed Army Institute of Research
WRBU	Walter Reed Biosystematics Unit
YL	Yuen Long Park
μg	microgram
μL	micro-litre

CHAPTER 1

INTRODUCTION

1.1 Background

Dengue fever (DF) is a disease first reported in the early 18th century in Asia, Africa and North America [Gubler, 1998]. This disease is caused by dengue viruses (DV), which were first isolated during World War II. DF epidemics occurred in Asia and Southeast Asia in the 1900s and resulted in 218,859 cases and 2075 deaths [Kautner et al., 1997; Thomas et al., 2003; Mackenzie et al., 2004]. According to the World Health Organization (WHO), it is estimated that over 50 million cases of DF and dengue haemorrhagic fever (DHF) are reported annually worldwide [WHO, 2004a]. DV are transmitted to humans by infected female *Aedes aegypti/albopictus* mosquitoes [Holtzclaw, 2002]. In the past, DF was prevalent in tropical and subtropical areas since *Aedes* mosquito vectors mostly colonised warm climates. From the discovery of cold-resistant *Aedes* mosquitoes which become more adapted to low temperatures, this implies that both vectors and DV are expanding their geographical boundaries [Kautner et al., 1997; Tsai, 2000; Mairuhu et al., 2004].

DF is one of the most important arthropod-borne infections since patients with DF may be asymptomatic or present flu-like symptom which impedes immediate diagnosis [Tsai, 2000]. Without adequate supportive treatment, the disease may progress to severe DHF and lethal dengue shock syndrome (DSS) [Kauthner et al, 1997; Groen et al., 2000;]. More importantly, vaccination and specific treatment for dengue infection are still unavailable. Vaccination and

anti-viral drugs are research areas with high priority for the WHO [WHO, 2006]. With the advancement of recombinant DNA technology, the development of treatment and preventive measures for dengue is in progress. In order to control the disease, development of rapid and affordable diagnostic assays is warranted so as to ensure early treatment, since most of the dengue endemic countries are under-developed. In addition, epidemiological information such as the distribution of vectors and seroepidemiology of the general population are essential for the planning of preventive measures [Kauthner et al., 1997; Tsai, 2000].

In the remaining section of this Chapter, various aspects of dengue infection will be reviewed, which include background regarding the clinical and epidemiological aspects of dengue infection, current approaches for DV diagnosis, and the development of dengue vaccines. Moreover, the concepts and rationale of the present study will be discussed. In the last section, the aims of the study will be addressed.

1.2 Dengue infections

1.2.1 Dengue virus

1.2.1.1 General properties of dengue virus

DV is a single positive-stranded RNA virus of the family *Flaviviridae*. This family contains five major human pathogenic viruses: yellow fever virus, tick-borne encephalitis virus, St. Louis encephalitis, Japanese encephalitis virus and DV [Tsai, 2000]. There are four serotypes (DEN-1 to DEN-4) of DV [Holtzclaw, 2002]. The immature and mature virions are spherical with a diameter of 60 nm and 50 nm (500 Å), respectively [Perera & Kuhn, 2008] (Figure 1.1). The

virion particle consists of nucleocapsid which is enveloped by an outer glycoprotein shell and an inner lipid bilayer. The nucleocapsid contains capsid (C) and the viral genome. The composition of viral lipid is similar to that of the host cell, as the lipid is derived from the host cell membrane. The virion surface is covered with surface projections, which consists of a glycoprotein shell composed of 180 copies each of envelope (E) and membrane (prM/M) glycoprotein [Perera & Kuhn, 2008; Tsai, 2000]. In electron micrographs (EM), the virus particle is constructed in icosahedral asymmetry, and every two icosahedral asymmetric units consists of three subunits of E protein (Figure 1.1A ii, iv) [Kuhn et al., 2002]. In their immature status, E and prM proteins are arranged in 60 vertical trimers with spiky morphology (Figure 1.1A i, ii). During maturation and before virion release from the host cell, the conformation of E and prM glycoprotein orientates horizontally into 90 anti-parallel homodimers in a low pH environment (pH 5.8-6.0), and the morphology changes to a smooth appearance [Perera & Kuhn, 2008] (Figure 1.1A iii, iv). Cleavage of the precursor (pr) subunit from prM by host-encoded furin protease is accompanied by the process of virion maturation in the trans-Golgi network. The cleaved pr remains associated with the E glycoprotein as a cap for protection until the mature virion is released into the extracellular space (Figure 1.1B) [Perera & Kuhn, 2008].

The natural hosts of DV are arthropods and vertebrates, including humans and lower primates. These hosts complete a cycle of transmission of DV which increases viral survival [Tsai, 2000]. Mosquitoes of the genus *Aedes (Ae)* are responsible for DV transmission. *Ae. aegypti* is the most important vector, which is abundant in tropical and subtropical areas (Figure 1.2). *Ae. albopictus* and *Ae.*

polynesiensis can also be vectors in some geographic locations [Malavige et al., 2004]. Also, the ova of *Aedes* mosquitoes are able to survive in dry conditions, which facilitates the recurrent nature of dengue outbreaks during the warm season [Kauthner et al., 1997].

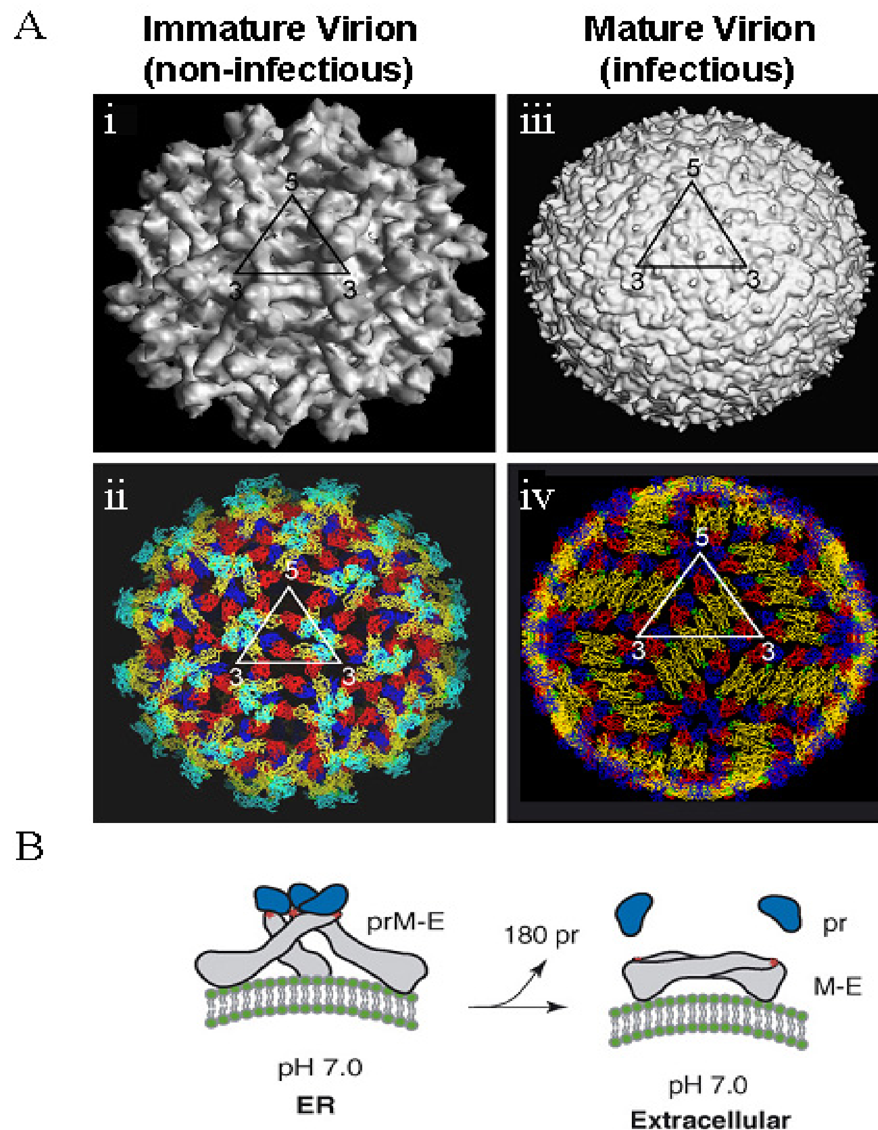


Figure 1.1 Structure of dengue virion in immature and mature state.

[Adopted and modified from Figure 2 in Perera & Kuhn, 2008]. A) Structure of dengue virion under electron microscope. i, & ii indicated the immature virion which arranged in trimers with 60 nm in diameter. iii & iv indicated the mature virin which arranged in dimmers with 50 nm in diameter. Outline of the triangle on virion indicated an icosahedral asymmetric unit. On ii and iv, domains ED1, ED2 and ED3 of E glycoprotein were shaded in red, yellow and blue, respectively. The pr of prM protein was shown in cyan n ii and the fusion loop was coloured in green on iv. B) Under lower pH change (pH 5.8-6.0, not shown in the figure), cleavage of pr from prM was induced in ER during maturation before virion release extracellularly.

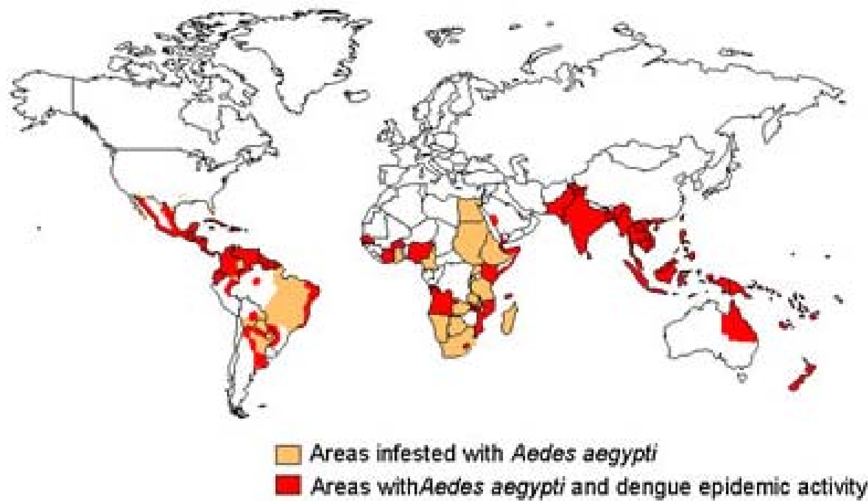


Figure 1.2 World distribution of dengue vector *Aedes aegypti* in 2005.
[Adopted from Centres for Disease Control and Protection, 2006]

1.2.1.2 Structure of the dengue virus

The genome of DV is a positive single-stranded RNA about 11 kilobases (kb) and is organised sequentially as 5'-C-prM(M)-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3' [Guzman & Kouri, 1996]. The genome encodes a long open reading frame made up of three structural genes (C, prM and E) and seven non-structural (NS) genes (NS1, NS2A, NA2B, NS3, NS4A, NS4B and NS5). NS1, NS3 and NS5 are responsible for encoding various virus enzymes. The genome is flanked by the 5' and 3' untranslated regions (UTR) without a poly(A) tail [Lindenbach & Rice, 2003]. During replication, the RNA genome is transcribed and translated into a single large polyprotein in the membrane of the perinuclear region. The polyprotein is a transmembrane protein in the endoplastic reticulum (ER), and co-translational cleavages at several sites of the polyprotein occur both in the cytoplasm and in the lumen of the ER (Figure 1.3). In the cytoplasm,

cleavages occur after one pair of amino acids at the junction of NS2A-NS2B, NS2B-NS3, NS3-NS4A, and NS4B-NS5. The serine protease encoded by NS3 and its cofactor NS2B are responsible for these cleavages. In lumen of the ER, cleavages between C-prM, prM-E, E-NS1, and NS4A-NS4B are mediated by a cell-signal peptidase. One more cleavage between NS1 and NS2A is mediated by an unknown protease. All cleaved proteins undergo virus assembly and are eventually released from the host cell [Westaway & Blok, 1997]. The properties and functions of each viral protein are elaborated in the following paragraphs.

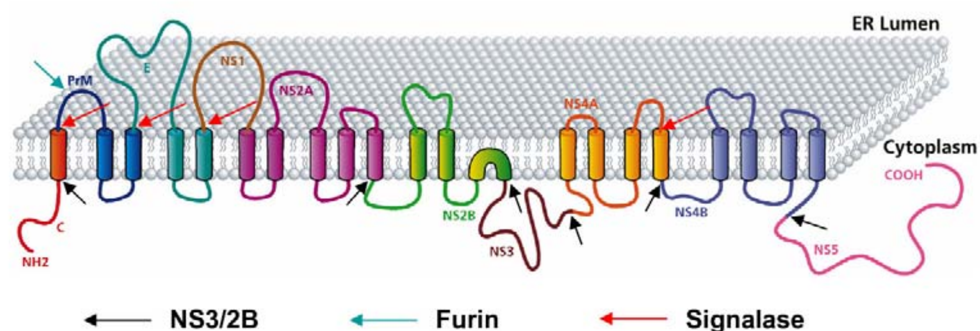


Figure 1.3 Polyprotein of DV in ER.

[Adopted from Figure 1 in Umareddy et al., 2007]. Arrows shaded in cyan and red indicated the cleavage sites by host enzyme protease furin and signalase, respectively. Arrow shaded in black indicated the cleavage site by viral protease.

Structural proteins

Capsid

Capsid (C) is a basic protein with a size of ~11 kiloDaltons (kDa). The hydrophobic property of the C-terminal anchor acts as signal sequence to mediate the translocation of prM into the ER. Removal of this anchor protein in mature C is achieved by the viral serine protease [Lindenbach & Rice, 2003]. It is believed that C protein is not involved in eliciting neutralising antibodies, based on the studies of previous researchers [Chang, 1997].

Precursor membrane

The precursor membrane (prM) protein is made up of 166 amino acid residues with a size of ~26kDa. As mentioned in the previous paragraph, cleavage of prM from C is activated until the removal of the C-terminal anchor signal peptide from C protein. The actual function of prM is for the protection of the E glycoprotein in the early secretory pathway. As mentioned in Chapter 1.2.1.1, ninety-one amino acid residues of the N-terminal pr are cleaved from the M glycoprotein during virion maturation. The cleaved pr remains attached to the E glycoprotein for stabilisation in a reduced pH environment (pH 5.8-6.3). Wang and co-workers have suggested that the major binding sites for prM are located in the membrane-anchoring carboxyl terminus without involving the ectodomain of the E protein [Wang et al., 1999]. The interaction between prM and E proteins had not been identified until a recent study which confirmed that pr protein interacts with the fusion loop of the ectodomain for E stabilisation. In this study, the recombinant protein model for cryo-EM was constructed from prM (residues 1-166) and E (residues 1-394) from a DEN-2 strain. The transmembrane region was replaced with a Tobacco Etch virus linker, and the furin cleavage site was mutated to prevent digestion by the intracellular protease of *Drosphilia melanogaster* Schneider 2 (S2) cells. The cryo-EM findings determined that pr protects immature virions from premature membrane fusion with host cells by covering the fusion loop [Li et al., 2008].

Apart from its protective function, current evidence has shown that prM mediates proper folding of the E glycoprotein by playing a chaperone-like role. The cleavage of pr-M by furin is important for the initiation of subsequent

rearrangements of the E glycoprotein into mature virions [Heinz & Allison, 2003]. Furin cleavage could be blocked by acidotropic reagents, but the resultant virions released extracellularly were less infectious and unable to induce membrane fusion in an acidic pH environment. Infectivity reduction of DV is the least effective among the other flaviviruses, although the reasons for this are not understood [Randolph et al., 1990; Keelapang et al., 2004]. In contrast, it was found that enhancing prM cleavage could delay and reduce the export of infectious virions, which was related to the charged residues in the proximal region to the pr-M cleavage site [Keelapang et al., 2004].

In spite of the importance of prM cleavage for DV conformation, virions containing uncleaved prM have been found extracellularly in certain cell lines [Murray et al., 1993; Wang et al., 1999; Keelapang et al., 2004]. This phenomenon is unique to DV and not seen in other flavivirus, and the biological significance of the presence of extracellular uncleaved prM-containing virions is still unclear [Puttikunt et al., 2008]. Keeplapang and colleagues hypothesised that the presence of prM-containing virions can act as a regulator of cell fusion by preventing the E protein of M-containing virions from fusion with newly infected cells [Keelapang et al., 2004]. Alternatively, Kuhn et al., as cited in Keelapang et al., suggested that the prM protein on extracellular virions might restrict the E protein from outward expansion before the re-arrangement of E dimers is completed [Kuhn et al., 2004 cited in Keelapang et al., 2004]. Apparently, the co-existence of extracellular M-containing and prM-containing dengue virions is not a defect of viral assembly. There must be a functional role of prM but it is still unclear, although it seems to not be involving in either cell binding [Keelapang et al., 2004] or as a target for

virus neutralisation [Puttikunt et al., 2008]. However, monoclonal anti-prM antibodies likely furnished passive protection from infection with the recruitment of antibody-dependent cytotoxic T cells [Falconar, 1999]. Kaufman et al. indicated that there was evidence the prM protein might be responsible for the generation of neutralising antibodies [Kaufman et al., 1989]. These findings indicated that prM could be a potential candidate for vaccine development. Moreover, it is important that prM and E glycoproteins are required to maintain the native structure of the conformationally-dependent E-glycoprotein epitopes [Konishi & Manson, 1993]. Thus, a subunit vaccine candidate targeted to the E glycoprotein should also take into consideration the co-synthesis of prM protein. Therefore, in the present study, prM will be co-expressed with a known epitope, envelope domain 3 protein (ED3), to investigate whether it is necessary for maintaining the native conformation of ED3, not only the whole E glycoprotein. Additionally, the expression of prM and ED3 expression alone will also be investigated for the antigenic and neutralisation properties of their corresponding polyclonal antisera.

Envelope

The E glycoprotein (~53kDa) is a surface protein of the virion. It acts as an antigen to elicit host immunity and is a major target of neutralisation by host antibodies. The E glycoprotein consists of an ectodomain, a stem domain and a membrane-anchor domain. The ectodomain is sub-divided into three distinct domains, envelope domain 1 (ED1) (residues 1-51, 132-192 & 280-295), ED2 (residues 52-131 & 193-279) and ED3 (residues 296-394) [Modis et al., 2003]. ED2 is elongated in appearance and ends with a tip. The tip is composed of a fusion peptide loop structure which mediates membrane fusion by exposure of the

internal fusion loop [Heinz & Allison, 2003]. Exposure of the fusion loop requires a change in the orientation of ED1 and 2 to support the dramatic conformational changes on the surface of virion [Modis et al., 2003; Bressanelli et al., 2004]

ED3 is recognised as an antigenic epitope due to its immunoglobulin -like structure and is responsible for cell adhesion [Lindenbach & Rice, 2003]. Several studies have shown that ED3 is a potential candidate for vaccine design against DV. Anti-ED3 antibodies of DEN-1 and -2 raised in animals were able to completely neutralise homologous serotypes but were ineffective against heterologous infections [Chiu & Yang, 2003; Chin et al., 2007]. Recently, a tetravalent candidate vaccine was constructed by production of a single tandem ED3 protein which included all serotypes; however, protection from DEN-3 was not guaranteed [Chen et al., 2007]. Ineffective cross-protection might have been due to differences in critical residues for host cell receptor binding between serotypes. These critical residues can be localised and characterised by epitope mapping using either monoclonal antibodies (mAbs) or recombinant ED3 fragment peptides [reviewed by Roehrig, 2003]. Gromowski & Barrett recognised four critical epitopes of DEN-2 by ED3 site-directed mutant proteins against ED3 type-specific mAbs [Gromowski & Barrett, 2007]. A similar approach was utilised for type-specific epitope mapping of DEN-1 [Chen et al., 2007]. The above data supported the idea that ED3 rather than the whole E glycoprotein is sufficient to elicit immunity against DV. Furthermore, ED3 is a truncated E glycoprotein with lower molecular weight which facilitates its expression in higher yield compared to the full length E-glycoprotein [Zhang et al., 2007]. Based on these two reasons, only ED3 was expressed and not the whole E protein in the present study. Heterologous expression of E glycoprotein has been performed in various systems,

including *Escherichia coli* (*E. coli*), *Pichia* yeast, baculovirus and insect cells [Sugrue et al., 1997; Kelly et al., 2000; Etemad et al., 2008]. For studies using the *E. coli* system, most expressed the protein as insoluble inclusion bodies [Khanam et al., 2006; Chen et al., 2007; Pattnaik et al., 2007; Zhang et al., 2007; Tripathi et al., 2008]. However, native protein folding might not been achieved after solubilisation and re-folding procedures. This might account for the discrepancies in the ability of the expressed ED3 proteins to produce neutralising antibodies [Khanam et al., 2006; Chen et al., 2007].

Non-structural proteins

There are seven NS proteins. NS1 and NS2A are suggested to play essential roles in viral RNA replication. NS3 and NS2B encode for the serine protease and its co-factor, respectively. NS5 encodes for several enzymes, including RNA-dependent RNA polymerase, S-adenosyl-methionine methyltransferase and guanglyl transferase, which are essential for viral propagation [Lindenbach & Rice, 2003]. The functions of NS4A and NS4B have not yet been identified. However, the presence of anti-NS4A antibodies in dengue patient sera indicates the antigenic nature of NS4A [AnandaRao et al., 2005]. There is limited information of NS4B, which is a trans-membrane protein that co-localises with ER markers [Miller et al., 2006].

Untranslated regions (UTR)

5’ & 3’ UTR

The function of 5’ and 3’ UTR is believed to be important for cyclisation during viral replication. There are cyclisation sequence (CS) structures with conserved sequences downstream of the 5’ end near the beginning of the open

reading frame. The 3’ end of the genome contains a similar structure, but with a sequence complementary to the 5’ end CS named CS1. CS1 is a well-conserved region containing 25 nucleotides. During replication, the CS1 and CS sequences complement with each other to form a circle for RNA replication [Lindenbach & Rice, 2003]. Deletion of CS1 was found to be lethal for DEN-4 virus replication [Men et al., 1996]. Another feature which is conserved among flaviviruses is the 3’ stem-loop (3’SL), made up of 90-120 nucleotides and shaped into a secondary structure near the terminus of the 3’ end of the viral genome. The actual function of 3’SL is not fully identified yet, but previous studies have revealed its interaction with several functional viral proteins, such as NS3 and NS5 *in vitro* [Lindenbach & Rice, 2003]. Two additional conserved structures, CS2 and RCS2, are upstream of CS1 sequentially, but their function has not yet been established. They do not appear to be directly related to viral replication as deletion of these structures is not lethal for DEN-4 [Lindenbach & Rice, 2003].

Due to the well-conserved property of the 3’UTR, it may facilitate the development of a molecular detection assay based on dengue- and serotype-specific sequences. However, a high secondary structure may render difficulties for primer hybridisation. In this study, the in-house real time RT-PCR assay was developed based on the conserved sequence among all serotypes and the unique serotype-specific sequences of the 3’ UTR. The details of the assay design are illustrated in Chapter 2.

1.2.2 History and Epidemiology

The earliest record of dengue-like illness can be traced back to the Chin Dynasty (265 to 420 A.D) of China. The symptoms of dengue-like illness were recorded in a Chinese medical encyclopaedia of "disease symptoms and remedies" [Gubler, 1998]. Periodic epidemics of dengue were reported in 1779-1780 in Asia, Africa and North America. Afterward, dengue cases increased with the development of the international shipping industry in the 18th and 19th centuries. Transportation of mosquito vectors and DV-infected people played an important role in the spread of dengue. Before 1940, dengue-like illness was reported spontaneously in a large epidemic [Holtzclaw, 2002]. Dengue became epidemic and widespread during World War II (1939-1945). The effect of World War II was to not only increase the number of dengue epidemics, but it also resulted in the interchange of dengue serotypes between Asia and America. Serological studies carried out during World War II discovered that there was an association between co-circulation of different serotypes of DV in the same geographic region and epidemics of severe dengue cases [Thomas et al., 2003]. Dengue haemorrhagic fever (DHF) was first recognised in 1953 to 1954 in Manila, the Philippines. The severity of the DHF outbreak in the Southeast Asia was the major cause of hospitalisation and death among children in the 1970s [WHO, 2009b]. Due to the development of rapid transportation (air travel and high-speed trains) in the 20th century, travel-acquired cases dramatically increased the frequency of dengue epidemics in various geographic regions and promoted global resurgence of the disease [Hoktzclaw, 2002].

Generally, several factors are responsible for the increase in incidence of DF. These include 1) climate changes, particularly global warming [Mairuhu et al.,

2004]; 2) continued global population growth associated with uncontrolled urbanisation, which has increased the number of mosquito breeding sites in the domestic environment [Groen et al., 2000; Ellerin et al., 2003]; 3) changes in agricultural practices, which expand the vector populations; 4) viremic travellers who return from dengue-endemic areas carrying the virus to their homeland [Jelinek, 2000]; and 5) lack of public health policy and education to enforce effective vector control programmes and educate the general public regarding the importance of mosquito control measures associated with dengue and other mosquito-borne infections [Gubler, 1998].

WHO has estimated that 50-100 million cases of DF and 400 thousand cases of DHF are reported annually worldwide [WHO, 2004b]. Globally, DF is endemic in more than 100 countries, which are mostly distributed among the three continents of Central and South America, Africa and Asia (Figure 1.4). In addition, dengue has invaded into countries of Europe, the Mediterranean and the Middle East, but most cases have been imported from Southeast Asia or Latin America [Reviewed by Thomas et al., 2003].

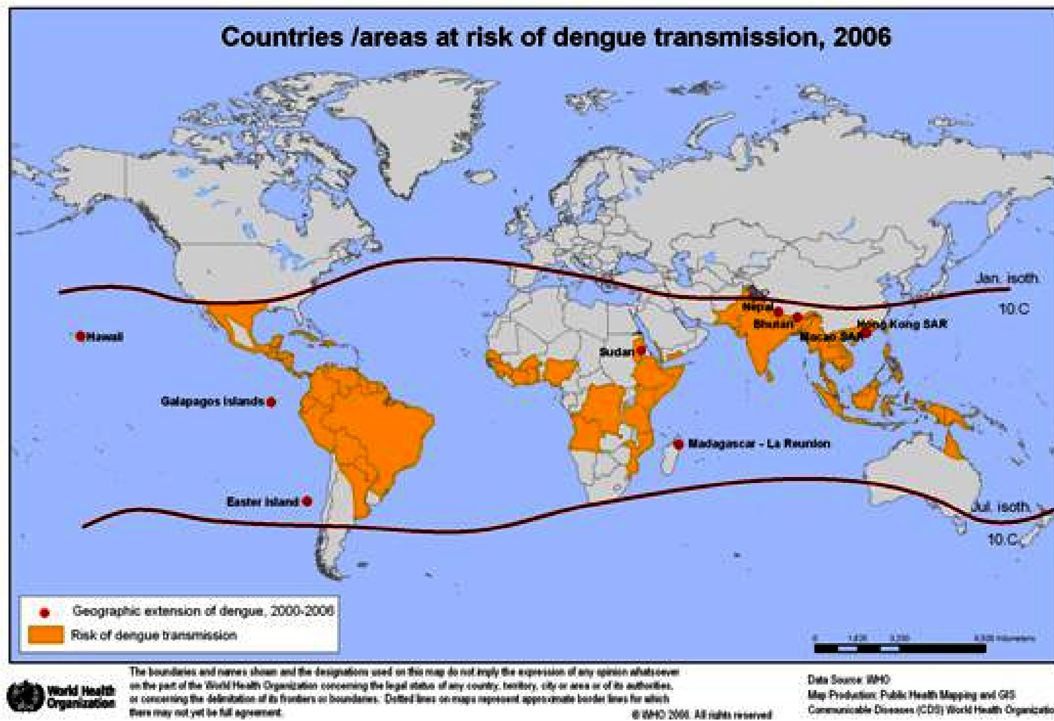


Figure 1.4 Geographical transmission of dengue.

[Adopted from WHO, Impact of dengue, http://www.who.int/csr/disease/dengue/impact/en/]

In America, endemic countries are located in the Central and South American regions. The first major epidemic of DF was recorded in Cuba in 1977-78 [Reviewed by Malavige et al., 2004]. Significant outbreaks of DF and DHF have been reported in the last two decades in Puerto Rico, Brazil, Ecuador, Guatemala, French Guiana, Panama, Costa Rica, El Salvador, Nicaragua and Guadeloupe. Severe dengue cases in Brazil, El Salvador and Honduras were reported by WHO. Brazil has experienced dengue outbreaks due to co-circulation of DEN-1 and 2. However, introduction of the DEN-3 serotype in 2000 had increased epidemics of dengue in this country [Reviewed by Thomas et al., 2003]. In 2007, over 890,000 cases of dengue were reported in the Americas, of which 26,000 cases were DHF [WHO, 2009a]

Four dengue serotypes co-circulated throughout Africa during the 1980s; DEN-1 and -2 were early isolated in the 1960s-70s, while DEN-3 and -4 were isolated ten years later in 1983 and 1984, respectively. By the mid-1970s, a few significant outbreaks of DEN-2 were recorded in the Seychelles and Reunion Island leading to high morbidity and fatality. In the 1980s and 1990s, more countries reported DV in isolation, which demonstrated the wide spread of DF in Africa. Moreover, dengue epidemics have increasing in the last decade, but rare cases of DHF were recorded. The association of co-circulation of serotypes and DHF might be related to poor reporting systems and diagnosis in Africa. [Reviewed by Thomas et al., 2003].

Dengue in Asia was transmitted rapidly after World War II due to the destruction of the water transportation system, since rebuilding the water system facilitated breeding and provided habitats for the larvae of *Aedes* mosquitoes. DHF was confirmed in several countries including Thailand, the Philippines and Vietnam during the 1950s-60s. Currently, the incidence of DF and DHF have increased steadily and epidemics continue to occur in numerous Southeast Asian regions including Cambodia, China, Indonesia, Malaysia, Myanmar, Taiwan, Thailand and Singapore [Reviewed by Thomas et al., 2003]. The incidence of DHF has increased 5-fold in the past 30 years [reviewed by Malavige et al., 2004]. During the period 2001-2005, the infection rate intensified in Southeast Asia with over 715,616 cases; more than 50% of the people living in tropical and subtropical countries are at risk for acquiring this infection [WHO, 2004]. Thailand, Indonesia, Calcutta, Kho Samui and some Southeast Asian countries have experienced co-circulation of all four serotypes. Since the global incidence of

dengue was found to be dramatically increased in 1998, WHO has predicted that dengue epidemics will become active in a 3-years cycle [WHO, 2001].

The incidence of DF has been increasing annually in Hong Kong. From 2000-2003, the number of notifications progressively increased from 11 to 49 cases [Mok, 2001; Sin & Tse, 2002; Fung et al., 2003; Leung & Tsang, 2004]. The majority of cases were imported from nearby Asian countries. From 2004-2010, over 30 of imported cases were reported annually [Centre for Health Protection, 2010]. The first local case was reported in 2002 during an outbreak in Ma Wan, with a total of 16 people infected [Tsang, 2002; Auyeung et al., 2003]. In the nearby region of Macau, a large outbreak was documented in 2001, where more than 1000 cases were reported [The Health Bureau, Macao SAR, 2001]. In China, several outbreaks were reported from Guangdong Province and Hainan Island between 1978 and 2003. The serotypes DEN-1, -3 and -4 were involved in these outbreaks, respectively [Fan et al., 1989; Qiu et al., 1991 & 1993; Zhang et al., 2005]. Between 1990 and 1996, dramatic changes in of the incidence were observed, which ranged from 0 to 6812 recorded cases [Wang, 1997].

1.2.3 Pathogenesis of dengue virus infections

1.2.3.1 Lack of animal models for pathological studies of dengue virus

The actual pathogenesis of DV is still unknown since no suitable animal model is available to demonstrate the symptoms observed in humans. DV is capable of replication in humans and some non-humans primates only. In the past, a mouse model had been used for studying the pathogenesis of DV. It was discovered that irrelevant symptoms produced by the mouse model impeded the

investigation of DV pathogenesis. Transgenic mouse model AG129 which lacks IFN- α / β and γ receptors has been used as potential model for study DV vaccine and pathogenesis [Johnson and Roehrig, 1999; Schul, et al., 2007]. A novel non-mouse adapted DV strain (D2Y98P) which enable demonstration of clinical symptoms observed in human, this model provided an impact for investigation of dengue and related vaccine testing [Tan et al., 2010].

Primates, including monkeys and chimpanzees which provide the closest model to humans, have been used for DV pathogenesis studies [Rothman, 1997]. However, many of the serious symptoms seen in humans were not observed in those non-humans primates. Further investigations are difficult due to the unavailability of the animals, heavy expenses and ethical issues [Rothman, 1997].

There are some limitations to studying DV pathogenesis in animal models. First, since replication of DV is restricted to humans and lower primates, and mouse is not a natural host of DV, this factor has increased the difficulty of conducting *in vivo* studies of DV compared to similar studies on other viruses. Secondly, the DV strains used in tissue culture and other non-human animal models have been modified for adaptations of viral replication. Those modified DV strains were definitely genetically and phenotypically different from the native DV strains, so the results found in many *in vitro* studies may not represent the situation *in vivo*.

1.2.3.2 Hypothesis on the pathogenesis of dengue virus

The actual pathogenesis of dengue virus infection and related disease remains unclear. By reviewing the results of extensive pathologic studies from the

past decades, it is believed the pathogenesis of DV infections is attributed to the overproduction of cytokines and mediators released from the infected cells of the host [Rothman, 1997; Lei et al., 2001; Fink et al., 2006].

After being bitten by a DV-infected mosquito, DV is believed to mostly infect the host's dendritic cells (DC) in the skin, and sometimes infect monocytes or macrophages [Fink et al., 2006]. DCs express specific ICAM3-grabbing non-integrin receptors (DC-SIGN) which mediate endocytosis by membrane fusion with DV [Fink et al., 2006; Heinz & Allison, 2003]. Before DV is eliminated by the host's immune response, DV circulates in the host's blood circulation system. During that period, the host is considered to be viremic, and is able to transmit DV via another mosquito bite.

During the early stage of elimination of DV-infected cells, the innate immune response is initiated which increases the production of interferon (IFN) from infected monocytes or fibroblasts and the activation of natural killer cells [Rothman, 1997]. At the same time, the dengue antigen is presented by infected DCs on major histocompatibility complex (MHC) molecules, which in turn trigger the specific cell-mediated immunity of the host [Rothman, 1997; Fink et al., 2006]. DV-specific T-cells may responsible for releasing various cytokines and mediators such as IL-2 and IFN-$\gamma$and TNF-β, and resulting in lysis of DV-infected cells [Rothman, 1997]. In addition, B-cells are responsible for the production of DV-specific neutralising antibodies and complement-fixing antibodies for further clearing of DV-infected cells [Rothman, 1997]. The mechanism of antibody-mediated neutralisation is based on blocking the attachment of DV to the

receptors of target cells by means of structural changes, steric and membrane fusion interference [Halstead, 2003].

In the case of secondary infection by another DV serotype, the infection will be more severe due to the phenomenon of antibody-dependent enhancement (ADE). Although ADE may not completely explain the increased severity of secondary infection, it is believed that ADE plays a key role in the pathogenesis of DHF [Rothman, 1997; Fink et al., 2006]. The pre-existing non-neutralising antibodies against the DV serotype of the primary infection cross-react with another DV serotype in the secondary infection. Non-neutralising antibodies bind to the E protein of DV and form an antigen-antibody complex, which cannot be neutralised. Therefore, the antigen-antibody complex may bind to Fc receptor-bearing monocytes, which in turn increases DV uptake and enhances the infection of monocytes [Rothman, 1997; Fink et al., 2006].

"Original antigenic sin" is a phenomenon allows for memory T-cell from a previous DV infection to respond more rapidly and to produce T-cell clones which are specific for the first DV serotype. However, this low affinity T-cell is not only less effective in the elimination of the second serotype of DV, but more importantly, it promotes an immuno-pathological response [Mongkolsapaya et al., 2003; Fink et al., 2006]. Failure to clear the secondary DV challenge leads to increase viral load in the circulation. As a result, the death of a relativity low proportion of high-affinity specific T-cells produced in response to the current DV infection results in the release of a large amount of cytokines. A significantly increased level of cytokines in the serum, such as IL-2, IL-6, IL-8, TNF-α and

IFN-γ, induces endothelial permeability. This results in increased vascular leakage, which is a typical clinical manifestation of DHF. Severe plasma leakage from capillaries during DHF may rapidly progress to DSS and death [Rothman, 1997; Malavige et al., 2004; Fink et al., 2006]. In addition, the production of antibodies against the dengue viral protein (NS1) cross-react with platelets, thereby leading to the destruction of platelets (thrombocytopenia) which further enhances vascular leakage [Rothman, 1997]. However, mortality of DHF is less than 1% [Malavige et al., 2004] in developed countries because patients are generally rapidly treated by the intensive replacement of fluid and electrolytes in hospital [Holtzclaw, 2002]. The rapid recovery from DHF suggests that DV is not cytopathic for endothelial cells. Several *in vitro* studies [Rothman, 1997] on the cytopathic effect of DV on various human cells indicated that no typical cytopathic effect had been observed.

1.3 Diagnosis of dengue infections

Since there is no specific therapy for dengue infection, vector control and surveillance are the major strategies to prevent spreading of the infections. Before a vaccine for prevention is successfully developed and widely applied to the community, the development of a rapid laboratory detection method of acute-phase dengue infections associated with all serotypes is necessary. In combination with adequate medication, rapid virus identification helps in preventing the spread of DF infections and reducing the incidence of DHF and lethal DSS in patients with suspected DF. Meanwhile, the development of a vaccine and drugs against DV are equally important in the fight against dengue.

1.3.1 Clinical manifestations of dengue infection

DV infection can be classified into DF, more severe dengue hemorrhagic fever (DHF) and lethal dengue shock syndrome (DSS). More than half of reported DF cases are asymptomatic or subclinical [Burke et al., 1988; Cobelens et al., 2002; Endy et al., 2002; Jelinek et al., 1997; Lopez-Velez et al., 1996].The clinical symptoms of DF are related to the strain of virus and age of patient, normally infection in young children are mild compared to adult [Bennett et al., 2010; Ooi et al., 2006; Rico-Hesse, 2003]. The incubation period of DV in humans is about 3-5 days after a mosquito bite. The onset is accompanied by high fever (39°C-40.5°C), severe headache, arthralgia (joint pain), myalgia (muscle pain), acute abdominal pain, vomiting, anorexia and a characteristic rash that looks like "an island of white in a sea of red", commonly observed on the face and limbs of patients [Holtzclaw, 2002; Kautner et al, 1997; Malavige et al, 2004]. The symptoms are self-limiting and last for 2-7 days from the day of onset [Malavige et al, 2004]. Following the febrile phase of DF is a critical moment during which the DF patient will either recover or progress into DHF. An indicator of progression to severe DHF is seen when patients with persistent illness return to normal body temperature [Malavige et al, 2004].

DHF and DSS are more severe manifestations, which are observed in 2-4% of patients experiencing a secondary DV infection [Guzman& Kouri, 2002; Malavige et al, 2004], but some cases have also been reported in patients with primary dengue infection. Several risk factors which include age, gender, racial, genetic differences and the immune response of an individual host are involved in the pathogenesis of DHF [Fink et al 2006; Malavige et al, 2004; Rothman, 1997],

the major clinical manifestation is plasma leakage due to increased vascular permeability [Holtzclaw, 2002; Malavige et al, 2004]. Excessive capillary leakage during DHF may progress into DSS and cause death rapidly within a day due to dehydration, hypotension and, finally, a failure of circulation [Holtzclaw, 2002]. However, most cases of DHF can be quickly recovered from by fluid and electrolyte replacement [Holtzclaw, 2002; Malavige et al, 2004; Rothman, 1997]. More details on the pathogenesis have been discussed in the Chapter 1.2.3.

1.3.2 Laboratory methodologies for dengue diagnosis

The ability to immediately type dengue virus will predict the occurrence of more severe secondary infections with another serotype different from the one seen in primary infection. Developing a highly sensitive and specific method for the rapid detection and serotyping of DV is a goal of many researchers. Application of a successful protocol to routine hospital laboratories will be beneficial to the community. Currently, methodologies for DV diagnosis are mainly classified into three aspects consisting of virus isolation, serological diagnosis and nucleic acid hybridisation.

1.3.2.1 Virus isolation

1.3.2.1.1 Cell line inoculation

Cell/tissue culture for virus isolation or immunoassays for virus-specific antibodies detection are currently used for DF diagnosis in hospital laboratories [Guzman & Kouri, 1996 & 2004; Kauthner et al., 1997; Tsai, 2000]. Serum, peripheral blood leucocytes or homogenised tissues obtained by autopsy can be used for culture. The cytopathic effect (CPE) induced in the cells indicates the

presence of virus [Tsai, 1999]. Cell/tissue culture can be carried out in a flask, 96-well plate, coverslip or shell vial [Tsai, 1999; Wang et al., 2000]. A clinical sample is seeded onto a monolayer of cells and maintained in medium with bovine serum albumin and antibiotics. CPE is caused by complex cell changes, including altered cell shape and membrane permeability, membrane fusion, presence of inclusion bodies and apoptosis. Viral replication from harvested cells is usually confirmed by immunofluorescence assays using mAbs for antigen detection [Tsai, 1999; Wang et al., 2000]. Application of low speed centrifugation has been shown to be effective for virus isolation from cultured cells [Roche et al., 2000; Guzman & Kouri, 2004].

Cell culture is regarded as the "gold standard" for the isolation of dengue virus, but time consuming procedures and high standards of safety requirements have limited its application in the routine laboratories [Harris et al., 1998; Mairuhu et al., 2004]. Since the short period of viremia due to virus neutralisation by virus-specific antibodies limits the accuracy of the assay, viral isolation is recommended for samples obtained within the early ($\leq$6 days) onset period of symptoms.

Viruses can be isolated in various cell lines from mammals and mosquitoes, or by the inoculation of suckling mice or mosquitoes. The monkey kidney cell lines LLCMK2 and Vero have been used for DV isolation. LLCMK2 is the most sensitive among mammalian cells for DV recovery [Guzman & Kouri, 1996 & 2004; Tan et al., 1981]. Mosquito cell lines such as AP-61 and C6/36 (*Ae. albopictus*) are more sensitive than mammalian cells, and can also support the growth of all DV

serotypes [Guzman & Kouri, 1996; Kauthner et al., 1997]. C6/36, which has been evaluated in several research studies, is currently used for routine diagnosis [Tesh, 1979; Tan et al., 1981; Gubler et al., 1984; Kauthner et al., 1997]. Immortal mosquito cell lines are the most sensitive and are used routinely. However, the recognition of CPE is difficult and depends on the different DV serotypes and strains used in the assay, and may hence delay viral diagnosis [Guzman & Kouri, 1996 & 2004].

1.3.2.1.2 Mosquito and mouse inoculation

Mosquito inoculation is the most sensitive system among all DV isolation methods, but the high cost of technical assistance has restricted the application to the identification of emergency and important DV cases only [Tan et al., 1981; Guzman & Kouri, 1996 & 2004]. *Ae. aegypti, Ae. albopictus* and *Toxorhynchites* mosquitoes are useful candidates for dengue isolation [Rosen et al., 1981; Tan et al., 1981; Guzman & Kouri, 1996 & 2004; Kauthner et al., 1997]. The large size and non-blood taking properties of *Toxorhynchites* mosquitoes assure the easy recognition of infected mosquitoes and enhances the safety of the assay, thus they are preferable for viral isolation [Rosen et al., 1981; Guzman & Kouri, 2004]. Alternatively, intracerebral inoculation of suckling mice is a traditional method for virus isolation, since the presence of DV can be identified rapidly and readily by the observed pathological symptoms with central nervous system involvement [Guzman & Kouri, 1996].

1.3.2.2 Serological diagnosis

For routine confirmation, serological diagnosis is the most widely used technique in the laboratory. Serological diagnosis is based on antigen or antibody

detection, including haemagglutination, enzyme immunoassays and immunochromatographic assays [Bundo & Igarashi, 1985; Innis et al., 1989; Kittigul et al., 1998; Branch & Levett, 1999; Martin et al., 2002; Yamada et al., 2003]. Conventional haemagglutination inhibition (HAI) has been used for a long time.

Virus isolation by the culture method is unable to give any information on the serotype and past infections associated with flaviviruses. However, the infection progression and the possibility of re-infections can be indicated from the significantly increased antibody titres measured by the serological test. Typically, a four-fold increase of antibody titre between acute and convalescent (paired) serum samples indicates a recent infection; a titre of >2560 suggests the possibility of a recent secondary infection [Stevens, 1996; Tsai, 1999]. However, cross-reactivity between related flaviviruses leads to decreased specificity of HAI and difficulties in interpreting results. Therefore, HAI has been replaced by a more accurate IgM capture enzyme-linked immunosorbent assay (ELISA) for serological diagnosis [Bundo & Igarashi, 1985; Innis et al., 1989; Branch & Levett, 1999; Tsai, 1999; Martin et al., 2002]. ELISA is a highly sensitive assay with >95% sensitivity for DV diagnosis [Bundo et al., 1985; Innis et al., 1989; Kittigui et al., 1998; Branch et al., 1999]. It is based on the principle that virus-specific IgM antibodies from patient serum are captured by a solid-phase anti-human IgM [Stevens, 1996]. Biotin-labelled sandwich ELISA assays have been development for DV detection. This type of assay is applicable for the capture antigen in low concentrations or with multiple epitopes [Stevens, 1996; Kittgul et al., 1998].

Recently, a cellulose membrane-based IgM capture ELISA (MAC-ELISA) and MAC dot enzyme immunoassay (MAC-DOT) with simplified procedures and much higher sensitivity have been developed [Cardosa et al., 1995; Martin et al., 2002]. The principle of the MAC-DOT assay is similar to conventional ELISA, but the observable colour change by the naked eye has eliminated the requirement for specialised equipment, such as a spectrometer used for conventional ELISA. These two assays are more preferable and widely accepted in hospitals for DV diagnosis using serum samples [Tsai, 2000]. An extremely high specificity (99.2%) of this assay offers a great advantage in the differential diagnosis of DV infections. In spite of the high sensitivity (>95%) of ELISA diagnostic tests, a review has reported such high sensitivity is only achieved with specimens obtained more than one week after the onset of infection [Tsai, 2000]. A review on the related flavivirus Japanese encephalitis virus came to a similar conclusion on the sensitivity of ELISA. However, the author clarified that with a lower sensitivity (75%), ELISA could detect infection in samples taken on the third day after onset, while 100% sensitivity could be achieved for samples obtained 7 days after the onset of infection [Rao, 2001].

There are several commercial kits available for DF and their performance has been evaluated [Groen et al., 2000]. The sensitivities and specificities of these tests varied and no information on DV serotype was revealed [Groen et al., 2000]. Similar evaluation of commercial rapid immunochrmatographic assays for DV infections were conducted [Blacksell et al., 2006]. It was discovered that inadequate accuracy of these kits with poor sensitivity (6.4-65.3%) and moderate specificity (69.1-100%) on characterised serum samples [Blacksell et al., 2006].

Serological diagnosis can only detect dengue viruses after specific antibodies have begun to circulate, thereby increasing the risk of DHF or/and DSS due to delayed diagnosis and inadequate treatment [Lemmer et al., 2004]. In addition, the accuracy depends on the timing of sample collection, so a negative reaction from serological test is inadequate for excluding infections due to the seroconversion period. During that period, virus-specific antibodies may not be produced in sufficient amount for detection [Stevens, 1996].

Apart from ELISA assays developed based on intact virus particles, recombinant viral proteins have recently been applied to serological assay development for dengue diagnosis. NS1 is a novel diagnostic marker of acute DV infection [Bessoff et al., 2008; Tripathi et al., 2007]. NS1 antigen capture ELISA reagent kits are commercially available recently, such as the Panbio® Dengue Early ELISA [Panbio] and Dengue Duo Rapid Test [Standard Diagnostics]. The performance of a few NS1 detection assays were evaluated with clinical sera which were cases confirmed by PCR and virus isolation. However, the overall sensitivities of these kits varied from 60-82% [Bessoff et al., 2008; Hang et al., 2009; Tricou et al., 2010]. Recombinant E protein is also a potential target for serological diagnosis of DV in an immunochromatographic assay [Cuzzubbo et al., 2001]. This assay is also available as a commercial reagent kit, the Panbio Dengue Duo Rapid Strip Test [Panbio]. The performance of the kit based on sensitivity (90%) and specificity (86%) of clinical sera was quite promising, however, the high costs (~US$13/test) of the kit prevent its use for seroprevalence screening. The above findings suggest that recombinant subunit proteins are able to replace whole virus for developing diagnostic assays, which then minimises many problems regarding safety issues.

1.3.2.3 Nucleic acid detection

The reverse transcription polymerase chain reaction (RT-PCR) technique has been used for the diagnosis of flavivirus infections [Lanciotti, 2003]. Available protocols are mainly consensus assays for non-species specific identification of all flaviviruses since the primer sequences were designed based on the conserved region common among them [Lanciotti, 2003]. However, dengue-specific nucleic acid amplification is necessary for the rapid diagnosis and immediate treatment and clinical care for patients. RT-PCR is also an effective and reliable method for DV detection in the acute phase of the infection. The development of conventional PT-PCR [Lanciotti et al., 1992; Harris et al., 1998; De Paula et al., 2004] and real-time PT-PCR [Callahan et al., 2001; Warrilow et al., 2002; Shu et al., 2003] methods for DV detection have been described in several studies.

As there are four DV serotypes (DEN-1 to DEN-4), serotyping of DV is very important because it provides information on the possibility of secondary infection by another DV serotype. RT-PCR provides a rapid means for simultaneous detection and serotyping and rapid RT-PCR detection can minimise progression to DHF and DSS due to secondary infection. The current problem encountered in RT-PCR assay design is the high degree of genomic homology between members of the flavivirus family. Thus, current RT-PCR assays for DV serotyping are performed in 4 separate reactions [Lanciotti et al., 1992; Warrilow et al., 2002; Lindenbach & Rice, 2003; Markoff, 2003, Shu et al., 2003;]

There are various published protocols for dengue diagnosis, some of which have simply detected the presence of DV and other which have identified serotypes

as well, which is useful for dengue surveillance through monitoring the introduction and circulation of dengue serotypes [Lanciotti, 1992 & 2003; Seah et al., 1995a; Sudiro et al., 1997; Harris et al., 1998; De Paula et al., 2002]. Generally, nucleic acid amplification together with serotyping is based on two approaches: 1) the combination of four serotype-specific primers are applied in a single step approach or 2) universal primers specific for dengue virus, plus four serotype-specific primers for the second round nested PCR or probes for hybridisation are applied [Deubel et al., 1990; Lanciotti, 2003; Guzman et al., 2004].

Single step RT-PCR was demonstrated by Harris et al., and the application of a universal forward primer and four serotype-specific reverse primers as well as the rTth DNA polymerase, which is a bi-functional enzyme, worked for reverse transcription and PCR. This technique made the detection and serotyping of DV possible in a single run. This simplified protocol reduced the number of primers in a multiplex so as to increase the sensitivity as compared with two-step PCR protocols [Harris et al., 1998]. However, many of the current assays are in-house methodologies which require further assay validation. In order to achieve the maximum performance of RT-PCR for detection, commercial RT-PCR reagent kits would be an alternative to ensure assay quality. One study [De Paula et al., 2004] conducted a validation on five commercial RT-PCR reagents kits including one-step and two-step kits on serum samples from the acute phase of DF. It revealed that the Qiagen® one-step kit showed the best performance among the others based on the agreement with results from the MAC-ELISA assay and the sensitivity on serial diluted samples. It was discovered that the Qiagen® one-step

kit showed a 100% result agreement with MAC-ELISA assay which was related to the high effectiveness and specificity of the enzymes for reverse transcription [De Paula et al., 2004].

To quantify viral load and reduce the turn-around time for diagnosis, real-time PCR would be preferable owing to the shorter reaction time and post-PCR procedures. The available assays based on TaqMan, hybridisation probes and SYBR Green theories have been applied as detection formats, and the TaqMan probe is the most widely used detection strategy [Laue et al., 1999; Callahan et al., 2001; Warrilow et al., 2002; De Paula et al., 2004]. Real-time RT-PCR assays, similar to conventional RT-PCR, can be briefly classified into i) one-step which assays which are achieved by commercial kits or the bi-functional polymerase rTth or ii) two-step real-time RT-PCR [Houng et al., 2001]. In general, some of the assays covered a particular serotype only [Houng et al., 2000; Wang et al., 2002], while most of them amplified the four serotypes of DV in four separate tubes rather than a multiplex assay in a single tube [Laue et al., 1999; Shu et al., 2003; Warrilow et al., 2002]. Moreover, an assay which amplified all four serotypes in a single tube was not able to differentiate the four serotypes [Warrilow et al., 2002]. The hurdles are related to the high degree of sequence conservation among the four serotypes and the light source excitation of the real-time PCR detection instrument which limits the fluorophores available for detection by colour discrimination in multiplex assays [Mackay et al., 2002]. All these factors make the development of a multiplex real time RT-PCR in a single tube for DV detection and serotyping more difficult than for other pathogens. However, this goal has been achieved recently [Shu et al., 2003].

Recently, a one-tube fourplex TaqMan assay for the detection of DV serotypes has been developed [Johnson et al., 2005]. Each serotype was amplified and typed by individual specific primers and probes. The assay was highly specific and sensitive. The detection limits for DEN-1 to DEN-4 ranged from 0.5 to 0.016 plaque forming units (PFU) per reaction. However, four pairs of primers and four distinct fluorophores for dual-labelled probes increased the complexity and the cost of the assay. The LightCycler has very rapid thermal cycling, and allows multiplexing based on melting temperature (T_m) and colour [Tan et al, 2004]. Chien and co-workers in Taiwan developed another multiplex TaqMan assay for detecting and serotyping DV [Chien et al, 2006]. Multiplexing was achieved by RT-PCR with a universal primer pair specific for flavivirus and four fluorophore-labeled TaqMan probes for individual typing of each serotype. Compared with virus isolation, this multiplex assay was more sensitive and capable of detecting specimens coinfected with more than one DV serotype.

The specificity of a PCR assay is mainly based on primer specificity. Since flaviviruses share certain homology between their genomes, non-specific hybridisation should be considered in designing the primers. The conserved regions of C, M, E, NS1, NS3 and NS5 genes and 3'UTR have been used for primer designed for DV by several researchers [Deubel et al., 1990; Morita et al., 1991; Lanciotti et al., 1992 & 2003; Seah et al., 1995a & b; Sudiro et al., 1997; Harris et al., 1998; De Paula et al., 2002]. The 3'UTR, which is a highly conserved region among all serotypes of dengue virus, was widely used for primers designed for real-time RT-PCR [Callahan et al., 2001; Houng et al., 2001; Warrilow et al., 2002]. The specificity of primers based on different regions varied from 83% to

100% [Houng et al., 2000; Callahan et al., 2001; Shu et al., 2003; De Paula et al., 2004]. To ensure that the results of RT-PCR assays provide high agreement with the current ‘gold standard’ for DV diagnosis, RT-PCR protocols were compared with MAC-ELISA or virus isolation [Wang et al., 2000; De Paula et al., 2002; Lanciotti, 2003]. Several assays showed variable results of sensitivity and specificity for DV [Wang et al, 2000; De Paula et al, 2002; Lanciotti, 2003].

The sensitivity of a PCR depends on the time for blood sample collection. To prevent dengue virus eliminated by neutralising antibodies, it is suggested that blood samples are collected early, within 5 days from the day of onset and before the development of seroconversion [De Paula et al., 2002]. Therefore, the RT-PCR technique is restricted to the early detection of dengue virus in acute-phase serum. A study showed that DV could be detected by RT-PCR in samples collected more than 7 days after onset, but the detection rate was significantly higher in peripheral blood leucocytes than in serum [Wang et al., 2000]. Thus, it is advisable to apply RT-PCR on peripheral blood leucocytes for the detection of dengue virus in convalescent samples. Apart from blood samples, RT-PCR can be applied *in situ* for DV detection on autopsy tissue sections obtained from cases of suspected DSS death [Killen & O’Sullivan, 1993]. Since different units for detection limits were used in various protocols, it is difficult to directly compare the sensitivity of these assays. In summary, the detection limit is approximately 10 PFU for conventional RT-PCR and 0.1 PFU or less for real-time RT-PCR [Lanciotti, 2003].

RT-PCR is a technique which can detect and distinguish between related flaviviruses at the same time. It was undeniable that RT-PCR and real-time

RT-PCR offer a rapid, specific and sensitive means for flavivrius diagnosis. The capability for rapidly identifying concurrent infection has not been easily achieved with other methods [Gubler, 1998; Guzman & Kouri, 2004]. Moreover, it is superior to immunoassays since a single serum sample is sufficient for diagnosis rather than paired sera. However, PCR requires a long time for primer design, especially for real-time RT-PCR for multiplex. Although RT-PCR is a specific and sensitive tool, it cannot totally replace the other diagnosis methods, since the timing of sample collection is very important to ensure the success of an RT-PCR assay. If the sample collected in during the late stage of clinical symptoms, ELISA is recommended for diagnosis rather than RT-PCR or viral isolation, because the neutralising antibodies generated by human body may eliminate the virus.

In summary, limitations are present in the various current DV diagnostic methods. An ideal methodology for this diagnosis has not yet been developed. Nucleic acid hybridisation is becoming the trend for effective, specific and sensitive diagnosis. It is superior to the other diagnostic methods since it is capable of identifying coinfection and only a single serum sample is sufficient for diagnosis [Gubler, 1998; Guzman & Kouri, 2004]. However, it is still under investigation for further validation before clinical application. Owning to similar epidemic patterns, including peak season, vector (mosquitoes) transmission and the symptoms of the early phase, multiplex real-time RT-PCR has the potential to be useful for DV detection in a single reaction. In addition, the application of an effective PCR method for vector surveillance is important for preventing epidemics outside Southeast Asia. Therefore, it is worthwhile to further investigate

these assays with the goal of wider application of DV detection in routine hospital laboratories.

1.4 Treatment and management of dengue infections

Treatments for dengue infection are only non-specific, since there is no specific drug against DV. As mentioned in Ch 1.3.1, the symptoms of DF are mild and self-limited; symptoms such as fever and headache can be relieved by Paracetamol. This antipyretic is recommended instead of aspirin to minimise the risk of gastrointestinal bleeding [Malavige et al., 2004]. For DHF, adequate replenishment of bodily fluids and electrolyte balance are important to prevent shock syndrome due to plasma leakage. Assessment of basic vital signs, urine output (volume and frequency), haematocrit, platelet counts and liver enzymes are necessary [Malavige et al., 2004; WHO, 2009a]. Once there is an abnormality in cardiac index, pulse pressure, urine output or haematocrit, isotonic solutions such as normal saline, Ringer's lactate or Hartamann's solution should be given intravenously for the recovery of these parameters. Cases with severe plasma leakage and organ impairment must be immediately admitted for intensive care and blood transfusion in hospital. Management should be continued to maintain effective circulation during the critical phase of 24-48 hours [WHO, 2009a]. Patients who develop shock syndrome should be managed vigorously and immediately. Intravenous fluid resuscitation with crystalloid or colloid solutions (dextran 70 or protein digest gelafundin 35000) should be given during the next 24-48 hours to expand vascular volume. Patients should be monitored frequently for vital signs every 5-30 minutes until out of shock and parameters are stabilised [Malavige et al., 2004; WHO, 2009a].

Generally, management at home is sufficient for mild dengue cases. Admission into hospital is unnecessary unless DHF symptoms develop, such as bleeding, severe abdominal pain, persistent vomiting or presentation of unconsciousness [Malavige et al., 2004; WHO, 2009a]. Infants under the age of one, which is a high-risk population for DHF, should be monitored frequently for the development of severe complications [Malavige et al., 2004]. Dengue patients who are pregnant, infant, elderly, obese, diabetic, in renal failure or with chronic haemolytic diseases should be observed carefully by professional health care providers, since management of these patients them may be complicated [WHO, 2009a]

1.5 Control and prevention of dengue infections

1.5.1 Surveillance of dengue infections

DV infection is endemic in Southeast Asia, but the dynamics of dengue transmission are not understood. In order to monitor and estimate the resurgence of infections, a strong surveillance system is necessary, especially in endemic areas. A great number of DV infections are asymptomatic and subclinical, thereby facilitating the silent spread of DV in the community. DV infected patients who do not show typical symptoms may not be aware of being infected. In order to obtain an accurate figure on previous exposure to DV in the general population, seroprevalence studies should be conducted in the community. Mosquito vectors and DV are closely related in controlling transmission. Vector surveillance provides information for emergency or reinforced vector control measures.

1.5.1.1 Seroepidemiology

Seroepidemiological studies have been carried out periodically in some endemic countries including Singapore, India, Vietnam, Indonesia and Brazil [Graham et al., 1999; Ooi et al., 2001; Singh et al., 2000; Bartley et al., 2002; Teixeira et al., 2002; Wilder-Smith et al., 2004; Thai et al., 2005;]. In most developing countries, antibodies against DV were detected in over 50% of the screened population [Singh et al., 2000; Kurukumbi et al., 2001]. Recently, two studies with one targeted to primary school children and the other assessing a broad range of age groups, were performed in southern Vietnam [Bartley et al., 2002; Thai et al., 2005]. Both indicated that over 65% of the subjects were seropositive. Seropositivity was significant associated with increasing age among children, but not among the adults population [Bartley et al., 2002; Thai et al., 2005]. In Singapore, which is an urbanised and developed country, about 50% of the subjects of the ages 18-45 enrolled in the seroepidemiological study were confirmed seropositive [Wilder-Smith et al., 2004].

It is believed that several risk factors are associated with seroprevalence. Sex, age, race, population density, residential area and standard of living were investigated in several studies [Ooi et al., 2001; Bartley et al., 2002; Teixeira et al., 2002; Wilder-Smith et al., 2004; Thai et al., 2005;]. Age was found to be significantly associated with seroprevalence, with an increasing risk of seropositivity for every 10 years of age [Wilder-Smith et al., 2004]. In Germany and Singapore, seropositivity was detected in non-Europeans and non-Singaporeans, respectively, who were immigrants from dengue endemic areas [Wilder-Smith et al, 2004; Wichmann et al., 2005]. It was suggested that these

seropositive subjects acquired dengue infections from their home countries, rather than suggesting an association with race [Wilder-Smith et al., 2004; Wichmann et al., 2005].

So far, there has been no sero- and molecular epidemiolgical dengue study performed in Hong Kong and Macau, but limited seroepidemiological studies on Chinese society are available. A seroprevalence study was carried out among Chinese in Taiwan, with 5-10% of dengue IgG prevalence recorded from serum samples collected from an outbreak in 1987-8 [Chen et al., 1997]. Although Hong Kong is not endemic for dengue, a 4-fold increase in the incidence rate was observed from 2000-2003. From 2004 up to June 2010, over 30 cases of imported DF were reported annually in Hong Kong and progressively increased [Centre for Health and Protection, 2010]. An outbreak in Ma Wan and a few sequentially confirmed cases were reported from other districts in 2002. No linkage between was found these cases because the DV serotype of the Ma Wan outbreak was different from that of the locally-acquired cases. These findings implied these DF cases were acquired from different sources, and that silent spreading of DV may exist. In Macau, there was a large outbreak in 2001, causing more than 1400 cases of DF, and sporadic incidence is still recorded every year.

Hong Kong is located at Southeast Asia and is surrounded by countries endemic for dengue. According to the Department of Health of the Hong Kong Special Administrative Region (HKSAR), most dengue fever reported in Hong Kong is from imported cases. Hong Kong residents travel frequently to nearby epidemic areas, which is believed to be a risk factor associated with seroprevalence.

According to the information of two infectious disease surveillance networks in European countries, about 500 cases of travel-acquired dengue fever were confirmed from 1999-2002 [Wichmann et al, 2003]. The majority (70%) of cases were imported from Southeast Asia, with Thailand as the leading country.

Several studies indicated that travellers who have returned from dengue epidemic countries have a higher prevalence of dengue antibodies and number of travel-acquired dengue infection [Potasman et al., 1999; Jelinek, 2000; Wichmann et al., 2003 & 2005]. A total of 7%-45% of febrile travellers were serologically confirmed with dengue infection [Lopez-Velez et al., 1996; Schwartz et al., 1996; Jelinek et al., 1997 & 2000]. Travellers who returned from Southeast Asia showed the highest risk of dengue seropositivity compared to other endemic countries in Africa, Central and South America [Potasman et al., 1999; Wichmann et al., 2003 & 2005]. The importance of travel-acquired dengue might be underestimated due to the asymptomatic or subclinical presentation of the infection [Wichmann et al., 2003]. Seventy-six percent of travel-acquired dengue-infected patients returned from endemic areas did not display typical febrile symptoms of dengue fever [Cobelens et al., 2002]. The major concern is that dengue-infected travellers may carry DV from endemic areas and be responsible for silent spreading of DV in their home countries [Lopez-Velez et al, 1996; Schwartz et al, 1996; Cobelens et al, 2002; Wichmann et al., 2003].

1.5.1.2 Vector surveillance

Vector surveillance provides a means for estimating the emergence of dengue infection in a geographical area. Colonisation and expansion of vectors can

be reflected by the mosquito's ovitrap index. An increasing ovitrap index in a particular area indicates increased risk for dengue infection. In order to comprehend the mechanism of transmission of DV, it is important to understand the relationship between virus, vector and host.

The natural hosts for DV are humans, non-human primates and some species of mosquitoes. *Ae. aegypti* and *Ae. albopictus* are mosquito vectors for DV transmission, but *Ae. aegypti* plays a more important role of DV transmission than *Ae. albopictus* [Gratz, 2004]. *Aedes* mosquitoes are distributed between latitudes of 35 degrees north and 35 degrees south [WHO, 1997]. *Ae. aegypti,* which originated in Africa, spread into tropical Asia and the Pacific Islands in the nineteenth and the beginning of twentieth century. Currently, *Ae. aegypti* is widespread in the tropical and subtropical areas of Southeast Asia, and tends to expand geographically into other non-dengue endemic countries. *Ae. aegypti* is highly adapted to human environments, and is capable of breeding in many artificial sites. These factors facilitate the transmission of DV among human populations in urbanised areas [Rodhain & Rosen, 1997].

Ae. albopictus is an Asian species which is distributed mainly in Southeast Asia, but in the last decades, it spread into the Americas , the Pacific Island and even into Southern Europe [Rodhain & Rosen, 1997]. This mosquito species was originally found in rural areas, such as forests and jungles [Rodhain & Rosen, 1997]. *Ae. albopictus* is believed to be involved in transmission of DV to lower primates, such as monkeys and chimpanzees [Rodhain & Rosen, 1997; Holtzclaw, 2002].

Since the survival and reproduction of *Ae. aegypti* depends on humans*, Ae. albopictus* is less important than *Ae. aegypti* for DV transmission in humans for the following reasons. First, the ability of *Ae. albopictus* to adapt to a human environment is superior to *Ae. aegypti* [Rodhain & Rosen, 1997; Gratz, 2004]. *Ae. albopictus* is able to breed both in natural (tree holes, fallen leaves) and artificial sites (flower pots, vases, water containers), in both rural and urban areas [Gratz, 2004]. However, the breeding activity of *Ae. aegypti* is restricted to urban areas. *Ae. aegypti* can only colonise in a low density environment with high resources, while *Ae. albopictus* can exist in an environment with a high density of population [Gratz, 2004]. The habitat of *Ae. aegypti* was found to be restricted to lower altitudes, whereas *Ae. albopictus* which was found in a diversity of places, from valleys to mountain peaks. Therefore, the above factors have offered *Ae. aegypti* a higher opportunity than *Ae. albopictus* to survive in a human environment [Gratz, 2004]. Additionally, *Ae. albopictus* does not feed exclusively on humans; it can feed on blood meals from other primates [Sullivan et al., 1971]. However, *Ae. aegypti* solely feeds on human blood. Nevertheless, the role for DV transmission becomes obvious in the absence of *Ae. aegypti* due to the above properties of *Ae. albopictus*.

Apart from the mosquito-human relationship mentioned above, transmission of DV also depends on relationships between virus and vector. For *Ae. albopictus*, males who do not feed on blood can transmit DV during mating, and the female is then able to vertically transmit DV to her offspring. The vertical transmission of DV to offspring and sexual transmission complete the transmission cycle of DV in *Ae. albopictus*. It has been implied that *Ae. albopictus* probably

plays an important role for the maintenance of DV in nature where dengue recurs periodically. Conversely, *Ae. aegypti* does not effectively transmit DV vertically [Khin & Than, 1983; Choochote et al., 2001; Vazeille et al., 2003]. In different geographical areas, it is reported that *Ae. albopticus* and *Ae. aegypti* may be replacing each other. It is important to assess if *Ae. albopticus* is really expanding its population, because *Ae. albopticus* is not only the vector for DV but also the reservoir required to maintain DV continuously through vertical transmission.

Ae. albopictus plays a less important role for DV transmission, but surprisingly it is a better host for DV than *Ae. aegypti.* According to previous studies on the oral receptivity for DV in mosquito vectors, *Ae. aegypti* was demonstrated to have a lower oral receptivity, which suggested that *Ae. aegypti* had to be infected by a higher viral load than *Ae. albopictus*. This result indicated that *Ae. aegypti* was less susceptible to DV than *Ae. albopitcus* [Rodhain & Rosen, 1997; Gratz, 2004]. It seems that *Ae. albopticus* is more able to carry DV and has a high potential to replace *Ae. aegypti* for dengue transmission. Lambrechts et al has suggested that we have to consider the displacement of *Ae. aegypti* by *Ae. albopictus* for dengue transmission in the future, the vector displacement might be accompanied by virus adaptation including virus susceptibility [Lambrechts et al., 2010]. Recently, it was demonstrated in *Ae. aegypti* showed a significantly higher oral receptivity for dengue serotype 2 virus than that *Ae. albopticus* [Vazeille et al, 2003]. However, this finding was only discovered in recently collected *Ae. albopticus* from Cambodia, so it might not reflect the overall trend in other Asian countries [Vazeille et al, 2003]. Nevertheless, *Ae. albopictus* probably plays an important role for the maintenance of DV in nature.

Despite the fact that *Ae. albopictus* is a vector for the maintenance of DV in rural areas and seldom for infecting humans, *Ae. albopictus* is capable of DV transmission in countries where *Ae. aegypti* is absent. In the Guangdong province of China, where *Ae. aegypti* is absent, *Ae. albopictus* has been considered as vector for DV transmission. Several dengue outbreaks were reported in Guangdong between 1978 and 2003, and *Ae. albopictus* was confirmed as the vector for transmission [Fan et al, 1989]. All four DV serotypes were isolated from both mosquito species, of which DV was frequently isolated from *Ae. albopictus* in China. These findings implied that *Ae. albopictus* is the primary vector in China [Gratz, 2004]. A study in China discovered that, *Ae. albopictus* and *Ae. aegypti* are vectors for dengue in inland areas and along the coast, respectively [Fan et al, 1989]. In some endemic countries where *Ae. aegypti* and *Ae. albopictus* have colonised, DV has also been isolated from both mosquito species. In a study in Singapore screening the infection rate of vectors, 6.9% and 2.9% of *Ae. aegypti* and *Ae. albopictus* was found to be DV positive, respectively [Chung & Pang, 2002]. In another longitudinal study conducted in South India, DV was also isolated from *Ae. albopictus* [Tewari et al, 2004].

Ae. albopictus is the only *Aedes* vector in Hong Kong, where *Ae. aegypti* has not been detected since 1950 [Chang, 2003]. Up to the end of 2009, there were a total of 20 local dengue cases; most of the cases were reported in a local outbreak in Ma Wan and three other cases were reported outside Ma Wan in 2002. Despite the isolation of DV from *Ae. albopictus,* this was not found to correlate with these local cases (DEN-1). *Ae. albopictus* is recognised as the sole vector in Hong Kong for dengue transmission [Tsang, 2002]. Guangdong and Macau are regions

neighbouring Hong Kong, and local outbreaks have been recorded in these places [Fan et al., 1989; Qiu et al., 1993; The Health Bureau Macao SAR, 2001]. The above evidence clearly shows that *Ae. albopictus* is a potential vector for DV transmission. Compared with other near by countries, Hong Kong has reported a lower number of locally acquired dengue cases, but the reasons for that have not been investigated. The major concern is whether silent dengue transmission among *Ae. albopictus* already exists or not in Hong Kong and leads to an increasing risk for a large local outbreak. To address this issue, vector surveillance should be conducted in Hong Kong.

DF ovitrap indices have been set up in Hong Kong since 2000 to detect the presence of adult female *Ae. albopictus*. There are two kinds of indices: Area Ovitrap Index (AOI) indicates the distribution of *Ae. albopictus* in a particular area and Monthly Ovitrap Index (MOI) indicates the average value of overall AOI [Food and Environment hygiene Department, HKSAR, 2006]. The ovitrap indices provide essential information for vector surveillance, but infection of DV in *Ae. albopictus* is more important to reflect the risk of outbreak. Therefore, it is required to conduct longitudinal surveys on DV infection in *Ae. albopictus* collected from various districts in Hong Kong, as this can serve as an additional indicator of DV surveillance.

1.5.2 Vaccine development for dengue infections

Vaccination has been widely applied for the prevention and control of infectious diseases worldwide. For viral infections, thirteen viral vaccines have been licensed up to now, of which ten are attenuated live vaccines, two are

inactivated and only one is a subunit vaccine [Ada, 2001; Berzofsky et al., 2004]. Development of vaccine for DV began in the 1920s [Hombach, 2007], but a licensed vaccine has not yet been launched. As extensive research on the pathogenesis of dengue infections has been carried out, new findings and molecular technology have made advances in the progress of vaccine development. Two dengue vaccine candidates are currently in phase 2 clinical trials [Hombach, 2007; Durbin & Whitehead, 2010]. The following sections summarise the issues of dengue vaccine development and various vaccines that are currently being investigated.

1.5.2.1 Obstacles to dengue vaccine development

There are several hurdles which have made development of a dengue vaccine more complicated. First, due to the presence of four serotypes of DV, a successful dengue vaccine must be tetravalent to protect against all DV serotypes. However, previous studies have shown that interference between serotypes might occur in the tetravalent formulation of a live vaccine. Additionally, the immunogenicity of either serotype could be dominant such that careful adjustment of the individual components in a tetravalent formulation is required.

Second, the pathogenesis of severe symptoms in heterologous dengue infection is not fully understood. More importantly, there is inadequate evidence to prove that a vaccine candidate is capable for preventing DHF [Chaturvedi et al., 2005]. Administration of a vaccine to populations with previous exposure might not be applicable due to the possibility of ADE phenomena. Hence, further vaccine evaluation is required [Hombach, 2007].

Third, there is an absence of suitable animal models for investigations on the pathogenesis of disease and the evaluation of a vaccine. Severe clinical symptoms which have appeared in humans were not found in animal models of infection. Therefore, during clinical evaluation, post-vaccination responses including reactiogenicity, immunogeneticity and protection induced by a vaccine candidate in an animal model might not be the same in humans [Chaturvedi et al., 2005; Hombach, 2007;].

1.5.2.2 Types of vaccine

Several dengue vaccine candidates are being developed in various stages. Two live attenuated vaccines are in phase 2 clinical trails; another six live vaccine candidates, which are either chimeric or with mutations in their viral genome, are undergoing phase 1-2 or preclinical trials. One recombinant subunit vaccine has been evaluated in preclinical tests [Hombach, 2007].

Apart from the mentioned dengue vaccine candidates, many vaccines have been developed by employing new molecular technologies and different attenuated strategies. Generally, vaccines are simply classified as live or non-live. Live vaccines are preferable since full protection by humoral and cellular immunity is generated in the recipients. Live vaccines can be typical virus particles, mutated or chimeric virus particles or virus-vectored vaccinia virus. Whether the virus particles are typical, mutated or chimeric, attenuation is necessary for live vaccines. Non-live vaccines include inactivated virus, DNA vaccines and recombinant subunit vaccines. Non-live vaccines provide some advantages over live vaccines.

Basically, a successful tetravalent vaccine should achieve a good balance between high immunogenicity and low reactiogenicity, and persistent protection against DV challenge. With the advancement of molecular technology and the application of recombinant DNA technology, more flexible vaccine design can be allied to the development of novel dengue vaccine candidates.

1.5.2.2.1 Live attenuated virus particles

In early vaccine development, whole virus was attenuated by successive passage in tissue culture. Virus particles collected from cell-free medium of tissue culture were purified, attenuated and injected into the host with adjuvant. Live dengue vaccine development began in 1940s. The DEN-1 and DEN-2 serotypes were attenuated by serial passage involving intracerebal inoculation of mice. These vaccines were shown to elicit neutralising antibodies in recipients and they were protected from challenge with wild-type DV [Durbin & Whitehead, 2010].

Currently there are two tetravalent live attenuated dengue vaccines undergoing clinical trails. The two vaccines have been developed by Mahidol University in Thailand with the collaboration of Sanofi Pasteur and by the Walter Reed Army Institute of Research (WRAIR) with the collaboration of GlaxoSmithKline (GSK), respectively [Chaturvedi et al., 2005]. The former employs DEN-1, -2 and -4 viruses attenuated by serial passage in primary dog kidney (PDK) cells, while DEN-3 was passaged in primary green monkey kidney (PGMK) cells [Bhamarapravati & Sutee, 2000]. The vaccine was respectively evaluated in children and adults in Thailand. In 105 flavivirus-naïve child volunteers, two doses were given followed by a booster 12 months afterward. Over

50% tetravalent seroconversion was observed after the second dose and reached 85-100% after the booster [Sabchareon et al., 2004]. The reactiogenicity was mild and acceptable. Since DEN-3 exhibited interference with the other components and was not attenuated, reformulation of the vaccine was evaluated in 7 Thai flavivirus-naïve adults [Sanchez et al., 2006]. However, unacceptably high reactiogenicity was observed in adults which led to the suspension of the development of this vaccine candidate.

Another dengue vaccine developed by WRAIR and GSK was attenuated by serial passages in fetal rhesus lung (FRhL) cells. Since interference was observed when individual monovalent components were combined into tetravalent formulations, adjustments were made to produce several different formulations. One of the formulations was selected and evaluated in seven Thai children aged 6-9 years. The vaccine induced a 100% tetravalent seroconversion rate after the second dose with acceptable reactiogenecity [Edelman et al., 2003].

1.5.2.2.2 Live recombinant virus or vaccinia viral vector vaccines

Live virus undergoes recombination and can be classified into recombinant virus or recombinant vaccinia viral vectors. The former is attenuated by modification or deletion of virulence sequences or genes, making virulence unlikely. Recombinant vaccinia viral vectors are modified by the insertion of foreign genes or sequences of interest from other pathogens into the genome for vaccination. Adenovirus, poxvirus and influenza virus have been incorporated into vaccinia viral vectors [Ellis, 1999].

Since live attenuated virus vaccines replicate inside the host in order to acquire an effective humoral and cellular immune response in recipients, novel vaccines have been developed based on this approach which employs new recombinant biotechnology. This allows for intracellular replication and expression, but exerts minimum risk to the recipient. [Ellis, 1999; Ada, 2001; Eckels & Utnak, 2003]

As mentioned, live vaccines must be attenuated before administration into human recipients. Apart from attenuation by a conventional serial passage strategy, vaccine candidates can be attenuated by introduction of mutations into the viral genome or production of a chimeric virus. A dengue vaccine developed by the National Institutes of Health in the United States incorporated a wild type DEN-4 virus attenuated by the deletion of 30 nucleotides (172-143) in the 3' UTR, known as the Δ30 mutation [Durbin et al., 2005]. This vaccine was found to be immunogenic and had acceptable reactiogenicity in monkeys and in human volunteers. This Δ30 mutation was applied on other dengue serotypes, but DEN-2 and -3 were not attenuated to an acceptable safety level [Blaney et al., 2004a & b]. Therefore, these serotypes were further developed into a chimeric DV. In order to create rDEN2/4Δ30 and rDEN3/4Δ30 chimeric viruses, the non-structural genes of attenuated DEN-4 virus with the Δ30 mutation (rDEN4Δ30) were integrated with the structural genes, PrM and E, of DEN-1, -2 and -3 [Blaney et al., 2004a; Whitehead et al., 2003]. A monovalent vaccine candidate has been evaluated in a phase 1 clinical trail [Blaney et al., 2008; Durbin et al., 2006a&b; Durbin et al., 2005]. Tetravalent formulations consisting of rDEN1Δ30, rDEN4Δ30,

rDEN2/4Δ30 and rDEN3/4Δ30 has been evaluated preclinically in monkeys [Blaney et al., 2005].

Another chimeric vaccine using a similar strategy has been developed. This vaccine was engineered with the structural genes, prM and E, of DEN-1 to -4 into a yellow fever 17D vaccine virus background. Preliminary results of the monovalent and tetravalent formulations were quite promising, as the vaccine was highly immunogenic and successfully induced neutralising antibodies. The vaccine was found to have mild reactiogenicity [Guirakoo et al., 2004 & 2000; WHO, 2006]. Moreover, one of the advanced chimeric vaccine candidate using wide-type DEN-2 16681 virus (PDK-53 strain) as backbone for tetravalent vaccine development has been studied extensively [Hunag et al., 2003; Kinney et al., 1997].

Recombinant vaccinia viral vector vaccines are an alternative type of live vaccine. They are mainly constructed by homologous recombination. Cells are co-transfected with recombinant plasmids containing foreign genes of interest, promoters, vaccinia virus DNA flanking sequences and vaccinia virus helpers. During viral replication, the vaccinia virus DNA undergoes homologous recombination with the flanking vaccinia sequence of the plasmid vector inside the transfected cell, and the recombinant genome which is packaged intracellularly and released as a recombinant vaccinia virus [Broder et al., 1999]. Some features of recombinant vaccinia viral vectors make them useful tools for vaccine delivery. These viruses infect cells efficiently within 10 min, and thus facilitate antigen expression. The ability to directly infect antigen presentation cells (APC) increases

the priming of cytotoxic T lymphocytes (CTL) to induce a specific adaptive immune response.

Several researchers have inserted the DV structural prM and E genes in recombinant vaccinia viruses; however, the immunogenicity of them varied. Some constructs failed to induce neutralising antibodies, but others gave promising results. Two recombinant adenovirus vectors expressed prM and E genes of DEN-1 and -2 or DEN-3 and -4 in mice, and neutralising antibodies of tetravalent seroconversion were elicited after a primary dose [Holman et al., 2007; Raja et al., 2007]. These constructs were also evaluated in monkeys and similar results were demonstrated [Raviprakash et al., 2008].

The possibility of the integration of foreign genes from vaccinia virus into the human genome is the main concern of all vaccines constructed by genetic engineering technology, including DNA vaccines. To minimise this risk, the vaccinia viral vector should be highly attenuated by successive passage and multiple gene deletion of the genome. However, recipients who have previously been exposed to the viral vector due to infections or vaccination will encounter difficulties in transfection, because the vaccine will be eliminated by the pre-existing immunity of recipient. [Srivastava & Liu, 2003]

1.5.2.2.3 Inactivated virus vaccine

Inactivated viruses were the earliest approach for the development of non-live vaccines. Viruses can be inactivated chemically by formalin treatment [Ellis, 1999]. However, inactivated viruses restrict immunogenicity to stimulation

of the humoral immune response, so protection against the vaccinated pathogen is not fully covered. Due to the inability of these viruses to replicate, these vaccines may be administered to immunocompromised recipients [Durbin & Whitehead, 2010].

The U.S. Army has developed an inactivated DEN-2 virus vaccine [Putnak et al., 1996]. The DEN-2 strain S16803 was propagated in Vero cells, concentrated and finally inactivated by formalin. The vaccine candidate was evaluated using a monkey model and neutralising antibodies were induced after a second vaccination.

1.5.2.2.4 DNA vaccine

Live vaccines, including attenuated virus vaccines and recombinant vaccinia viruses, are an effective and successful means of mimicking viral infection, so as to generate full protection to the host [Henke, 2002]. However, drawbacks due to insufficiently attenuated viruses have lead to the development of a novel DNA vaccine technology [Henke, 2002].

Other non-live vaccines such as recombinant subunit proteins or inactivated viruses can only stimulate immune responses in the host through the major histocompatibility complex II (MHC II) pathway. Apart from live vaccines, DNA vaccines are an alternative approach which also induce MHC I-restricted CTL immune responses. DNA vaccines which transcribe inside cells without replication express protein antigens in the cytoplasm which will form a complex and be presented by the MHC I molecule [Corr et al., 1996; Giese, 1998; Ada, 2001;

Gregersen, 2001; Srivastava & Liu., 2003]. Therefore, protection from DNA vaccines is fully covered since they provoke immune responses through both the MHC I and MHC II antigen processing pathways.

DNA vaccines can be naked DNA or plasmid DNA engineered to carry a sequence or gene of interest which is expressed intracellularly as an immunogenic antigen for vaccination. The structures of DNA vaccines are composed of a bacterial plasmid backbone, the foreign viral gene or coding sequence of interest and regulatory elements including strong mammalian promoter or enhancer sequences, an origin of replication, selective marker genes and transcription terminator sequences [Montgomery et al., 1994; Giese, 1998]. Commercial plasmid vectors carrying all these components and a multiple cloning site (MCS) for the insertion of coding sequences or genes of interest are also available [Ertl & Thomsen, 2003].

DNA vaccines can be administered alone without vehicle or with an adjuvant such as in a complex with liposome, attached to gold beads or biodegradable nanoparticles, in virus-like particles or as an aerosol for increased uptake by cells. [Hasan et al., 1999; Srivastava & Liu, 2003] The mechanism of DNA uptake is not fully understood yet, but it has been suggested in previous studies that naked DNA was taken up directly by myofibres [Montgomery et al., 1994]. Zhou & Huang suggested that a DNA-liposome complex vaccine was internalised into the cytoplasm by endocytosis, followed by transferral from endosome to cytoplasm [Zhou & Huang, 1994]. The level of expression of encoded protein antigen in eukaryotic cells is directly related to the efficiency of a

DNA vaccine [Donnelly et al., 2003]. Different routes for administration facilitate plasmid DNA uptake, which varies the expression of the DNA vaccine antigen. The pathways and mechanisms of internalisation are different between DNA vaccines in naked and complex forms. It was discovered the expression of naked DNA is more effective than DNA in complexed forms [Montgomery et al., 1994].

DNA vaccines can be administered by intramuscular injection with a syringe or by particle bombardment with a “gene gun” [Yang et al., 1990; Giese, 1998; Hasan et al., 1999]. Various administration methods are sufficiently potent to prime immune responses by different mechanisms. DNA plasmid vaccines administered through intramuscular injection will prime the CLT response by non-antigen presenting cells (non-APC) by an alternative mechanism [Donnelly et al., 2000]. Plasmid DNA transfects non-APC cells (ie. muscle cells) directly. The antigen expressed by DNA plasmids inside muscle cells is most likely taken up by dendritic cells, and degraded into peptides. The MHC I-peptide complex will then be presented to CTL on the surface of professional bone marrow-derived APC [Giese, 1998]. However, non-APC have restricted efficiency of expression due to indirect cross-priming of immunity by bone marrow-derived antigen presenting cells [Hasan et al., 1999].

For dengue vaccine development, Konishi et al. developed a tetravalent DNA vaccine which consisted of four plasmids expressing prM and E of the four individual serotypes [Konishi et al., 2006]. This vaccine successfully induced neutralising antibodies in mice, and the antibody titre was comparable to those induced by the monovalent formulation. Before that, a plasmid DNA which

expressed prM and full-length E of DEN-1 was evaluated in mice and in rhesus macaques and Aoytus monkeys, and is currently being evaluated in human volunteers in the United States [Kochel et al., 2000; Raviprakash et al., 2000a & b]. Another DNA vaccine focussed on expression of the NS-1 protein, but since antibodies elicited by this vaccine did not have neutralising properties, it prevented immunity complications due to ADE. The vaccine was evaluated in mice which were partially protected from challenge by wild-type DV [Wu et al., 2003; Costa et al., 2007]. It was believed that protection was related to the complement-dependent manner of the Fc portion of anti-NS1 antibodies. In addition, NS1 was demonstrated to elicit strong cellular immune responses for cytotoxic activity [Wu et al., 2003; Costa et al., 2007]. Structural proteins fused with liposome-associated membrane proteins (LAMP) were found to induce protection against DV and other flaviviruses [Dhalia et al., 2009 review]. LAMP is naturally found on the outer membrane of lysosomes [Lippincott-Schwartz & Fambrough, 1986]. Raviprakash and colleagues fused prM and E of DEN-2 with LAMP. prM-E/LAMP fusion proteins and prM-E proteins expressed by DNA plasmids were utilised individually for immunisation of mice. It was discovered prM-E-LAMP elicited seroconversion but prM-E protein alone did not [Raviprakash et al., 2001].

The problem of integration of antigen sequences or genes into the human genome is highly concerning. In accordance with guidelines from the U.S. Food and Drug Administration, sequences homologous to the human genome should be avoided so as to minimise the possibility of integration by homologous recombination [Ertl & Thomsen, 2003]. The induction of allergy or autoimmune diseases by DNA vaccines has also been investigated. A study by Mor et al.

indicated that DNA vaccines are unlikely to induce or accelerate autoimmunity [Mor et al., 1996].

Although safety concerns have not been fully investigated, DNA vaccines indeed provide many advantages over the others. Compared with live attenuated viral vaccines, plasmid DNA is very stable, with no special requirements for storage and delivery, so it would be more appropriate for worldwide distribution, especially in developing countries [Giese, 1998; Srivastava & Liu, 2003]. Plasmid DNA can also be produced readily and inexpensively in large quantities by cell culture [Giese, 1998; Henke, 2002]. Moreover, the commercial availability of DNA plasmids with MCS sequence insertion provides flexibility, hence, facilitates wide application in vaccine design [Hasan et al., 1999; Ertl & Thomsen, 2003; Srivastava & Liu, 2003].

1.5.2.2.5 Recombinant subunit vaccine

DNA vaccines are indeed a good approach for vaccine development. However, safety concerns of the integration of viral sequences into the human genome require further investigation. Recombinant subunit protein vaccines are an alternative providing a proper balance of pros and cons between DNA vaccines and live attenuated vaccines, since live attenuated vaccines in tetravalent formulation may have interference phenomena. At the same time, the balance between the immunogenicity and reactogenicity of live vaccines is difficult to adjust. Recombinant protein subunit vaccines eliminate the problems mentioned above.

E-glycoprotein has been expressed in various hosts, including bacteria, yeast, baculovirus and mammalian cells in extensive studies. The strategy of production of a subunit protein is summarised in chapter 1.5.3. The principle is similar to the production of recombinant vaccinia virus as described in chapter 1.5.2.2.2; the insertion sequence is selected and generated from the immunogenic protein. Expressed recombinant proteins in heterologous host cells are replicated in large amounts and finally purified as a vaccine. [Ellis, 1999]

In early studies of recombinant vaccine candidate, entire or carboxy-terminal (C-terminal) truncated E-glycoprotein was expressed. Generally, due to the hydrophobic property of this protein, the anchor and transmembrane proteins of the carboxy-terminal were removed to increase the solubility and secretion of protein from the expressing host cells. E-glycoprotein was found to be necessary for co-expression with prM to maintain the native structure of conformationally-dependent E-glycoprotein epitopes [Konishi & Mason, 1993]. Previous studies demonstrated that solely expressing E-glycoprotein failed to induce neutralising antibodies. A study which expressed the entire E-glycoprotein in baculovirus without prM was only found intracellularly, and hence failed to elicit neutralising antibodies in mice [Feighny et al., 1994]. Sugrue and co-workers expressed DEN-1 E protein with truncation at the C-terminal in *E.coli*. Large portions of the protein were expressed as insoluble proteins. A small portion expressed in soluble form was found to be highly sensitive to degradation by a bacterial protease. Antiserum raised by this protein in rabbits did not show neutralisation of DV [Sugrue et al., 1997]. More recently, a study evaluated and compared two non-live vaccines and one live attenuated vaccine candidate in

rhesus macaques monkeys [Robert Putnak et al., 2005]. Another non-live vaccine using recombinant proteins consisted of E and prM (r80E). It was found that a high level of neutralising antibodies was induced from r80E and able to protect against challenge with wild-type DEN-2.

The envelope domain 3 (ED3) protein was recognised as an immunoglobulin-like structure for cell adhesion, which can elicit neutralising antibodies without the presence of domains 1 and 2 of the envelope. Since the molecular weight of a protein is directly related to its solubility [Koschorreck et al., 2005] and expression yield [Zhang et al., 2007], ED3 instead of the whole E protein was expressed and investigated. Several studies showed that ED3 was a potential candidate for vaccine design against DV. Chiu & Yang expressed ED3 of DEN-2 in *E.coli* to block infection by DEN-2 in BHK-21 cells [Chiu & Yang, 2003]. Antibodies against the ED3 proteins of DEN-1 & -2 inhibited virus entry into the human hepatocyte cell line HepG2 and mosquito C6/36 cells. Meanwhile, polyclonal antiserum raised from BALB/c mice after ED3 immunisation was able to completely neutralise dengue infection of the same serotype; however, heterologous protection was not achieved [Chin et al, 2007]. Chen and colleagues produced a recombinant DNA fragment which contained ED3 of four serotypes in a single construct and proteins were then expressed into a single polypeptide. This polypeptide successfully elicited tetravalent seroconversion in mice, but protection from the DEN-3 serotype was not guaranteed [Chen et al., 2007].

Apart from the abovementioned subunit vaccine candidates, many ED3 proteins were generated in other studies, but not all of them were able to induce neutralising antibodies. Most of them were expressed in the form of insoluble inclusion bodies so the folding of protein might not be maintained during the refolding process [Khanam et al., 2006; Chen et al., 2007; Zhang et al., 2007]. Moreover, epitope mapping of ED3 has been carried out for two decades. It has revealed a small shift of the nucleotide sequences for peptide production leading to a significant difference in neutralising ability [Roehrig, 2003]. As mentioned, co-expression of prM protein was found to be necessary for maintaining the native structure of E-glycoprotein [Konishi & Mason, 1993]. However, the interaction between prM and ED3 has not yet been investigated. Therefore, co-expression of prM and ED3 could be the next step for these investigations. In summary, there is no protein subunit vaccine candidate that is currently being evaluated in clinical trials.

Despite full-length E or ED3 having the ability to induce seroconversion in mice, their performance varied between studies. This may be related to the strategies of protein production including: 1) the terminal sequences of the DNA fragment, 2) the fusion tag and linker proteins attached and 3) the expression system utilised which is directly related to post-translational modifications and native folding of the protein. These factors will be discussed in the following chapter.

In summary, since little knowledge on the mechanisms of human immunity against DV in the past decades have contributed to preventing effective vaccine

development., Recombinant DNA techniques render vaccines not only for prevention against infection, but also for therapeutic strategies. However, a dengue vaccine unable to provide full protection against all serotypes can increase the risk of more serious symptoms due to the ADE phenomenon. Meanwhile, safety problems with DNA-based and recombinant vaccines are an issue which has aroused public concern. No evidence has been found for the integration of foreign genes from viral pathogens or viral vectors into the human genome, but this uncertainty becomes a barrier against production of licensed DNA or recombinant vaccines for therapeutic purpose.

1.5.3 Strategies for the production of recombinant proteins and polyclonal antibodies by immunisation

In the present study, recombinant DNA technology was used for the production of recombinant proteins to hopefully be used as vaccine candidates. The following sections will introduce the major steps and various strategies involved in the production of recombinant proteins and polyclonal antibodies. It aims to give a better understanding of the particular strategies used in the present study, and which will be described in Chapter 2.2.

1.5.3.1 Production of recombinant proteins

1.5.3.1.1 Construction of recombinant DNA

Recombinant DNA is required prior to recombinant protein production. In principle, particular genes coding for proteins of interest are isolated and incorporated into to an expression system. The extracted mRNA contains the gene of interest and is then reverse transcribed into complimentary DNA (cDNA). High

fidelity *Taq* polymerase that provides 3' to 5' end proofreading activity is utilised to reduce the error rate during PCR amplification.

Prior to designing the plasmid constructs, it is necessary to choose the N- and C-terminal boundaries for the recombinant protein. Since the terminal of the protein is highly related to the solubility of the protein after expression, there are some general guidelines for choosing the boundaries of the recombinant protein. In general, membrane-spanning and hydrophobic regions should be avoided. The region which belongs to the globular domains and form the secondary structure should not be disrupted [Structural Genomics Consortium et al, 2008]. To avoid those unwanted protein regions, production of truncated forms of protein may be required for some cases. Many online tools (ExPASY, PHYRE protein fold recognition server, PSIPRED protein structure prediction servers, STRAP free computer program) are available for prediction of the hydrophobic regions, epitopes or domains as well as the three dimensional structure of the protein. More importantly, in-frame reading of the nucleotide sequences is a critical point for successful expression of the protein of interest.

1.5.3.1.2 Cloning of recombinant DNA into *E. coli* cells

Cloning can be carried out in a number of host cells, such as bacteria, viral, yeast, insect, plant and mammalian cells. The choice of host cell depends on the expression system utilised for the protein of interest. *E. coli* is the most common host cell for recombinant protein expression due to easy, efficient and economical of cultivation as well as the well-characterised genome [Sledjeski & Gares, 1998; Walsh, 2002]. Whether the recombinant protein is prokaryotic or eukaryotic, the

first attempt of protein expression should be performed in *E. coli* [Structural Genomics Consortium et al, 2008]. More detailed information on *E. coli* expression systems will be discussed in the following section of this chapter. Brief details on baculovirus-mediated insect cell expression and other expression systems will also be covered.

A vector is a vehicle for carrying the recombinant DNA or gene of interest. The structural features of a plasmid vector must consist of an origin of replication site (*ori*), elements for transcription and translation, a cloning site or multiple cloning sites for introduction of foreign DNA and genes for selection [Balbas & Bolivar, 1998]. The genes for selection are mainly antimicrobial resistance genes [Sørensen & Mortensen, 2005a]. A powerful promoter system is an essential element for transcription as it controls the transcription of the plasmid vector by the inducer. Plasmid vectors carrying the recombinant constructs are finally transfected by means of electroporation or calcium chloride-mediated methods [Sambrook & Russell, 2001a].

Expression systems can be simply classified into prokaryotic and eukaryotic systems. The *E. coli* expression system is the most commonly used prokaryotic system. In the *E. coli* expression system, pET is a common plasmid vector for heterologous protein expression involving a T7 promoter system. Expression of the pET plasmid relies on the *E. coli* host cell that harbours a DE3 lysogen which is a DNA fragment of lambda phage. This DNA fragment is responsible for encoding T7 RNA polymerase under the control of the *lacUV5* promoter. Introduction of the chemical inducer isopropyl-β-D-thiogalactoside

(IPTG) into the cultivation medium can drive the *lacUV5* promoter, so as to trigger the transcription of the T7 RNA polymerase [Søresen & Mortensen, 2005a]. Since the T7 promoter is not recognised by *E. coli* RNA polymerase, only T7 RNA polymerase can trigger the protein expression of the plasmid vector. However, low-level expression of T7 RNA polymerase from plasmids can drive recombinant protein expression in the absence of the inducer. This problem can be overcome by co-expression with T7 lysozyme or by adding 1% glucose to the cultivation medium during expression [Søresen & Mortensen, 2005a]. The *E. coli* strains for protein expression are mainly BL21(DE3) and its derivatives. These strains are characterised by their deficiency of two proteases, *lon* and *ompT*, which protect the expressed protein from degradation [Søresen & Mortensen, 2005a].

Baculovirus-mediated insect cell expression systems have been used in many laboratories for protein production in the past [Hunt, 2005]. This is a eukaryotic system for expression with post-translational protein modification. This expression system makes use of a helper-independent virus, baculovirus, for carrying the gene of interest and high levels of propagation in culture medium. The baculovirus expression system is currently commercially available [Invitrogen, Novagen, Merck Bioscience and BD Biosciences], and some systems allow direct transfection of recombinant plasmids into insect cells without the help of transfer virus. These systems offer more rapid and efficient protein expression and purification procedures than ordinary baculovirus expression systems. However, due to the more complicated nature of this system compared to the *E. coli* expression system, more resources including labour and time are required and multi-parallel expression may not be achieved [Hunt, 2005].

There are other expression systems including yeast, fungi, plants, mammalian cells and even transgenic animals that can be a source for heterologous protein expression. However, the relatively low expression level is not comparable to the *E. coli* system. The post-translational modifications may not be identical to those of higher eukaryotic systems, so expression of recombinant proteins from animals may not be suitable [Shatzman, 1998; Walsh, 2002]. For mammalian protein expression, pcDNA vectors and the high expression cell lines CHO and HEK 293 are frequently used [Hunt, 2005]. However, it is worth considering that the complexity of these higher eukaryotic systems increase the difficulty of protein production due to highly labour intensive, time consuming and technically demanding criteria.

1.5.3.1.3 Protein expression

Protein expression conditions vary for different proteins and expression systems. Time course, temperature and aeration during induction, the concentration of inducer and the conditions of cultivation medium can be optimised [Søresen & Mortensen, 2005b]. Further optimisation is necessary for large scale production since these conditions can be different from those for small scale production [Hunt, 2005].

Expression with fusion tags can improve the solubility of proteins. Glutathione S-transferase (GST) is one of the most popular affinity tags for enhancing soluble protein expression. Maltose binding protein (MBP) will also increase the solubility of protein, however, its large size (40kDa) may adversely affect the nature and hence the activity of the recombinant protein [Structural

Genomics Consortium et al., 2008]. Ubiquitin and small ubiquitin-like modifier (SUMO) are other fusion partners to improve solubility, but they cannot facilitate downstream protein purification as GST and MPB tags can [Hunt, 2005]. Apart from using parent strain BL21(DE3) for expression, *E. coli* mutant strains such as C41 (DE3) and C43 (DE3), can be used instead to improve soluble protein expression [Søresen & Mortensen, 2005b].

1.5.3.1.4 Recombinant protein purification

Based on the expression system used for protein production, protein can be harvested from culture supernatants in mammalian systems or by extraction from cell lysis in bacterial systems. Mostly, cells can be disrupted by mechanical means, such as repeating freeze-thaw cycles, needle and syringe aspiration and sonication. Enzymatic digestion by lysozyme is employed for complete cell lysis in some cases. The principles for protein purification are based on protein size or shape, charge, hydrophobicity and the affinity tag fused with the protein. Protein can be purified by chromatography or gel filtration according to the above protein properties. Subsequent purification with more than one type of chromatography may be required for high purity [Anonymous, 2007].

Protein expression with a fusion tag is used not only for improving the solubility of recombinant proteins, but also as an efficient tool for purification. Hexahistidine (6xHis) and GST are frequently used affinity tags which facilitate protein purification. The size of the 6xHis tag is the smallest among all affinity tags, so it rarely affects the nature and characteristics of the protein; therefore, removal of the fusion tag from the recombinant protein is not necessary. The 6xHis

tag can be purified by immobilised metal affinity chromatography (IMAC) using Ni^{2+} ions which show the strongest binding affinity with the nitrilotriacetic acid ligand or Sepharose matrix [Arnau et al, 2006]. Protein purification systems are available for small scale manual methods to the fully automatic ÄKTA system [Anonymous, 2007]. Spin-columns or columns in combination with a syringe or peristaltic pump are sufficient for protein purification in the microgram to milligram scale. The automatic ÄKTA design chromatography system is a robust and convenient system. It allows for real-time monitoring of the purification and multiple chromatography steps with the additional system unit [Anonymous, 2007].

1.5.3.1.5 Protein characterisation

After purification, all protein fractions collected during elution are analysed by denaturing SDS-PAGE or Western blotting, or by mass spectrometry if necessary. The total protein concentration can be quantified using the Bradford protein assay [Bradford, 1976]. The protein's native activities can be evaluated by ELISA with specific antibodies [Abhyankar et al., 2008]. In recombinant proteins with failed native function restoration, refolding experiments may be required.

The production of recombinant proteins involves tedious steps. There is no particular protocol for producing all recombinant proteins, because many aspects depend on the nature of the protein. The critical point for successful production is choosing the most suitable tool from the vast array of available tools for producing the protein of interest.

1.5.3.2 Production of polyclonal antisera by animal immunisation

1.5.3.2.1 Types of antibodies

Antibody preparation can be either monoclonal or polyclonal. Production of polyclonal antibodies is faster and cheaper than for monoclonal antibodies, but the specificity of polyclonal antibodies is lower due to the mixture of antibodies. Purified protein as an immunogen is injected into an animal host to stimulate the production of polyclonal antibodies [Ladyman & Ritter, 1995].

1.5.3.2.2 Host organisms

Antibodies can be raised in several animals, of which rabbits, goats and mice are the commonly used [Hanly et al., 1995]. Normally, rabbits at an age of four to six months are suitable for inducing antibodies, and the half sandy lop and New Zealand white species are frequently used [Green & Manson, 1992]. For industrial scale production, such as antibody production for passive immunisation, large animals including horses and humans have been used as antibody-producing sources [Walsh, 2002].

1.5.3.2.3. Immunisation and boosting schedules

For polyclonal antibody production, the route of immunisation is mainly subcutaneous, but intraperitoneal, intramuscular and intradermal routes can also be chosen [Hanly et al., 1995; Ladyman & Ritter, 1995]. An adjuvant is required for administration together with the protein immunogen. It increases immunogenicity of the immunogen and allows slowly release the immunogen in small amount for a period of time after administration [Ladyman & Ritter, 1995]. There are several choices of adjuvant, where Freund's adjuvant (Complete and Incomplete) is the

most common one and has been widely used for more than five decades. TitreMax and Ribi Adjuvant Systems are other choices of commercially available adjuvants [Cooper & Paterson, 1995]. In general protocols, the intact protein or peptide conjugated with the carrier are mixed thoroughly with Complete Freund's adjuvant and injected into the animal as a priming immunisation, but Incomplete Freund's adjuvant which does not contain killed mycobacteria is used in the boosting emulsion [Ladyman & Ritter, 1995]. Boosting is provided two weeks after the priming immunisation and is continued for 2-3 times at three to four week intervals. Further boosting may be required for a weak immunisation response [Carter, 2003].

1.5.3.2.4 Detection and purification of antibodies

For producing polyclonal antibodies, serum samples are collected from animals about seven to ten days after the last injection. Anti-protein/peptide antibodies can be screened by the Ouchterlony diffusion technique, ELISA or Western blotting [Green & Manson, 1992; Reen, 1994]. Purification of specific antibodies can be achieved by affinity-based methodologies. Further purification such as gel filtration can be used to increase purity to over 95% [Roque et al., 2007]. Neutralising potential of the antisera can be evaluated by a plaque reduction neutralisation assay with intact virus. It is believed that the application of antibodies will be more extensive in the near future for prevention and therapeutic purposes.

In summary, the background information of dengue infection and the techniques utilised in the present study were briefly reviewed. The detailed

experimental procedures will be described in Chapter 2 (Materials & Methods), followed by Chapter 3 (Results) and Chapter 4 (Discussion).

1.6 Aims of the study

In the present study, we investigated the development of diagnostic tools, the development of a vaccine candidate and the epidemiology of DV and its vectors in Hong Kong. This study consists of four parts:

PART 1: The development of a rapid one-step molecular diagnostic PCR assay based on T_m and colour multiplexing for simultaneous detection and serotyping of DV. Apart from diagnostics purpose, the assay was further modified to be cost-effective; it will be applied for surveillance purpose on vector and general population. The validation of the assay will be performed with clinical virus strains isolated from Hong Kong, Mainland China, and Brazil.

PART 2: Generate a soluble recombinant protein consisting of the membrane precursor (prM) and envelope domain 3 (ED3) of DV in an *E. coli* expression system. Individual prM and ED3 proteins will be generated as well for comparison purposes. Their ability to elicit neutralising antibodies by rabbit immunisation will be assessed by mean of inhibitory recombinant subviral particles (RSPs) binding assay. The recombinant proteins will also be used to develop a serological diagnostic assay in an inexpensive and non-biohazardous way.

PARTS 3 and 4: Conduct two individual epidemiological studies of DV infection including the distribution and DV infection rate of mosquito vectors as well as

seroepidemiology in the general population of Hong Kong. Vector surveillance will be investigated by field-caught mosquito sampling from 15 geographic locations in Hong Kong. The DV infection rate of mosquito will be detected using a conventional RT-PCR assay and the in-house RT-PCR assay developed in PART 1, while seroepidemiology will be studied using a commercial ELISA assay kit.

CHAPTER 2

MATERIALS & METHODS

2.1 Development of a rapid molecular RT-PCR assay for simultaneous detection and serotyping of DV

2.1.1 Assay design

2.1.1.1 Colour and melting temperature multiplexing

Two strategies offered by the LightCycler were applied for developing a one-step assay for detecting and typing DV. The first strategy (Figure 2.1.1 A) was to increase the T_m of an amplicon by means of a primer tagged with a GC tail (5′-CGC GCC GGC CGC GG-3′), which allowed for distinguishing between DEN-2 and DEN-4 in channel F1 (T_m multiplexing). The second strategy (Figure 2.1.1B) used to induce fluorescence resonance energy transfer (iFRET) to detect DEN-1 in channel F2 and DEN-3 in channel F3 (colour multiplexing). Fluorogenic primers specific to DEN-1 and DEN-3 were incorporated at the ends of the amplicons by means of the PCR. The principle of generation of a fluorescence signal from an iFRET-excited fluorophore is illustrated in Figure 2.1.2. SYBR Green I (SGI) molecules [Molecular Probes/Invitrogen] intercalating with the duplex amplicons acted as an energy donor for the acceptor fluorophores tagged to the primers. As a result, light with a longer wavelength was emitted from the excited acceptor fluorophore. During melting-curve analysis, the progressive rise in temperature diminished the interaction between SGI and the acceptor fluorophore as the duplex amplicons dissociated. A dramatic reduction in the fluorescence signal occurred at the T_m of the amplicon.

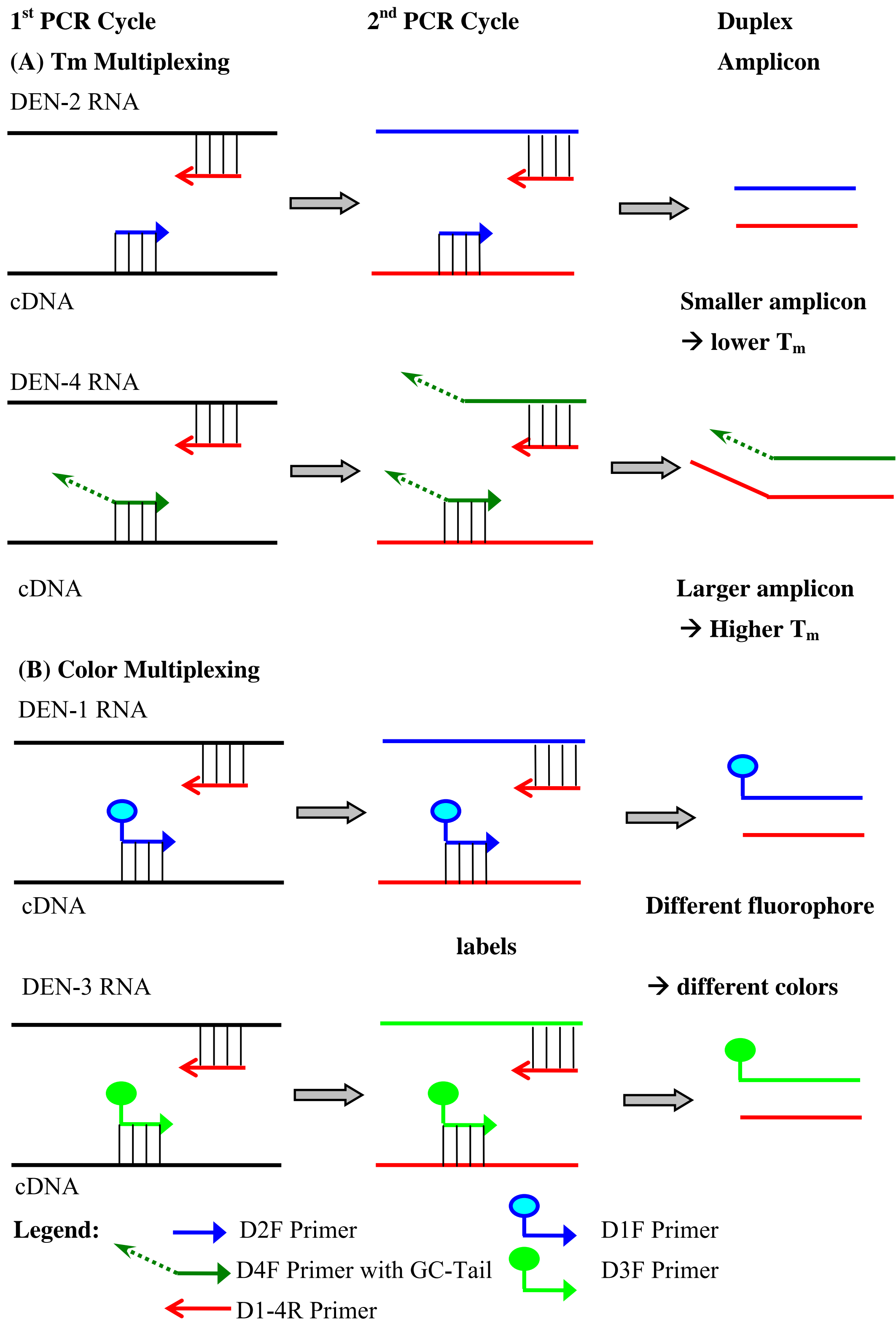


Figure 2.1.1 T_m and colour multiplexing detection strategies for the one-step RT-PCR LightCycler assay.

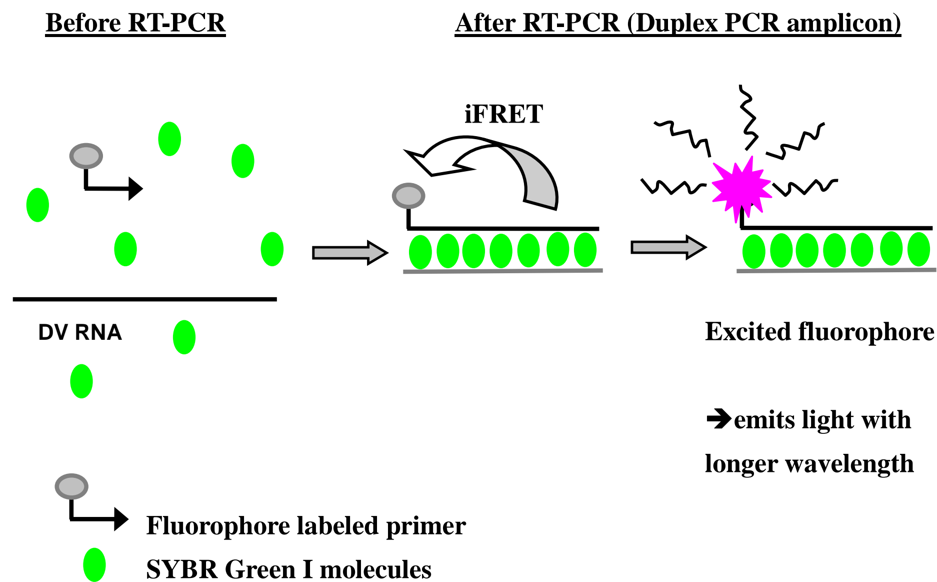


Figure 2.1.2 Generation of fluorescence signal from iFRET excited fluorophore.

2.1.2 Primer design

2.1.2.1 Genome alignment

Complete genome sequences of DV (n = 112) from GenBank were retrieved and aligned with MAVID [Bray & Pachter, 2004]. The moderately conserved 3′ untranslated region (3' UTR) was selected for primer design (Figure 2.1.3A) with OLIGO software [Molecular Biology Insights].

(A)

```
                            Forward Primer Region
DEN-1 AY277666  10560 AA-CACCAGG GGAAGCTGTA CCTTGGTGGT AAGGACTAGA GGTTAGAGGA GACCCCCCGC 10618
DEN-2 AY858035  10549 AAGGTGAGAT G-AAGCTGTA GTCTCACTGA AAGGACTAGA GGTTAGTGGA GACCCCCCC- 10606
DEN-3 AB189128  10533 GAGCACTGAG GGAAGCTGTA CCTCCTTGCA AAGGACTAGA GGTTAGAGGA GACCCCCCGC 10592
DEN-4 AF326573  10475 GAAGCCAGGA GGAAGCTGTA CTCCTGGTGG AAGGACTAGA GGTTAGAGGA GACCCCCCCA 10534
                       *          * ********            ********** ****** *** ********
                                                        Reverse Primer Region
DEN-1 AY277666  10619 ACAACAACAA ACAGCATATT GACGCTGGGA GAGACCAGAG ATCCTGCTGT CTCTACAGCA 10678
DEN-2 AY858035  10607 GAAATAAAAA ACAGCATATT GACGCTGGGA AAGACCAGAG ATCCTGCTGT CTCCTCAGCA 10666
DEN-3 AB189128  10593 AAA--CAAAA ACAGCATATT GACGCTGGGA GAGACCAGAG ATCCTGCTGT CTCCTCAGCA 10650
DEN-4 AF326573  10535 ACA--CAAAA ACAGCATATT GACGCTGGGA AAGACCAGAG ATCCTGCTGT CTCTGCAACA 10592
                         *   * ** ********** **********  ********* ********** ***  ** **
```

(B)

Primer sequence alignment (5'→ 3')

DEN-1

D1F	Bo-GGAAGCTGTACCCTGGTGGT	No. of strains with the sequence (%)
	............T.......	16/31 (52)
	....	11/31 (36)
	.A........T.........	1/31 (3)
	.A..................	1/31 (3)
	..........T.........	1/31 (3)
	...T................	1/31 (3)

DEN-2

D2F	GAGATGAAGCTGTAGTCTC**I**CTG	
	...................A...	27/52 (52)
	...................G...	23/52 (44)
	A..................G...	1/52 (2)
	...................G..T	1/52 (2)

DEN-3

D3F	Cy-AGGGAAGCTGTACCTCCTTGCA	No. of strains with the sequence (%)
	....	24/25 (96)
	..........C...........	1/25 (4)

DEN-4

D4F	Tail-GAAGCCAGGAGGAAGCTGT**G**CT	
	..................A..	4/4 (100)

DEN-1 ~ DEN-4

D1-4R	GAGACAGCAGGATCTCTGGTC	
	....	108/112 (96)
	..A..................	1/112 (1)
	.....G...............	1/112 (1)
	...........G.........	1/112 (1)
	.................T...	1/112 (1)

Figure 2.1.3 Primer design for the 1-step RT-PCR LightCycler assay.

(A), alignment of amplified regions of DEN-1 to DEN-4 and positions of forward and reverse primers. Underlined sequences indicate the primer regions, and asterisks indicate the bases conserved among the 4 serotypes. (B), alignment of primer regions of all primers. **Bo** indicates the BODIPY 630/650 fluorophore; **I**, deoxyinosine base; **Cy**, Cy5.5 fluorophore; **Tail**, the GC tail (5′-CGC GCC GGC CGC GG-3′). There are 112 aligned DV genome sequences: 31 DEN-1, 52 DEN-2, 25 DEN-3, and 4 DEN-4. The numbers of viral sequences perfectly matching or possessing 1–2 mismatches with the respective forward primers (D1F to D4F) and the common reverse primer D1-4R, their proportions, and the percentages within each serotype are indicated to the right of the aligned sequences. In all forward primers, the 8 bases (AAGCTGTA) highly conserved in dengue viral genomes are underlined. Of the 112 aligned genome sequences, only 1 DEN-1 and 1 DEN-3 genome differ from this consensus sequence (by a single base). In the D4F primer, the boldface **G** base (near the 3′ end of the primer and the 3′ base of the 8-base consensus sequence) is a deliberate mismatch with the genome sequence to enhance the DEN-4 specificity of this primer. Most (96%) of the genome sequences show a perfect match with the common reverse primer, and only 4 genome sequences (4%) show a single mismatch.

2.1.2.2 PCR Primers

All primers were purchased from Integrated DNA Technologies. The four serotypes were amplified with four serotype-specific forward primers and a common DV-specific reverse primer (Table 2.1.1 and Figure 2.1.3B). Forward primers for DEN-1 and DEN-3 were labeled at the 5′ end with BODIPY 630/650 and Cy5.5, respectively. Deoxyinosine was introduced in the DEN-2 forward primer (D2F) to overcome the genotypic variations within DEN-2. A GC tail was added to the 5′ end of the DEN-4 forward primer (D4F) to give a product T_m that was higher than that of DEN-2 (predicted difference, 3.6 °C; Table 2.1.1). Because 9 of 10 consecutive nucleotides upstream of the 3′ end of the D4F primer were identical among all 4 DV serotypes (marked by asterisks in Figure 2.1.1A), we applied a simple strategy of sequence-specific PCR for accurate typing of DEN-4 to minimise nonspecific annealing of the D4F primer to non–DEN-4 templates. The specificity of the D4F primer was enhanced with a deliberately destabilising mismatched G base close to the 3′ end of the primer [Newton et al., 1989]. This change ensured that only DEN-4 was amplified under stringent PCR conditions. The amplicons were between 103 bp and 111 bp in length (Table 2.1.1). A BLAST analysis demonstrated all primers to be specific for DV.

Table 2.1.1 Primer and amplicon characteristics of the 1-step RT-PCR LightCycler assay.

Serotype	Primer sequence (5′-3′)[a]	Positions (accession no.)	Amplicon: Size, bp	Amplicon: Predicted T_m, °C[b]	Amplicon: Observed T_m, °C[c]
DEN-1	D1F: **Bo**-GGA AGC TGT ACC CTG GTG GT	10569-10588	103	84.4	83.3 (0.5), 0.6%
	D1-4R: GAG ACA GCA GGA TCT CTG GTC	10651-10671			
		(AY277666)			
DEN-2	D2F: GAG ATG AAG CTG TAG TCT C**I**C TG	10554-10639	105	82.4	81.0 (0.5), 0.6%
	D1-4R: GAG ACA GCA GGA TCT CTG GTC	10639-10659			
		(AY858035)			
DEN-3	D3F: **Cy**-AGG GAA GCT GTA CCT CCT TGC A	10541-10562	103	84.0	83.9 (0.2), 0.3%
	D1-4R: GAG ACA GCA GGA TCT CTG GTC	10623-10643			
		(AB189128)			
DEN-4	D4F: **Tail**-GAA GCC AGG AGG A AG CTG T**G**C T	10475-10496	111	86.0	85.7 (0.2), 0.2%
	D1-4R: GAG ACA GCA GGA TCT CTG GTC	10565-10585			
		(AF326573)			

[a] Note that the reverse primer, D1-4R, was common for all serotypes, although the base positions were different in the genome sequences of different serotypes. Refer to Figure 2.1.3 legend for details.
[b] Predicted amplicon melting temperatures (T_ms) were obtained with OLIGO software.
[c] Data are presented as the mean (SD), CV. Observed amplicon melting temperatures (T_ms) were the mean of 8 readings measured in separate runs. The T_ms were obtained from channel F1 for DEN-2 and DEN-4, channel F2 for DEN-1, and channel F3 for DEN-3. Although not used for typing, T_ms were also obtained from channel F1 for DEN-1 (mean, 83.1 °C; SD, 0.3 °C; CV, 0.4%), and for DEN-3 (mean, 82.9 °C; SD, 0.2 °C; CV, 0.2%).

2.1.3 One-step RT-PCR assay

2.1.3.1 Commercial reagent kit assay

The RT-PCR assay in this study was performed with the LightCycler RNA Amplification Kit HybProbe [Roche Diagnostics] in glass capillaries in a LightCycler 1.5 System. The 20 μL reaction mixture contained the following components: 1× Roche LightCycler RT-PCR reaction mix HybProbe, 5 mM $MgCl_2$, 0.5 μM D1-4R primer, 0.4 μM D1F primer, 0.3 μM each of D2F and D3F

primers, 0.5 μM D4F primer, 1× SGI, and 0.5 μL RNA sample. The 1× SGI concentration used was as suggested in the protocol of the LightCycler RNA Amplification Kit SYBR Green I. Reaction conditions were as follows: a reverse transcription step at 55 °C for 30 min and denaturation at 95 °C for 1 min, followed by 45 PCR cycles of 95 °C for 10 s, 59 °C for 15 s, and 72 °C for 20 s. The assay ended with a melting-curve analysis: heating to 95 °C without a hold, rapid ramping to 59 °C and holding for 30 s, gradual heating to 95 °C at 0.1 °C/s and final cooling to 40 °C. The identities of the specific products were confirmed by agarose gel electrophoresis and DNA sequencing with an ABI 3130 Genetic Analyzer [Applied Biosystems]. Sequence analysis is described in Chapter 2.1.3.3.

2.1.3.2 In-house developed assay

An in-house assay was also developed based on modification of the kit-based assay of Chapter 2.1.3.1 with reduced costs for detecting and typing of DV. This cost-effective assay could be applied on vector surveillance and large scale screening of suspected cases. The assay was validated with clinical sera collected from three different geographical regions.

The assay strategy and primer design were the same as described in Chapter 2.1.1 & 2.1.2. In the in-house RT-PCR assay, the LightCycler RNA Amplification Kit HybProbe [Roche Diagnostics] was replaced with an in-house PCR cocktail. The assay was performed on the same LightCycler 1.5 System as the commercial reagent kit assay. The 20 μL reaction mixture consisted of 1×PCR reaction buffer [Roche Diagnostics], 4 mM $MgCl_2$, 0.3 mM of each dNTP [Invitrogen], 0.5 μM D1-4R primer, 0.4 μM D1F primer, 0.3 μM each of D2F,

D3F and D4F primers, 1× SGI [Molecular Probes], 0.25 U/μL MultiScribe reverse transcriptase [Applied Biosystems], 0.4 U/μL RNase inhibitor [Applied Biosystems], 2U FastStart Taq polymerase [Roche Diagnostics], 10 mg bovine serum albumin [New England Biolabs], 5% (v/v) dimethyl sulfoxide (DMSO) [Sigma Aldrich] and 1 μL RNA sample. Reaction conditions were as follows: a reverse transcription step at 52 °C for 30 min, inactivation of reverse transcriptase and denaturation at 95 °C for 10 min, followed by 45 cycles of 95 °C for 5 s, 59 °C for 15 s, and 72 °C for 20 s. Upon completion of the PCR cycles, melting-curve analysis was performed as described in Chapter 2.1.3.1. Four DV serotypes were detected by the corresponding detection channels according to the emitted fluorescence from SGI or the fluorogenic primers. Specific products were confirmed by agarose gel electrophoresis and DNA sequencing with an ABI 3130 Genetic Analyzer [Applied Biosystems]. Sequence analysis of the PCR products is described in Chapter 2.1.3.3.

2.1.3.3 Cycle sequencing and sequence analysis of PCR products

Prior to cycle sequencing, the PCR products were purified by digestion of excessive primers and dNTPs. Five μL of each PCR product was mixed with 40U shrimp alkaline phosphatase and 1U *ExoI* endonuclease. This was incubated at 37°C for 30 min, then enzymes were inactivated at 80°C for 20 min. The reaction mixture was kept on ice until further processing. The purified PCR product was mixed with 2 μL BigDye Terminator Ready Reaction Mix V1.1 [Applied Biosystems] and 1.6 pmol of primer. The reaction tube containing a total volume of 10 μL reaction mixture was heated at 95°C for 5 min, then followed by 35 cycles of 96°C for 30 s, rapid thermal ramp to 50°C for 15 s and 60°C for 4 min.

The DyeEx 2.0 spin kit [Qiagen] was used to remove the unincorporated dye terminators from the sequencing reactions according to the manufacturer's instructions. DNA was precipitated by vacuum drying for 15 min in a SpeedVac [Savant Instruments Inc, N.Y.], and then resuspended in 15 μL of Hi-Di™ formamide solution [Applied Biosystems]. This was followed by incubation at room temperature (RT) for 10 min in the dark. Before loading samples into the sequence analyser, DNA was denatured at 95°C for 3 min followed by quick cooling on ice. DNA sequence analysis was performed on a ABI 3130 Genetic Analyzer [Applied Biosystems].

2.1.4 Determination of assays performance

Total of eight RNA samples of known DV serotype (n=2 for each serotype) and one RNA sample of a Japanese encephalitis virus strain provided by the Public Health Laboratory Centre (PHLC) of the Hong Kong Special Administrative Region (HKSAR) were used as control (Table 2.1.2). The DV RNA samples were extracted from cell culture supernatants by the Qiagen QIAamp viral RNA extraction reagent kit [Qiagen]. Viral titres for DEN-1, DEN-2, DEN-3, and DEN-4 were 3.27×10^6, 2.10×10^8, 1.63×10^7, and 1.16×10^8 plaque-forming units (PFU)/L, respectively. Only one of each DV serotype (n=1 for each serotype) were provided as control for in-house assay performance determination (Table 2.1.2).

The detection limit of each serotype was determined using 10-fold serial dilutions of the RNA samples. Four replicates were run for each dilution. T_m of the in-house assay was compared with the original kit-based assay using a paired t-test.

Table 2.1.2 Viral strains analysed with the 1-step RT-PCR LightCycler assay.

Virus	Serotype	No. of strains extracted			
		Viral culture		Clinical sera	
		Kit-based (n=9)	In-house (n=5)	Kit-based (n=16)	In-house (n=12)
Dengue	DEN-1	2	1	4	2
	DEN-2	2	1	4	3
	DEN-3	2	1	4	3
	DEN-4	2	1	4	4
Japanese encephalitis	—	1	1	—	—

2.1.5 Clinical serum samples for assays validation

Sixteen DV-positive and typed clinical sera (DEN-1 to DEN-4) were provided by PHLC of the HKSAR for kit-based assay validation (Table 2.1.2). Of these sixteen samples, only twelve were used for in-house assay validation due to the limited sample volume available.

More clinical sera were collected from two other geographical regions for in-house assay validation. Thirty-six DV-positive clinical sera were collected from the cities of Aracaju and Goiãnia in Brazil. Serotypes and viral load of the samples from Goiãnia were provided, but only serotype information was available for the samples from Aracaju. Viral load of the samples varied from 3.82×10^7 to 1.30×10^{13} RNA copies/L (Appendix I). In addition, 125 clinical sera from suspected dengue cases were collected from Guangzhou, China, from 2006 to 2008. These samples were collected within day 1-10 of symptom onset. Anti-DV IgM results were available for 88 samples (Appendix I). Sixty-two samples were IgM-positive and 26 samples were IgM-negative confirmed by either ELISA or

immunochromatography (IC) assay. Of these 26, 22 were IgG-negative and four was IgG positive. Twenty out of these 26 were collected second samples after three to six days from the first sample for IC assay re-confirmation in the Guangzhou hospital. In 125 samples, there were total seven cases finally confirmed as a non-dengue diagnosis. The RNA samples were extracted from sera using the QIAmp viral RNA extraction kit [Qiagen].

2.1.6 Assay validation by a conventional nested RT-PCR assay

Clinical serum samples were detected for DV RNA with the kit-based assay or in-house assay and a widely-used conventional nested RT-PCR assay developed by Lanciotti and co-workers [Lanciotti et al., 1992] with slight modification. Difference in the detection rates between the two assays was compared using McNemar's test. Moreover, two important values, the positive predictive value (PPV) and negative predictive value (NPV), for evaluation of diagnostic method were determined. PPV and NPV represent the proportion of patients with positive / negative results are correctly diagnosed. With reference to the results of the conventional nested RT-PCR assay, PPV and NPV were calculated respectively on evaluation of clinical sera collected from Hong Kong and Brazil and screening of clinical sera from China.

The conventional nested RT-PCR assay consisted of two rounds of PCR. The first round of RT-PCR detected the presence of DV by consensus primers (D1-D2), and the second round of PCR identified the serotype by DV serotype-specific primers (TS1-TS4). Primer sequences are shown in Table 2.1.3. Reverse transcription of RNA samples was performed separately prior to PCR. A

20 μL volume for the reverse transcription reaction contained 1×PCR reaction buffer II [Applied Biosystems], 5 mM $MgCl_2$ [Applied Biosystems], 1 mM of each dNTP [Invitrogen], 0.15 μM D2 primer, 2.5 U MuLV reverse transcriptase [Applied Biosystems], 0.4 U RNase inhibitor [Applied Biosystems] and 1 μL RNA sample. Reaction conditions were as follows: 42 °C for 30 min, inactivation of reverse transcriptase and denaturation at 99 °C for 5 min, followed by cooling on ice. We used MuLV reverse transcriptase [Applied Biosystems] to replace rav-2 recombinant reverse transcriptase which was no longer commercially available.

The reaction of the first round of PCR was prepared by adding the following reagents. The 80 μL reaction mixture contained 8 μL of 10×PCR reaction buffer II [Applied Biosystems], 4 μL of 2 mM $MgCl_2$, [Applied Biosystems], 0.3 μM D1 primer, 2.5 U AmpliTaq Gold DNA polymerase [Applied Biosystems]. This reaction was then mixed with 20 μL of RT reaction to make up to 100 μL of the final first round PCR reaction. PCR was run with an initial activation step at 95 °C for 10 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 1 min, and 72 °C for 2 min. The PCR was ended with a final extension at 72 °C for 7 min.

The nested round PCR contained 1×PCR reaction buffer II [Applied Biosystems], 1.5 mM $MgCl_2$ [Applied Biosystems], 0.2 mM of each dNTP [Invitrogen], 0.5 μM each of D1, TS1, TS2, TS3 and TS4 primers, 2.5 U AmpliTaq Gold DNA polymerase [Applied Biosystems], and 10 μL of the first-round PCR reaction end product diluted 1:100. PCR was performed with the

following conditions: initial activation step at 95 °C for 10 min, followed by 20 cycles of 95 °C for 30 s, 55 °C for 1 min, and 72 °C for 2 min. The PCR was ended with a final extension at 72 °C for 7 min. All amplicons were identified by agarose gel electrophoresis and visualised under UV exposure after ethidium bromide staining.

Table 2.1.3 Primer sequences of a conventional nested RT-PCR assay.

Primer	Primer sequence (5′-3′)[a]	Positions[b]	Amplicon Size, bp
D1	TCA ATA TGC TGA AAC GCG CGA GAA ACC G	134-161	511
D2	TTG CAC CAA CAG TCA ATG TCT TCA GGT TC	616-644	511
TS1	CGT CTC AGT GAT CCG GGG G	568-586	482
TS2	CGC CAC AAG GGC CAT GAA CAC	232-252	119
TS3	TAA CAT CAT CAT GAG ACA GAG C	400-421	290
TS4	CTC TGT TGT CTT AAA CAA GAG A	506-527	392

[a] Primer sequences were adopted from an assay designed by Lanciotti et al., 1992.
[b] Accession numbers of the particular DV strains please refer to Lanciotti et al., 1992.

2.2 Generation of recombinant DEN-2 proteins for investigating the neutralising potential by their corresponding antisera and developing a serological diagnostic assay

In the following sections of this chapter, the methods for production and confirmation of the DNA fragments for generation of the recombinant proteins are described. Generation of recombinant proteins and their characterisation are also covered. Afterward, detailed methods on the use of recombinant proteins for immunisation in rabbits to generate polyclonal antisera and the development serological assays, followed by antisera characterisation and assay validation are described.

2.2.1 Production of DNA fragments

2.2.1.1 Genes selected for production of PCR products

The whole prM protein sequence containing 166 amino acids (nucleotides 1-498 of the *prM* gene) and the ED3 containing 98 amino acids (nucleotides 296-394 of *E* gene) were selected for construction of recombinant proteins. The sequences encoding for linkers, stem-anchors or trans-membrane regions of the *ED3* gene were not included due to their hydrophobic nature [Modis et al., 2003]. Removal of the hydrophobic region facilitated antibody access and thus enhanced immunogenicity [Dodson et al., 2007]. The amino acid sequences of *prM* and *ED3* genes were back-translated to nucleotide sequences by tblastn software available on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Corresponding nucleotide sequences were then Blasted to find the correct reading frame and to ensure the sequences did not share great homology with other proteins. The

nucleotide sequences was then aligned with all available sequences of the complete genome or coding sequences of DEN-2 serotypes in GenBank (95 DEN-2 strains, accessed on January 2008) to compare the homology among the same serotypes.

2.2.1.2 Amplification of *prM* and *ED3* genes by PCR

2.2.1.2.1 Synthesis of cDNA from the RNA template

RNA was reverse transcribed into cDNA in a 20 μL reaction mixture containing 1x first strand buffer [Invitrogen], 200U MMLv reverse transcriptase [Invitrogen], 40U RNase OUT [Invitrogen], 0.01 M DDT [Invitrogen], 3 μg random primers [Invitrogen], 0.5 mM dNTPs [GE healthcare] and 1 μL of DEN-2 RNA, which was equivalent to 2.1 PFU of dengue virus. The RNA of DEN-2 was provided by PHLC of the HKSAR. RNA, random primers and dNTPs were heated at 65°C for 5 min. After heating, the remaining components were added and then incubated at 25 °C for 10 min, followed by 37 °C for 50 min. The reverse transcriptase enzyme was finally inactivated by heating at 70 °C for 15 min.

2.2.1.2.2 PCR amplification by degenerate primers

The RNA of a clinical DEN-2 strain was provided by the PHLC of the HKSAR. Since the nucleotide sequence of this strain was not provided, an extra PCR step to flank the target region had to be performed. Gene products of *prM* (644-bp) and *ED3* (404-bp) were amplified respectively (Figure 2.2.1A). The primer used in this PCR study was designed based on the alignment of all available sequences of the complete genome or coding sequences of DEN-2 serotypes deposited in the GenBank database. In view of nucleotide variations among the same serotype, degenerated primers were designed (Table 2.2.1).

A 50 μL reaction mixture was prepared from 1x HF PCR buffer, 4 mM $MgSO_4$, 1U Platinum Taq DNA polymerase High Fidelity [Invitrogen], 0.2 mM dNTP, 0.5 μM forward (prM_F_de or ED3_F_de) and reverse (prM_R_de or ED3_R_de) primers [Invitrogen]. Five μL of cDNA was used as template. PCR was performed in the GeneAmp PCR system 9700 thermal cycler [Applied Biosystems]. Taq DNA polymerase was activated at 94 °C for 2 min, followed by 40 cycles of 94 °C for 30 s, 50 °C for 1 min and 72 °C for 2 min. Final extension at 72 °C for 7 min was applied. PCR amplicons were subjected to sequence analysis by an ABI3130 Genetic Analyzer [Applied Biosystems] and described in Chapter 2.1.3.3.

2.2.1.2.3 PCR amplification by specific primers to produce in-frame *prM* & *ED3* products

After cycle sequencing, the sequence of the clinical DEN-2 strain was known, and specific primers for producing in-frame PCR products were designed (Table 2.2.1). A stop codon was included in the 5'end of each reverse primer, and for the *prM* and *ED3* genes, 501 bp and 297 bp in-frame products were amplified, respectively (Figure 2.2.1 B). The PCR conditions were the same as described in Chapter 2.2.1.2.2, except that the annealing temperature was increased to 58 °C for more specific amplification. Purity of the product was visualised by agarose gel electrophoresis.

2.2.1.2.4 PCR amplification of in-frame *prM-ED3* chimeric fusion product

For generation of the fusion *prM-ED3* product, a PCR-driven overlap extension technique was applied [Heckman & Pease, 2007]. First, two individual

PCRs were carried out to generate two intermediate chimeric gene products (Figure 2.2.1B). The *prM* gene product was 516 bp in size, while the *ED3* gene product was 315 bp in size. This *prM* gene product carried a segment of the *ED3* gene, and vice versa, which was achieved by the specific design of the primers. For the *prM* gene, the forward primer was the same as that used to generate the in-frame *prM* product, but the 3'end of the reverse primer of *prM* (prM_R_fu) contained segments of sequences belonging to *ED3*. For the *ED3* gene, the 5'end of forward primer of *ED3* (ED3_F_fu) contained segments of sequences belonging to *prM*, while the reverse primer was the same primer used for producing the in-frame ED3 product.

Second, the two products were mixed and acted as templates in another PCR, known as fusion PCR (Figure 2.2.2). Products were purified by the QIAquick PCR purification kit [Qiagen] according to the manufacturer's instructions. One µL of each purified product was used as DNA template. During fusion PCR, the two products hybridised to each other and generated an overlapping sequence. Extension at the 3' ends of the overlapping sequences segment generated a full-length double-stranded DNA. In the presence of the *prM* forward primer (prM_F_in) and the *ED3* reverse primer (ED3_R_in) only, the fusion product *prM-ED3* (795 bp) was generated. The PCR conditions for amplification of the two intermediates and the *prM-ED3* chimeric product were similar to those used in the amplification of the in-frame *prM* and *ED3* products as described in Chapter 2.2.1.2.2. The fusion product was purified by the QIAquick gel extraction kit [Qiagen] and then visualised on an agarose gel for purity.

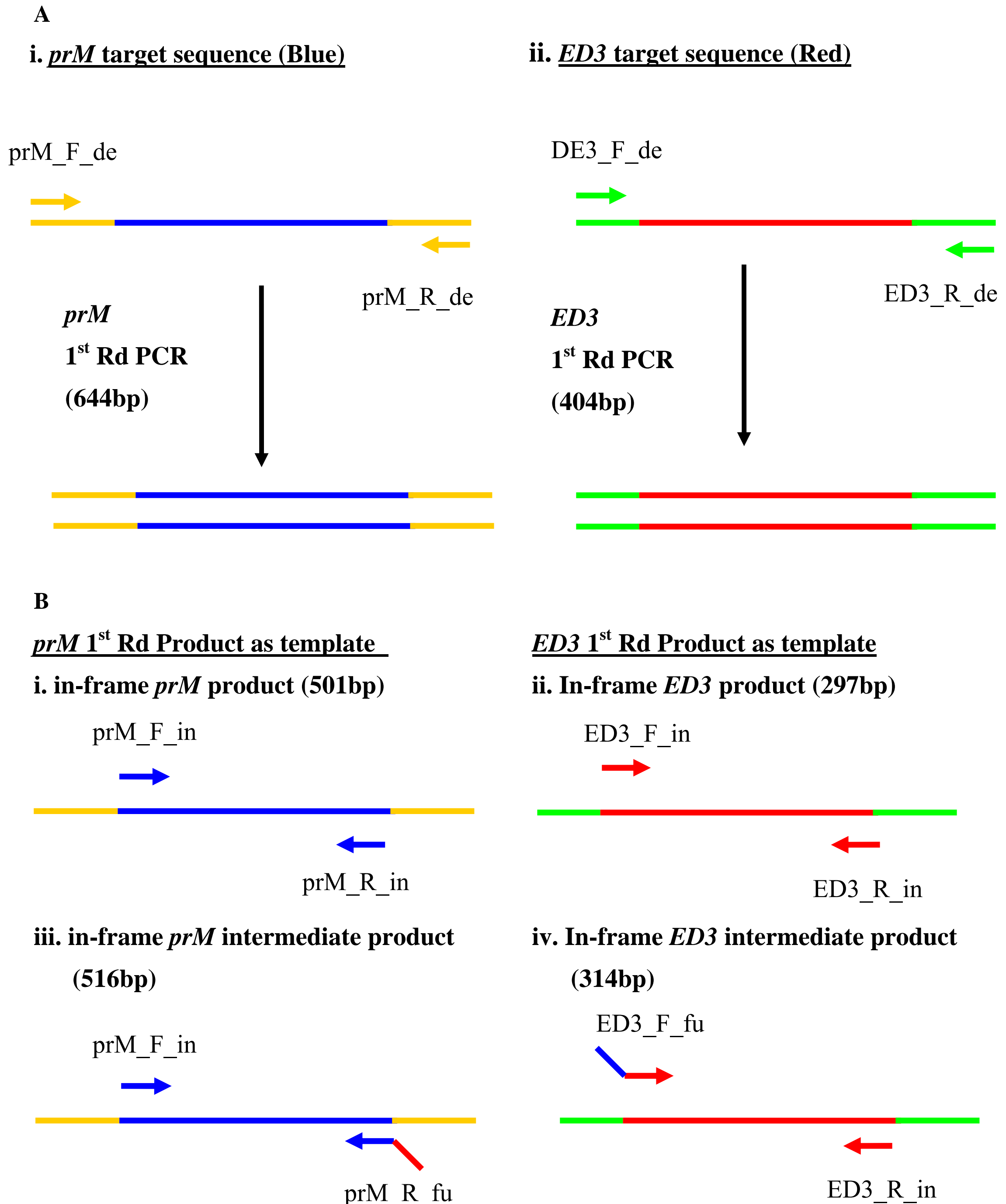


Figure 2.2.1 Generation of in-frame amplicons by amplification by degenerated primers and in-frame primers.

A) To produce in-frame amplicon for cloning, the sequences of our DEN-2 strain were identified by a PCR amplification with degenerated primers. Region in blue (i) and red (ii) indicated target sequences of *prM* and *ED3* to be amplified, respectively. Amplicons were analyzed by sequencing. B) In-frame primers were designed from known sequences of *prM* and *ED3* target. The in-frame products of *prM* & *ED3* were amplified in second round of PCR. i & ii indicated the in-frame *prM* & *ED3*, respectively. For generation of fusion amplicon *prM-ED3*, two intermediate chimeric products (iii & iv) were generated. Introduction a segment of sequences on 5' end of prM_R_fu and ED3_F_fu primers allowed fusion PCR. iii) Red region of prM_R_fu primer indicated sequence belonged to *ED3*. iv) Blue region of ED3_F_fu primer indicated sequence belonged to *prM*. All primer sequences please refer to Table 2.2.1

Table 2.2.1 Oligonucleotides for production of DNA fragments for cloning.

Name of primers	[a] Oligo Sequences 5'→ 3' (n)	Product size (bp)	Purpose of primers
prM_F_de prM_R_de	ATG CTG AAC ATY TTG AAY AGG AGA C (25) AAG ACD ATR TCA ACC CAG CTY CCT C (25)	644	Degenerate primers, product was used for design in-frame primers
ED3_F_de ED3_R_de	CTG AGR ATG GAC AAR CTR CAR CTY AAA GG (29) TCC CAR GCT GTG TCD CCY AAR ATG GC (26)	404	Degenerate primers, product was used for design in-frame primers
prM_F_in prM_R_in	TTC CAT TTA ACC ACA CGT AAC GGA (24) CTA TGT CAT TGA AGG AGC GAC AG (23)	501	Amplified in-frame PCR product
ED3_F_in ED3_R_in	ATG TCA TAC TCT ATG TGC ACA GGA AA (26) CTA TTT CTT AAA CCA GTT GAG CTT CAA T (28)	297	Amplified in-frame PCR product
prM_R_fu ED3_F_fu	GCA CAT AGA GTA TGA CAT TGT CAT TGA AGG AGC GAC AGC (39) GTC GCT CCT TCA ATG ACA ATG TCA TAC TCT ATG TGC ACA GGA AA (44)	-	Introduction of overlapping segment to two intermediate chimeric products for fusion PCR

[a] R= A or G; Y= C or T; D= A or G or T

Underline sequence indicated the sequence segment introduced into PCR product for generating overlapping segment during fusion PCR.

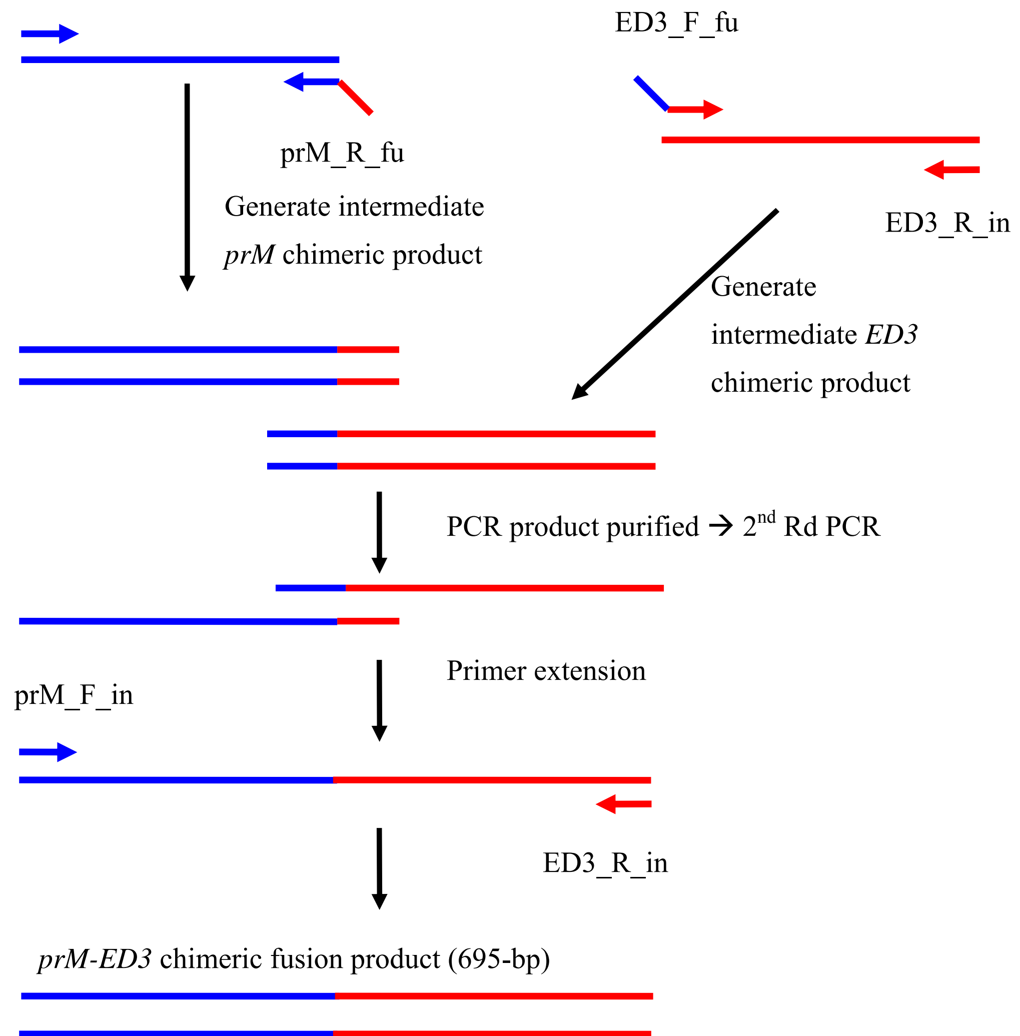


Figure 2.2.2 Fusion PCR amplification for generation of *prM-ED3* fusion product from two in-frame intermediate *prM* and *ED3* chimeric products.

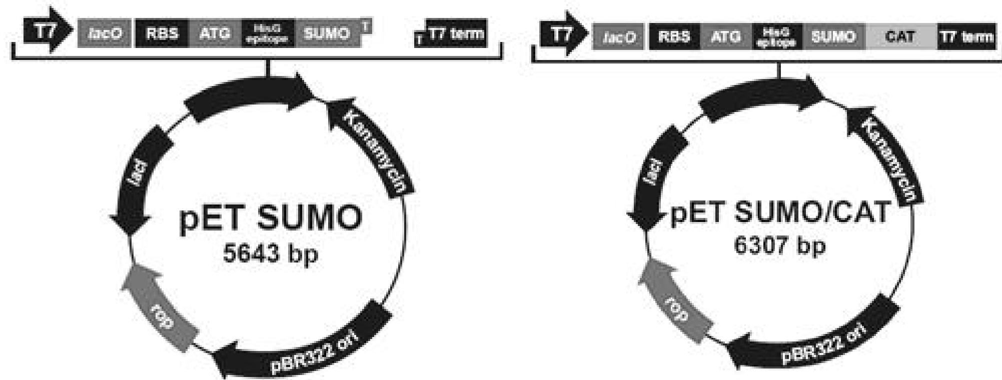
2.2.1.2.5 Cycle sequencing of PCR products

All in-frame PCR products including *prM*, *ED3* and fusion *prM-ED3* were subjected to cycle sequencing by an ABI3130 Genetic Analyzer [Applied Biosystems]. Details of purification and cycle sequencing of PCR products were described in Chapter 2.1.3.3.

2.2.2 Production of recombinant proteins

Recombinant proteins were expressed using the Champion pET SUMO protein expression system [Invitrogen]. The map of the pET SUMO vector and positive control SUMO/CAT vector is shown in Figure 2.2.3 A. Detailed sequence information of the pET SUMO vector is included in Figure 2.2.3 B. Overhang adenine (A) at the 3' end of the PCR product was complementary to thymine (T) of the pET SUMO vector for ligation. The recombinant protein expressed by the system was a fusion protein that was composed of an N-terminal 6x histidine tag and a small ubiquitin-like modifier (SUMO) protein upstream of the insert. The SUMO contained in the vector was a yeast *Saccharomyces cerevisiae* Smt3 protein which regulates some cellular functions and attaches covalently to modify the target protein and regulate protein stability [Müller et al., 2001; Seeler & Dejean, 2001].

A



B

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121  ATAGGCGCCA GCAACCGCAC CTGTGGCGCC GGTGATGCCG GCCACGATGC GTCCGGCGTA GAGGATCGAG ATCTCGATCC

                   T7 promoter                 lac operator
201  CGCGAAATTA ATACGACTCA CTATAGGGGA ATTGTGAGCG GATAACAATT CCCCTCTAGA AATAATTTTG TTTAACTTTA
                                                          HisG epitope
      RBS                                 Polyhistidine region
281  AGAAGGAGAT ATACAT ATG GGC AGC AGC CAT CAT CAT CAT CAT CAC GGC AGC GGC CTG GTG CCG CGC GGC AGC
                       Met Gly Ser Ser His His His His His His Gly Ser Gly Leu Val Pro Arg Gly Ser
                                                   SUMO fusion protein
354  GCT AGC ATG TCG GAC TCA GAA GTC AAT CAA GAA GCT AAG CCA GAG GTC AAG CCA GAA GTC AAG CCT GAG ACT
     Ala Ser Met Ser Asp Ser Glu Val Asn Gln Glu Ala Lys Pro Glu Val Lys Pro Glu Val Lys Pro Glu Thr

426  CAC ATC AAT TTA AAG GTG TCC GAT GGA TCT TCA GAG ATC TTC TTC AAG ATC AAA AAG ACC ACT CCT TTA AGA
     His Ile Asn Leu Lys Val Ser Asp Gly Ser Ser Glu Ile Phe Phe Lys Ile Lys Lys Thr Thr Pro Leu Arg
                                                                         SUMO forward priming site
498  AGG CTG ATG GAA GCG TTC GCT AAA AGA CAG GGT AAG GAA ATG GAC TCC TTA AGA TTC TTG TAC GAC GGT ATT
     Arg Leu Met Glu Ala Phe Ala Lys Arg Gln Gly Lys Glu Met Asp Ser Leu Arg Phe Leu Tyr Asp Gly Ile

570  AGA ATT CAA GCT GAT CAG ACC CCT GAA GAT TTG GAC ATG GAG GAT AAC GAT ATT ATT GAG GCT CAC AGA GAA
     Arg Ile Gln Ala Asp Gln Thr Pro Glu Asp Leu Asp Met Glu Asp Asn Asp Ile Ile Glu Ala His Arg Glu

642  CAG ATT GGT GGT  PCR product  AGACAAG CTTAGGTATT TATTCGGCGC AAAGTGCGTC GGGTGATGCT
     GTC TAA CCA CCA               TCTGTTC GAATCCATAA
     Gln Ile Gly Gly
                    ^
                    SUMO cleavage site
701  GCCAACTTAG TCGAGCACCA CCACCACCAC CACTGAGATC CGGCTGCTAA CAAAGCCCGA AAGGAAGCTG AGTTGGCTGC

             T7 reverse priming site
781  TGCCACCGCT GAGCAATAAC TAGCATAACC
```

Figure 2.2.3 Map of pET SUMO and SUMO/CAT vector.
(Maps were adopted from user manual of Invitrogen Champion^{TM} pET SUMO protein expression system).

A) Map and features of pET SUMO and SUMO/CAT vector. B) Map of pET SUMO with detail nucleotide sequence.

2.2.2.1 Cloning of PCR products into the pET SUMO vector

2.2.2.1.1 Ligation of the PCR product to the cloning vector

The ligation reaction must be completed with freshly prepared PCR products. To ensure the presence of an overhang A at the 3'end of the PCR product, the PCR product was incubated with 1 U of AmpliTaq Gold DNA polymerase [Applied Biosystems] at 72 °C for 30 min. The ligation reaction consisted of 1x ligation buffer, 50 ng of pET SUMO vector, 4 Weiss units of T4 DNA ligase and 1 μL of fresh PCR product. This mixture was incubated at 15 °C overnight and kept at -20°C until transformation.

A control reaction for the cloning procedures was set up using control DNA and primers supplied by the manufacturer and according to the manufacturer's instructions. A control DNA template was supplied with the cloning kit. A 750 bp control PCR product was amplified with 1x HF PCR buffer, 4 mM $MgSO_4$, 1 U of Platinum Taq DNA polymerase [all from Invitrogen], 0.2 mM of dNTPs [GE healthcare], 0.1 μg each of control forward and reverse primers [Invitrogen] and 1 μL control DNA template [Invitrogen]. PCR was performed in the GeneAmp PCR system 9700 thermal cycler [Applied Biosystems]. The product was amplified with the following parameters as suggested by the manufacturer: Taq DNA polymerase activation at 94 °C for 2 min, followed by 30 cycles of 94 °C 1 min, 55 °C 1 min and 72 °C 2 min. The reaction was stopped with a final extension at 72 °C for 7 min. Five μL of PCR product was removed for agarose gel electrophoresis.

2.2.2.1.2 Preparation of competent One Shot Mach1^{TM}-T1^{R} and BL21(DE3) competent *E. coli* cells

One vial (50 µL) of One Shot Mach1-T1 or BL21(DE3) competent *E. coli* cells [Invitrogen] was inoculated into 10 mL of LB broth [Sigma Aldrich] in a 50 mL centrifuge tube. The culture was incubated at 37 °C overnight with agitation at 250 rpm. One hundred mL of LB broth in a 1 L conical flask were inoculated with 1 mL of the *E. coli* culture which had been left overnight. The culture was incubated at 37 °C for 3 hr with agitation at 250 rpm until absorbance at 600 nm reached 0.6. Fifty mL of the culture were transferred to a centrifuge tube and harvested at 2,700 x g for 10 min at 4 °C. The supernatant was removed as much as possible. The *E. coli* cells were resuspended in 25 mL pre-chilled 50 mM calcium chloride solution, and then incubated on ice for 20 min. Cells were harvested again at 2,700 x g for 10 min at 4 °C, and then resuspended in 100 µL pre-chilled 50 mM calcium chloride solution. Cells were kept on ice until transformation. Fifty µL of competent cells were used for each transformation.

2.2.2.1.3 Transforming to One Shot ® Mach1^{TM}-T1^{R} competent *E. coli* cells for stable propagation and maintenance of recombinant plasmids

Four µL of the ligation reaction and 10 ng of pET SUMO/CAT plasmid which was a positive control vector for expression were gently mixed with 50 µL of competent *E. coli* cells and incubated on ice for 15 min. Cells were heat-shocked at 42 °C in a water bath for 40 s without shaking. The tube was then immediately placed on ice and 1 mL of S.O.C medium was added with gentle mixing. The tube was incubated at 37 °C for 1 hr with agitation at 200 rpm for phenotypic expression of the selection marker. One hundred µL of cells were spread on pre-warmed selective LB agar containing 50 µg/mL kanamycin. The agar plate was incubated overnight at 37 °C.

2.2.2.1.4 Analysing transformants

At least 10 colonies were selected from each overnight culture on LB agar. Part of a colony was cultured on LB agar with kanamycin for further analysis, while another part of the colony was directly inoculated into the PCR reaction mixture to confirm the presence and the orientation of the insert.

The 50 µL PCR reaction contained 1x PCR gold buffer, 4 mM of $MgCl_2$, 1.5 U of AmpliTaq Gold DNA polymerase, 0.2 mM of dNTPs and 0.5 µM each of forward and reverse primers. One primer must be located in the SUMO vector, which could either be the SUMO forward (5'-AGATTCTTGTACGACGG TATTAG-3') or the T7 reverse primer (5'-TAGTTATTGCTCAGCGGTGG-3') provided with the kit, while the other primer would be annealed to the insert of the gene product of interest (Figure 2.2.4). Two PCRs were set up for each colony. One PCR was amplified by the SUMO forward primer and the reverse primer for the insert of the gene product of interest. The other PCR was amplified by the forward primer for the gene product of interest and the T7 reverse primer (Figure 2.2.4). The choices of primers for the gene product of interest depended on the insert used in the analysis. The cycling conditions for PCR were: activation of Taq DNA polymerase at 94 °C for 10 min, followed by 40 cycles of 94 °C for 30 s, 55 °C for 1 min and 72 °C for 1 min and a final extension step of 72 °C for 7 min. The size of the product was confirmed by agarose gel electrophoresis. All products showing the correct amplicon size were subjected to sequence analysis as described in Chapter 2.1.3.3. Frozen stock of all confirmed positive transformants was kept in LB broth with 20% glycerol at -80 °C.

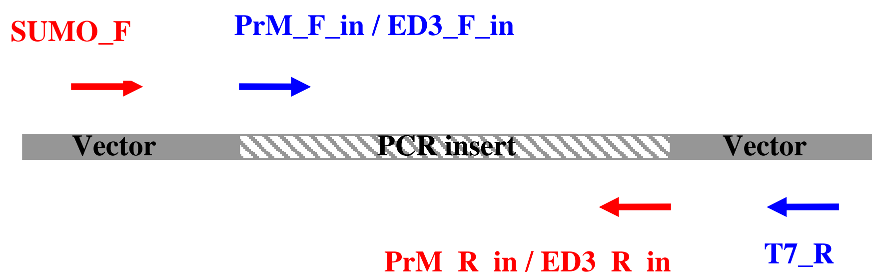


Figure 2.2.4 PCR setting for analysis positive transformants.
Primers pair in red indicated one PCR and primers pair in blue indicated another PCR.

2.2.2.1.5 Plasmid extraction from positive transformants for sub-cloning

Plasmid DNA was extracted from positive transformants for sub-cloning into BL21(DE3) *E. coli* cells. A single colony was picked from the cultured LB agar with the positive transformants and sub-cultured into 5 mL LB broth with 50 μg/mL kanamycin at 37 °C and incubated for 15 hr. Plasmid DNA was extracted from 5 mL of culture by the Qiagen Plasmid Mini kit [Qiagen] according to the manufacturer's instructions. All extracted plasmid DNA was quantified at A_{260} by a NanoDrop® spectrophotometer [NanoDrop].

2.2.2.1.6 Sub-cloning to BL21(DE3) competent *E. coli* cells for recombinant protein expression

Sub-cloning to BL21(DE3) was performed in a similar way to the transforming method for One Shot® Mach1™-T1^R competent *E. coli* cells. Two μL of extracted plasmid (instead of ligation reaction) was added to 50 μL BL21(DE3) *E. coli* cells. For the detailed methodology for transformation and analysis of transformants, please refer to Chapters 2.2.2.1.3 and 2.2.2.1.4, respectively.

2.2.2.2 Expression of protein fragments

2.2.2.2.1 Preparation of mid-log phase BL21(DE3) *E. coli* cells for expression

BL21(DE3) strains transformed with the pET SUMO construct were cultured from frozen stock into LB agar with 50 µg/mL kanamycin. A single colony was inoculated into 10 mL LB broth with 50 µg/mL kanamycin and 1% glucose (w/v) for 18 hr at 37 °C with 250 rpm agitation. Overnight culture was inoculated into LB medium with 1% glucose in a conical flask in a ratio of 1:100, and then incubated at 37 °C for 3-4 hr with shaking at 250 rpm to allow the cells to reach mid-log phase. *E. coli* growth was monitored by measuring absorbance at 600 nm, with absorbance at 0.6 indicating that *E. coli* cells had reached mid-log phase.

2.2.2.2.2 Pilot expression at different time points

The mid-log phase *E. coli* cells were divided into two aliquots: one induced with isopropyl-ß-D-thiogalactopyranoside (IPTG) [Invitrogen] and one non-induced control. IPTG was added to one of the aliquots to a final concentration of 1 mM. Before incubation, 500 µL was removed from each aliquot as T_0. The two aliquots were incubated at 37 °C with agitation at 220 rpm for 4 hr. 500 µL was removed from each culture at 1-hr intervals. All cell aliquots were harvested by centrifuging at 14,000 x g for 1 min. The supernatant was removed and cell pellets were kept at -20°C for protein analysis.

2.2.2.2.3 Optimisation of protein expression conditions

Temperature and IPTG concentration were optimised for each pET SUMO construct (*prM*, *ED3* and *prM-ED3* fusion products). Two expression temperatures,

30 °C and 37 °C were investigated. IPTG made up to a final concentration of 0.2 mM, 0.5 mM and 1 mM were tested. Optimisation experiments were carried out on 6-well culture plates. Each well was inoculated with 4 mL of cultured cells. After *E. coli* growth reached the mid-log phase, IPTG with different final concentrations as described was added to the corresponding wells and incubated either at 30 °C or 37 °C to induce protein expression. An aliquot of 500 μL of the cultured cells at T_0-T_4 were collected for protein analysis.

2.2.2.2.4 Protein expression using scaled-up culture volumes

The expression volume was scaled-up progressively from 5 mL to 50 mL and finally to 500 mL in a conical flask. For the large-scale production of recombinant protein, an aliquot of 500 mL of culture volume was used. Cells were divided into 50 mL each in centrifuge tubes and harvested by spinning at 4,500 x g for 10 min at 4 °C.

2.2.2.3 Analysis of recombinant protein samples

2.2.2.3.1 Extraction and purification of recombinant proteins

Protein was extracted from cell pellets using lysis buffer (Appendix II) which was prepared in-house according to the manufacturer's instructions from the cloning kit, but with the exclusion of imidazole. Cell pellets were thawed at RT, and 1 mL of lysis buffer and 200 μg of lysozyme solution (20 mg/mL) were added. The tube was incubated on ice for 20 min and the cells were then lysed in a sonicator at 4-6 amplitudes for 6 cycles of 10 s, followed by a 5 s break in every cycle. The viscosity of the lysate was further reduced by trituration with a 20 gauge needle and syringe. The cell lysate was harvested by centrifugation at

14,000 x g for 30 min at 4 °C. The supernatant (soluble protein) was aspirated and the pellet (insoluble protein or inclusion bodies) was resuspended in 1 mL lysis buffer. All fractions were stored temporarily at 4 °C.

For the large-scale production of recombinant proteins, the supernatant of extracted cells was purified by immobilised metal affinity chromatography (IMAC) on a HisTrap-FF [GE Healthcare] column before characterisation analysis. A His buffer kit [GE Healthcare] containing 2 M of imidazole solution and 8x phosphate buffer stock solution at pH 7.4 was utilised for the purification of histidine-tagged proteins. Supernatant samples were mixed with imidazole to a final concentration of 20 mM in 1x phosphate buffer (binding buffer) before binding to the column. Proteins were eluted with an increasing gradient of imidazole (40 mM, 60 mM, 100 mM and 500 mM) in 1x phosphate buffer (elution buffer). All flow-through fractions were collected for analysis.

2.2.2.3.2 Characterisation of recombinant proteins

2.2.2.3.2.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-polyacrylamide gels consisted of 4 mL of 12% separating gel, at pH 8.8 overlain with 2 mL of 5% stacking gel at pH 6.8 (Appendix II) and were set in a gel casting module (1.0 mm) Mini-PROTEAN® system [Bio-Rad]. A 10-15 µL of protein sample was boiled for 3 min and mixed with 5 µL of 6x SDS sample loading buffer (Appendix II). A standard protein marker, the Novex® sharp standard [Invitrogen] with a known molecular weight ranging from 3.5-260 kDa was run in parallel on each gel. Protein fragments were separated at 200V for 1 hr in electrophoresis buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, 0.1% SDS)

inside the Mini-PROTEAN® tetra cell [Bio-Rad]. The gel was then stained in 0.25% (w/v) coomassie brilliant blue for 1 hr. Protein bands were visualised after overnight de-staining in methanol:acetic acid (4:1) solution. The gel was dried at 80°C for 30 min on 3MM chromatography paper [Whatman].

2.2.2.3.2.2 Western blotting

After electrophoresis of the protein samples as described in Chapter 2.2.2.3.2.1, proteins on the gel were electroblotted onto polyvinylidene fluoride (PVDF) membrane [GE Healthcare] at 100V for 1 hr. Non specific binding was blocked with 5% (w/v) dried non-fat milk in Tris-buffered saline (TBS) (10 mM Tris-HCl, 150 mM NaCl, pH 8.0) with 0.05% of Tween-20 for 1 hr. The membrane was blotted with a primary antibody against anti-HisG [Invitrogen] at a dilution of 1:5000 in TBS with 0.05% Tween-20 (TBST) overnight at 4 °C. Other primary antibodies, a rabbit polyclonal against dengue virus serotype 1-4 (1:100) and mouse monoclonal against prM glycoprotein (1:100) [both from Abcam] were used for specific detection on different membranes.

After overnight blotting, the membrane was washed for 10 min each with three changes of TBST with rocking. For membranes blotted with anti-HisG and the mouse monoclonal against prM glycoprotein, a rabbit anti-mouse IgG (H+L) secondary antibody (1:10,000) conjugated to horseradish peroxidase (HRP) [Zymax] in TBST was applied. For membranes blotted with a rabbit polyclonal antibody against dengue virus serotype 1-4, a secondary goat anti-rabbit IgG (H+L) antibody conjugated to HRP [Zymax] diluted 1:10,000 in TBST was used. Membranes were blotted at RT for 1 hr. After washing the membrane three times

with TBST as previously described, the specific protein bands on the membrane were detected by BM chemiluminescence blotting substrate [Roche]. The detection reagent was freshly prepared by mixing 1 mL of luminescence substrate solution A with 15 μL of starting solution B. The membrane was covered with mixed detection reagent. The excess reagent was drained off before the membrane was wrapped in a plastic sheet. Hyperfilm ECL [GE Healthcare] was exposed for 5 min in an x-ray film cassette. The image was developed under premixed developer and fixer solution [Fuji] and the film was left to dry in a 55 °C oven for 5 min.

2.2.2.3.2.3 Protein identification and mass measurement by mass spectrometry

For protein identification, the target protein bands were excised from the SDS-PAGE gel and the gel plugs and washed with Milli-Q water for 15 min. The protein was recovered from the gel by immersing it in two changes of 50 mM ammonium hydrogen carbonate/acetonitrile (NH_4HCO_3/ACN, 1:1 v/v) for 15 min followed by two changes of 100% ACN for 5 min. Proteins were then dried for 5 min in a SpeedVac. The dried gel plugs were digested using 10 μL of 10 ng/μL trypsin [Promega] in 25 mM NH_4HCO_3 at 4°C for 30 min. Peptides were separated by means of an Ultimate 3000 nano liquid chromatography system [LC Packings, Dionex] and analysed by HCTultra Electrospray (ESI)-ion trap mass spectrometry (MS) [Bruker Daltonics]. The target peptides were detected in positive ion mode and fragmented by collision-induced dissociation using helium gas. The two most abundant precursor ions were selected for MS/MS study within a mass-to-charge (m/z) range of 300-1500. The MS/MS mass spectrum was obtained after three

scannings. Data obtained from MS and MS/MS study was submitted through the MASCOT web-based search engine and the protein identity could be determined by searching for it in the NCBI protein database (NCBIni_20081017). A score was calculated by the MS computer MASCOT for each peptide fragment. The ranking of each peptide was shown in descending order based on the calculated score. The proteins were considered as identical when the peptide ion score was above 25.

For accurate molecular weight measurement of the proteins, analysis was performed by ESI Q-TOF2 Quadrupole-Time-of-Flight MS [Waters]. Purified protein samples underwent pre-concentration, desalting and buffer exchange with Amicon® Ultra-4 (MW = 10,000) centrifugal filter devices [Millipore]. Two hundred µL of purified sample were exchanged twice with 4 mL of 20 mM ammonium acetate (pH 7.0) by spinning at 4,000 x g for 15-20 min. The treated protein was then mixed with equal volumes (v/v) of ACN with 2% formic acid and injected into the mass spectrometer for analysis via the ESI interface at a flow rate of 5 µL/min. For data acquisition, the mass spectrometer was operated in scanning mode to determine any multiply-charged protein ion peaks within the m/z range of 700–1600. The m/z axis was calibrated externally with 10 µM of horse heart myoglobin (MW = 16,950.5). The raw multiply-charged spectra were finally analysed and converted into exact mass by the MassLynx 4.1 Transform Program.

2.2.3 Production of polyclonal antibodies by rabbit immunisation

2.2.3.1 Sample preparation and quantification for rabbit immunisation

Purified proteins were simultaneously concentrated and buffer-exchanged with Amicon® Ultra-4 (MW = 10,000) centrifugal filter devices [Millipore]. The

membrane of the column was pre-rinsed twice with 2 mL deionised water for removal of triethylene glycol on the membrane filter unit. The entire volume of purified protein sample eluted with elution buffer containing 500 mM imidazole as described in Chapter 2.2.2.3.1 was added into the device, which was then centrifuged in swinging bucket rotors at 4,000 x g for 15-20 min until a volume of ~100 μL was retained. Four mL of 1x phosphate buffer (pH 7.4) was added into the His buffer kit [Invitrogen] for buffer exchange. The device was centrifuged again as described. The concentrated protein sample was aspirated immediately. For maximum recovery of protein, the sample was removed by gently rinsing the membrane and the bottom of the filter unit with a pipette. Concentrated samples were kept at 4°C for temporary storage and later underwent SDS-PAGE for purity confirmation.

In order to calculate the absolute quantity of protein for immunisation, concentrated samples were quantified by the Bradford method (Bradford, 1976). The Bradford assay dye reagent [Bio-Rad] was diluted 1:5 with distilled water. A standard curve was set up using serial dilutions of 1 mg/mL bovine serum albumin (BSA) solution. Protein concentrations corresponding to 1, 0.5, 0.4, 0.25, 0.1 and 0.05 mg/mL were prepared. Two hundred μL of diluted reagent were mixed with 10 μL of each sample or protein standard. The mixture was incubated at room temperature for 5 min and absorbance was measured at 595 nm. Absorbance of the BSA protein standard was plotted against its concentration, and the protein concentrations of samples were determined from the standard curve.

Since the concentrated sample did not consist solely of the target protein, the presence of other protein bands became obvious after concentration using ultracentrifugation. For the purposes of immunisation, a purity of 80% was sufficient. The purity of target protein was estimated by Image J (a freely available software, at http://rsb.info.nih.gov/ij/index.html), which could be used for quantifying the blotting image [Miller, 2007]. The SDS-PAGE gel image was scanned and converted to 8-bit grayscale and pixel scale. Each gel band on the same lane was analysed by its area and relative intensity compared to the background (mean gray value). The proportion of the protein of interest was estimated and presented as an integrated density (multiplication of the area and mean gray value) of gel bands.

Once the concentrations of the concentrated protein samples and the purity (%) of the target protein were obtained, the absolute concentration of target protein was calculated. The absolute amount of target proteins (~150 μg) for immunisation was adjusted and prepared. The protein samples were kept on ice before they were administered into the rabbits.

2.2.3.2 Rabbit immunisation with purified protein samples

Four 12-week old female New Zealand White rabbits were used for immunisation [Dodson et al., 2007]. They were supplied by the Laboratory Animal Services Center of the Chinese University of Hong Kong and kept individually in the Central Animal Facility Laboratory of the Hong Kong Polytechnic University. Each of them was respectively administered with a different combination of target proteins: 1) prM protein, 2) ED3 protein, 3) prM-ED3 fusion protein and 4) a

mixture of prM and ED3 proteins. Pre-immunised blood was collected from each rabbit to act as a control.

The anesthetic agent used was a mixture of xylazine (250 µL of 20 mg/mL) [AlfaMedic] and ketamine (500 µL of 100 mg/mL) [AlfaMedic] in a ratio of 1:2. Each rabbit was sedated with the anesthetic agent injected intramuscularly in the thigh muscle and was then administered subcutaneously with 150 µg of protein at multiple sites along the back. Prior to injection, the protein was mixed with an equal volume of Freund's adjuvant to a maximum volume of 1 mL and the contents were mixed until the formation of a stable emulsion. Complete Freund's adjuvant [Sigma Aldrich] composed of inactivated mycobacteria was used for the priming injection and incomplete Freund's adjuvant [Sigma Aldrich] was used for the boosters. Booster immunisation was repeated every 4-6 weeks after the first injection. Ten mL of blood was collected from the central auricular artery of the ear of each rabbit on day 10 and day 24 after each booster injection [Dodson et al., 2007]. Blood samples were transferred into a sterile blood collection tube with clot activator [Terumo]. Complete blood clotting was achieved by keeping the samples at 4°C for 1 hr. Sera were prepared by centrifugation at 2,700 x g for 10 min at 4 °C and kept in a 0.5 mL aliquot at -80 °C. The rabbits were euthanised when there was no further increase in antibody titre, using sedation with the anesthetic agent and CO_2 asphyxiation in a closed chamber.

2.2.3.3 Characterisation of antisera from rabbits

2.2.3.3.1 Immunoblotting

Fifteen µL of purified prM protein was loaded in one lane of a 12% SDS-PAGE gel. In an adjacent lane, 3 µL of Novex® sharp pre-stained standard

[Invitrogen] protein marker was loaded. Four identical sets of these samples were loaded on the same gel. Similarly, two more gels were loaded with ED3 and prM-ED3 proteins. The gel was electrophoresed and electroblotted onto PVDF membrane as described in Chapters 2.2.2.3.2.1 and 2.2.2.3.2.2.

After the gel was electroblotted onto the PVDF membrane, the membrane was cut into four strips so that each contained identical samples. Each strip was blotted overnight at 4°C with different serum samples: 1) pre-immunised serum, 2) crude antiserum from rabbit, 3) pre-adsorbed antiserum with purified protein and 4) pre-adsorbed antiserum with SUMO-CAT fusion protein. Dilution of antiserum was optimised from 1:1,000, 1:5,000, 1:10,000 and 1:20,000. Pre-adsorbed serum was prepared by incubation with ~1 µg/mL purified proteins or SUMO-CAT fusion protein at RT with rocking for 1 hr. The purpose of pre-adsorption with SUMO-CAT fusion protein was the removal of anti-SUMO and anti-His tag antibodies. A detailed method of washing and blotting was described in Chapter 2.2.2.3.2.2. The strip blotted with anti-His primary antibody was then incubated with HRP-conjugated rabbit anti-mouse IgG (H+L) antibody (1:10,000). For other strips blotted with rabbit antisera, HRP-conjugated goat anti-rabbit IgG (H+L) antibody (1:10,000) [Zymax] was used as the secondary antibody. The film was developed as described in Chapter 2.2.2.3.2.2. Each serum sample collected from the rabbits over the immunisation period was evaluated by immunoblotting.

2.2.3.3.2 ELISA assay

2.2.3.3.2.1 Adsorption of purified protein to a microtitre plate

The protocol of adsorption of purified protein was performed according to the chapter on Production of polyclonal antibodies in rabbits in Methods in

Molecular Biology: Immunochemical Protocols [Hancock & O'Reilly, 2005]. Solutions of purified protein with a concentration of 10 µg/mL were prepared in adsorption buffer (0.1 M sodium bicarbonate, pH 9.6). One hundred µL of protein solution were added into each well of a high-binding flat bottom microtitre plate [Jet Biofil]. The plate was incubated overnight at RT in a moist chamber. Any unabsorbed solution was removed from the microwells, and each well was washed with several changes of TBS (25 mM Tris-HCl and 144 mM NaCl, pH 8.1). For blocking of non-specific binding of antibodies, 150 µL of blocking buffer, TM (TBS containing 2% dried non-fat milk powder) was added and incubated at RT for 1 hr. The TM buffer was removed, the plate was coated with the desired protein and was ready for use.

2.2.3.3.2.2 Indirect ELISA

The procedures for the in-house ELISA assay were based on the chapter from Hancock & O'Reilly as described in Chapter 2.2.3.3.2.1 [Hancock & O'Reilly, 2005], and with slight modifications based on the manufacturer's instruction for the Panbio Dengue IgG Indirect ELISA kit [Panbio]. Regents of substrate and stop solution provided in the ELISA kit were used for this assay. Antisera were pre-adsorbed with SUMO-CAT fusion protein as described in Chapter 2.2.3.3.1. Pre-adsorbed antisera were then serially diluted in doubling dilutions with serum diluents TMT (TBS containing 2% dried non-fat milk powder and 0.2% Tween-20). Two-fold serial dilutions ranging from 1:50 up to 1:102,400 were prepared. The TM blocking buffer was removed and 100 µL of diluted antiserum were added. The plate was incubated for 1 hr at RT in a moist chamber. The antiserum solution was removed and each microwell was washed with several changes of TBS.

Secondary antibody, lyophilised HRP-conjugated goat anti-rabbit IgG (H+L) [Invitrogen] was reconstituted to 1.5 mg/mL in PBS (pH 7.2). Working dilutions of 1:1,000 and 1:5,000 were prepared for optimised signal. One hundred µL were added to each well. The plate was incubated for 1 hr at RT in a moist chamber. After a washing step, 100 µL of tetramethylbenzidine (TMB) substrate (3,3',5,5'-tetramethylbenzidine and hydrogen peroxide in a citric-acid citrate buffer, pH 3.5-3.8) [Panbio] was pipetted into each microwell. One hundred µL of stop solution (1M phosphoric acid) [Panbio] were added in the same order in which TMB substrate was added after 10 min incubation at RT. Obvious colour change from blue to yellow was noted. Absorbance was measured by the Benchmark plus microplate spectrophotometer [Bio-Rad] within 30 min at a wavelength 450 nm with 620 nm as the reference wavelength.

Controls were set up for each purified protein (prM, ED3 and prM-ED3). The positive and negative control human sera provided in the Panbio Dengue IgG Indirect ELISA kit [Panbio] were utilised as control serum samples. Control sera were diluted with serum diluent (TBS with 0.1% Proclin^{TM} preservative, pH 7.2-7.6) [Panbio] at a dilution of 1:100. HRP-conjugated anti-human IgG [Panbio] was used as the secondary antibody. Microwells without adsorption of purified protein acted as uncoated controls for analysing the background signal.

2.2.3.3.3 DEN-2 recombinant subviral particle (RSP) binding assay

2.2.3.3.3.1 Harvest and confirmation of RSPs from cell culture

DEN-2 RSPs were kindly provided by the Pasteur Research Centre of the University of Hong Kong. Details information including the construction of RSPs

was described in Wang et al [Wang et al., 2009]. Harvest of RSPs was performed according to the protocol of the Pasteur Research Centre of the University of Hong Kong. Supernatants of transfected 293T cell culture were harvested for RSP isolation. Supernatant was clarified by centrifugation at 3,000 x g for 10 min, followed by 13,000 x g for 30 min. RSPs were isolated from a 20% sucrose solution with ultracentrifugation at 28,000 rpm (Beckman SW-32 rotor) for 2.5 hr. All centrifugation steps were carried out at 4 °C. The pellet fraction was resuspended in 100 μL of 20 mM HEPES buffer [Gibco] with 30 min incubation on ice. Concentrated RSPs were stored at -80 °C. The presence of RSPs was confirmed by dot blot. A small volume of DEN-1 RSPs was also provided by the Pasteur Research Centre of the University of Hong Kong as a control. Two μL of RSPs were blotted directly onto a PVDF membrane. Non-specific sites were blocked with 5% milk in TBST for 1 hr. The membrane was incubated for 1 hour with anti-E 4G2 monoclonal antibody (mAb; 1:5,000) (provided by the Pasteur Research Centre of the University of Hong Kong) followed by an additional 1 hr with HRP-conjugated goat anti-mouse IgG antibody (1:10,000) [Invitrogen]. The RSPs were quantified by a Bradford assay as described in Chapter 2.2.3.1.

2.2.3.3.3.2 Culture of Vero E6 cells for RSPs binding

Vero E6 cells were cultured overnight at 37 °C, 5% carbon dioxide (CO_2) in Dulbecco's modified Eagle's medium (DMEM) [Gibco] with 10% fetal bovine serum (FBS) [Gibco]. Cells in a T-75 flask were detached with 2 mL of 0.25% trypsin-EDTA solution [Gibco] and incubated at 37 °C for 5 min. Eight mL of pre-warmed DMEM were added to cells to neutralise the effect of trypsin-EDTA and harvested by centrifugation at 1,500 x g for 3 min. Vero E6 cells were counted

and adjusted with DMEM to 6-9 x 10^4 cell per 100 µL, and each 100 µL aliquot was used for a single binding assay.

2.2.3.3.3.3 Binding assay of RSPs to Vero E6 cells

Ten µg of RSPs were inoculated individually into 40 µL of antisera (neat concentration) and incubated at RT (20 °C) for 1 hr. Controls either without RSPs or antisera were set up. The mixture was inoculated into a vial of 6-9 x10^4 Vero E6 cell (100 µL) in DMEM with 2% FBS for binding at 4 °C for 1.5 hr. The cells were washed with 1 mL ice-cold PBS (pH 7.4) and centrifuged at 1,500 x g for 4 min at 4 °C. Binding of RSPs was detected by incubation with anti-E 4G2 mAb (1:100) for 1 hr at 4 °C. The cells were washed again as previously described followed by incubation for 1 hr with fluorescein isothiocyanate (FITC)-conjugated horse anti-mouse IgG (H+L) secondary antibody (1:100) [Vector Laboratories]. Cells were washed again and resuspended in 300 µL ice-cold PBS.

2.2.3.3.3.4 Flow cytometric analysis of RSP binding to Vero E6 cells

Cells resuspended in ice-cold PBS were analysed by a flow cytometer FC-500 system [Beckman Coulter]. Measurements were performed with an argon laser at 488 nm for excitation of the FITC conjugate. The fluorescence signal generated by FITC was detected on the FL1 channel at a wavelength of 520-525 nm. Twenty thousand events were acquired for the negative control (Vero E6 cells only); however, each sample was acquired for 300 s. Data were analysed by CXP software [Beckman Coulter] and expressed by histograms.

2.2.3.3.3.5 Microscopy imaging of RSP binding to Vero E6 cells

In order to show qualitative evidence of the green fluorescence signal generated by to RSP binding to Vero E6 cells, fluorescence images were captured by an inverted research microscope Nikon ECLIPSE Ti [Nikon] under laser illumination at 488 nm. The green fluorescence was detected by the filter unit at 590 nm. Cell images were captured and analysed by the software EZ-C1 version 3.90 [Nikon].

Monolayers of Vero E6 cells ($4x10^5$) were inoculated and cultured in shell vial containers overnight at 37 °C, 5% CO_2 in DMEM [Gibco] with 10% FBS [Gibco]. Cells were gently washed twice with 1 mL ice-cold PBS (pH 7.4) and then made up to 100 μL in DMEM with 2% FBS for the evaluation of RSP binding as described in Chapter 2.2.3.3.3.3. Negative controls with Vero E6 cells only and controls without RSPs were also set up. Cell supernatants were removed and gently washed twice with 1 mL ice-cold PBS (pH 7.4). Cells were then fixed with 100% ethanol for 5 min, followed by a washing step as described. Cells were incubated with anti-E 4G2 mAb and FITC-conjugated horse anti-mouse IgG (H+L) secondary antibody as described in Chapter 2.2.3.3.3.3. The coverslip from the shell vial was taken out and mounted upside down on another coverslip with a drop of 90% glycerol in between. The fluorescence signal could be visualised under the inverted microscope Nikon ECLIPSE Ti [Nikon].

2.2.4 Development of a diagnostic tool using recombinant proteins

Apart from rabbit immunisation with the recombinant proteins to investigate the neutralisation potential of their corresponding polyclonal antisera,

another application of the recombinant proteins was attempted to develop a serological diagnostic tool.

2.2.4.1 Serum samples for assay validation

Eleven dengue IgG-positive sera collected from community in Hong Kong were used for ELISA assay validation. The presence of dengue IgG was confirmed by a commercial ELISA kit, the Panbio Dengue IgG Indirect ELISA [Panbio]. This ELISA assay was mostly applied to assess primary dengue infections or seroconversion, seroepidemiology and surveillance. Details of this part of study and the samples collected from community in Hong Kong are described in Chapter 2.4. Another 36 clinical sera collected from Brazil was used for validation. These sera were PCR positive for DV with the Dengue LC RealArt™ RT-PCR assay [Artus]. One hundred dengue IgG negative samples collected from the community in Hong Kong were randomly selected by SPSS 11.5 and acted as a control group. The required number of control samples were calculated according to the formula below [Flahault et al., 2005]:

$N_{control} = N_{cases}$ [(1-Prevalence)/Prevalence]

$N_{control} = 40$ [(1-0.3)/0.3] = 93

Since the seroprevalence varied from 9.2-89.0% [Kurukumbi et al., 2001; Singh et al., 2001; Texeria et al., 2004; Wilder-Smith et al., 2004] based on different geographical areas, the average global prevalence was roughly estimated as 30%. Based on the limited number of cases (~40) available, the minimum acceptable lower confidence limit was specified to be 0.7 and the expected sensitivity or specificity of the assay for validation was 0.9 according to the table provided by Flahault et al. [Flahault et al., 2005]. The absorbance measured from

the control group would be used to estimate the cut-off value for the in-house assay. Additionally, measured absorbance from all samples would be converted into the corresponding Panbio unit according to the manufacture's instruction for performance comparison of the assays.

For information on the sera from Brazil, please refer to Appendix II. Sera were thawed at RT and diluted 1:10 and 1:100 with TMT. Similarly, the positive and negative control sera provided in the Panbio Dengue IgG Indirect ELISA kit [Panbio] were diluted 1:100 with the serum diluent provided in the kit [Panbio]. In addition, all sera were tested in parallel with the in-house ELISA assay and the Panbio Dengue IgG Indirect ELISA kit assay [Panbio]. The PPV and NPV were also determined with reference to the results of the Panbio ELISA kit.

2.2.4.2 Serological ELISA assay

Adsorption of three individual purified recombinant proteins to the microtitre plate and non-specific blocking was performed as described in Chapters 2.2.3.3.2.1 and 2.2.3.3.2.2. The plate was used immediately. Each human serum sample was tested against three individual purified proteins antigen as well as one uncoated well without any purified protein. The positive and negative control sera and cut-off calibrator serum provided in the Panbio Dengue IgG Indirect ELISA kit were run in parallel. One hundred μL of diluted sera samples and control sera were added to each well. The plate was incubated at RT for 1 hr and then washed four times with TBS. The following procedures for the ELISA assay were performed according to the manufacturer's instructions for the Panbio Dengue IgG Indirect ELISA kit. One hundred μL of HRP-conjugated anti-human IgG

antibody for detecting the presence of dengue IgG in the sample were added per well. The plate was incubated at 37 °C for 1 hr. Microwells were washed six times with 1x wash buffer (PBS with Tween-20 and 0.1% Proclin preservatives, pH 7.2-7.6) [Panbio]. One hundred μL of TMB substrate (3,3',5,5'-tetramethylbenzidine and hydrogen peroxide in citric-acid citrate buffer, pH 3.5-3.8) [Panbio] were added to each microwell. After a 10 min incubation, 100 μL of stop solution (1M phosphoric acid) [Panbio] were added to each well to cease the HRP enzymatic reaction . The absorbance for each well was read by the Benchmark plus microplate spectrophotometer [Bio-Rad] within 30 min at 450 nm and with 620 nm as the reference wavelength.

The absorbance measured from control group would be used to determine the cut-off value of local population for the in-house assay. The absorbance measured from the triplicate cut-off calibrator serum provided in the Panbio kit would be analyzed as well for cut-off value estimation. Additionally, measured absorbance from all samples would be converted into corresponding Panbio unit according to the manufacture's instruction for assays performance comparison.

2.3 Vector surveillance from *Aedes* mosquitoes collected in Hong Kong by RT-PCR assays

2.3.1 Sample collection

2.3.1.1 Mosquitoes species

Aedes (Ae.) albopictus is the only *Aedes* mosquito in Hong Kong and it is responsible for dengue transmission.

2.3.1.2 Collection sites

There were fifteen sites for mosquito collection. These sites were distributed in various geographical locations in Hong Kong and included eight urban and seven rural locations (Table and Figure 2.3.1). Permission was obtained from the Agriculture Fisheries and Conservation Department (AFCD) of the HKSAR for country parks and the Leisure and Cultural Services Department (LCSD) of the HKSAR for parks and campsites. Two campsites managed by a non-profit organisation were also included for sampling. The Hong Kong Polytechnic University campus represented one of the urban locations and was included as a field collection site. Each site was sampled once a month and over one year (12 visits). Mosquito sampling at six places (QB, KC, ST, ND, YL & ML) governed by the LSCD was held from March 2007 to February 2008, and the rest was held from January 2007 to December 2007. The temperature and rainfall on sampling days were recorded according to the Hong Kong Observatory.

Table 2.3.1 Field-catching sites for mosquito sampling in Hong Kong.

Hong Kong	No.	Area	Mosquito Sampling Venue (code)	District
Hong Kong Island	1	Urban	Quarry Bay Park (QB)	Eastern
	2	Rural	Pok Fu Lam Country Park (PF)	Southern
	3	Rural	Tai Tam Country Park (TT) (Near Hong Kong Parkview)	Southern
Kowloon	4	Urban	The Hong Kong Polytechnic University (PU)	Yau Tsim Mong
New Territories	5	Urban	Central Kwai Chung Park (KC)	Kwai Tsing
	6	Urban	Sha Tin Park (ST)	Sha Tin
	7	Urban	North District Park (ND)	North
	8	Urban	Yuen Long Park (YL)	Yuen Long
	9	Urban	Tuen Mun Town Center (TM)	Tuen Mun
	10	Rural	Shing Mun Country Park (SM)	Tsuen Wan
	11	Rural	So Kwun Wat Valley (SV)	Tuen Mun
	12	Rural	Lions Nature Education Centre (LN)	Sai Kung South
	13	Rural	Lady MacLehose Holiday Village (LM)	Sai Kung West
Lantau Island	14	Urban	Tung Chung Outdoor Recreation Camp (TC)	Islands
	15	Rural	Silvermine Bay Outdoor Recreation Camp (SB)	Islands

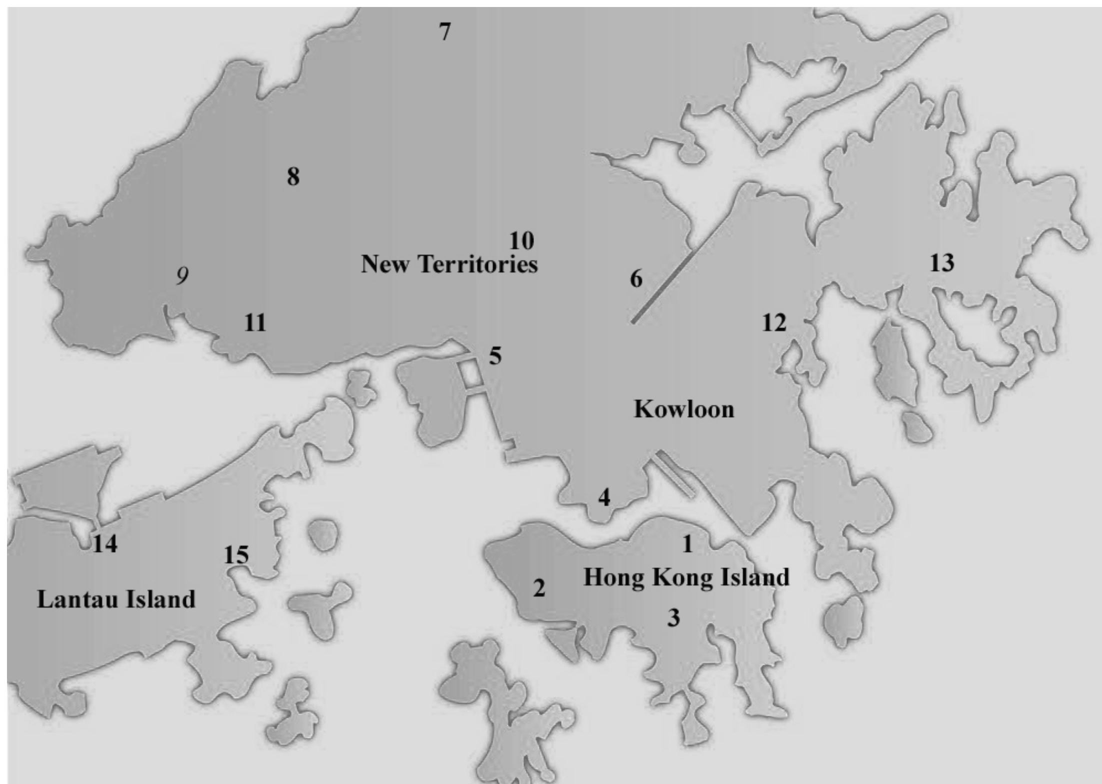


Figure 2.3.1 Sites for mosquito sampling on the map of Hong Kong.

(Hong Kong map background was adopted from nursery.bgca.org.hk and modified in present study http://nursery.bgca.org.hk/general/image/hk-map.gif)

2.3.1.3 Mosquito traps

Mosquitoes were collected using four CDC Wilson traps [John W. Hock Company, USA] (Figure 2.3.2) specific for *A. aegypti and A. albopictus* sampling. The trap was composed of a battery unit, a black cylinder and a bucket on top for placing dry ice. Dry ice released carbon dioxide (CO_2) to attract mosquitoes. Inside the black cylinder, there was a white chamber with a metal nest at the bottom and a metal funnel on the top. A fan under the chamber would suck mosquitoes flying over the trap into the chamber. The metal funnel prevented trapped mosquitoes from flying away. The traps were switched on for a 24 hr for mosquito collection. The mosquitoes collected were kept at -80 °C until further processing.

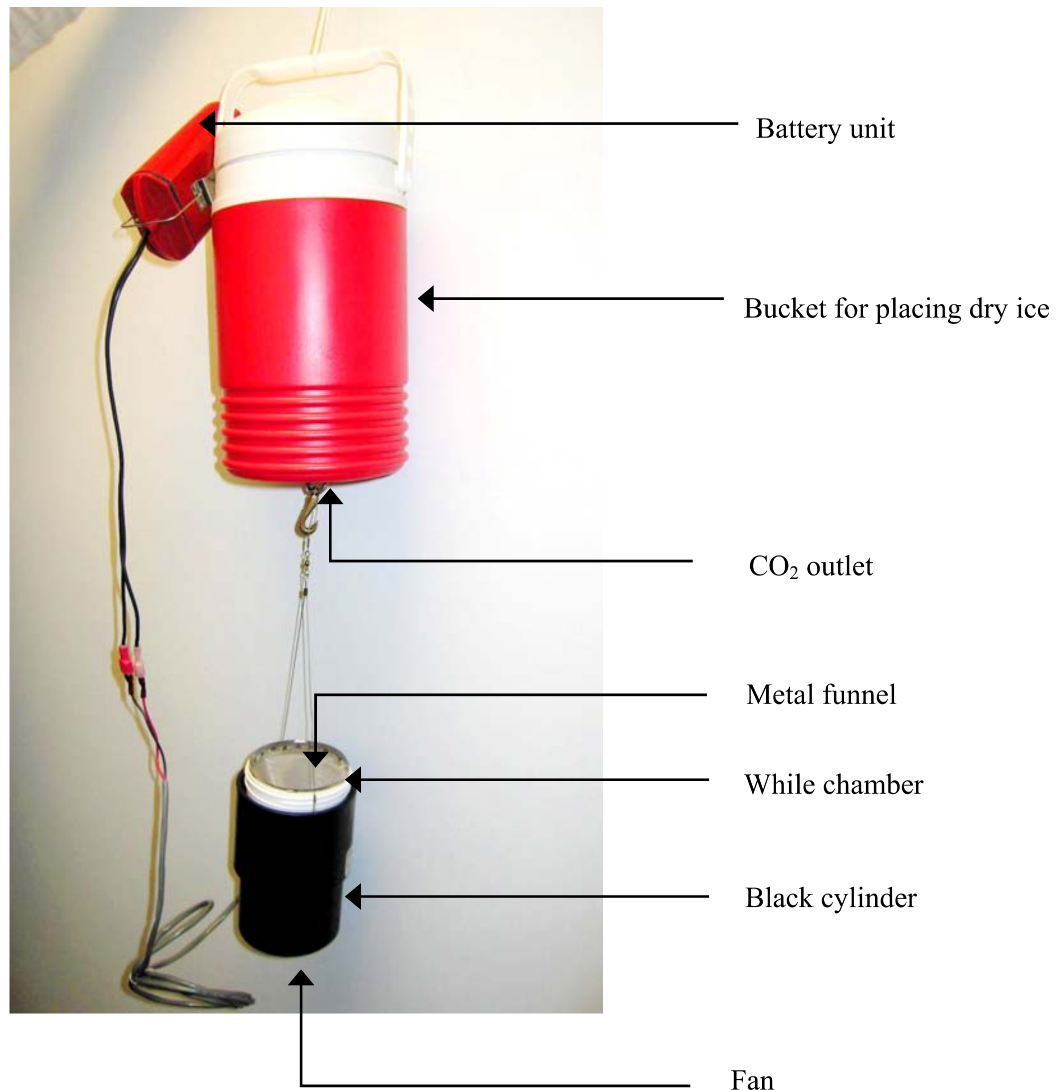


Figure 2.3.2 Schematic figure of a CDC Wilson trap

2.3.2 Mosquito identification by morphological examination

Mosquitoes placed on ice were identified by morphological examination under a low power microscope (10x) according to 1) the Mosquito ID Guide on the website of The Florida Medical Entomological Laboratory (FMEL), the University of Florida's Institute of Food and Agricultural Sciences (IFAS) [Cutwa-Francis MM and O'Meara GF, 2006]; and 2) The mosquito identification key provided by The Walter Reed Biosystematics Unit (WRBU), which is a unique national

resource of the United States Government's entomological research system [The Walter Reed Biosystematics Unit]. *Ae. albopictus* was characterised by some typical features (Figure 2.3.3): 1) the scutum has one silvery-white median stripe down the middle. 2) the palps of the head have silvery-white scales at the tips 3) white bands on the legs. *Ae. albopictus* were pooled according to geographical locations for each month after mosquito identification and kept at -80 °C until RNA extraction. Other mosquito species were also identified into genera by the same mosquito identification guides.

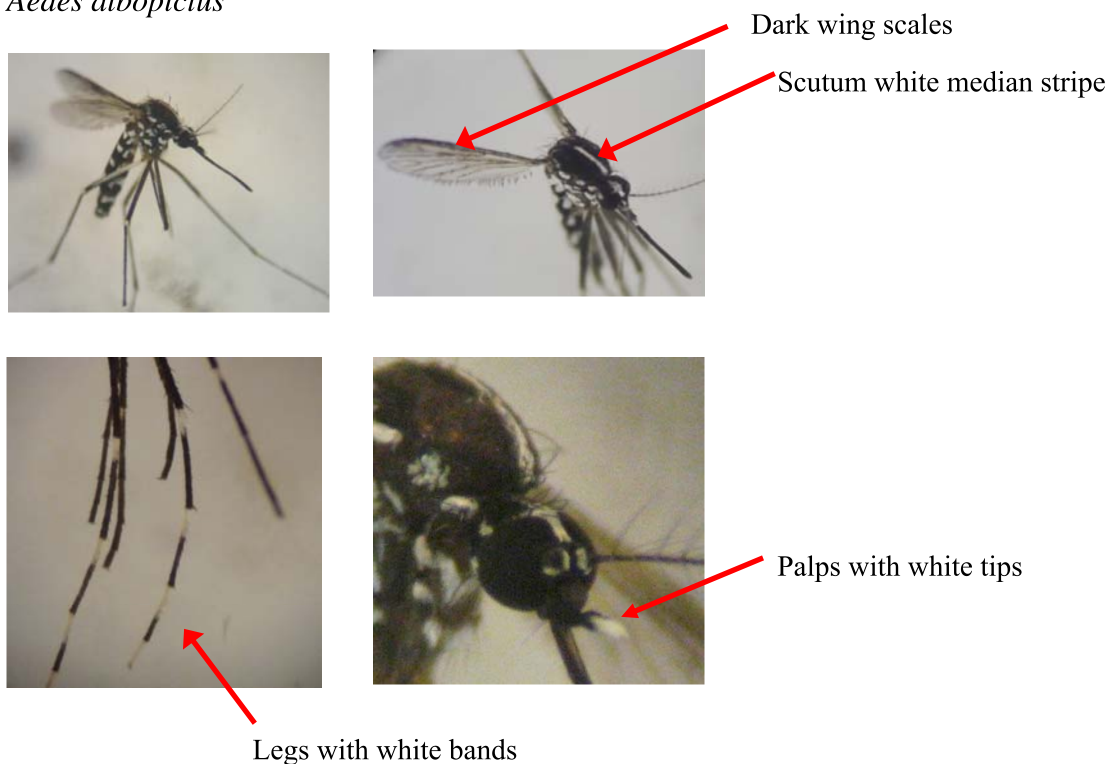


Figure 2.3.3 Identification keys of *Aedes albopictus*

2.3.3 Mosquito screening for DV by RT-PCR assays

2.3.3.1 RNA extraction from mosquito pools

Each mosquito pool represented one RNA sample. Frozen mosquitoes were homogenised with a cryo-cup grinder [Bio-Gene], which is a mortar and pestle that allowed the sample to be ground in liquid nitrogen. Each mosquito pool was put into a stainless steel mortar which was pre-cooled with liquid nitrogen. Mosquitoes were ground into a powder using a gently circular motion of the plastic pestle. In between different mosquito pools, the mortar and pestle were cleaned with deionised water and soap, followed by disinfection with absolute ethanol and RNAzap for the removal of nucleic acid contaminants. The spatulas used for sample transfer were cleaned using the same method as the mortar and pestle, followed by autoclave sterilization [The Institute for Genomic Research, 2004].

Ground mosquitoes were immediately transferred to 100 µL BA-1 diluent (1x Medium 199 with Hank's balanced salt solution and $NaHCO_3$ [Sigma], 50 mM Tris buffer at pH 7.6, 1% BSA [New England Biolab], 100 µg/L streptomycin [Sigma] and 1 µg/L of amphotericin B [Sigma] [Lanciotti et al., 2000]. Eighty µL of the ground mosquitoes in BA-1 diluent were transferred to 300 µL buffer RLT [Qiagen] with ß-mercaptoethanol for complete cell and tissue lysis. The lysate was mixed by pulse-vortexing for 15 s followed by RT incubation for 10 min. All suspensions were transferred to a QIAshredder homogeniser [Qiagen]. After 2 min centrifugation at full speed in a microcentrifuge, the homogenised lysate was subjected to RNA extraction.

Total RNA was extracted from the homogenised lysate with the QIAmp viral RNA mini kit [Qiagen] according to the manufacturer's instructions. Every 180 µL of lysate was mixed with 560 µL of buffer AVL with freshly-added carrier RNA provided by the kit. Finally, the RNA was eluted with 60 µL buffer AVE and kept at -80 °C. The RNA samples were subjected to RT-PCR assays for dengue virus detection.

2.3.3.2 RT-PCR assays for DV screening

Each RNA sample was screened for DV with two RT-PCR assays. One assay was an in-house real time assay for rapid detection, and the other assay was a widely used conventional nested RT-PCR assay. RNA was thawed on ice and then digested with DNase I [Invitrogen] as described in the manufacturer's instructions. Five µL of the DNase I reaction mixture were used as the template. Details of the two PCR assays are found in Chapters 2.1.3.2 and 2.1.6.1, respectively. Any suspected positive cases were confirmed by DNA sequencing. The minimum infection rate (MIR) of *Aedes* mosquitoes in Hong Kong could be calculated by the following formula [Urdaneta et al., 2005]:

$$\text{MIR} = \frac{\text{number of positive tests}}{\text{total number of mosquitoes species tested}} \times 100$$

2.3.3.3 Determination of recovery of DV RNA from the RNA extraction method

Since procedures for RNA extraction from mosquitoes were tedious, RNA recovery after RNA extraction was important to identify, so as to validate the RNA

extraction method and confirm truly negative RT-PCR results. Ten μL of mosquito lysate suspended in BA-1 diluent (30μL in total) was taken out from three randomly-selected *Aedes* mosquito pools which were collected in April 2007 (KC, LN and ML). DEN-1 RNA corresponding to 0.3 PFU of virus was spiked into the mosquito lysate and then subjected to further extraction procedures as described in Chapter 2.3.3.1. The extracted RNA was amplified with the in-house real-time RT-PCR assay. One more reaction with the same amount of pure DEN-1 RNA was run in parallel as a control. The RNA recovery rate could be calculated based on the Ct values and the standard curve of pure DEN-1 RNA.

2.3.3.4 Determination of the effect of contaminants and PCR inhibitors from mosquitoes

In order to test for the presence of contaminants and PCR inhibitors from mosquito samples, DV RNA was spiked into RNA that was extracted from the mosquito pool. Five μL each of RNA that was extracted from the mosquito pools (collected in the same month) were pooled, then 5 μL of the pooled RNA was used as the PCR template and was spiked with DEN-2 RNA (corresponding to 0.21 PFU of virus). Another control PCR was run in parallel using the same amount of DEN-2 RNA alone as the positive control.

To investigate the effect of contaminants and PCR inhibitors from mosquitoes on detecting different serotypes of DV with the PCR assay, RNA extracted from all collected mosquitoes was pooled into a single mixture. Five μL of RNA mixture were aspirated and spiked individually with RNA of 1) DEN-1 corresponding to 0.03 PFU; 2) DEN-2 corresponding to 0.21 PFU; 3) DEN-3 corresponding to 0.16 PFU and 4) DEN-4 corresponding to 1.16 PFU into four

PCR reaction tubes. Another four positive control PCR reactions were set up using pure DV RNA as templates.

The detection limit of assay for DV in the presence of mosquito extract was also investigated. RNA pool extracted from all mosquitoes was individually spiked with six dilutions of 10-fold serial dilution of DEN-2 RNA (2.10×10^{2} to 2.10×10^{-5} PFU). Five μL of DEN-2 RNA-spiked mosquito extract were used as the template. Another set of serially-diluted DEN-2 RNA was set up as the positive control.

The obtained Ct values were compared, and each reaction was subjected to agarose gel electrophoresis for confirmation of the presence of specific PCR products.

2.3.3.5 DNA sequencing of PCR positive samples

Details of sequencing analysis are described in Chapter 2.1.3.3.

2.4 Seroepidemiological study of dengue in general population of Hong Kong with a commercial ELISA assay

2.4.1 Samples collection

2.4.1.1 Subject recruitment

Two groups of subject were recruited from different sources. One group consisted of samples from the community via various routes collected by the researcher (researcher-collected). They included subjects recruited from a university campus, a community center and a hospital. Subjects from university and community center were recruited by poster advertisement (Appendix III). Those recruited in hospital were normal subjects randomly selected based on a clinical research subject recruitment database of the Prince of Wales hospital in Hong Kong. The age range of subjects recruited from the university campus was 18-60 years. Subjects recruited from two other routes were all adults over the age of 18 years. Information sheets were sent and consent forms were completed by each subject. A questionnaire survey (Appendix III) was conducted to investigate the risk factors associated with seropositivity. Those risk factors included gender, age, residential district in Hong Kong, residence in Hong Kong, original home town and travel history of the subjects. Past vaccination history for Japanese encephalitis and yellow fever was also recorded for interpretation of false positive results.

The other group consisted of healthy blood donors from the Hong Kong Red Cross. Subjects were randomly selected and kindly provided by the Hong Kong Red Cross. The age of subjects was within the range of 16-65 years which

are the eligibility criteria for blood donation in Hong Kong. Only information on the age and gender of each subjects were provided.

2.4.1.2 Blood sample collection

For subjects recruited at the university campus and community centers, 2 hr fasting before blood collection was requested to prevent lipaemic serum. Six mL of blood was drawn by venipuncture by an experienced phlebotomist with a sterile syringe. Blood was aliquoted into two blood collection tubes. For serum, 4 mL of blood was collected into a tube with a clot activator and gel barrier [Terumo]; while 2 mL of blood was collected into a tube with ethylenediaminetetraacetic acid (EDTA) anticoagulant for plasma [Terumo]. Blood samples from subjects recruited at the Prince of Wales hospital were collected on site by a experienced phlebotomist with a Vacutainer® blood collection system [BD]. Four mL of blood was collected into a tube with a clot activator [Terumo]. Blood collection tubes were labeled with a code corresponding to each subject. Subjects could be later identified by codes which were known only to the researcher.

For blood samples collected from the Hong Kong Red Cross, 4 mL of blood was aliquoted in blood collection tubes with the anticoagulant EDTA. Every tube was identified with a bar-code label representing a particular subject. All blood samples were delivered at 4 °C to the laboratory.

2.4.2 Determination of past dengue infections by a commercial indirect ELISA assay

2.4.2.1 Sample preparation

For researcher-collected samples, blood was allowed clot completely by allowing the samples to sit at RT for 1 hr. Serum was retrieved by centrifugation with a swing-out rotor [Thermo] at 2,700 x g for 15 min at 4 °C. Serum was kept in small aliquots at -80 °C. For samples collected from the Red Cross, blood samples were collected in collection tubes with the anticoagulant EDTA. Plasma instead of serum was harvested after centrifugation as described above. Plasma was converted into a serum analogue by defibrination as followed [Johnstone & Thorpe, 1996]: One mL of plasma was pre-warmed to 37 °C, 10 μL of thrombin solution (100 U of thrombin in 1 mL of 1 M $CaCl_2$) [Johnson & Johnson] was added to the plasma and stirred vigorously with a sterile wooden stick to induce clot formation. The mixture was incubated at 37 °C for 10 min followed by incubation at RT for 1 hr. The tube was centrifuged at 18,000 x g for 15 min, and supernatants were kept at -80 °C as serum.

2.4.2.2 Indirect ELISA assay

Sera and serum analogues were tested for IgG antibodies against DV using a commercial ELISA kit, the Panbio Dengue IgG Indirect ELISA. This kit detects the presence of IgG in serum from past and active dengue infections, so it could be applied for a seroepidemiological study. The microwells of the microtitre plate were pre-coated with dengue antigen serotypes 1 to 4. The presence of anti-dengue IgG in serum samples was captured and then detected by horseradish peroxidase (HRP)-conjugated anti-human IgG. The signal was then visualised by a

colourimetric change after the addition of substrate and stop solution. The absorbance of each well was measured by a Benchmark plus microplate spectrophotometer [Bio-Rad] at 450 nm with the reference filter at 620 nm.

The ELISA protocols were performed according to the manufacturer's instructions. Briefly, serum samples, positive and negative control sera and the cut-off calibrator serum were diluted 1:100 with 1 x serum diluent (TBS with 0.1% Proclin^{TM} preservative, pH 7.2-7.6) provided in the kit [Panbio]. One hundred μL of each diluted serum was pipetted into a microwell. One each of the positive and negative controls and three cut-off calibrator sera were included in each assay. The plate was incubated at 37 °C for 30 min and then washed six times with 1x wash buffer (20x concentrate of phosphate buffer saline with Tween-20 and 0.1% Proclin^{TM} preservative, pH 7.2-7.6) [Panbio]. One hundred μL of HRP-conjugated anti-human IgG antibody were added per well. The plate was incubated at 37 °C for 30 min. Microwells were washed six times with wash buffer. One hundred μL of tetramethylbenzidine (TMB) substrate (3,3',5,5'-tetramethyl-benzidine and hydrogen peroxide in citric-acid citrate buffer, pH 3.5-3.8) [Panbio] was pipetted into each microwell. After a 10 min incubation, 100 μL of stop solution (1 M phosphoric acid) [Panbio] was pipetted to cease the HRP enzymatic reaction. Each microwell was read for absorbance by the microplate spectrophotometer [Bio-Rad] at a wavelength 450nm with a reference filter of 620 nm within 30 min. Absorbance was converted into Panbio units for analysis by the formula provided: Panbio unit = absorbance of sample / mean of cut-off value x 10.

In order to investigate any adverse effect of the defibrination of plasma on the interpretation of results from the Panbio Dengue IgG Indirect ELISA kit, all seropositive sera were tested in parallel using their defibrinated plasma. Moreover, an aliquot of a pool of negative plasma samples collected from the Hong Kong Red Cross was converted to a serum analogue by defibrination as described in Chapter 2.4.2.1, and then tested parallel with the untreated plasma by the ELISA kit. Absorbance collected from both kinds of samples by two assay measurements was analysed for the degree of variability by the coefficient of variation (CV).

2.4.3 Statistical method for potential risk factor analysis

All data were analysed by the SPSS statistics programme (V11.5, for Windows). Individual univariate qualitative variables (potential risk factors) were analysed individually for association with dengue seropositivity using the *Chi*-squared test. The odds ratio (OR) and 95% confidence interval (CI) were also calculated for each potential factor to estimate the strength of risk related to seropositivity. Factors which were statistically significant ($P \leq 0.05$) were subjected to multivariate analysis by logistic regression with seropositivity as the dependent variable [Chan, 2004].

CHAPTER 3

RESULTS

3.1 Development of a rapid RT-PCR assay for simultaneous detection and serotyping of DV

3.1.1 Commercial reagent kit assay

The RT-PCR assay specifically detected the four DV serotypes, with no cross-reactivity between the primers (Figure 3.1.1). The Japanese encephalitis virus was not amplified by the assay. Twelve (75%) of the sixteen clinical serum samples known to be DV positive were accurately identified and serotyped (specificity 100%). The F1 channel gave melting peaks due to SGI fluorescence for all four serotypes. In particular, the T_m difference between DEN-2 and DEN-4 was 4.7 °C, which was sufficient to differentiate these two serotypes. DEN-1 and DEN-3 had very similar T_ms in the F1 channel; however, DEN-1 was characterised by its T_m (83.3 °C) in the F2 channel owing to the iFRET between SGI and BODIPY 630/650, and DEN-3 was characterised by its T_m (83.9 °C) in channel F3 because of the iFRET between SGI and Cy5.5.

The measurement of T_m was very precise, with within-run CVs ranging from 0.1% to 0.3% and between-run CVs ranging from 0.2% to 0.6% (Table 3.1.1). The detection limits in plaque forming units per reaction and the corresponding threshold cycle (Ct) value for DEN-1 (1.64×10^{-4} and 30.8) and DEN-3 (8.15×10^{-4} and 27.8) were one log unit lower than those for DEN-2 (1.05×10^{-3} and 33.7) and DEN-4 (5.80×10^{-3} and 30.7). The assay had a dynamic range of detection

(10^3–10^8 plaque forming units/L). Amplification plots for each serotype detected in the F1 channel due to the SGI signal are shown in Figure 3.1.3. The correlation coefficients for the correlation between viral concentration and Ct value were –0.99 for both DEN-1 and DEN-3, –0.98 for DEN-4, and –0.95 for DEN-2 (Figure. 3.1.2). The amplification efficiency was highest for DEN-1 and lowest for DEN-4, as calculated using the slope of the calibration curves based on the equation: efficiency = $10^{(-1/slope)}$ [Rasmaussen, 2001]. The PCR products of each serotype were visualized by agarose gel electrophoresis (Figure 3.1.4A)

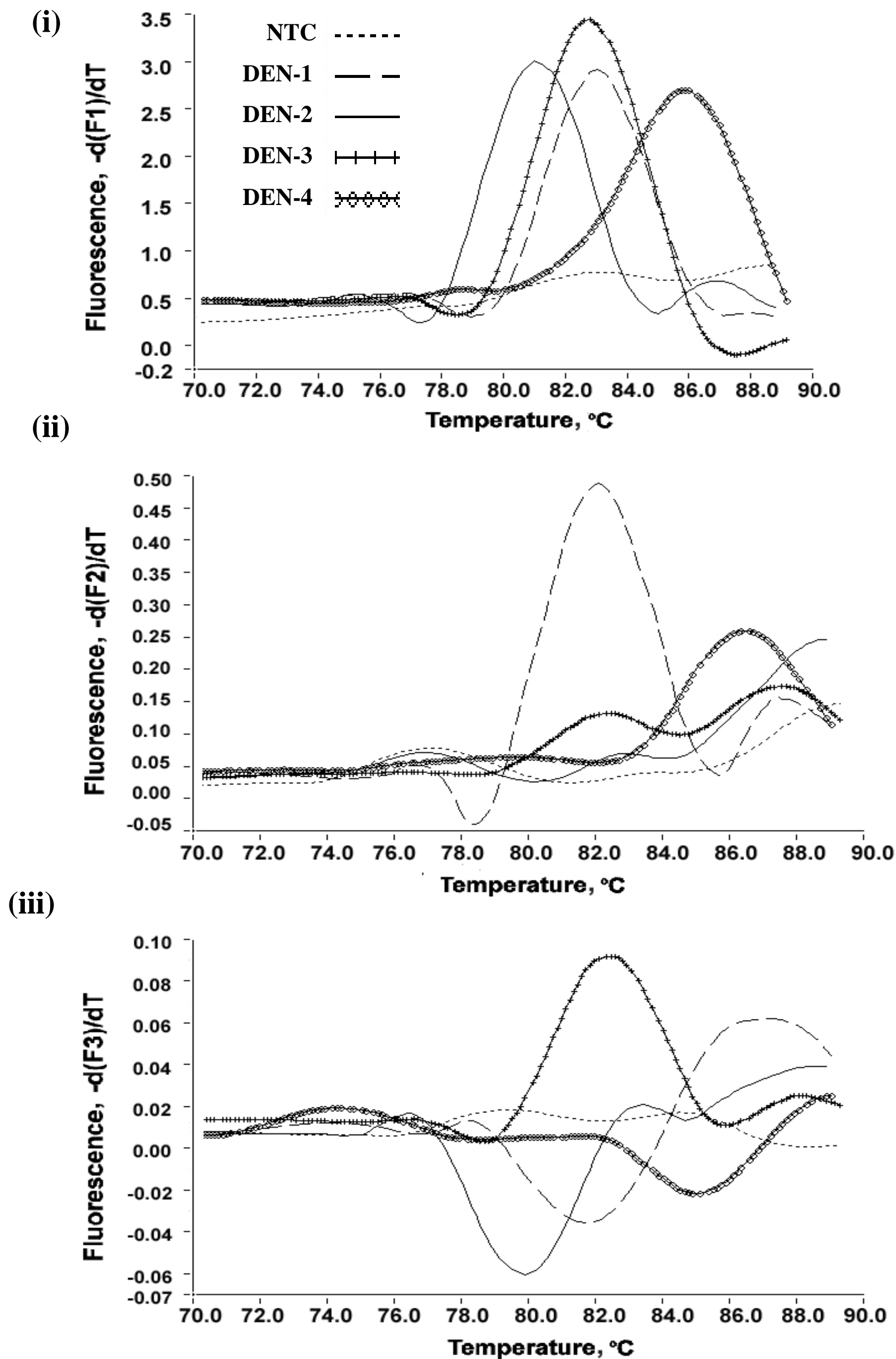


Figure 3.1.1 Derivative melting curves for each serotypes of DVs of the kit-based RT-PCR LightCycler assay.

Derivative melting curves showing the melting peaks characteristic of the 4 serotypes detected in 3 LightCycler channels (F1–F3). NTC indicates no-template control. Amplicon T_ms are measured in channel F1 (SGI fluorescence) for DEN-2 and DEN-4 (*i*), in channel F2 (BODIPY 630/650 fluorescence) for DEN-1 (*ii*), and in channel F3 (Cy5.5 fluorescence) for DEN-3 (*iii*). In channel F3, inverted melting peaks due to DEN-2, and sometimes to DEN-1 and DEN-4, may be detected. The reason for these inverted peaks is not known; however, they do not interfere with DEN-3 typing.

Table 3.1.1 Comparison of assay performance of the original kit-based and in-house RT-PCR LightCycler assays.

Serotype	Predicted T_m[a], °C	Observed T_m Mean ± SD, °C (CV, %)		T_m difference between original and in-house assays[b] Inter-assay (Intra-assay)		Detection limit of assay, PFU per reaction (Ct value)	
		Kit-based	In-house	*P*-value	Correlation	Kit-based	In-house
DEN-1	84.4	83.3 ± 0.5 (0.6%)	82.0 ± 0.3 (0.7%)	0.23 (0.31)	0.52 (0.45)	1.64×10^{-4} (30.8)	3.27×10^{-3} (28.9)
DEN-2	82.4	81.0 ± 0.5 (0.6%)	80.82 ± 0.3 (0.3%)	0.79 (0.82)	-0.12 (-0.11)	1.05×10^{-3} (33.7)	2.10×10^{-3} (29.8)
DEN-3	84.0	83.9 ± 0.2 (0.3%)	82.29 ± 0.3 (0.4%)	0.95 (0.66)	-0.02 (-0.20)	8.15×10^{-4} (27.8)	1.63×10^{-3} (28.4)
DEN-4	86.0	85.7 ± 0.2 (0.2%)	85.08 ± 0.3 (0.3%)	0.16 (0.14)	-0.60 (0.62)	5.80×10^{-3} (30.7)	1.16×10^{-2} (27.6)

[a] Predicted amplicon melting temperatures (T_m) were calculated by OLIGO 6.0 software (Molecular Biology Insights, USA).

[b] *P*-value and correlation were calculated by paired T-test by SPSS 11.5 statistics program.

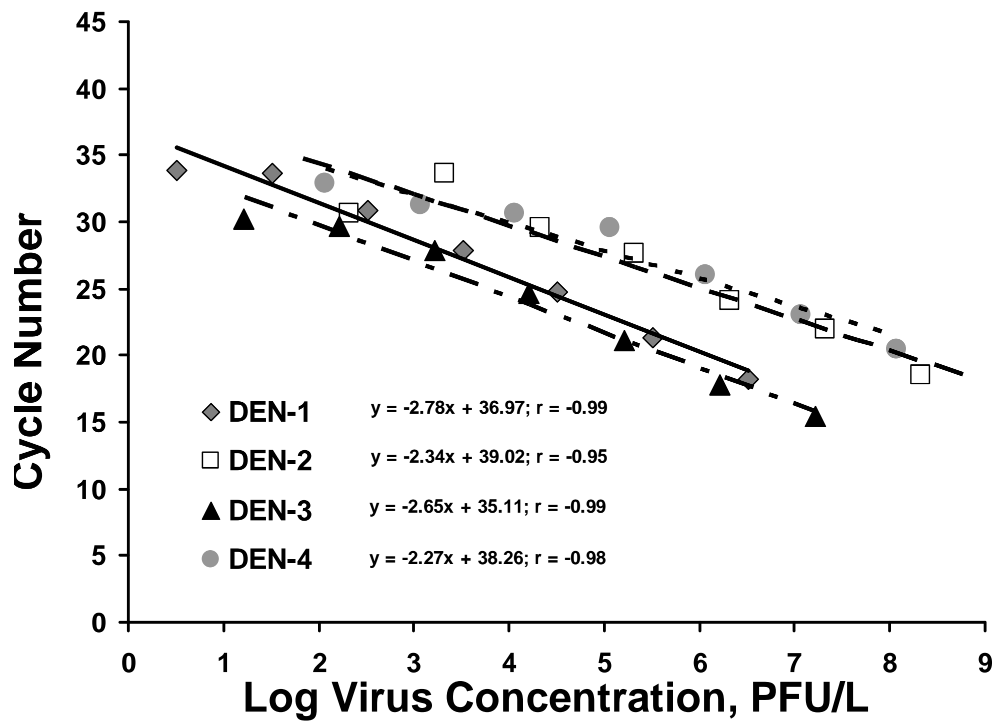


Figure 3.1.2 Calibration curves for the four serotypes of DVs of the kit-based RT-PCR LightCycler assay. (PFU, plaque-forming unit.)

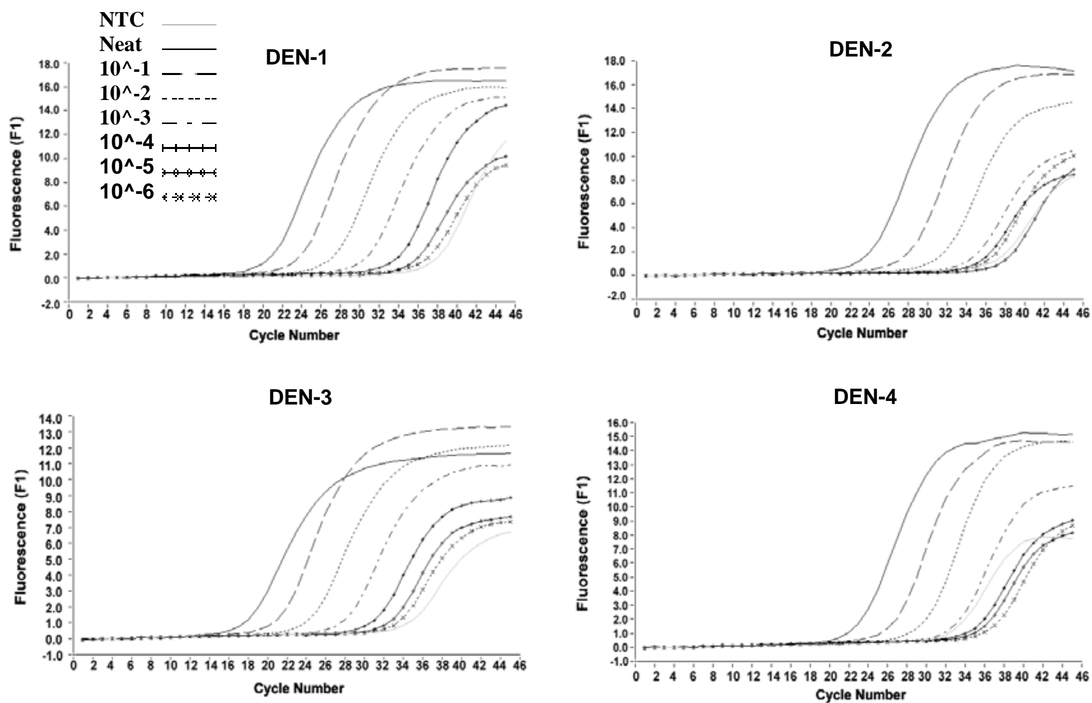


Figure 3.1.3 Amplification curves for each DV serotype detected by F1 channel using the kit-based RT-PCR LightCycler assay.

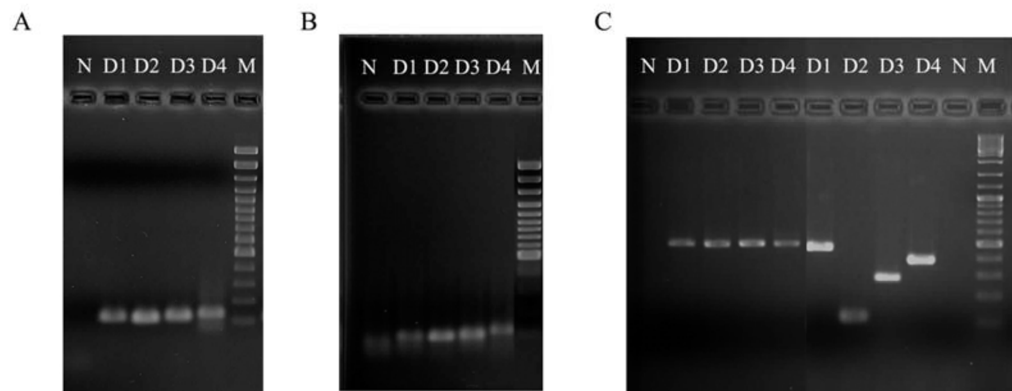


Figure 3.1.4 Agarose gel electrophoresis of RT-PCR products amplified by the RT-PCR LightCycler assays and the conventional nested RT-PCR assay.

N, no template control; D1-4, DEN-1 to -4 serotypes; M, molecular weight marker, 100 bp. A) PCR products amplified by the kit-based RT-PCR LightCycler assay. B) PCR products amplified by the in-house RT-PCR LightCycler assay. The product sizes for DEN-1 to -4 were 103 bp, 105 bp, 103 bp and 111 bp, respectively. C) PCR products amplified by the conventional nested RT-PCR assay. Products from the first round RT-PCR (Lane 1-5), 511 bp and the nested round PCR (Lane 6-10) of DEN-1 to -4 were showed in sequence (DEN-1, 482 bp; DEN-2, 119 bp; DEN-3, 290 bp; DEN-4, 392 bp).

3.1.2 In-house developed assay

Four serotypes of control DV were specifically detected by the corresponding detection channels of the LightCycler 1.5 system and there was no cross-reaction with Japanese encephalitis virus RNA. As in the original kit-based assay, the F1 channel was mainly used for detecting DEN-2 and DEN-4 since the amplicons were not labeled with fluorophore and the fluorescence signals were emitted from SGI (Figure 3.1.5). The mean T_m was 80.8 °C (n=8; SD, 0.3; CV, 0.3%) for DEN-2 and 85.1 °C (n=8; SD, 0.3; CV, 0.3%) for DEN-4. The mean T_m difference between DEN-4 and DEN-2 was 4.3 °C (n=8; SD, 0.2; CV, 4.5%). The T_ms of DEN-1 and DEN-3 were respectively measured by the F2 and F3 detection channels, depending on the particular fluorophore label of the primer (BIODPY 630/650 for DEN-1 and Cy5.5 for DEN-3). The mean T_m of DEN-1 measured in

the F2 channel was 82.0 °C (n=8; SD, 0.5; CV, 0.7%) and the mean T_m of DEN-3 measured in the F3 channel was 82.3 °C (n=8; SD, 0.3; CV, 0.4%). There was no statistically significant difference ($P>0.05$) of the measured T_m (inter- and intra-assay) between the in-house and original assays (Table 3.1.1). The detection limits of the in-house assay ranged from 3.27×10^{-3} to 1.16×10^{-2} PFU/reaction. Except for the DEN-2 serotype, they were one log unit higher than the original kit-based assay. The dynamic range of detection of the in-house assay was 10^4-10^8 PFU/L compared to the original assay. The linear correlation (r) between viral concentration and Ct was -0.97 for DEN-3, -0.95 DEN-2, -0.93 for DEN-1, and -0.92 for DEN-4 (Figure 3.1.6). The agarose gel electrophoresis image of PCR products from each serotype was showed in Figure 3.1.4B.

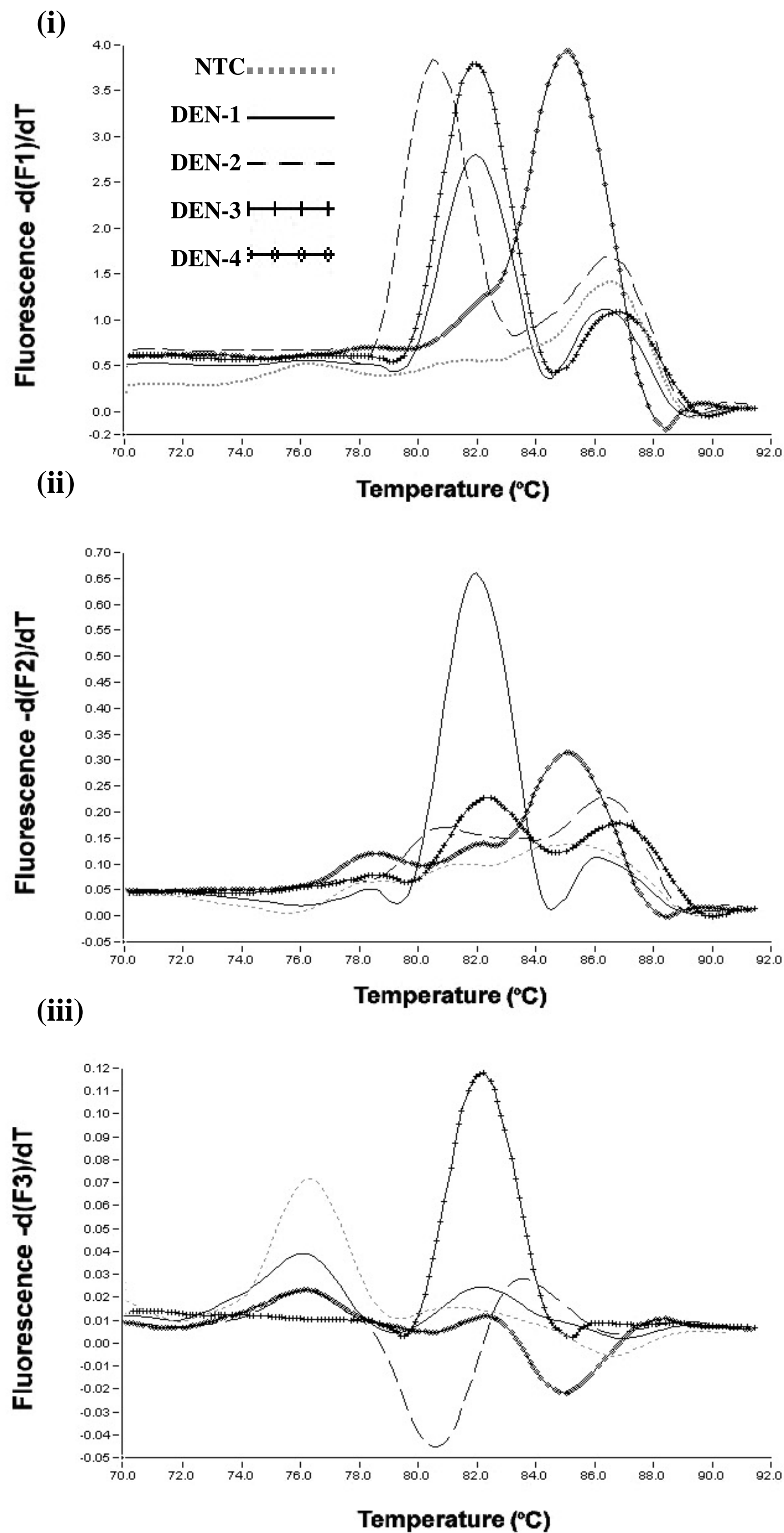


Figure 3.1.5 Derivative melting curves of each serotypes of DVs of the in-house RT-PCR LightCycler assay.

Derivative melting curves showing the melting peaks characteristic of the 4 serotypes detected in 3 LightCycler channels (F1–F3). NTC indicates no-template control. Amplicon T_ms are measured in channel F1 (SGI fluorescence) for DEN-2 and DEN-4 (*i*), in channel F2 (BODIPY 630/650 fluorescence) for DEN-1 (*ii*), and in channel F3 (Cy5.5 fluorescence) for DEN-3 (*iii*). In channel F3, inverted melting peaks due to DEN-2, and sometimes to DEN-1 and DEN-4, may be detected. The reason for these inverted peaks is not known; however, they do not interfere with DEN-3 typing.

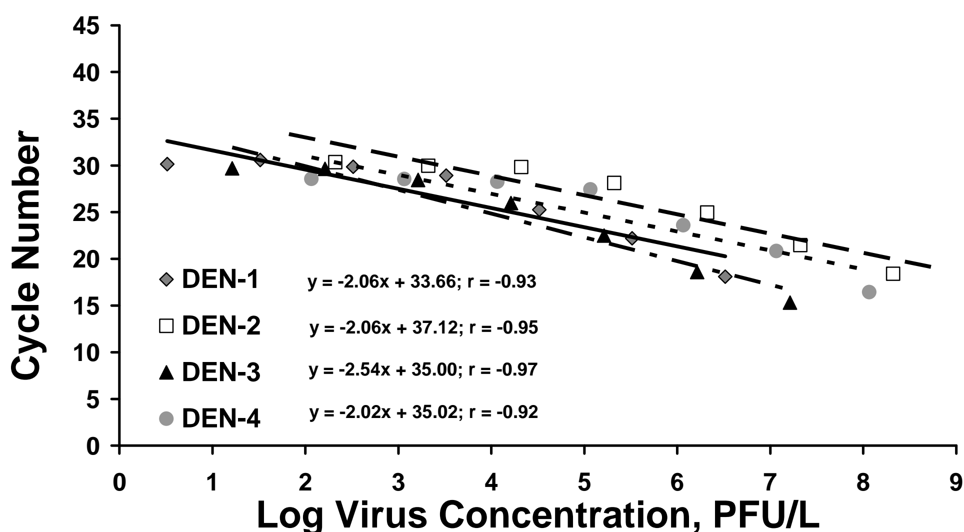


Figure 3.1.6 Calibration curves of the four serotypes of DVs of the in-house RT-PCR LightCycler assay. PFU, plaque-forming unit.

3.1.3 Assay validation with clinical sera

The kit-based RT-PCR assay was validated with clinical sera provided by the PHLC of Hong Kong. The positivity was confirmed by RT-PCR assay. The sensitivity of the kit-based assay was 75.0% (12/16) and all positive detected sera were correctly serotyped. The sensitivity was increased by 12.5% (2/16) with increased RNA template volume from 0.5 μL to 5 μL. Four negative samples were tested again with the original RNA stock in PHLC for confirmation. It was found that one was PCR-positive, one was weakly PCR-positive and the other two were PCR negative by their usual molecular detection method.

For the in-house RT-PCR assay, the assay was validated with clinical sera collected from three geographical regions. For samples from Brazil, 26 out of 36 (72.2%) serum samples were detected by our in-house assay; three samples were identified as DEN-2 and 23 samples as DEN-3 (Table 3.1.2). The serotyping

results matched with the results provided. Ten samples could not be detected by our in-house assay. Of these, five samples collected from Goiãnia city had viral loads less than 6.75×10^{8} RNA copies/L and the other five from Aracaju city had unknown viral loads (Appendix I). The conventional nested RT-PCR assay detected 29 out of 36 (80.6%) sera. PCR positive of the conventional nested RT-PCR was based on the specific bands visualized on agarose gel electrophoresis (Figure 3.1.4C). One DEN-3 strain was typed as DEN-2 by the conventional RT-PCR (Table 3.1.2). For samples from Hong Kong, the in-house assay detected and correctly typed eleven out of twelve (91.6%) samples, and the conventional RT-PCR detected and correctly typed all twelve (100%) samples (Table 3.1.2). The overall sensitivity of the in-house and the conventional assays were 77.1% (37/48) and 85.4% (41/48), respectively; there was no significant difference (P=0.219, McNemar's test) between the assays. The in-house and conventional assays correctly serotyped 100% (37/37) and 97.6% (40/41), respectively. Moreover, the PPV and NPV for evaluation of sera collected from Hong Kong and Brazil was 100% (37/37) and 63.6% (7/11), respectively. These results indicated all positively detected sera by the in-house assay were truly positive. While negatively detected sera by the in-house assay had about 30% of chance which was falsely unidentified.

For the 125 samples from Guangzhou, 20 samples (16.0%) were positively detected and typed as DEN-1 by the in-house assay (Table 3.1.3). When tested with the conventional nested RT-PCR, fourteen samples were detected as positive (11.2%); thirteen were typed as DEN-1 and one was typed as DEN-2. Compared to the conventional RT-PCR assay, in-house assay falsely detected 6 sera (PPV =

14/20 = 70%); however, the in-house assay showed a good result as a screening assay for dengue (NPV = 105/105 = 100%). We discovered that another fifteen samples were positive only in the first-round PCR. Notably, when these amplicons were DNA-sequenced and searched against the GenBank database, all sequences were aligned to DEN-1 strains. There were five nucleotide variations within the priming sequence of the DEN-1-specific reverse primer TS1 used in the second-round PCR (Figure 3.1.7), so this might account for the negative result in the second-round PCR. Of the 62 anti-DV IgM-positive samples, eight (12.9%) were detected as positive by both assays. Of the 26 anti-DV IgM negative samples, twelve (46.2%) and five (19.2%) were detected as positive by the in-house assay and the conventional RT-PCR assay. Of these 26 IgM-negative samples, a second serum specimen was collected from 20 patients (three to six days apart) for repeating the serological assay in the hospital of Guangzhou. It was found that twelve specimens were IgM-positive, which indicated that those patients were in the acute phase of DV infection; eight specimens were still IgM-negative. All seven non-dengue cases of the total 125 samples were detected as negative by both assays. These indicated the specificities of two assays were 100% (7/7). Of the 37 samples without anti-DV IgM information, only one sample was detected as positive by the conventional RT-PCR.

Table 3.1.2 Information and PCR results of clinical sera collected from Brazil and Hong Kong for in-house and conventional nested RT-PCR assays validation.

Sample source	Serotype	No. of strains	No. of strains positively detected and typed [Sensitivity, %]	
			In-house assay	Conventional nested PCR assay 1st round (nested round)
Aracaju, Brazil	DEN-2	4	3	2 (2)
	DEN-3	4	0	0 (1)
Goiania, Brazil	DEN-3	28	23	24 (26)
	Total	**36**	**26** [**72.2**]	**26 (29)** [**80.6**]
Hong Kong SAR, China	DEN-1	2	2	2 (2)
	DEN-2	3	3	3 (3)
	DEN-3	3	3	3 (3)
	DEN-4	4	3	4 (4)
	Total	**12**	**11** [**91.6**]	**12 (12)** [**100.0**]

Table 3.1.3 Information and PCR results of clinical sera collected from Guangzhou, China for in-house and conventional nested PT-PCR assay validations.

Anti-dengue IgM status	No. of strains	No. of strains positively detected and typed	
		In-house assay	Conventional nested PCR assay 1st round (nested round)[a]
Positive	62	8	10 (8)
Negative	26	12	12 (5)
Unknown	37	0	0 (1)
Total	**125**	**20**	**22 (14)**

[a] There were 15 samples positively detected by the 1st round PCR only but not by the nested round PCR. And 7 more samples were positively detected after nested round PCR.

```
                TS1 primer     3'--//GGGGGCCTAGTGACTCTGT//--5'
Complementary priming sequence 5'--//CCCCCGGATCACTGAGACG//--3'
                Sample no.
                    3699       5'--//CCCTCGAATCACTGAAGCT//--3'
                  3741         5'--//CCCTCGAATCACTGAAGCT//--3'
                  4072         5'--//CCCTCGAATCACTGAAGCT//--3'
                  4162         5'--//CCCTCGAATCACTGAAGCT//--3'
                  4189         5'--//CCCTCGAATCACTGAAGCT//--3'
                  4255         5'--//CCCTCGAATCACTGAAGCT//--3'
                  4369         5'--//CCCTCGAATCACTGAAGCT//--3'
                  4375         5'--//CCCTCGAATCACTGAAGCT//--3'
                  4430         5'--//CCCTCGAATCACTGAAGCT//--3'
                  4432         5'--//CCCTCGAATCACTGAAGCT//--3'
                  4506         5'--//CCCTCGAATCACTGAAGCT//--3'
                  4571         5'--//CCCTCGAATCACTGAAGCT//--3'
                  4590         5'--//CCCTCGAATCACTGAAGCT//--3'
                  4603         5'--//CCCTCGAATCACTGAAGCT//--3'
                  4669         5'--//CCCTCGAATCACTGAAGCT//--3'
                                     *** ** *******    *
```

Figure 3.1.7 Alignment of TS1 primer priming sequence region of clinical sera from Guangzhou, China.

Underlined base indicates nucleotide different from the TS1 primer of the conventional RT-PCR assay.

3.2 Generation of recombinant DEN-2 proteins for investigating the neutralising potential by their corresponding antisera and developing a serological diagnostic assay

3.2.1 DNA fragments of *prM, ED3* and *prM-ED3* generated by PCR amplifications

For PCR amplification by degenerate primers, 404 bp and 644 bp of *ED3* and *prM* products was amplified, respectively, with an annealing temperature of 50 °C for the primers (Figure 3.2.1A). The sequences of the target regions of the two genes for amplification were identified by ABI3130 [Applied Biosystems] and shown in Figure 3.2.2. In-frame products were amplified based on the specific primers designed from identified sequences (Figure 3.2.2).

The 297 bp and 501 bp of *ED3* and *prM* in-frame products were amplified, respectively. Annealing temperatures of 55 ^{o}C and 58 ^{o}C were able to amplify the specific product (Figure 3.2.1B); however, a non-specific product was amplified at 55 ^{o}C for the *ED3* gene. Prior to the generation of a chimeric *prM-ED3* fusion DNA fragment, two intermediate chimeric *prM* and *ED3* products were amplified. Two intermediate products with sizes of 516 bp for *prM* and 315 bp for *ED3* were amplified (Figure 3.2.1C). These two intermediate products were purified by the QIAquick PCR purification kit followed by a second round fusion PCR (the principle of fusion PCR is illustrated in Figure 2.2.2). A 795 bp product was visualised on an agarose gel. We observed a non-specific product of ~1.3 kb, so the specific product was purified by a QIAquick gel extraction kit and then subjected to sequence analysis. All in-frame freshly prepared PCR products were cloned into the vector.

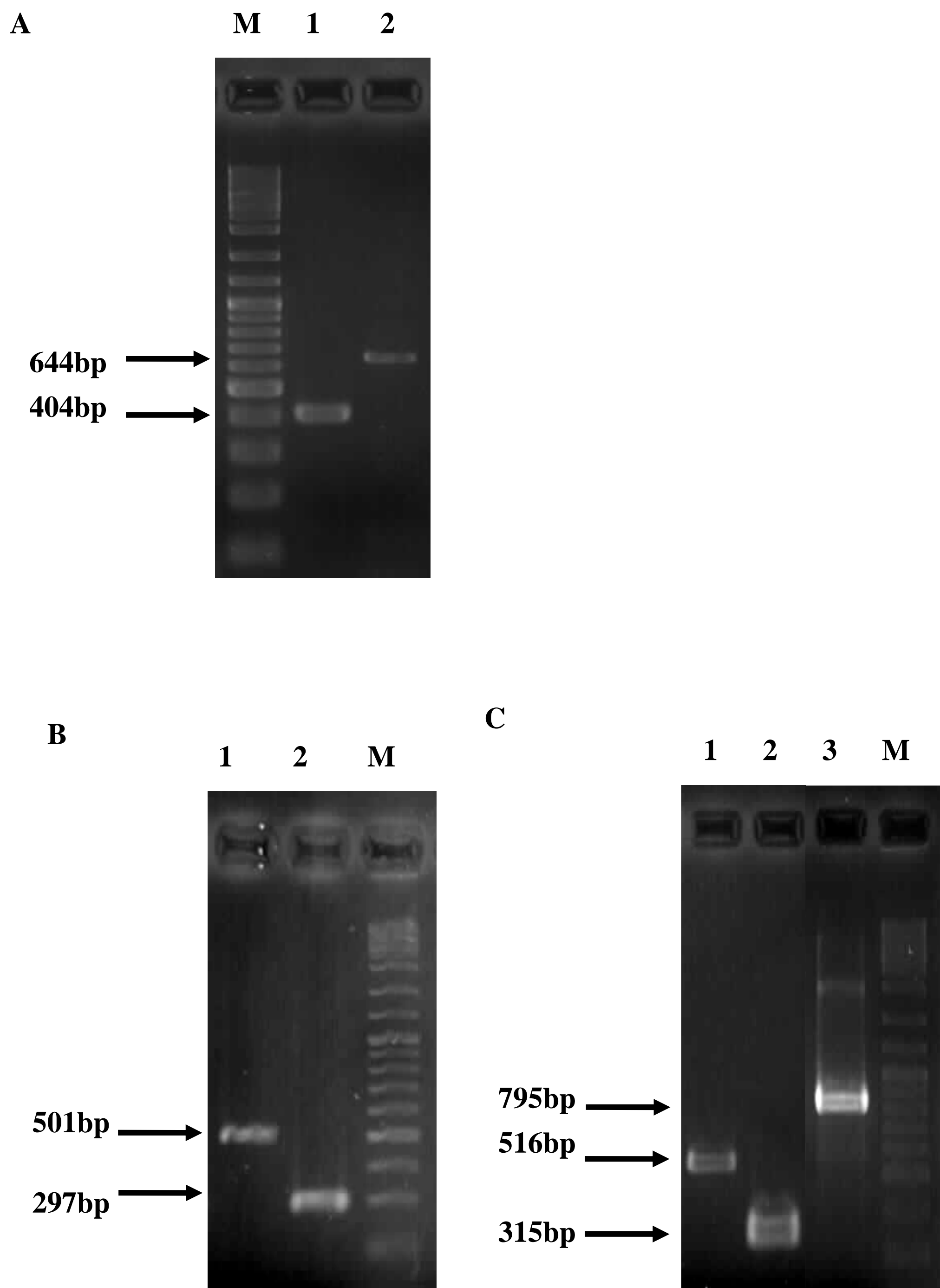


Figure 3.2.1 PCR amplification of *prM* and *ED3* genes by different primer set.

M, molecular weight marker, 100bp. A) *ED3*, 404 bp (Lane 1) and *prM*, 644 bp (Lane 2) genes were amplified by degenerated primers. B) In-frame *prM*, 501 bp (Lane 1) and *ED3*, 297 bp (Lane 2) were amplified by sequence specific primers. C) In-frame *prM-ED3* chimeric fusion product, 795 bp (Lane 3) was amplified by fusion PCR. Two chimeric intermediate product of *prM* (Lane 1) and ED3 (Lane 2).

***prM* fragment amplified by degenerate primers (5'→ 3')**

```
1    TGCCAGTGCGGGGCTTGAGCTATATGGTGATTCCGACATTGAAGGCGTTCCATTTAACCA
                                                        prM_F_in
61   CACGTAACGGAGGAACCTGGTGATCGTCAGTCTTAAAGAGAAAGGGAAAAGTCTTCTGTT
121  TAAAACAGAGGATGGTGTGAACATGTGTACCCTCATGGCCATGGACCTTGGTGAATTGTG
181  TGAAGATACAATCACGTACAAGTGTCCTCTTCTCAGGCAGAATGAACCAGAAGACATAGA
241  TTGTTGGTGCAACTCTACGTCCACATGGGTAACTTATGGGACGTGTACCACCACAGGAGA
301  ACACAGAAGAGAAAAAAGATCAGTGGCACTCGTTCCACATGTGGGAATGGGACTGGAGAC
361  ACGAACTGAAACATGGATGTCATCAGAAGGGGCCTGGAAACATGCCCAGAGAATTGAAAC
421  TTGGATCTTGAGACATCCAGGCTTTACCATAATGGCAGCAATCCTGGCATACACCATAGG
481  AACGACACATTTCCAAAGAGCCCTGATTTTCATCTTACTGACAGCTGTCGCTCCTTCAAT
                                                    prM_R_in (rc)
541  GACAATGCGTTGCATAGGAACATCAAATAGAGACTTTGTAGAAGGGGTTTCAGGAGGGAG
601  CTGGGTTGATATTGTCTTATTA
        prM_R_de (rc)
```

***ED3* fragment amplified by degenerate primers (5'→ 3')**

```
1    CTTCTGAGAATGGACAAGCTGCAGCTCAAAGGAATGTCATACTCTATGTGCACAGGAAAG
                  ED3_F_de                    ED3_F_in
61   TTTAAAGTTGTGAAGGAAATAGCAGAAACACAACATGGAACAATAGTTATCAGAGTACAA
121  TATGAAGGGGACGGTTCTCCATGTAAGATCCCTTTTGAGATAATGGATTTGGAAAAAAGA
181  CATGTTTTAGGTCGCCTGATTACAGTCAACCCAATCGTAACAGAAAAAGATAGCCCAGTC
241  AACATAGAAGCAGAACCTCCATTCGGAGACAGCTACATCATCATAGGAGTAGAGCCGGGA
301  CAATTGAAGCTCAACTGGTTTAAGAAAGGAAGTTCTATCGGCCAAATGTTTGAGGCAACA
           ED3_R_in (rc)
361  ATGAGGGGAGCGAAGAGAATGGCCATTTTGGGCGACACAGCTTGGGAA
                               ED3_R_de (rc)
```

Figure 3.2.2 Nucleotides sequences of *prM* and *ED3* of a clinical DEN-2 strain utilized in the present study.

The viral strain was clinical strain of an imported case in Hong Kong. The PCR products were amplified by degenerated primers. Sequences identified by ABI3130 sequence analyzer was subjected to primer design for amplification of in-frame products. rc represented reverse complement. Underline sequences indicated the degenerated primer sequences, but position of primer prM_F_de was not shown in the figure. Sequences in framed box indicated primers designed for amplification of in-frame products. For in-frame primer sequences, stop codon was not shown in the figure.

3.2.2 Cloning of PCR products into the pET SUMO vector and transformation into *E. coli* cells

The appropriate ratio of *E. coli* cells to ligation product was optimised; we found that a ratio of 50 µL *E. coli* cells to 4 µL of ligation product was the best combination to produce more colonies by overnight LB agar culture. For vectors transformed to Mach1^{TM}-T1$^{®}$ *E. coli* cells, positive clones and proper orientation of the insert were analysed by two PCRs with two set of primers (Figure 2.2.4). The specific bands from one PCR are shown in Figure 3.2.3. Only clones which showed PCR positivity with specific products on two PCR reactions were recognised as positive clones with the correct orientation of the DNA insert. All positive clones were subjected to sequence analysis. Nucleotide sequences are shown in Figure 3.2.4. Successful clones with correct transformants were sub-cultured for plasmid DNA extraction. The plasmid DNA concentration was measured by a Nanodrop spectrophotometer and the clones E6, P6 and PE3 corresponding to the DNA inserts *prM*, *ED3* and *prM-ED3*, respectively, were selected for sub-cloning to BL21(DE3) *E. coli* for protein expression.

Extracted plasmids were subjected to sub-cloning in BL21(DE3) *E. coli* cells. Similar to cloning in Mach1^{TM}-T1$^{®}$ *E. coli* cells, positive clones were cultured overnight on LB agar plates and were analysed by PCR with the same primer pairs as previous described (Figure 2.2.4). Specific bands of positive clones were visualised and confirmed by agarose gel electrophoresis.

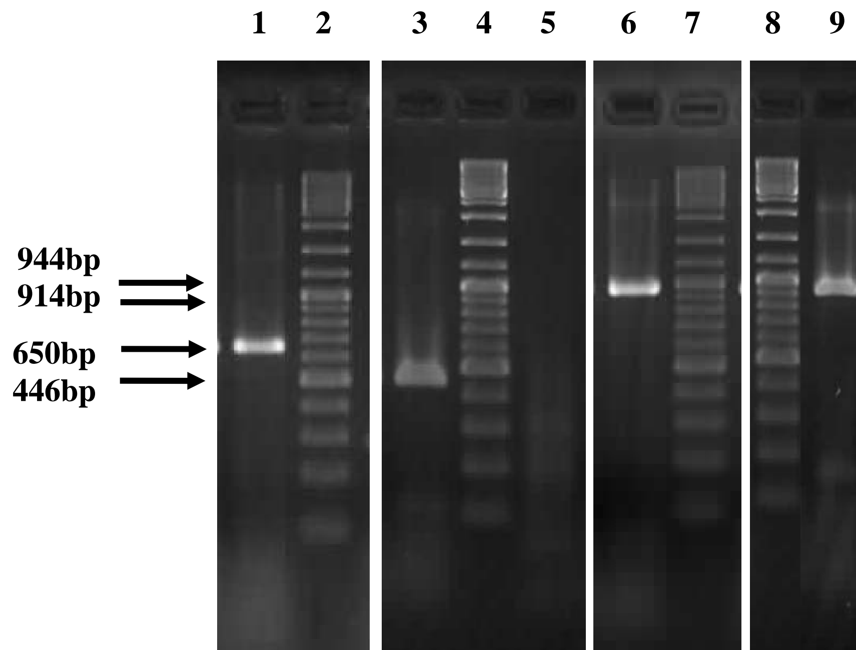


Figure 3.2.3 Confirmation by PCR for the presence of various inserts in the transformants.

PCR products were amplified by prM_F_in / ED3_F_in and T7 reverse primer. Molecular weight marker, 100 bp (Lane 2, 4, 7 & 8); For transformants with *PrM* insert, 650 bp (Lane 1), PCR products were amplified by prM_F_in and T7 reverse primer; For transformants with *ED3* insert, 446 bp (Lane 3) PCR products were amplified by ED3_F_in and T7 reverse primer; For transformants with *prM-ED3* chimeric fusion insert, 944 bp (Lane 6), PCR products were amplified by prM_F_in and T7 reverse primer; For control SUMO-CAT vector, 914 bp (Lane 9), PCR products were amplified by SUMO forward and T7 reverse primers. A no template control was also set up (Lane 5).

In-frame *prM* DNA fragment (5'→ 3')

```
1    ttccatttaaccacacgtaacggagaaccacacatgatcgtcagtagacaagagaaaggg
      F  H  L  T  T  R  N  G  E  P  H  M  I  V  S  R  Q  E  K  G
61   aaaagtcttctgtttaaaacagaggatggtgtgaacatgtgtaccctcatggccatggac
      K  S  L  L  F  K  T  E  D  G  V  N  M  C  T  L  M  A  M  D
121  cttggtgaattgtgtgaagatacaatcacgtacaagtgtcctcttctcaggcagaatgaa
      L  G  E  L  C  E  D  T  I  T  Y  K  C  P  L  L  R  Q  N  E
181  ccagaagacatagattgttggtgcaactctacgtccacatgggtaacttatggacgtgt
      P  E  D  I  D  C  W  C  N [S] T  S  T  W  V  T  Y  G  T  C
241  accaccacaggagaacacagaagagaaaaagatcagtggcactcgttccacatgtggga
      T  T  T  G  E  H  R  R  E  K  R  S  V  A  L  V  P  H  V  G
301  atgggactggagacacgaactgaaacatggatgtcatcagaaggggcctggaaacatgcc
      M  G  L  E  T  R  T  E  T  W  M  S  S  E  G  A  W  K  H  A
361  cagagaattgaaacttggatcttgagacatccaggctttaccataatggcagcaatcctg
      Q  R  I  E  T  W  I  L  R  H  P  G  F  T  I  M  A  A  I  L
421  gcatacaccataggaacgacacatttccaaagagccctgattttcatcttactgacagct
      A  Y  T  I  G  T  T  H  F  Q  R  A  L  I  F  I  L  L  T  A
481  gtcgctccttcaatgacatag
      V  A  P  S  M  T  -
```

In-frame *ED3* DNA fragment (5'→ 3')

```
1    atgtcatactctatgtgcacaggaaagtttaaagttgtgaaggaaatagcagaaacacaa
      M  S  Y  S  M  C  T  G  K  F  K  V  V  K  E  I  A  E  T  Q
61   catggaacaatagttatcagagtacaatatgaaggggacggttctccatgtaagatccct
      H  G  T  I  V  I  R  V  Q  Y  E  G  D  G  S  P  C  K  I  P
121  tttgagataatggatttggaaaaagacatgttttaggtcgcctgattacagtcaaccca
      F  E  I  M  D  L  E  K  R  H  V  L  G  R  L  I  T  V  N  P
181  atcgtaacagaaaaagatagcccagtcaacatagaagcagaacctccattcggagacagc
      I  V  T  E  K  D  S  P  V  N  I  E  A  E  P  P  F  G  D  S
241  tacatcatcataggagtagagccgggacaattgaagctcaactggtttaagaaatag
      Y  I  I  I  G  V  E  P  G  Q  L  K  L  N  W  F  K  K  -
```

Figure 3.2.4 Nucleotide sequences of in-frame *prM*, *ED3* and *prM-ED3* chimeric fusion fragments from positive clones of Mach1^{TM}-T1^{R} *E. coli* cell.

The sequences were in 5' to 3' direction. Nucleotides was translated to corresponding amino acid sequences (in capital letters) and shown below nucleotides sequences. Amino acid residue in framed box indicated N-linked glycosylation site. '–' indicated stop codon. The numbers on the left indicate the positions of the first nucleotide in each line.

In-frame *prM-ED3* DNA fragment (5'→ 3')

```
1    ttccatttaaccacacgtaacggagaaccacacatgatcgtcagtagacaagagaaaggg
      F  H  L  T  T  R  N  G  E  P  H  M  I  V  S  R  Q  E  K  G
61   aaaagtcttctgtttaaaacagaggatggtgtgaacatgtgtaccctcatggccatggac
      K  S  L  L  F  K  T  E  D  G  V  N  M  C  T  L  M  A  M  D
121  cttggtgaattgtgtgaagatacaatcacgtacaagtgtcctcttctcaggcagaatgaa
      L  G  E  L  C  E  D  T  I  T  Y  K  C  P  L  L  R  Q  N  E
181  ccagaagacatagattgttggtgcaactctacgtccacatgggtaacttatgggacgtgt
      P  E  D  I  D  C  W  C  N  S  T  S  T  W  V  T  Y  G  T  C
241  accaccacaggagaacacagaagagaaaaaagatcagtggcactcgttccacatgtggga
      T  T  T  G  E  H  R  R  E  K  R  S  V  A  L  V  P  H  V  G
301  atgggactggagacacgaactgaaacatggatgtcatcagaaggggcctggaaacatgcc
      M  G  L  E  T  R  T  E  T  W  M  S  S  E  G  A  W  K  H  A
361  cagagaattgaaacttggatcttgagacatccaggctttaccataatggcagcaatcctg
      Q  R  I  E  T  W  I  L  R  H  P  G  F  T  I  M  A  A  I  L
421  gcatacaccataggaacgacacatttccaaagagccctgattttcatcttactgacagct
      A  Y  T  I  G  T  T  H  F  Q  R  A  L  I  F  I  L  L  T  A
481  gtcgctccttcaatgacaatgtcatactctatgtgcacaggaaagtttaaagttgtgaag
      V  A  P  S  M  T  M  S  Y  S  M  C  T  G  K  F  K  V  V  K
541  gaaatagcagaaacacaacatggaacaatagttatcagagtacaatatgaaggggacggt
      E  I  A  E  T  Q  H  G  T  I  V  I  R  V  Q  Y  E  G  D  G
601  tctccatgtaagatcccttttgagataatggatttggaaaaaagacatgttttaggtcgc
      S  P  C  K  I  P  F  E  I  M  D  L  E  K  R  H  V  L  G  R
661  ctgattacagtcaacccaatcgtaacagaaaaagatagcccagtcaacatagaagcagaa
      L  I  T  V  N  P  I  V  T  E  K  D  S  P  V  N  I  E  A  E
721  cctccattcggagacagctacatcatcataggagtagagccgggacaattgaagctcaac
      P  P  F  G  D  S  Y  I  I  I  G  V  E  P  G  Q  L  K  L  N
781  tggtttaagaaatag
      W  F  K  K  -
```

Figure 3.2.4   Continued

3.2.3 Expression of proteins fragments from BL21(DE3) *E. coli* cells

The positive clones P6, E6 and PE3, corresponding to the prM, ED3 and prM-ED3 inserts in BL21(DE3) *E. coli* cells, respectively, were subjected to protein expression. LB medium with 1% glucose was necessary during expression to suppress expression of the recombinant protein in the absence of the IPTG inducer. In pilot experiments, the expression of prM, ED3 and chimeric fusion prM-ED3 protein fragments measured by the amount of insoluble protein (pellet fraction) increased with induction time for protein expression for all protein fragments. An induction time of 1-3 hr (T_1-T_3) was sufficient to produce soluble protein with the least amount of insoluble protein production. The intensity of the Western blot for specific bands was similar between T_2 and T_3, so a 2 hr induction time was selected for protein expression. For optimisation of expression conditions, we expressed proteins individually at 30 °C and 37 °C with 0.2, 0.5 and 1 mM of IPTG. Proteins expressed at 37 °C with 1 mM IPTG shifted to insoluble protein expression in the form of inclusion bodies. Higher concentrations of ITPG (0.5mM and 1.0mM) at a lower temperature (30 °C) were associated with increased induction of soluble protein expression. Protein expression at 30 °C with 0.2 mM of IPTG were the optimal conditions for all three proteins (Figure 3.2.5). Proteins were expressed in scale-up culture progressively from 5 mL, 50 mL to 500 mL LB culture medium. Specific protein bands on SDS-PAGE increased in intensity, which indicated that protein expression was increased in the scaled-up culture volumes.

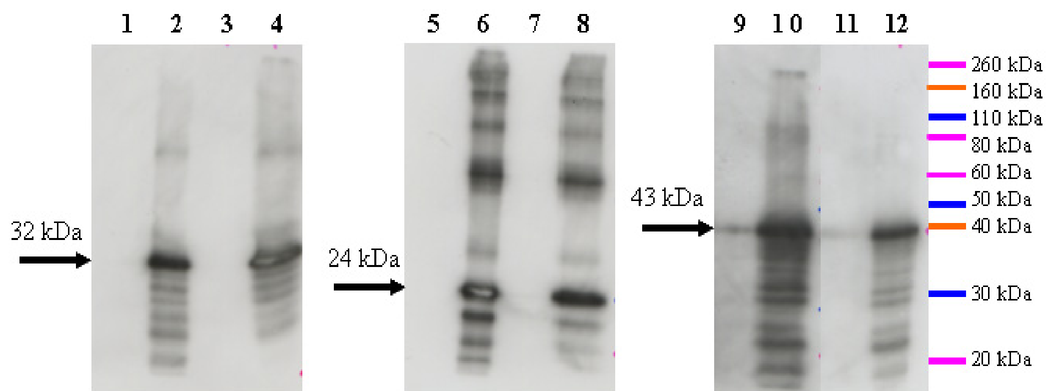


Figure 3.2.5 Western blot analysis of crude proteins for optimisation expression conditions.

Individual prM, ED3 and prM-ED3 proteins were expressed at 30°C with 0.3 mM of IPTG, 220 rpm of agitation. prM, ED3 and prM-ED3 with size 32kDa, 24kDa and 43kDa, respectively were expressed as fusion protein with SUMO and 6xHis-tag. Proteins were separated into supernatant and pellet after extraction. Uninduced control of supernatant (Lane 1, 5 & 9) and pellet fraction (Lane 3, 7 & 11). Proteins from supernatant (Lane 2, 6 & 10) and pellet fraction (Lane 4, 8 & 12) were induced for expression by 0.2 mM IPTG. Proteins transferred in PVDF membrane were blotted with 1:1000 anti-HisG antibody, and 1:10,000 rabbit anti-mouse HRP conjugate secondary antibody.

3.2.4 Characterisation of the expressed proteins

After protein extraction, specific protein fractions were eluted by a HisTrap-FF column using elution buffer with different concentrations of imidazole. For *prM* and *prM-ED3* protein fragments, 500 mM of imidazole in the elution buffer was used for elution. Similarly, the *ED3* protein fragment was eluted with 300 mM of imidazole in the elution buffer. The purity and identity of the proteins were analysed by SDS-PAGE and Western blotting (Figure 3.2.6). Western blotting was performed using anti-HisG antibody for all three proteins, while specific detection of the *ED3* and *prM* protein fragments was achieved by blotting with a rabbit polyclonal anti-dengue serotype 1-4 antibody and a mouse monoclonal anti-*prM* specific for DEN-2 antibody, respectively (Figure 3.2.6).

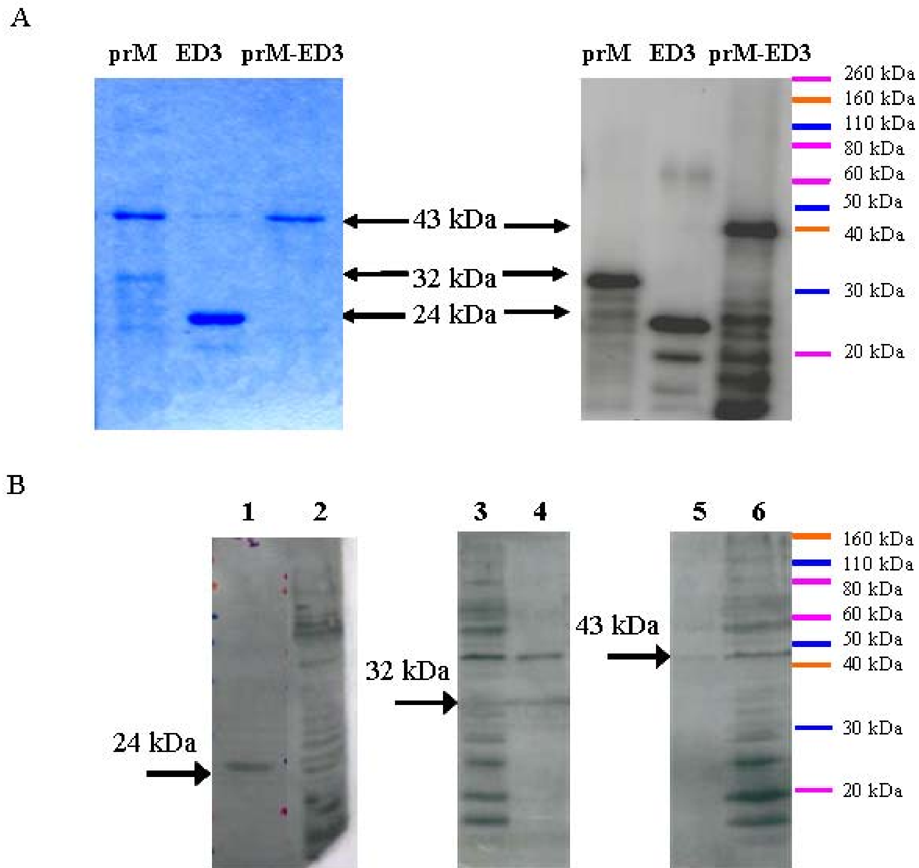


Figure 3.2.6 SDS-PAGE and Western blot analysis of purified proteins.

A) Analysis of purified prM, ED3 and prM-ED3 after HisTrapFF affinity purification Left: SDS-PAGE analysis. Right: Western blot analysis with 1:1000 anti-HisG antibody, and 1:10,000 rabbit anti-mouse HRP conjugate secondary antibody. B) Western blot analysis of crude and purified prM, ED3 and prM-ED3 with 1:200 polyclonal antibody against DV 1-4 serotypes, and 1:10,000 mouse anti-rabbit secondary antibody. Lane 2, 3 and 6 were ED3, prM and prM-ED3 protein, respectively before affinity purification. Lane 1, 4 and 5 were respectively represented purified ED3, prM and prM-ED3.

The signal from the anti-HisG antibody was very strong for all three proteins, but non-specific detection was noted in all of them (Figure 3.2.6 A, Right). prM was not detected by the anti-prM monoclonal antibody (mAb); this might be related to sequence variation between the DEN-2 strain used in the present study and the viral strain (Thailand DEN-2 18861 strain) used for

production of this commercialised mAb. The amino acid sequences of these two strains were aligned and identified with four sites of variation, of which two were along the sequence of the antigenic sites (residues 249-256 & 262-277 of the Thailand DEN-2 18861 strain) (Figure 3.2.7). Antigenic sites were predicted using the tool from MIF Bioinformatics (http://immunax.dfci.harvard.edu/Tools/antigenic.html). The polyclonal anti-dengue serotype 1-4 antibody was able to detect *ED3* protein but very weakly detected the prM and prM-ED3 fusion proteins (Figure 3.2.6B).

Specific bands of individual proteins were extracted from SDS-PAGE gels and subjected to protein identification and mass confirmation (accurate mass measurement) by HCTultra Electrospray (ESI)-Ion-Trap mass spectrometry [Dalton Bucker] and ESI Q-TOF2 Quadrupole-Time-of-Flight mass spectrometry [Waters], respectively. For protein identification, the extracted individual proteins were analysed by ESI-Ion-Trap MS operated in MS^n mode to obtain the mass spectrum. As a result, the resulting spectra of three proteins were compared and confirmed by the compound database to discover their identities. The detailed identification result was summarised in Table 3.2.1. Scores of each possible identity were over 100, which was much higher than the minimal score of 25 for identity confirmation. For mass confirmation of proteins, the purified proteins were subjected to accurate mass measurement performed by an ESI Q-TOF2 mass spectrometer. In this study, only the protein fragment *ED3* was detected, with a measured mass of 24311Da. (24310.80$\pm$0.95Da), which is very close to the theoretically predicted mass (24420.83Da) as performed on ExPASY software (http://au.expasy.org/cgi-bin/pi_tool), with less than 0.4% mass deviation. The other two proteins were not detected by this method because they failed to form the

multiple charged ions under ESI conditions. Therefore, they were not detected by the narrow mass range (maximum m/z 1700) of the mass analyser, or their signals were masked by non-specific protein fragments.

Purified proteins were quantified by means of the Bradford method. The average expression yield of prM was 149.9 μg/L of LB culture medium. The average yield of ED3 was 362.8 μg/L of LB culture medium, which was the highest among the three proteins. The average expression yield of the prM-ED3 chimeric fusion protein was 260.2 μg/L of culture medium.

```
Viral strain

HK DEN-2         //--------------FHLTTRNGEPHMIVSRQEKGKSLLFKTEDGVNMCTL   36
Thailand 16681   //SAGMIIMLIPTVMAFHLTTRNGEPHMIVSRQEKGKSLLFKTEDGVNMCTL   150
                                   ************************************

HK DEN-2           MAMDLGELCEDTITYKCPLLRQNEPEDIDCWCNSTSTWVTYGTCTTTGEH   86
Thailand 16681     MAMDLGELCEDTITYKCPLLRQNEPEDIDCWCNSTSTWVTYGTCTTMGEH   200
                   ********************************************** ***

HK DEN-2           RREKRSVALVPHVGMGLETRTETWMSSEGAWKHAQRIETWILRHPGFTIM   136
Thailand 16681     RREKRSVALVPHVGMGLETRTETWMSSEGAWKHVQRIETWILRHPGFTMM   250
                   *********************************.**************:*

HK DEN-2           AAILAYTIGTTHFQRALIFILLTAVAPSMT--------------------// 166
Thailand 16681     AAILAYTIGTTHFQRALIFILLTAVTPSMTMRCIGMSNRDFVEGVSGGSW// 300
                   *************************:****
```

Figure 3.2.7 Amino acid sequences of prM protein of the DEN-2 strain used in present study and the Thailand 16881 DEN-2 strain (for commercialised mAb production).

Alignment was performed by ClusterW (version 2.1) program. The upper sequence was DEN-2 strain used in the present study for protein production; the strain was collected from an imported case in Hong Kong. * indicated residues in the column were consensus. **:** indicated conserved substitution; and **.** indicated semi-conserved substitution in the sequence.

Table 3.2.1 Protein identity of individual fusion protein fragment by HCTultra ESI-Ion-Trap MS.

Fusion protein fragment[a]	**Protein name & species identified by HCTultra ESI-Ion-Trap MS database**	**Accession No.**	**Score**[b]
prM	Premembrane polyprotein [dengue virus type 2]	939865	355.01
	Ubiquitin-like protein of the SUMO family	6320718	230.82
ED3	Envelope protein SL77169	227009	338.55
	Polyprotein [Dengue virus type 2]	37674517	255.45
	Ubiquitin-like protein of the SUMO family	6320718	228.92
prM-ED3	Polyprotein [Dengue virus type 2]	18766557	310.15
	Genome polyprotein [contains: Protein C (Core protein) (Capsid protein); prM; Peptide pr; Small envelope protein M; Envelope protein E; Non-structural protein (NS1)]	266815	118.53
	Ubiquitin-like protein of the SUMO family	6320718	182.53

[a] All protein fragments were in fusion protein with 6xHis-tag and SUMO from the vector
[b] The minimal acceptance score of identification of compound is 25 as defined by HCTultra ESI-Ion-Trap MS database.

3.2.5 Characterisation of antisera from rabbits

3.2.5.1 Immunoblotting

All anti-sera collected from the four rabbits immunised with either prM, ED3, prM-ED3 or a mixture of prM and ED3 proteins at various time points were analysed by immunoblotting. From the immunoblotting results of pre-immunised sera, all rabbits were free of antibody against the prM and ED3 proteins of DV. This clearly demonstrated that the four rabbits were not previous exposed to DV (Figure 3.2.8). Antibodies against all protein fragments were elicited and detected on day 10 after the first booster injection. Several dilutions (1:5,000, 1:10,000 and 1:20,000) of crude anti-sera for immunoblotting were investigated; it was discovered that an antiserum titre against all protein combinations was detectable up to a 1:20,000 dilution. There was no significantly increased intensity of specific protein bands after the third booster injection. This indicated that no further increase in antibody production was elicited. For antisera pre-adsorbed with purified protein fragments (Figure 3.2.8), significantly diminished intensity of the specific bands demonstrated that the presence of competing protein antigen neutralised the corresponding antisera. For antisera pre-adsorbed with SUMO-CAT fusion protein (Figure 3.2.8), the intensity of the specific protein bands remained similar to those of crude antisera. These findings demonstrated that the signal of specific protein bands was mainly attributed to the antibody against the specific protein (prM, ED3 or prM-ED3 proteins). The non-specific antibodies against 6xHis tag or SUMO fusion proteins were removed from the rabbit's antisera after pre-adsorption with SUMO-CAT fusion protein (Figure 3.2.8).

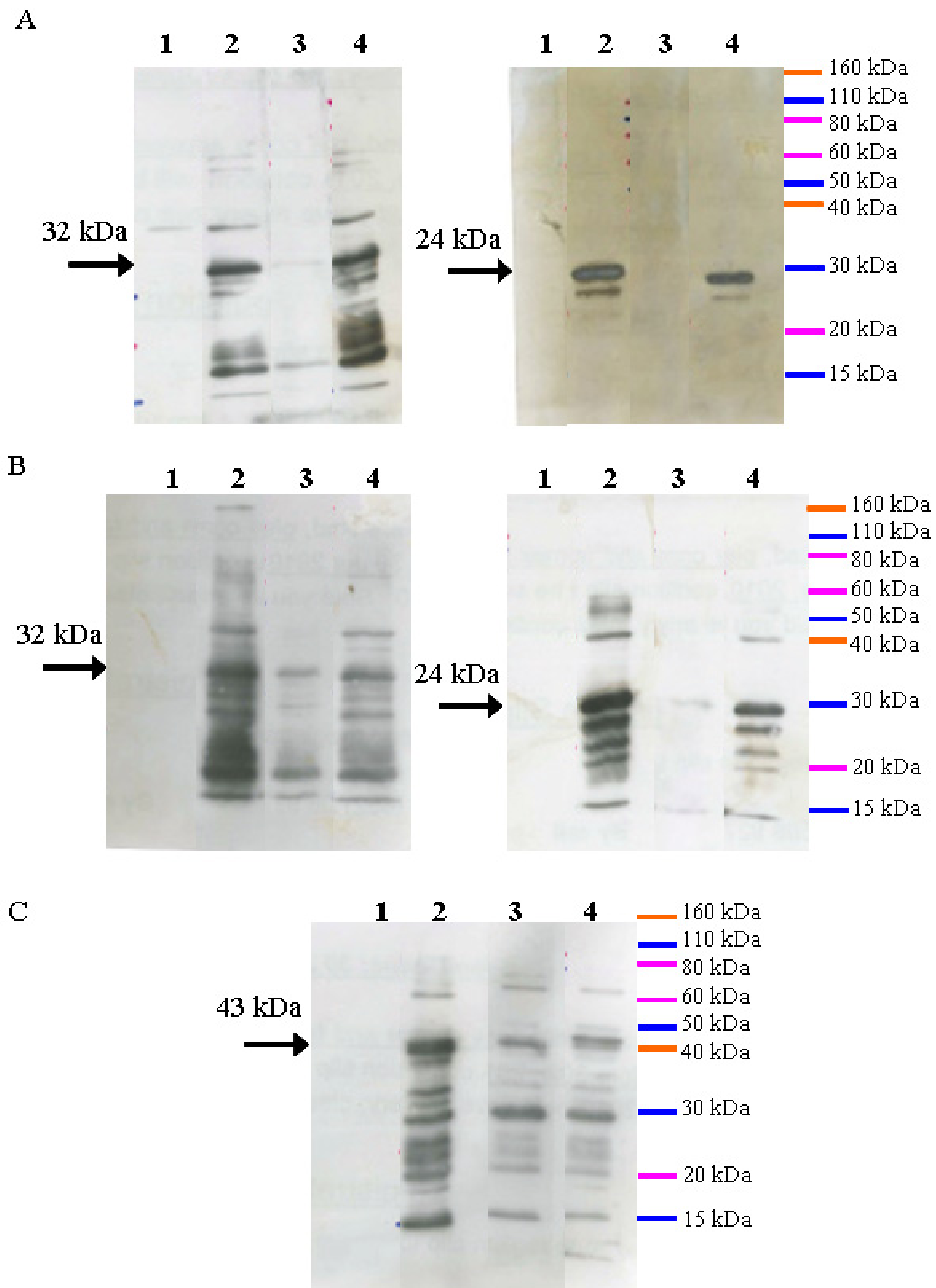


Figure 3.2.8 Immunoblot results from characterisation of the rabbit antisera.

Corresponding recombinant protein was transferred to PVDF after SDS-PAGE electrophoresis as follow. A) Left: prM protein. Right: ED3 protein, to investigate antiserum from rabbit immunised with prM and ED3 proteins mixture, two membranes were set up. B) Left: prM protein, to investigate rabbit antiserum immunised with prM protein. Right: ED3 protein, to investigate rabbit antiserum immunised with ED3 protein C) prM-ED3 chimeric fusion protein, to investigate antiserum from rabbit immunised with chimeric prM-ED fusion protein. Immunoblotting was then performed in parallel using following antibodies as primary antibody. Lane 1, pre-immunised sera; Lane 2, crude antisera; Lane 3, antisera pre-adsorbed with corresponding recombinant protein; Lane 4, antisera pre-adsorbed with SUMO-CAT fusion protein; followed by blotting with 1:10,000 goat anti-rabbit HRP conjugate secondary antibody.

3.2.5.2 Indirect ELISA assay

Antisera for ELISA assay analysis were pre-adsorbed with SUMO-CAT fusion proteins for removal of antibodies against 6xHis-tag and SUMO proteins. Titration of the crude anti-sera for ELISA was investigated by serial doubling dilution (started with 1:50, corresponding to log 1.7). The antibody titres of pre-adsorbed antisera were >25600 (corresponding to log 4.4) against prM-ED3 and the mixture of prM and ED3 proteins. The absorbance increase was directly proportional to antibody titre. Using the same dilution of antiserum, increased absorbance indicated a higher antibody titre in the serum. For each dilution, the antibody titre increased with the number of booster injections, but decreased after the third booster injection (Figure 3.2.9). This implied no further increase in antibody titre with further boosters. Similarly, the antibody titres of pre-adsorbed antisera were > 800 (corresponding to log 2.9) against prM and ED3 proteins, and the anti-prM titre was higher than that of anti-ED3. The titres were much lower than those for antibodies against prM-ED3 and the mixture of prM and ED3 proteins, since the rabbits had been euthanised due to illness before administration of the first booster.

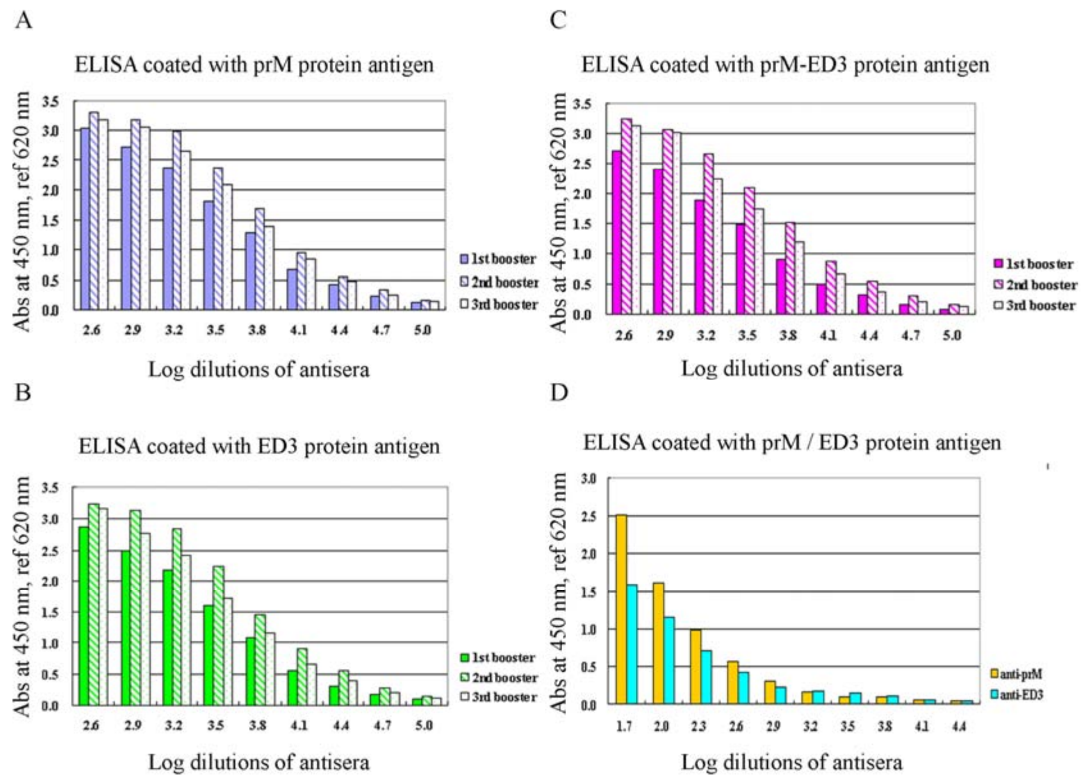


Figure 3.2.9 Antibodies titers of serially diluted antisera collected from immunised rabbits.

Number of booster indicated blood was collected on the Day 10 after that booster injection. A) prM protein and B) ED3 protein respectively coated as capture antigen on micro-well plate, in-house ELISA was performed with antisera against prM and ED3 proteins mixture. C) Chimeric prM-ED3 protein coated as capture antigen on micro-well plate, in-house ELISA was performed with corresponding antisera. D) Individual prM or ED3 protein coated as capture antigen on micro-well, in-house ELISA was performed with corresponding antiserum, respectively.

3.2.5.3 Inhibition of DEN-2 RSPs binding to Vero E6 cells by polyclonal antisera against prM, ED3 and prM-ED3 proteins

After harvesting and concentrating RSPs, the presence of RSPs was confirmed by dot blot. The x-ray film is shown in Figure 3.2.10. DEN-2 RSPs were specifically detected by the DEN-2 serotype-specific anti-E 4G2 monoclonal antibody (Figure 3.2.10). Anti-E 4G2 was not able to detect DEN-1 RSPs.

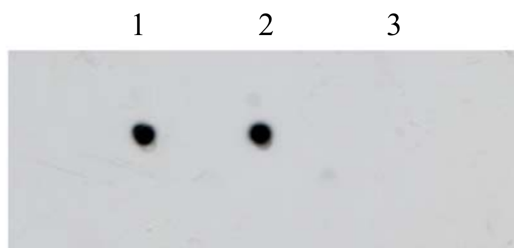


Figure 3.2.10 Dot blot analysis of DEN-2 RSPs.

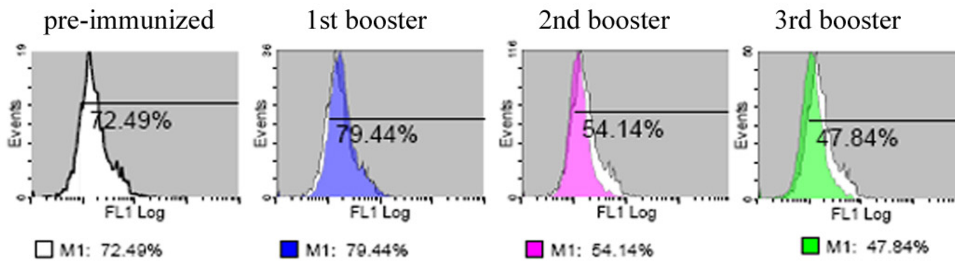
DEN-2 RSPs (Dots 1 & 2) and DEN-1 RSPs (Dot 3) were dot blotted on PVDF membrane. The membrane was blotted with anti-E 4G2 monoclonal antibody. DEN-1 RSPs was used as control for the specificity of DEN-2 serotype specific anti-E 4G2 mAb.

Vero E6 cells cultured overnight were adjusted and aliquoted to 8.2×10^4 cells in a 100 µL suspension. DEN-2 RSPs were incubated with polyclonal antisera against prM, ED3, prM-ED3 and the mixture of prM plus ED3 fusion proteins. DEN-2 RSPs were also incubated with pre-immunised sera as a negative control. Then the mixture of DEN-2 RSPs and antisera was further incubated with 8.2×10^4 Vero E6 cells. Inhibition of DEN- RSP binding to Vero E6 cells was indicated by the green fluorescence signal from the FITC conjugate of the secondary antibody. The fluorescence signal generated by the FITC conjugate was detected by the FL1 channel of the FC 500 flow cytometer. Decreased fluorescence signal to log 10^0 or below indicated that there was blocked binding of RSPs to the cells. The blockage of RSP binding to the Vero E6 cells was quantified by the decrease in percentage

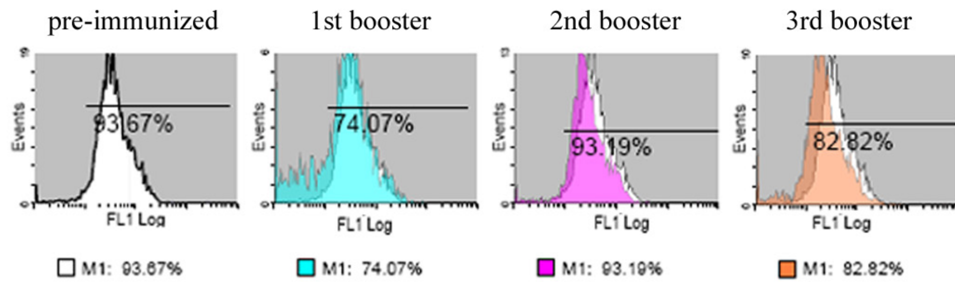
of cells having green fluorescence above log 10^0. Blockage of RSP binding to the cells indicated neutralising ability of the antisera.

Compared with the pre-immunised sera control, the percentage of cells with a FITC fluorescence signal decreased progressively with an increasing number of booster injections. For antiserum against the prM-ED3 chimeric fusion protein (anti-prM-ED3), cells having a fluorescence signal >log 10^0 detected by FL1 were decreased by 24.7% after the third booster injection (Figure 3.2.11 A). Similarly, for antiserum against the prM and ED3 protein mixture (anti-prM&ED3 mixture), cells having a fluorescence signal >log 10^0 were decreased by 10.9% after the third booster injection. There was a 19.6% (93.67%-74.07%) shift in the cell population with a fluorescence signal log 10^0 or below after the first booster; however, the data may not be valid since a low number of cells were acquired for this sample (Figure 3.2.11 B). Cells having a fluorescence signal >log 10^0 after treatment with the antisera against individual prM (anti-prM) and ED3 proteins (anti-ED3) were decreased by 18.6% and 5.0%, respectively (Figure 3.2.11 C). Interestingly, anti-ED3 showed the least ability, while anti-prM-ED3 showed the strongest ability to inhibit DEN-2 RSPs binding to Vero E6 cells among the four antisera. Various controls were set up for flow cytomtetry analysis (Figure 3.2.11D). Controls in absence of RSPs or antisera from rabbits generated fluorescence signal level (FL1 Log) as that in positive control. The measurement deviation for detection of the fluorescence signal from two duplicated positive controls (RSPs + Vero E6 cells) and antiserum against the prM and ED3 protein mixture (after the first booster) were 2.98% and 6.78%, respectively.

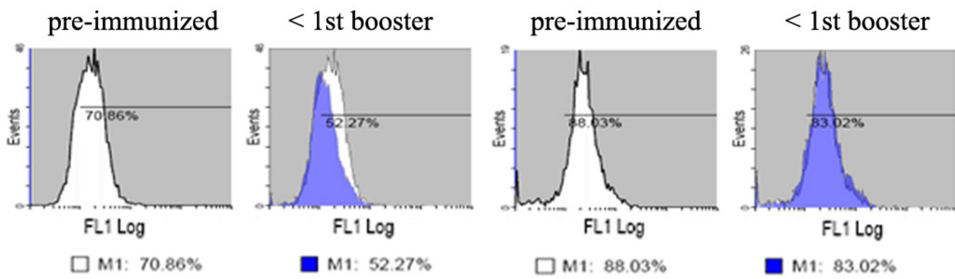
A Anti-prM-ED3

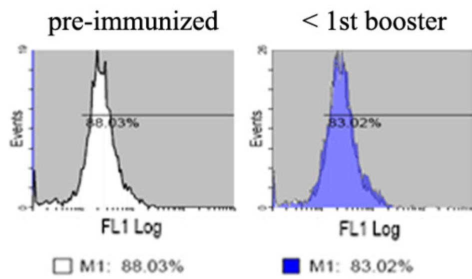


B Anti-prM&ED3 mixture



C Anti-prM Anti-ED3





D Controls

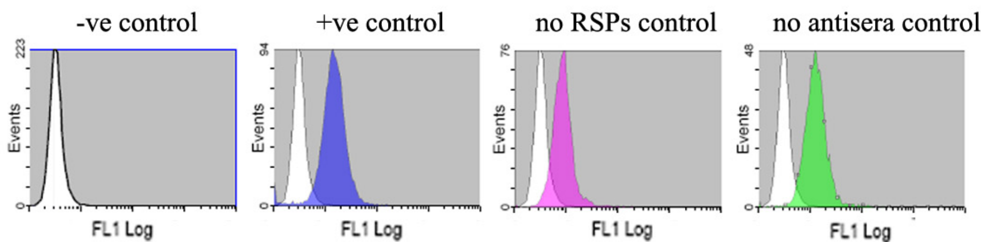


Figure 3.2.11 Histogram of flow cytometer of DEN-2 RSPs binding to Vero E6 cell.

Antisera were collected from four rabbits at different time points on pre-immunised, day 10 after 1st, 2nd and 3rd booster. Histograms were presented as overlay with pre-immunised serum for comparison. Ten micro-gram of RSPs were treated with individual antiserum for 1 hr at RT. Vero E6 cells were incubated with RSPs for 1.5 hr at 4°C, then stained with 1:100 anti-E 4G2 mAb followed by 1:100 horse anti-mouse IgG FITC conjugates antibody. A) Anti-prM-ED3. B) Anti-prM&ED3 mixture. C) Anti-prM and anti-ED3, antisera were collected pre-immunised and before the first booster injection. D) Various control samples were set up. Histograms were presented as overlay with the negative control. –ve control represented negative control with Vero E6 cells only; +ve control represented positive control with Vero E6 cells incubated with 10 μg of DEN-2 RSPs. No RSPs and no antisera control were control without DEN-2 RSPs or antisera, respectively.

Binding of RSPs to Vero E6 cells was visualised on the inverted microscope Nikon ECLIPSE Ti [Nikon]. The images are shown in Figure 3.2.12. The images demonstrate that the green fluorescence signal was visualised due to the binding of RSPs to Vero E6 cells. The binding of RSPs to Vero E6 cells was indicated by the green fluorescence signal emitted by the secondary antibody conjugated to FITC.

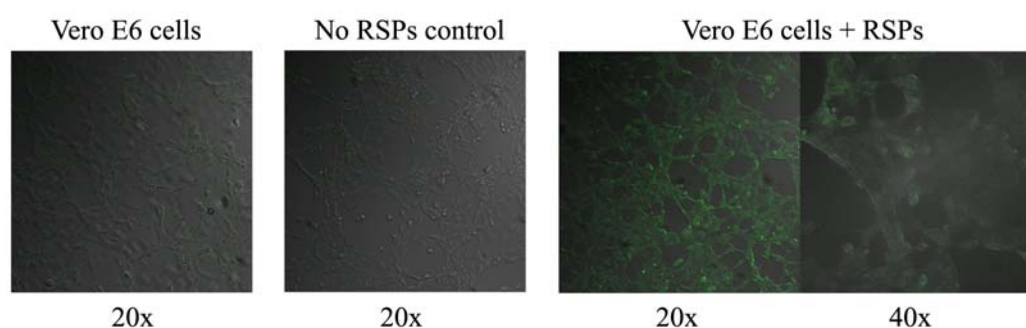


Figure 3.2.12 Visualisation of DEN-2 RSPs binding to monolayer Vero E6 cells by inverted research microscope.

The images were captured by Nikon ECLIPSE Ti inverted research microscope and analyzed by EZ-C1 version 3.90 software. Left: Vero E6 cells only incubated with 5µl PBS as a negative control. Middle: No DEN-2 RSPs control, Vero E6 cells were incubated with 5µl PBS, cells were then incubated with primary anti-E 4G2 mAb followed by secondary horse anti-mouse IgG FITC conjugate antibody. Right: Vero E6 cells incubated with 10 µg DEN-2 RSPs as positive control. Cells were then incubated with primary anti-E 4G2 mAb followed by secondary horse anti-mouse IgG FITC conjugate antibody. Magnification of 20x was captured for all samples, 40x was captured for Vero E6 cells + RSPs positive control.

3.2.6 Development of a serological diagnostic tool by recombinant proteins

3.2.6.1 Assay validation

The ELISA assay was validated with clinical sera collected from Brazil and sera collected from the community in Hong Kong. The optimal dilution of clinical serum was determined by titration of sera. A known positive clinical serum was serially 10-fold diluted in TMT buffer up to 10^5. One hundred micro-litre of serum

was reacted with 3 protein fragments individually coated in micro-plate. The procedures for in-house developed indirect ELISA were described in chapter 2.2.4.2. Absorbance was measured from each well. It was observed in Figure 3.2.13 that saturated absorbance was measured from serum with dilution $\leq$ 1:100 (corresponding to log dilution 2). This result was unable to define which protein fragment was more feasible as detection antigen. Serum with dilution 1:1000 (corresponding to log dilution 3) showed significant difference in absorbance among 3 protein fragments for readily determination of which protein fragment as detection antigen for the development of the in-house indirect ELISA assay. Therefore, a 1:1000 dilution was able to generate an optimal signal of absorbance for result interpretation (seropositive or seronegative).

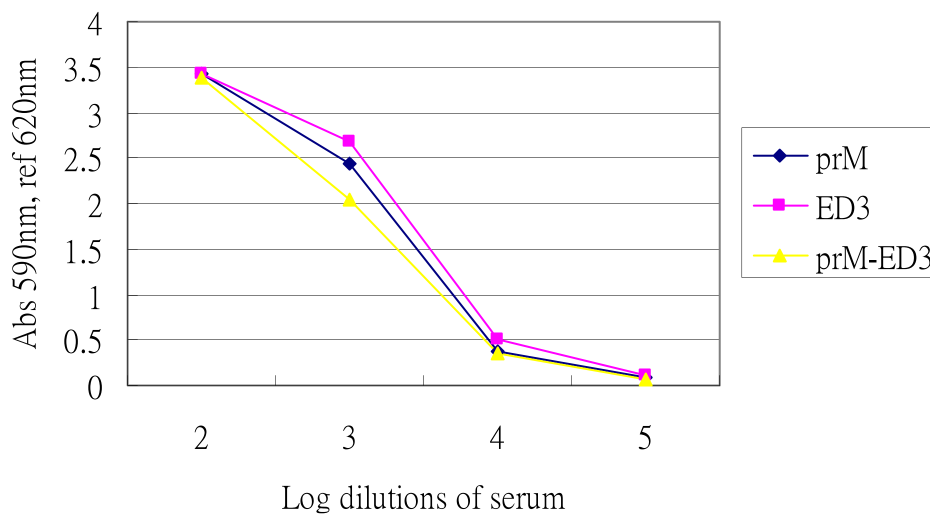


Figure 3.2.13 Dilution of clinical sera for in-house ELISA assay optimization.

The interpretations of the in-house ELISA were based on the cut-off values generated by two methods. First, measured absorbance was converted into Panbio units based on the mean absorbance of three calibrator serum provided in the assay

kit. For sensitivity, among the eleven seropositive sera confirmed by the Panbio Dengue Indirect IgG ELISA, only one (9.0%) serum was detected by the in-house ELISA assay using either ED3 or prM-ED3 as the capture antigen; two (18.1%) sera were detected using the prM protein fragment as the capture antigen (Table 3.2.2). However, for the in-house ELISA, either with prM or ED3 as the detection antigen, about 30% of the serum samples were interpreted as equivocal (Table 3.2.2). Since Panbio units were calculated by sample absorbance/mean cut-off value x 10, result interpretation totally relied on the mean cut-off value generated by the cut-off calibrator serum provided in the Panbio Dengue IgG Indirect ELISA kit. The average cut-off values for prM, ED3 and prM-ED3 were 2.4, 2.3 and 1.1, respectively.

Second, measured absorbance of 100 samples collected from the community in Hong Kong acted as local control group for cut-off value determination. Cut-off values were estimated as the mean plus three standard deviations (SD) for each protein fragment [Santos et al., 2007]. The cut-off values for prM, ED3 and prM-ED3 capture antigen were 2.4, 2.6 and 1.3, respectively; absorbance above these readings was regarded as seropositive. It was noted that one more positive sample of 11 confirmed positive was detected from prM and ED3 using this method compared to using Panbio's cut-off value (Table 3.2.2).

The specificity of the self-developed assay for all three protein fragments as detection antigens was over 90%, despite being interpreted by Panbio's cut-off calibrator serum or the local control group (Table 3.2.2).

Table 3.2.2 Sera collected from community in Hong Kong for validation of a self-developed ELISA.

Samples for in-house ELISA assay validations [a]	Interpretation of self-developed ELISA with various protein fragment as capture antigen [sensitivity or specificity, %][b]						
		Cut-off value based on Panbio cut-off calibrator serum[c]			Cut-off value based on Hong Kong control group sera[d]		
		prM	ED3	prM-ED3	prM	ED3	prM-ED3
Positive (n=11)	Positive	2	1	1	3	2	1
	Equivocal	3	4	2	/	/	/
	Negative	6	6	8	8	9	10
		[18.1]	[9.1]	[9.1]	[27.2]	[18.1]	[9.1]
Negative (n=100)	Positive	2	0	1	0	0	1
	Equivocal	8	6	0	/	/	/
	Negative	90	94	99	100	100	99
		[90.0]	[94.0]	[99.0]	[100]	[100]	[99.0]

[a] Serological interpretation was based on the Panbio units calculated from absorbance at 450nm with reference filter 620nm according to the manufacture's instruction of the Panbio Dengue IgG Indirect ELISA assay. Panbio units was calculated by sample absorbance / cut off value x 10.

[b] Number of equivocal was excluded from calculation of sensitivity or specificity.

[c] Interpretation was based on the cut-off value measured on every round of ELISA. Cut-off value was the mean of 3 measured absorbance of cut-off calibrator serum provided in Panbio Dengue IgG Indirect ELISA. The cut-off values for prM, ED3 and prM-ED3 capture antigen were 2.4, 2.3 and 1.1, respectively.

[d] Cut-off value was calculated by the mean absorbance plus 3 standard deviation of overall 100 samples collected from community in Hong Kong. The cut-off values for prM, ED3 and prM-ED3 capture antigen were 2.4, 2.6 and 1.3, respectively.

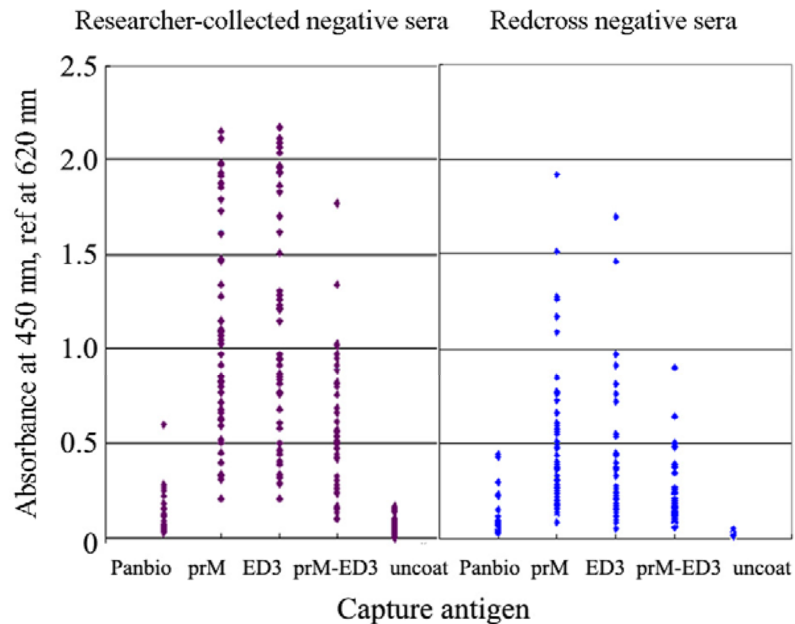
Meanwhile, results were also interpreted by the magnitude of measured absorbance instead of the Panbio unit; the distribution of overall negative sera (n=100) was scattered over the range of absorbance measured at 450 nm with a reference filter at 620 nm for prM 0.06-2.17 (mean=0.79, SD=0.55) ED3 0.05-2.17 (mean=0.74, SD=0.63) and prM-ED3 0.06-1.77 (mean=0.37, SD=0.31) capture antigen. Among the three protein antigens, the prM-ED3 fusion protein showed the least range of absorbance (Figure 3.2.13). In Figure 3.2.13 A, 49 sera were

researcher-collected samples from the community of Hong Kong, while the remaining 51 samples were plasma provided by the Hong Kong Red Cross. Plasma samples from the Hong Kong Red Cross were defibrinated to serum analogues as described in chapter 2.4.2.1. It was clearly shown that samples from the Hong Kong Red Cross were less diverse in distribution in terms the magnitude of absorbance for all three proteins. The mean and standard deviation of absorbance for prM were 0.79 and 0.55; for ED3 were 0.74 and 0.63; and for prM-ED3 were 0.37 and 0.31, respectively. Compared with the magnitude of absorbance measured with the Panbio Dengue IgG Indirect ELISA kit for all negative sera (n=100), less variation of absorbance was measured with the range 0.02-0.60 (mean=0.093; SD=0.091). For seropositive self-collected and Red Cross sera, the absorbance generated from prM and ED3 capture proteins was apparently similar to the that measured by the Panbio assay (Figure 3.2.13 B; Left). However, the low number of seropositive samples resulted from the high cut-off value (1.3-2.6) of the in-house ELISA assay.

The assay was also validated with 36 clinical sera collected from Brazil. All clinical samples were diagnosed cases collected during the viremic period of the acute phase and confirmed the presence of DV by RT-PCR. In 36 sera, all were detected to be negative for IgG against prM and prM-ED3. In accordance with the cut-off determined by Panbio calibrator serum, one (2.8%) serum was positively detected for IgG against ED3 protein and two (5.6%) samples were equivocal for the presence of IgG against ED3 protein fragment; however, no serum was positively detected by the cut-off value determined based on the local control group. Compared with the results of testing these 36 clinical sera by the Panbio

Dengue IgG Indirect ELISA assay, 23 (63.9%) sera were seropositive, 21 (58.3%) were equivocal and 11 (30.6%) were seronegative. Those sera identified as seropositive or equivocal for IgG against ED3 were also seropositve by the Panbio ELISA kit. It was discovered that the viral load (copy number/mL) was not related to the seropositivity of these 23 samples. Although a high number of samples were detected as seropositive, the optical density measured at 450 nm was scattered over a wide range of absorbance (Figure 3.2.13 B; Right). The PPV and NPV for the in-house ELISA assay on 47 evaluated sera (36 from Brazil and 11 from Hong Kong) were 100% (6/6) and 31.7% (13/41), respectively. These results indicated the in-house ELISA assay able to correctly indentify sera which were true positive; but poor in identified sera which did not contain anti-dengue antibody. However, due to the small sample size, reliability of the PPV and NPV will be affected.

A



B

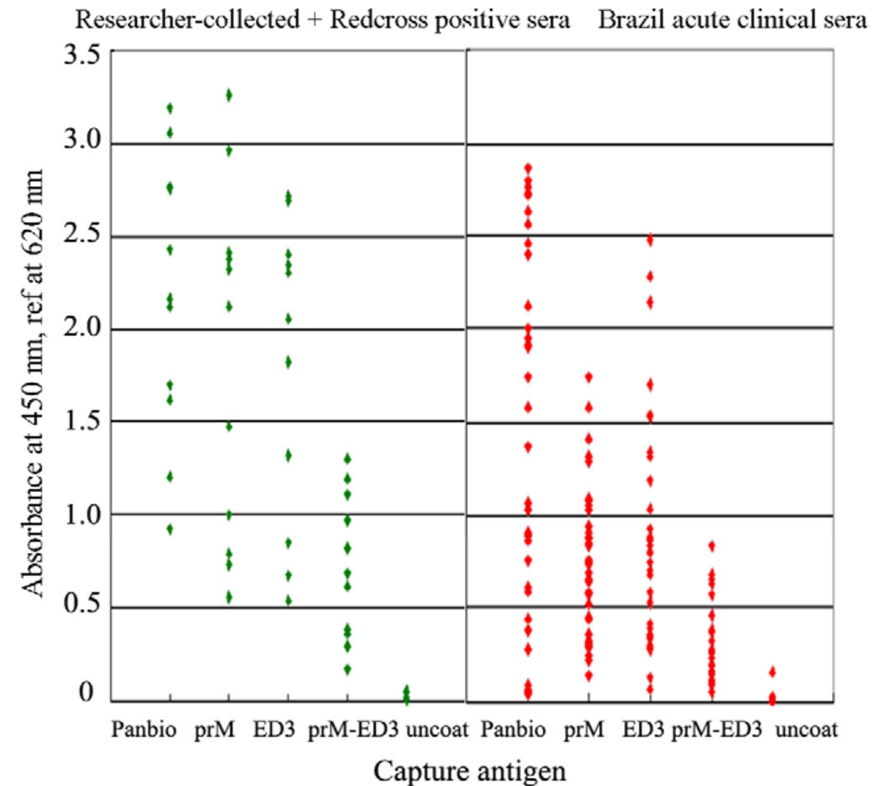


Figure 3.2.13 Distribution of absorbance measured from sera on validation of an in-house ELISA assay with prM, ED3 or prM-ED3 proteins as capture antigen.

Anti-DV IgG negative sera collected from community in Hong Kong randomly selected as control group. Left: Researcher-collected group (n=49); Right: Redcross group (n=51). Seronegativity was confirmed by the results of Panbio Dengue IgG Indirect ELISA assay. B) Left: Anti-DV IgG positive sera (n=11)collected from community in Hong Kong. Seropositivity was confirmed by the Panbio Dengue IgG Indirect ELISA assay. Right: Acute clinical sera collected from Brazil (n=36). Dengue was diagnosed by RealArtus RT-PCR assay.

3.3 Vector surveillance of *Aedes* mosquitoes collected in Hong Kong by RT-PCR assays

3.3.1 Mosquito species collected

A total of 1888 mosquitoes were collected in this pilot study. 31.4% (593) were *Ae. albopictus* (Table 3.3.1). The rest were *Culex, Armigeres* and *Anopheles* genera which represented 53.9%, 14.4% and 0.2% of collected mosquitoes, respectively. 0.2% mosquitoes were classified as unknown *Culicidae*. The peak month of field-caught mosquitoes was May 2007, and July 2007 was the peak month for the occurrence of *Aedes* mosquitoes. (Figure 3.3.1). The number of field-caught *Ae. albopictus* increased with mean temperature on the sampling day. Increased in the number of field-caught *Ae. albopictus* was not demonstrated obviously with rainfall (Figure 3.3.2)

The distribution of *Aedes* mosquitoes and other mosquito genera varied by geographical district, as shown in Figure 3.3.3. A relatively higher number of *A. albopictus* as a proportion of all species was collected from the residential areas of urban districts, such as QB, KC and YL. These sites were leisure parks located near to residential areas. An extremely low number of *Aedes* mosquitoes was collected from ND, SV and TM. The total number of mosquitoes collected from ND was also low. Most of the field-caught mosquitoes from SV and TM were *Culex* and *Armigeres* species, which indicated that these two sites might be habitats of these mosquito genera rather than *Aedes albopictus.*

Table 3.3.1 Mosquito genera of field-caught samples

Mosquito genera	No. of mosquito	(%)
Aedes albopictus / species	593	31.41
Culex	1018	53.92
Armigeres	271	14.35
Anopheles	3	0.16
Culicidae	3	0.16
Total	1888	100

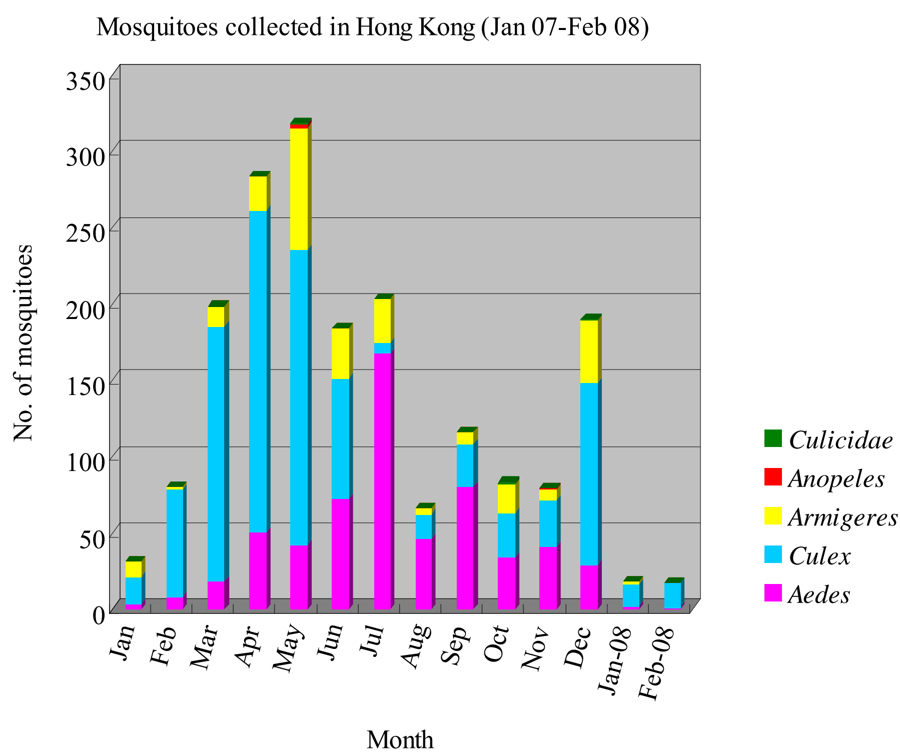


Figure 3.3.1 Monthly distributions of field-caught mosquitoes.

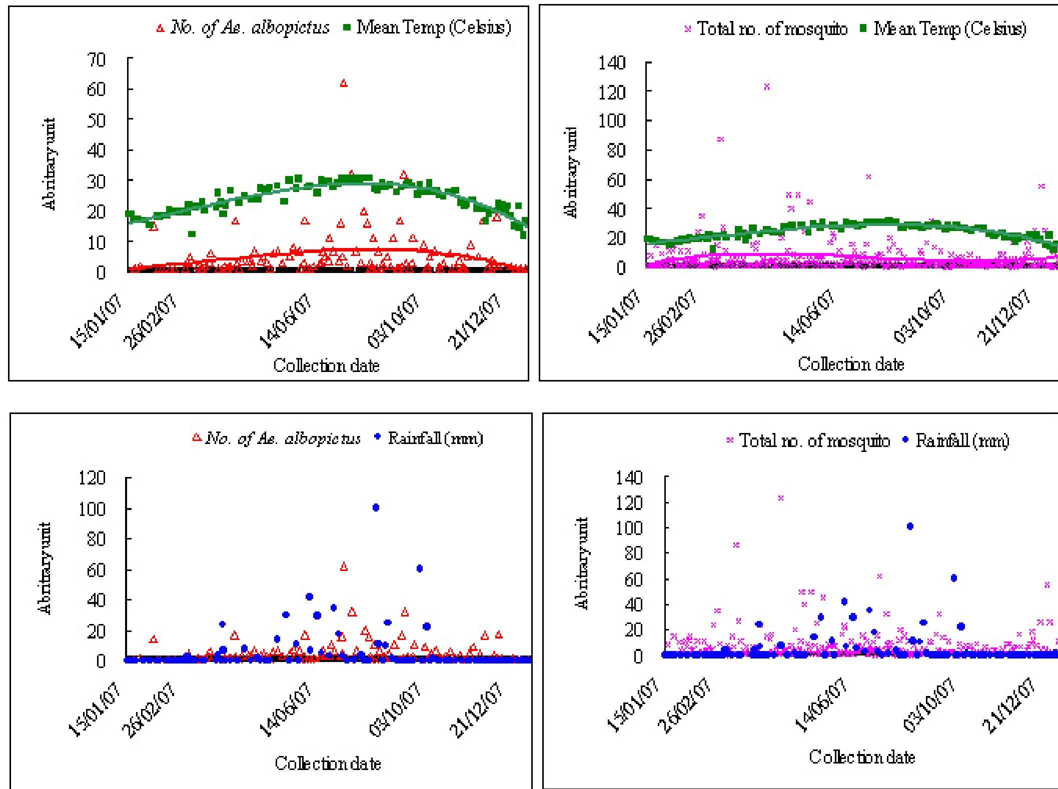


Figure 3.3.2 Relationship of temperature and rainfall with the number of field-caught mosquitoes and *Ae. Albopictus.*

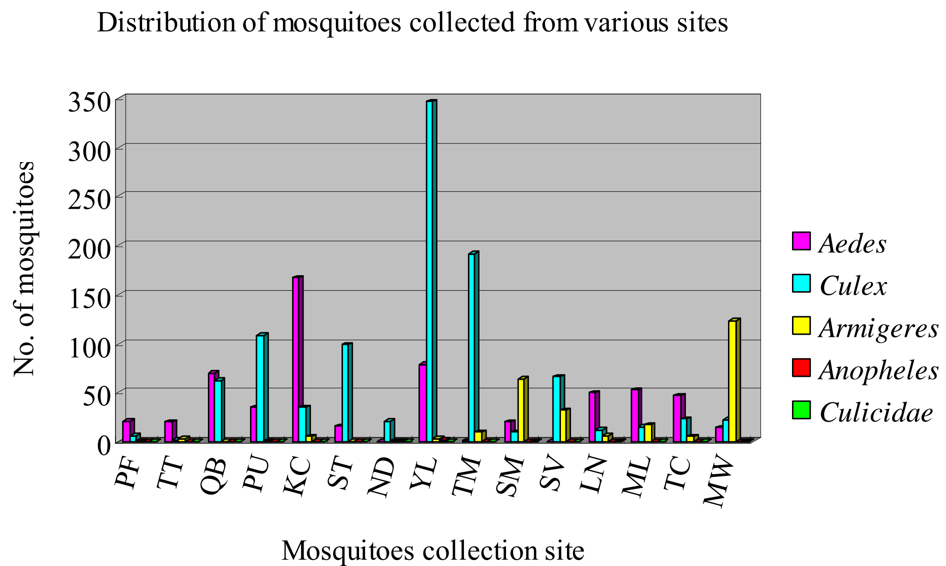


Figure 3.3.3 Distribution of field-caught mosquitoes in various sites.

3.3.2 RT-PCR assays for detection of DV in mosquito pools

All mosquito pools were RT-PCR negative in both assays, so MIR could not be calculated. In the conventional nested PCR assay, all RNA extracted from mosquito pools was negative in the first round of PCR (Figure 3.3.4B). After the nested round of PCR, non-specific bands with various sizes were observed from agarose gel electrophoresis (Figure 3.3.4B). Among those non-specific bands, there were four mosquito pools (SB in February, ST in August, SM in September and TC in December) showed a single product with a similar size to that of DEN-1 (482 bp) in agarose gel electrophoresis. Sequencing results of these four products indicated the absence of DV in the samples after matching with the database. Another six mosquito pools showed non-specific amplification with a product size ~600 bp and ~1100 bp after the nested PCR.

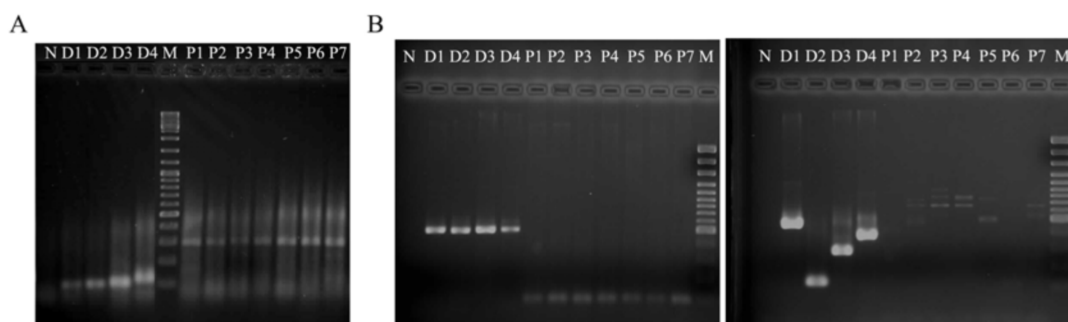


Figure 3.3.4 Agarose gel electrophoresis of products amplified from RNA extracted from mosquito pools by the in-house RT-PCR LightCycler assay and the conventional nested RT-PCR assay.

N, no template control; D1-4, DEN-1 to -4 serotype as positive control; M, molecular weight marker, 100 bp; P1-7; mosquito pools 1 to 7. A) RNA extracted from mosquito pools were amplified by the in-house RT-PCR LightCycler assay. The product sizes for DEN-1 to -4 were 103 bp, 105 bp, 103 bp and 111 bp, respectively. B) RNA extracted from mosquito pools were amplified by the conventional RT-PCR assay. Left: Products from the first round PCR, 511 bp. Right: Products from the nested round PCR. (DEN-1, 482 bp; DEN-2, 119 bp; DEN-3 290 bp; DEN-4 392 bp) PCR products from the first round were diluted in 1:100 with MiliQ water, 10 µl was used as template for the nested round PCR.

For real-time RT-PCR, it was discovered a ~300 bp non-specific product was consistently amplified in all mosquito pools, but not in the positive controls when observed by gel electrophoresis (Figure 3.3.4A). The presence of the non-specific product gave a false fluorescence signal in the F1 channel due to the non-specific saturated binding property of the SYBR green I dye to all double-stranded DNA. However, the serotype-specific T_m signals detected in the F1, F2 and F3 channels indicated the absence of DV in the mosquito pools. Four mosquito pools (SM in January, PF, TT in May & YL in June) and one mosquito pool (LN in July) detected a melting peak with similar T_m as DEN-4 and DEN-2 controls, respectively (Table 3.3.2). Specific product bands of those suspected positive cases were not observed by agarose gel electrophoresis. Suspected positive cases of two assays were compared; and there was no mosquito pool sample which showed overlap results.

Table 3.3.2 Suspected RT-PCR positive mosquito pools based on in-house real time assay.

No.	Mosquito pool	T_m of control RNA (°C)	Detected T_m in F1 Channel (°C)	Suspected serotype based on T_m
1	LN_Jan	85.48	85.45	DEN-4
2	PF_May	85.00	85.76	DEN-4
3	TT_May	85.00	85.89	DEN-4
4	YL_Jun	85.14	85.80	DEN-4
5	LN_Jul	81.46	82.14	DEN-2

3.3.3 Recovery of DV RNA from the RNA extraction method

Mosquito lysate spiked with pure DEN-1 RNA showed Ct values 3.77 cycles (Ct 25.90 versus 22.13) higher than that of the pure DEN-1 RNA. According to the standard curve of pure DEN-1 RNA ($y = -2.06x + 33.66$, $r = -0.93$) (Fig 3.1.4), the difference in Ct value of 3.77 cycles represented an RNA concentration (PFU/L) approximately one log unit lower after RNA extraction procedures. Meanwhile, T_m detected in the F2 channel of the mosquito lysate spiked with pure DEN-1 RNA was 0.48 ^{o}C (81.04 ^{o}C versus 81.52 ^{o}C) lower than that of the pure DEN-1 RNA.

3.3.4 Determination of contaminants and RNA inhibitors in mosquito pools

The average Ct values and T_m of monthly mosquito pools spiked with DEN-2 RNA (Jan 2007-Feb 2008) were 20.3 ± 0.5 and 80.4 ± 0.2 ^{o}C, respectively. Compared the same parameters for pure DEN-2 RNA, a higher Ct value (21.8) and T_m (80.8 ^{o}C) were found. All monthly mosquito pools except one (Jan 2008) gave a parallel slope at the exponential phase in the amplification curve on a logarithmic scale (Figure 3.3.5). The mosquito pool RNA extract from Jan 2008 showed a slightly decreased exponential phase slope and delayed Ct value among them. This finding implied that PCR inhibitors might be present in the mosquito pool collected in Jan 2008 [Kontanis & Reed, 2006].

Similarly, the Ct values and T_m of the total mosquito pool spiked with RNA were systematically lower than that of pure RNA for all serotypes (Table 3.3.3). Two individual standard curves were plotted by Ct values for the dilutions against concentration (Figure 3.3.6). The slope for the total mosquitoes pool spiked with

DEN-2 RNA (-1.93) was similar to that of pure RNA (-1.92). A similar slope of the standard curves represented the same PCR efficiency of the two sets of samples according to the equation: PCR Efficiency (E) = $10^{-1/slope}$ [Kontanis & Reed, 2006]. The same PCR efficiency implied that a PCR inhibitor was not detected from the mosquito RNA samples.

The detection limit of DEN-2 in the presence of mosquito lysate was 2.1×10^{-4} PFU, which was similar to that of pure DEN-2 RNA. A significant fluorescence melting peak signal with a DEN-2 specific T_m was detected in the F1 channel (80.9°C). However, the correlation coefficient was not desirable; linearity was only achievable to the fifth dilution (2.1×10^{4} PFU/L).

Table 3.3.3 Comparison of PCR performance on pure RNA and total mosquito pool spiked with RNA

Serotype	(PFU/Rxn)	Ct value		Tm (° C)	
		Pure RNA	Mosquitoes Spiked with RNA[a]	Pure RNA	Mosquitoes Spiked with RNA[a]
DEN-1	3.0×10^{-2}	24.4	22.1	82.0	81.7
DEN-2	2.1×10^{-1}	23.2	21.2	80.9	80.7
DEN-3	1.6×10^{-1}	23.7	22.4	82.0	81.6
DEN-4	1.2×10^{0}	24.4	23.3	84.5	84.5

[a] RNA extracted from total 593 *Aedes* mosquitoes were pooled into a single sample pool. 5 µl was used as template for RT-PCR

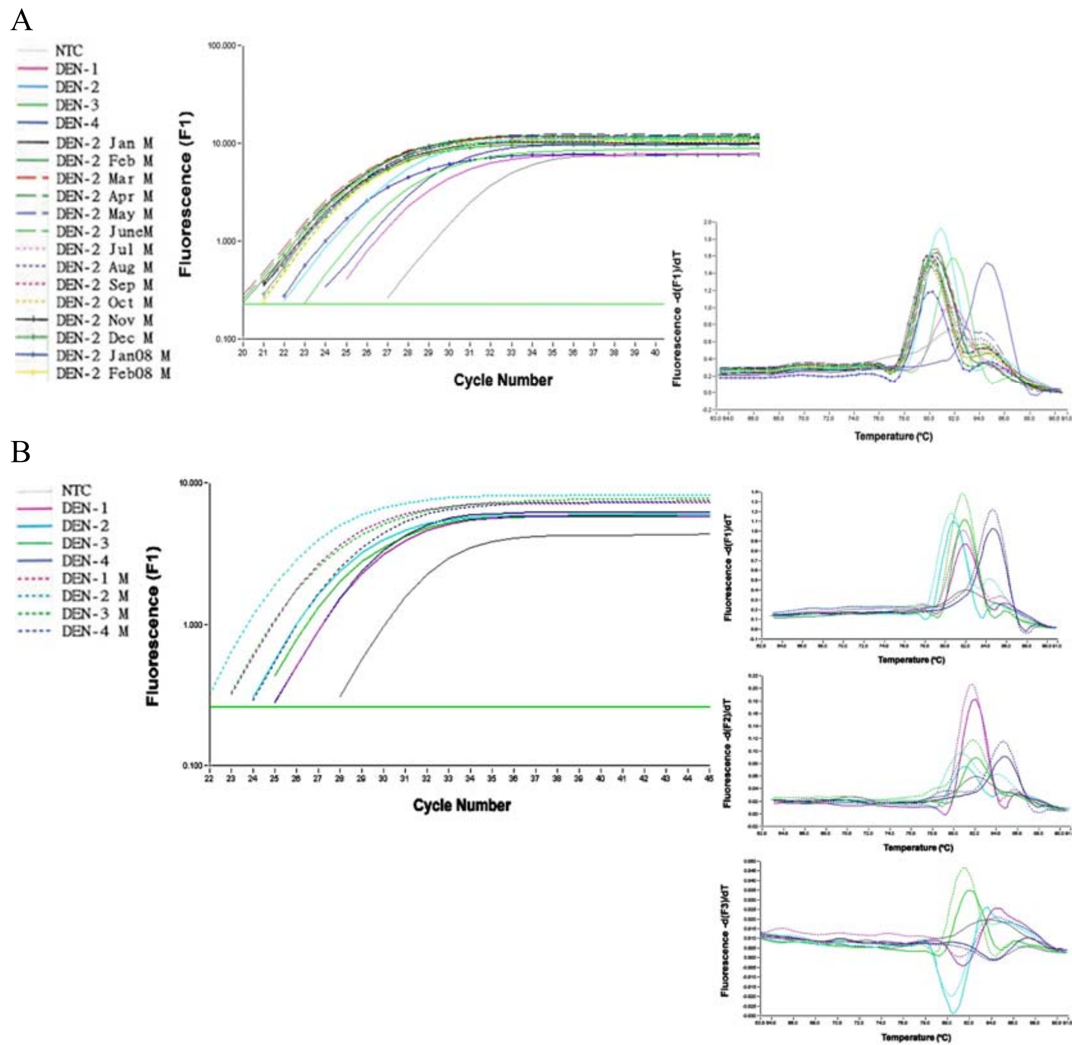


Figure 3.3.5 Logarithmic amplification curve and derivative melting curve of pure DV RNA and mosquito pools spiked with DV RNA

NTC, no template PCR control; M, mosquito pool. A) LC results of monthly mosquitos pool. Pure DEN-2 RNA was spiked into each pool. Upper: Logarithmic amplification curve detected by F1 channel due to SGI signal. Lower: Derivative melting curve of F1 channel, peak with specific T_m of DEN-2 was detected due to fluorescence signal of SGI. B) LC results of all collected mosquitoes in a single pool. Pure DEN-1 RNA was spiked. Left: Logarithmic amplification curve detected by F1 channel due to SGI signal. Right: Derivative melting curve of F1-F3 were shown respectively due to signal of SGI, BODIPY 630/650 and Cy5.5 fluorophore. Peaks with specific T_m of DEN-2 were detected in F1 channel due to the fluorescence signal of SGI. Peaks with specific T_m of DEN-2 and DEN-3 were respectively detected in F1 and F3 channels due to the fluorescence signal of BODIPY 630/650 and Cy5.5.

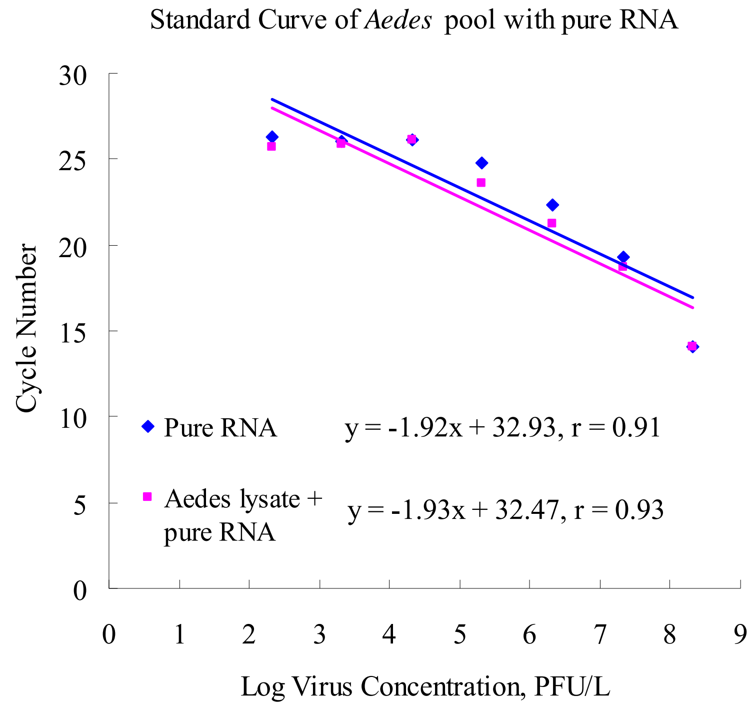


Figure 3.3.6 Standard curves of pure DEN-2 PCR and mosquito lysate pool spiked with DEN-2 RNA.

3.4 Seroepidemiological study of dengue in the general population of Hong Kong with a commercial ELISA assay

3.4.1 Number of subjects

A total of 685 subjects were recruited from April 2007 to July 2009 for this correlational study, in which the researcher-collected and Red Cross groups respectively accounted for 49.8% (341) and 50.2% (344) of the total subjects. According to the 2006 Population By-census of the Census and Statistics Department of the HKSAR, our sample size represented 0.01% of the total population of Hong Kong (6,864,346 people).

3.4.2 Demographical data of subjects

Among the 685 subjects, the number of females was 0.4 % higher than the number of males, with a sex ratio of male to female of 991:1000 (Table 3.4.1). More than half (57 %) of the subjects were within the age group of 16-34 years, and only 3.5% of the total subjects were 65 years of age or above. The mean age among all subjects was 35.2 years, while median age was 31 years. Compared with data from the 2006 Population By-census in Hong Kong, males and females accounted for 47.7% and 52.3%, respectively (sex ratio of male: female = 911:1000). Percentages of the total population for each age group were quite similar ~20% (15.3- 18.2%) except for the age group 55-64 years and 65 years or above, which were both less than 10% (Table 3.4.1).

The 341 subjects of the researcher-collected group, except for nine (2.6%) subjects who were unwilling to provide information on their residential area, were analysed and compared with the population in Hong Kong. Compared to the

population in Hong Kong by district council in 2006, the percentages of the sub-total population on Hong Kong Island, Kowloon and the New Territories of our researcher-collected subjects were appreciably close to those of the total population (Table 3.4.1). For researcher-collected subjects, about one-third of the subjects lived in the Kwai Tsing (19.1%) and Sha Tin (16.4%) districts. The North district had the least number of subjects, which accounted for only 0.9% of the total. Other subjects were quite evenly distributed in Hong Kong with less than 10% (1.8-8.2%) for each district (Table 3.4.1). Comparison of the population distribution in different districts in Hong Kong to that of the subjects recruited in the present study is shown in Figure 3.4.1.

Table 3.4.1 Comparison of distribution of population in Hong Kong and subjects recruited in present study by sex, age group and district council district.

Characteristics	2006 population by census[a]		Subjects collected in present study[b]	
	No.	% of population	No.	% of population
Sex				
Male	3272956	47.7	341	49.8
Female	3591390	52.3	344	50.2
Total	**6864346**	**100**	**685**	**100**
Age group				
0-14	939675	13.7	0	0
15-24	909005	13.2	225	32.9
25-34	1052126	15.3	165	24.1
35-44	1248855	18.2	109	15.9
45-54	1193788	17.4	102	14.9
55-64	668101	9.7	59	8.6
65+	852796	12.4	24	3.5
Total	**6864346**	**100**	**684**	**100**

(cont')

Characteristics		2006 population by census[a]		Subjects collected in present study[b]	
		No.	% of population	No.	% of population
District council district					
Hong Kong	Central & Western	250064	3.6	8	2.4
	Wan Chai	155196	2.3	5	1.5
	Eastern	587690	8.6	21	6.3
	Southern	275162	4.0	6	1.8
	Sub-total	**1268112**	**18.5**	**40**	**12.0**
Kowloon	Yau Tsim Mong	280548	4.1	9	3.0
	Sham Shui Po	365540	5.3	13	3.9
	Kowloon City	362501	5.3	27	8.4
	Wong Tai Sin	423521	6.2	24	7.2
	Kwan Tong	587423	8.6	21	6.3
	Sub-total	**2019533**	**29.4**	**94**	**28.8**
New Territories	Kwai Tsing	523300	7.6	63	19.0
	Tsuen Wan	288728	4.2	9	2.7
	Tuen Mun	502034	7.3	13	3.9
	Yuen Long	534192	7.8	19	5.7
	North	280730	4.1	3	0.9
	Tai Po	293542	4.3	19	5.7
	Sha Tin	607544	8.9	54	16.3
	Sai Kung	406442	5.9	11	3.3
	Islands	137122	2.0	5	1.5
	Sub-total	**3573635**	**52.1**	**196**	**59.0**
	Land total	**6861280**	**100**	**330**	**100**

[a] Data was adopted from 2006 Population By-census Office, Census and Statistics Department (Last review on 22 Feb 2007)
[b] Total researcher-collected subjects were 344. However, 1 and 11 unavailable data respectively on age group and residential area provided by subjects, comparison was made excluding missing data.

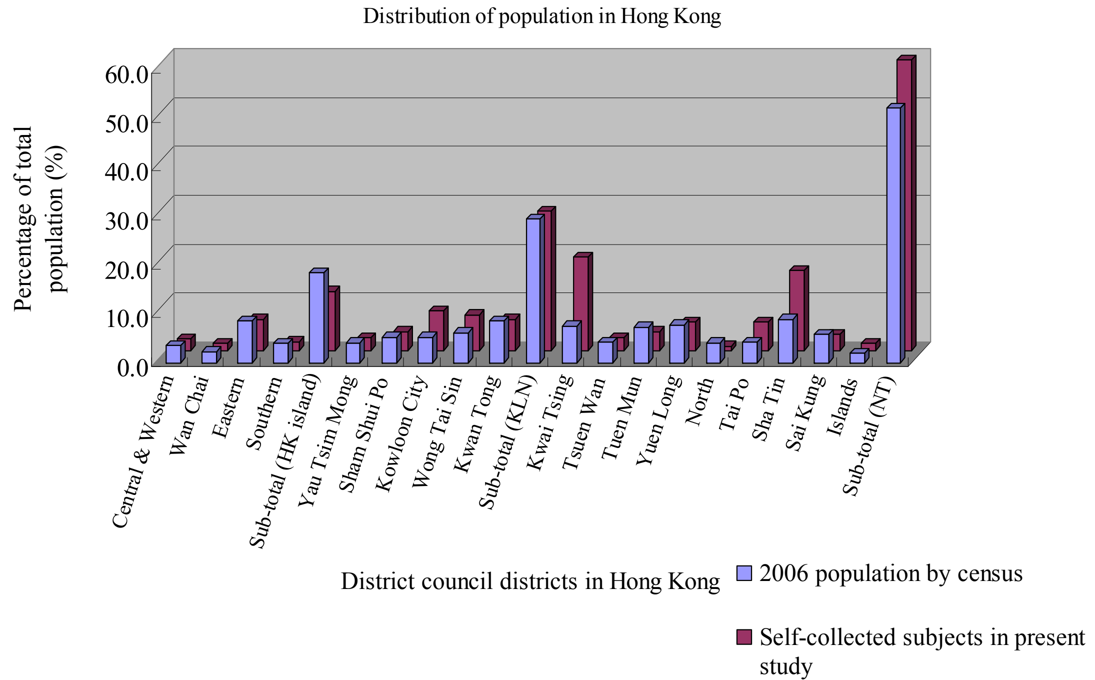


Figure 3.4.1 Distribution of population in Hong Kong.

3.4.3 Result of ELISA and prevalence of seropositivity

A total of eleven samples were positive by screening with the indirect IgG ELISA, and eight of them were researcher-collected subjects. Positive ELISA results represented the presence of detectable IgG antibodies against DV in serum, which was evidence of past or recent dengue infection. The overall prevalence of DV was 1.61%. Seroprevalence for the university group was 2.7-fold (2.35%) higher than that of the Red Cross group (0.87%).

Since samples from the Red Cross were plasma rather than serum, no validation was done for plasma samples (serum analogues) on this ELISA kit. For those sera from researcher-collected samples which showed positive results by ELISA, we also tested defibrinated plasma of the corresponding subjects with the

same kit for a preliminary validation. We found that the results from plasma samples agreed with those from sera. Panbio units calculated by the optical density at 450 nm for each positive sample are shown in Table 3.4.2. The degree of variability between the two assay measurements on the same group of samples was determined by the coefficient of variation (CV). The CV between the two types of sample for five samples was less than 4%, and the CVs of the remaining three samples were a bit larger with a range of 9.4-11.1%. For the pool of negative plasma, the CV between defibrinated plasma and untreated plasma was 46.6% (Mean 3.5, SD 1.63).

Table 3.4.2 Comparison of Panbio IgG Indirect ELISA results on positive serum and defibrination plasma samples

Sample no.[a]	Type of sample				Comparison of Panbio unit between 2 types of sample[d]		
	Serum		Defibrinated plasma[c]				
	Abs at 450nm	Panbio unit[b]	Abs at 450nm	Panbio unit	Mean	SD	CV, %
020	1.21	*15.2*	1.25	*14.7*	*15.0*	*0.35*	*2.4*
072	0.92	*12.0*	0.97	*11.4*	*11.7*	*0.42*	*3.6*
194	2.17	*42.3*	2.10	*40.9*	*41.6*	*0.99*	*2.4*
196	2.73	*53.2*	2.71	*52.7*	*53.0*	*0.35*	*0.7*
197	2.94	*57.2*	2.93	*56.9*	*57.1*	*0.21*	*0.4*
205	2.59	*50.4*	2.27	*44.1*	*47.3*	*4.45*	*9.4*
212	2.15	*41.7*	1.85	*36.0*	*38.9*	*4.03*	*10.4*
221	1.77	*34.3*	1.51	*29.3*	*31.8*	*3.54*	*11.1*

[a] Samples were researcher-collected group
[b] Panbio unit was calculated by formula, absorbance of sample / mean of cut-off value x 10. Patch specific calibration factor was not available for kit purchased before 2009. Panbio unit >11 indicated sero-positive of past or recent dengue infection.
[c] Plasma was defibrinated into serum analog as described in Chapter 2.4.2.1
[d] SD, standard deviation; %CV, coefficient of variation, which is calculated formula: SD/Mean x 100.

3.4.4 Association of seropositivity and risk factors

The association of seropositivity for univariate factors is showed in Table 3.4.3. It was observed that seropositivity was significantly associated with increased risk for subjects who were not born and did not grown up locally in Hong Kong (*P*<0.01, OR 18.00, CI 2.18-148.36). Among the seropositive individuals, one was local, five came from China and two from Indonesia (*P*<0.01, OR 19.71, CI 2.93-162.52). Travel in the past twelve months (*P*=0.29, OR 2.96 CI 0.36-24.39) and higher number of countries visited (*P*=0.15, OR 2.79 CI 0.65-11.98) indicated increased risk of seropositivity, although these parameters did not show a significant association. Interestingly, individuals who visited areas of Eastern Asia (*P*=0.07, OR 5.60, CI 0.68-46.03) or Southeast Asia (*P*=0.04, OR 4.27, CI 0.984-18.55) in the past twelve months were more likely to be dengue seropositive. However, only individuals who visited Southeast Asia were statistically significantly associated with seropositivity.

For multivariate analysis, the variable of individuals who visited countries in Southeast Asia in the past twelve months (*P*=0.03, OR 5.38, CI 1.13-25.54) was statistically significant associated with seropositivity. Individuals who visited Southeast Asia were 5.38 times more likely to be exposed to dengue infection. There was no significant association which contributed to seropositivity for individuals who were born and did not grow up in Hong Kong (*P*=0.63, OR 2.28, CI <0.01-18729.52) and also came from China and Indonesia (P=0.63, OR 2.28, CI <0.01-76183.17).

Table 3.4.3 Potential factors for association and risk estimation.

Factors for risk estimation[a]	Variables value	No. of subjects had the results of ELISA		Univariate analysis results		
		+ (%)	- (%)	*P*-value[b]	OR[c]	(95% CI)
Overall (n=685)						
Gender	Male	4 (36.4)	337 (50.0)	0.37	0.57	(0.17-1.97)
	Female	7 (63.6)	337 (50.0)			
Age group	>30	10 (90.9)	345 (51.3)	0.09	9.51	(1.21-74.68)
	= or <30	1 (9.1)	329 (48.1)			
Researcher-collected group (n=341)						
Demographical information						
Residential District	HK Island	0 (0.0)	45 (13.6)	0.46	-	-
	Kowloon	2 (25.0)	97 (29.2)			
	New Territory	6 (75.0)	190 (57.2)			
Race	Non-Chinese	0 (0.0)	1 (0.3)	0.88	1.00	(1.00-1.01)
	Chinese	8 (100)	324 (99.7)			
Born & grow up in HK	No	7 (87.5)	91 (28)	<0.01	18.00	(2.18-148.36)
	Yes	1 (12.5)	234 (72.0)			
Home Town	China & others	7 (87.5)	87 (27.6)	<0.01	19.71	(2.39-162.52)
	Hong Kong	1 (12.5)	245 (73.8)			
Travel history						
Travel within past 12 months	Yes	7 (87.5)	234 (70.3)	0.29	2.96	(0.37-24.39)
	No	1 (12.5)	99 (29.7)			
No. of countries visited in past 12 months	2 or more	3 (37.5)	59 (17.7)	0.15	2.79	(0.65-11.98)
	0-1	5 (62.5)	274 (82.3)			

(Cont')

Factors for risk estimation[a]	Variables value	No. of subjects had the results of ELISA		Univariate analysis results		
		+ (%)	- (%)	*P*-value[b]	OR[c]	(95% CI)
Destinations visited						
Eastern Asia 1	Yes	7 (87.5)	185 (55.6)	0.07	5.60	(0.68-46.03)
	No	1 (12.5)	148 (44.4)			
Eastern Asia 2	Yes	0 (0.0)	32 (9.6)	0.36	0.90	(0.87-0.94)
	No	8 (100)	301 (90.4)			
Southeast Asia	Yes	3 (37.5)	41 (12.3)	0.04	4.27	(0.98-18.55)
	No	5 (62.5)	292 (87.7)			
Southern Pacific	Yes	0 (0.0)	7 (2.1)	0.68	0.98	(0.96-1.00)
	No	8 (100)	326 (97.9)			
America	Yes	0 (0.0)	15 (4.5)	0.54	0.96	(0.93-0.98)
	No	8 (100)	318 (95.5)			
Europe	Yes	0 (0.0)	13 (3.9)	0.57	0.96	(0.94-0.98)
	No	8 (0.0)	320 (96.1)			
Middle East	Yes	0 (0.0)	4 (1.2)	0.76	0.99	(0.98-1.00)
	No	8 (100)	329 (98.8)			

[a] The number of researcher-collected subjects were 341 in total, however, number of subjects for potential factors "Residential District"& "Home Town" (n=340) and "Race" & "Born & grow up in HK" (n=333) were different due to missing data on questionnaire survey.
For travel history, Eastern Asia 1 represented China, Macau, Taiwan and Mongolia; Eastern Asia 2 represented Japan and Korea; Southern Asia represented Thailand, Singapore, Vietnam, Cambodia, Malaysia, Indonesia, New Guinea, Philippines; Southern Pacific represented Australia and New Zealand; America represented USA, Canada and South America. Africa was one of the separate categories, but no researcher-collected subject visited Africa within the past 12 months.
[b] *P*-value was calculated by Pearson *Chi*-square test.
[C] OR, odd ratio, was only valid for 2x2 contingency table analysis of which degree of freedom =1. CI, confidence interval.

CHAPTER 4

DISCUSSION

4.1 Development of a rapid RT-PCR assay for simultaneous detection and serotyping of DV

In order to control the spread of disease, it is necessary to develop a rapid and affordable diagnostic assay for DV detection to ensure early treatment and preventive measures.

4.1.1 Performance of the kit-based assay

Our assay was based on T_m and colour multiplexing for the detection and typing of DV. The unique amplicon T_m of each serotype in different channels (F1 to F3) allowed for specific detection of DV serotypes. Adding a GC tail improves the detection of single-nucleotide polymorphisms [Papp et al., 2003; Sheffield et al., 1989] and has a marked effect on T_m differentiation for small amplicons [Papp et al., 2003; Liew et al., 2004], such as the ~100 bp amplicons in this study. By contrast, minor base changes within amplicons typically do not produce detectable T_m changes. Because the amplicon sequence is highly conserved within the same DV serotype, a 4.7 °C T_m difference would be able to accommodate the possible minor genotypic variations in DEN-2 and DEN-4.

As expected, the amplicons from DEN-1 and DEN-3 had very similar T_ms (Table 3.1.1) and thus could not be distinguished in the F1 channel. Classically, two hybridisation probes (one donor and one acceptor) are required for FRET to

occur. Genotypic variations in the genome of the same serotype, however, make it difficult, if not impossible, to design two hybridisation probes for specific detection and typing. This study combined fluorogenic primers and SGI to achieve the same goal via iFRET. In fact, SGI is an effective energy donor for FRET, and the fluorescence generated by iFRET is 40 times greater than with the classic FRET assay [Howell et al., 2002]. This assay produced distinct melting peaks due to BODIPY 630/650 in the F2 channel for DEN-1, and due to Cy 5.5 in the F3 channel for DEN-3. We chose these two less-expensive dyes instead of LC Red 640 and LC Red 705, respectively, to reduce costs [Yip et al., 2003]. A one-step LightCycler assay based on the biprobe system and developed to detect only DEN-2 [Tan et al., 2002] is similar in principle to the iFRET assay.

Four (25%) of the serum samples known to be DV positive were not detected by this assay, but these false-negative samples tested either negative or weakly positive in the first-round PCR of a nested RT-PCR assay, which is a two-step nonquantitative assay. These results indicated that the DV titres in the samples might be too low to be detected. The RNA sample volume per reaction was believed and then confirmed to be the major factor affecting the sensitivity of our assay. The input sample volume for the nested RT-PCR assay was 10-fold larger than that for our assay. The sensitivity of our assay was improved by 12.5% (10 vs 12 of 16 samples) by increasing the RNA sample volume from 0.5 μL to 5 μL. This result showed that the sample-volume effect became magnified when the viral load in the serum was low. We suggest the use of larger sample volumes for clinical samples. For precious RNA samples from suspected DV-positive patients,

we suggest a 0.5 μL input sample volume for screening, followed by confirmation of a negative result with a larger sample volume.

The present assay gave a PCR efficiency >2, which is beyond the maximum theoretical value (efficiency = 2; slope = –3.3). The PCR efficiency was calculated from the slope of the calibration curve, –2.78 to –2.27 in this assay, which was generated from the Ct values of a dilution series of DV RNA. The presence of primer dimers in the reaction mixture increased the overall SGI signal, which in turn lowered the Ct values and yielded a PCR efficiency >2. Similar findings were noted in a recent study that used a Roche Diagnostics reagent set for amplifying *Chlamydia pneumoniae* using the LightCycler [Mygind et al., 2002]. Nevertheless, the sensitivity and specificity of our assay were not affected, because the PCR efficiency compared favourably with those of other available RT-PCR assays for dengue diagnosis [Lanciotti, 2003; Johnson et al., 2005].

Positive controls for all four serotypes and a no-template negative control should be run in parallel with test samples. This practice not only provides a reference for interpretation but also helps to monitor the accuracy of DV detection and typing, which is particularly important to recognise if deterioration of reagents has occurred. We once observed a systematic difference in results when we used a new batch of reagent sets; this systematic difference led to a T_m shift in all channels and for all serotypes. The T_m shift was determined to have been caused by improper storage conditions during delivery. When the samples were retested with another batch of the reagent sets that had been delivered under proper storage conditions, the T_m shift was negligible. Variations between different batches of

reagent sets have previously been reported and could lead to a 2.5-fold difference in mRNA quantification [Bustin, 2002].

The entire assay requires two hours (40 minutes for preparation plus 80 minutes for RT-PCR reaction/melting-curve analysis), compared with more than three hours for conventional TaqMan assays [Laue et al., 1999; Johnson et al., 2005]. Our assay is also more economical, because the primers are used for both detection and typing, only two primers have to be labelled with fluorophores, and no additional fluorescence probes are required.

4.1.2 Performance of the in-house developed assay and comparison with the original kit-based assay

In this study, an in-house one-step RT-PCR assay was developed by modification of the original kit-based assay for simultaneous detecting and typing dengue viruses. The in-house assay maintained the performance of the original kit-based assay in that it was efficient, sensitive and specific.

A number of assays have been developed recently using different approaches for cost-effective and rapid screening of DV. Gomes and colleagues [Gomes et al., 2007] modified the nested RT-PCR developed by Lanciotti et al. [Lanciotti et al., 1992] into a single-tube nested PCR. This assay utilised immobilised primers for the second-round PCR. Another team of researchers [Lai et al., 2007] reported two assays for separate screening and serotyping of DV, which included a rapid and low-cost SGI assay for DV screening and a FRET assay for subsequent serotyping of positive samples. However, these assays

utilised commercial reagents which increased the assay cost, and two-step RT-PCR was involved, which increased the reaction time and risk of cross-contamination.

Based on the T_m of the amplicon of each serotype, the performance of the in-house assay was apparently comparable with the original assay. However, we found that the T_m difference between DEN-4 and DEN-2 was reduced by 0.38 °C (T_m DEN-4 - DEN-2 = 4.7°C) when compared with that of the original assay (Table 3.1.1). Components of the PCR buffer and its concentration, such as potassium and magnesium ions, could affect the amplicon melting characteristics and alter the resolution of the melting peak [McPherson & Moller, 2000]. However, the temperature reduction here was small and would not lead to any difficulty in differentiation between these two serotypes. The amplification efficiencies (efficiency = $10^{-1/slope}$) of all serotypes were higher than those of the original assay. We believe that the presence of primer dimers was still the cause for PCR efficiency >2, but the difference in PCR reaction buffer components and DMSO concentration between the two assays might affect the Ct values of all serotypes systemically.

The turnaround time of the assay was only nine minutes longer than that of the original assay (~ two hours); this extra time was required for the inactivation of reverse transcriptase and template denaturation before PCR cycling. The running cost per reaction of the in-house assay was about half that of the original assay. Apart from the capillary reaction tubes, primers and probes (around US $1.30 per reaction), which were common to both assays, the main cost of the original assay came from the LightCycler RNA Amplification Kit HybProbe, which was about

US $3.75 per reaction. In the in-house assay, a combination of MultiScribe reverse transcriptase and FastStart Taq polymerase were used in the one-step RT-PCR. The cost for these enzymes was less than US $1 per reaction. This further reduced the running cost and was beneficial for large-scale routine screening, for example, for mosquito surveillance. Application of this assay on mosquito vector surveillance was described in Chapter 2.3.

The detection limits of the in-house assay ranged from 1.16×10^{-2} to 3.27×10^{-3} PFU/reaction, which is lower than a number of real-time RT-PCR assays reported recently [Houng et al., 2000; Johnson et al., 2005; Lai et al., 2007]. When compared with the conventional nested RT-PCR assay using known positive and serotyped samples from Hong Kong and Brazil, the in-house assay had sensitivity of 77% (37/48) and all sera were correctly serotyped, while the conventional RT-PCR had sensitivity of 85.4% (41/48) and only 97.6% of sera were correctly serotyped (Table 3.1.2). While not statistically significant, the higher sensitivity of the conventional RT-PCR was mainly contributed to by the nested second-round PCR. Relatively low sensitivity of our two RT-PCR assays is related to quality of RNA which might be degraded due to prolong storage and sample transportation. Nevertheless, our previous study showed that the conventional RT-PCR was more sensitive than our original kit-based assay and the in-house assay by one and two log units, respectively [Lo et al., 2007].

In assay validation with the clinical serum samples, result discrepancy was observed between our in-house assay and the conventional nested RT-PCR assay. The in-house assay detected and typed the same number of IgM-positive cases as

the conventional RT-PCR assay. Meanwhile, a more IgM-negative cases were detected by the in-house RT-PCR assay than by the conventional RT-PCR assay (twelve vs five). However, those 12 samples positively detected by the in-house assay were also detected by the first round of the conventional RT-PCR assay. Of these twelve samples, eleven of them were collected with the second sample (three to six days apart) for immunochromatography (IC) assay re-confirmation. All eleven samples were IgM-positive, which indicated that patients were in the acute phase of primary DV infection. A negative IgM reaction in the first sample for the IC assay might be related to the serum specimen collection time. The sensitivity of the serological assay was guaranteed only for serum specimens obtained more than one week after the onset of infection [Tsai, 2004]. Specimens collected before the adequate expression of anti-DV IgM might cause a false negative serological assay result. Of these 26 IgM-negative samples, eight samples were eventually shown to be IgM-negative after re-confirmation of the IC assay with the second serum specimens. Only one case out of eight was diagnosed as dengue, however. Two serum specimens were collected within seven days after the onset of infection, at time point at which seroconversion (anti-DV IgM) may not be detected. Four were diagnosed as dengue cases in addition to being diagnosed with hepatitis B or C infection. The presence of antibodies against hepatitis viruses might interfere with the detection of anti-DV IgM in the specimens. Three samples were finally identified as non-dengue cases.

In the conventional RT-PCR assay, total of fifteen samples which were PCR-positive in the first round were not detected by the nested round PCR. This might have been due to differences in the priming regions between the two assays.

It was discovered that five nucleotide variations fell within the priming sequence of the reverse primer TS1 for amplifying the DEN-1 serotype during the second round PCR (Figure 3.1.7). The priming sequence was located in the 5' UTR of the DV genome. This region was highly conserved, but there were some nucleotide variations within the same serotype. The locations of these five nucleotide variations commonly occurred among DEN-1 serotypes based on the alignment results. For the samples from Mainland China, since those clinical samples were collected from the same outbreak, some of the samples might contain the same sequence variation pattern. Thus, the TS1 primer might not anneal to DEN-1 strains with these sequences. This indicates that the primers of the conventional nested RT-PCR assay might be inadequate to cover some DV strains.

Compared with the conventional nested RT-PCR assay, the first round PCR assay detected two more positive sera of IgM-positive cases than the in-house RT-PCR assay, but same number of IgM-negative cases was detected by both assays. This finding indicated that our assay might not be able to detect DV after seroconversion. However, a positive result in the first round PCR of the conventional assay, which was only flavivirus specific, did not confirm the presence of DV. The confirmation of DV detection relied on the results of the nested round PCR. The sensitivity of our in-house assay was comparable to that of the nested round PCR. The same number of IgM-positive cases and a higher number of IgM-negative cases were detected by the in-house RT-PCR assay. The in-house assay detected more IgM-negative cases, which might be related a deficiency in the coverage of the primer as mentioned in the previous paragraph. For samples from Hong Kong and Brazil, the result discrepancy between the two

assays could be related to assay sensitivity. The assay, including a nested PCR, was apparently one log unit more sensitive than our in-house assay and the original kit-based assay [Lo et al., 2007].

Additionally, we observed that some DEN-1 samples collected from China showed a T_m shift to a lower temperature and towards the T_m of the DEN-2 serotype. However, this should not lead to incorrect result interpretation since the DEN-1 serotype was characterised by the specific fluorescence signal generated by the fluorophore label BODIPY 630/650 on the primer, and this fluorescence signal should only be detected by the F2 channel of the LightCycler. Three of these amplicons showing lower T_m were analysed by sequencing, and we found one variation, where a C nucleotide was substituted by a T nucleotide compared with the sequence of the positive control DEN-1 RNA. The effect on T_m of this nucleotide substitution became more significant in small amplicons of about 100 bp, and leading to a less than 1°C difference in T_m.

We encountered hurdles in the optimisation of the in-house assay. T_m shifting of amplicons to a lower temperature and a non-specific product with higher T_m were observed when compared with the original assay. Since the secondary structure of the 3’ untranslated region of dengue virus might inhibit primer annealing, we improved this condition by adjusting the concentrations of PCR additives, dNTPs and other components as well as the concentration of the PCR reaction buffer. DMSO is a PCR additive commonly used for enhancing PCR yield, specificity and amplification of DNA templates with high secondary structure and GC-content [Winship, 1989; Varadara & Skinner, 1994; Ralser et al.,

2006]. A GC-rich solution with a similar function as DMSO is provided as a supplementary reagent with the FastStart Taq polymerase. Final concentrations of 5%, 7% and 10% (v/v) DMSO and 1x GC-rich solution, according to the manufacturer's instruction, were initially tested with our in-house assay. 5% DMSO was found to be more efficient than the GC-rich solution in enhancing specificity, and also increased the T_m of the amplicons. It was believed that these two additives worked by a similar mechanism to lower the T_m of double-stranded DNA by altering primer-to-template hybridisation, and thus improved the PCR performance of DNA templates with high GC-content and secondary structure [Baskaran et al., 1996; Varadara & Skinner, 1994]. However, it was hard to compare these two additives since the content of the GC-rich solution was not available. Nevertheless, it was demonstrated that different combinations and concentrations of PCR additives could affect the performance of PCR [Ralser et al., 2006].

The PCR reaction buffer was an important factor in the development of our in-house RT-PCR assay, as the pH and content of working buffers for reverse transcriptase and Taq polymerase are usually different. Therefore, a common reaction buffer providing optimal conditions for both enzymes is critical for the success of one-step RT-PCR assay development. Our assay started with PCR Reaction Buffer II (500 mM KCl, 100mM Tris-HCl, pH8.3) from Applied Biosystems, since it was a reaction buffer compatible with RT and PCR. Eventually, Roche PCR Reaction Buffer (100 mM KCl, 500 mM Tris-HCl, 50 mM $(NH_4)_2SO_4$, pH 8.3; supplemented with FastStart Taq polymerase) was the final choice for our assay because of its compatibility with MultiScribe reverse

transcriptase and a higher T_m resolution between DEN-2 and DEN-4. The concentrations of the components were also different between the two buffers. A higher concentration of potassium chloride in PCR Reaction Buffer II might lower the stringency of PCR and lead to non-specific products under the high magnesium conditions in our assay [McPherson & Moller, 2000].

In summary, we developed two tetravalent molecular assays with the same strategy for simultaneous detection and typing of DV. One was a kit-based RT-PCR assay, and the other was a cost-effective in-house RT-PCR assay. The performance of the in-house assay was reproducible and comparable to that of the original kit-based assay. The in-house assay was validated with clinical sera collected from three geographical regions, including DEN-1 to -4. The sensitivity and specificity of the in-house assay were comparable to those of the conventional nested RT-PCR assay. The cost per reaction was only one third of the original assay. This in-house assay has potential for application in the routine screening of suspected cases and vector surveillance for DV. The kit-based assay is applicable for clinical cases, for example to re-confirm cases, due to less variability in the reagents and enhanced quality control found in commercialised products. We will continue collecting clinical serum samples, particularly for DEN-4, from more geographic regions for assay validation to ensure the assay is valid for most DV strains.

4.2 Generation of recombinant DEN-2 proteins for investigating the neutralising potential by their corresponding antisera and developing a serological diagnostic assay

4.2.1 Characterisation of expressed recombinant DEN-2 proteins

In this part of study, three recombinant protein fragments (prM, ED3 and prM-ED3) were expressed in soluble form in *E. coli* with average yields which varied from 149.9-362.8 μg/L of LB culture medium. The pET SUMO protein expression vector utilised in the present study facilitated the production of soluble protein since the SUMO fusion protein can increase the solubility of recombinant protein fragments. Additionally, SUMO fusion proteins increased the expression level of target proteins according to the research findings of the manufacturer. In addition, the hydrophobic transmembrane region of the target protein was omitted to further enhance the expression of soluble protein. There whereas no glycosylation sites along the sequence of ED3, and only one N-linked glycosylation site at the serine-70 residue of the pr portion of prM (Figure 3.2.4) [Chang, 1997]. We understood that expression in the *E. coli* expression system did not offer post-translational modification; however, efficient and high expression of recombinant proteins in *E. coli* cell favoured our first attempt at protein expression [Structural Genomics Consortium et al., 2008]. After protein purification, three protein fragments were applied for investigating the neutralisation potential of their corresponding polyclonal antisera and to attempt the development of a serological diagnostic tool. The neutralisation potential of the corresponding polyclonal antisera against the proteins were investigated by immunisation of rabbits to induce the production of polycloncal antibodies. Neutralisation activities of the anti-sera against individual protein fragments were evaluated by means of an assay

to detect the inhibition of RSP binding to Vero E6 cells. A serological diagnostic tool was developed based on an indirect ELISA assay.

During cloning into the pET SUMO vector, we experienced an unexpected loss of nucleotides after cloning of the DNA fragments. There was loss of one or two nucleotide bases (adenine or thymine) in several clones. The positions of the missing nucleotides were either between the junction connected primer region of the DNA insert into the vector sequence at the SUMO cleavage site (please refer to Figure 2.2.4 for a map of vector) or within the primer region of the DNA insert. The purity of the primers based on the number of nucleotides was investigated by means of denaturing high performance liquid chromatography. A single peak for each primer indicated they were synthesised in full-length and purified with assured quality. It was estimated that the missing nucleotides might be related to tiny amounts of primer which were not in full length during synthesis by the manufacturer. However, the sequences of all in-frame PCR products were analysed prior to ligation into the vector. The actual cause for the loss of nucleotides is still under investigation. In addition, we excluded the error rate of *Taq* DNA polymerase during PCR amplification as a reason for this, since the 3'→5' proofreading property of Platinum® *Taq* polymerase offered six times higher fidelity compared to standard *Taq* DNA polymerase.

Purified protein fragments were subjected to specific detection and protein identification by Western blotting and mass spectrometry. However, we encountered hurdles during the process of protein identity confirmation. prM protein was not detected by the prM mAb purchased from a commercial source. In

the present study, the relatively mild non-ionic detergent Triton X-100 was utilised in the preparation of lysis buffer for protein extraction. It ensured that protein recognition in Western blotting was under non-reducing conditions as specified by the manufacturer. However, only a negligible signal was detected. It was discovered that the weak and negligible signal detected by the prM mAb could be related to the reducing agent β-mercaptoethanol in the sample loading buffer. In any case, the results of Western blotting might be improved by protein extraction using Tris-HCl as a buffer component or by mechanical shearing.

In addition, there were four variations within the amino acid sequences between the DEN-2 strain used in the present study and the viral strain (Thailand DEN-2 18861 strain) utilised for production of this commercialised mAb. From the amino acid sequence alignment of these two strains, four residues of variations were identified. Two variations were located along the sequence of the antigenic sites (residues 249-256 & 262-277) (Figure 3.2.7). These two variations were considered to be conserved substitutions of amino acids. Conserved substitution represents an amino acid which was replaced by another amino acid containing a similar side chain [Davidson & Law, 2008]. The amino acid residues methioine-249 and threonine-276 of the Thailand DEN-2 18861 strain were substituted by isoleucine and alanine, respectively, in the strain utilised in the present study. Substitution of these amino acids may have caused altered biological activities, including antibody-antigen interactions due to changes in the overall polarity of the structure, the intra- and intermolecular binding and steric hindrance of the protein. Meanwhile, due to the structural specificity of recognition of the monoclonal antibody, the prM protein fragment produced in the present study

might not have been recognised by the commercialised prM mAb [Cater, 2003]. Nevertheless, the protein identities were finally confirmed by MS.

For accurate mass measurements of protein fragments, only ED3 was detected and measured by MS. The measured mass (24331 Da) of ED3 by accurate mass measurement was in agreement with the predicted mass (24421 Da) with a deviation of less than 0.4%. The discrepancy of mass could be explained as the neutral loss of an H_2O molecule (m/z 18) and the loss of the alkyl chain (C_5H_{12}, m/z 72) of the lysine (K) amino acid residue at the C-terminal of ED3 (Figure 3.2.4). Therefore, by including these, the measured mass of 24331 Da plus the neutral loss of molecules (18 + 72 Da) exactly agreed with the predicted mass of 24421Da, which confirmed the identity of ED3 by mass measurement as well as by protein identification. Since the mass of prM and prM-ED3 protein were beyond the detection limit of the Q-TOF2 MS, the identity of these proteins was also confirmed by protein identification based on information in a database.

4.2.2 Immunisation of recombinant proteins to produce polyclonal antibodies and their neutralising potential against RSPs

In the production of polyclonal antibodies from rabbits, two rabbits suffered from chronic diarrhoea and gastrointestinal disturbances after the priming injection of individual ED3 and prM protein. We accepted the suggestion of the veterinarian and decided that animal welfare should take priority over the experiment. Further booster injections were terminated and the two rabbits were euthanised humanely. Since only the priming injection was given to these two rabbits, a much lower antibody titre against ED3 and prM proteins was induced.

Interactions of the prM and E proteins in terms of affecting the conformation of E have been studied extensively. One metaphor has represented the *pr* subunit of prM as the drawstring of curtain, which can reversibly change the conformation of E similar to controlling the opening and closing of a curtain. However, once the string is removed, as when *pr* is cleaved by furin, the conformation of *E* can no longer be altered, even with pH changes [Li et al., 2008]. The importance of co-expression of both prM and E proteins to induce neutralising antibodies has been recently explored by other groups [Fonseca et al., 1994; Ocazionez Jimenez et al., 2001]. Since ED3 was recognised as the immunoglobulin-like epitope for cell adhesion, recent investigations have mainly focused on this envelope domain. Anti-ED3 antibodies have demonstrated strong protection against DV challenge in animals; however, the neutralisation potential of anti-ED3 in previous studies was variable [Khanam et al., 2006; Chin et al., 2007; Zhang et al., 2007; Etemad et al., 2008]. This could be explained by different expression systems in the production of recombinant ED3 proteins. However, the interaction of prM and ED3 affects its conformation and the impact of this on neutralisation potential has not been investigated. In the present study, we studied this hypothesis by evaluating a prM- ED3 chimeric fusion protein alongside a mixture of prM and ED3 to investigate the influence of prM-ED3 interactions on the neutralisation activities of the corresponding antibodies raised in rabbits.

Due to unavailable access to a class III biosafety level laboratory for performing neutralisation assays with native intact DV, the neutralisation activities of individual polyclonal antibodies were studied by means of a DEN-2 RSP

binding assay. RSPs or virus-like particles (VLPs) have been used as a tool for the evaluation of virus-cell binding and uptake in several studies. The inhibition of cell binding or entry was revealed by a decreased in fluorescence level which was detected by flow cytometric analysis [Bergsdorf et al., 2003; Bousarghin et al., 2005; Buonaguro et al., 2006; Qing et al., 2010]. In the present study, DEN-2 RSPs pre-treated with rabbit antisera were incubated with Vero E6 cells to evaluate the inhibition of cell binding. If an antiserum contained neutralising antibodies which bind to DV, it would interfere with the interaction between RSPs and the cell surface of Vero E6 cells and thus inhibit cell binding. The inhibition of RSP binding to Vero E6 cells was indicated by a change in the fluorescence signal of the FITC conjugate of the secondary antibody. The flow cytometry results demonstrated that the neutralisation potentials of the four antisera varied from 5.0-24.7%. Controls without either DEN-2 RSPs or rabbit's antisera generated fluorescence signal level similar as that of the positive control which indicated the integrity of the experimental set up. Antiserum against the prM-ED3 chimeric fusion protein (anti-prM-ED3) demonstrated the strongest neutralisation potential (24.7%). This could be due to two reasons. First, it was estimated that the prM protein facilitated retention of the native conformation of ED3, as we hypothesised. Second, it might be related to the prM fragment of the chimeric prM-ED3 protein which was responsible for elicitation of high affinity antiserum. Anti-prM demonstrated a very strong neutralisation potential (18.6%) even at a low titre, despite being elicited by a single dose of the priming injection. In a previous study, there was evidence to show that the prM protein might elicit neutralising antibodies since prM mAbs passively protected mice against DV challenge [Kaufman et al., 1989]. However, latest studies suggested opposite findings. They

suggested anti-prM enhanced internalization of immature prM-containing virion through Fc receptors of monocytes [Rodenhuis-Zybert et al., 2010]. Betramello et al. indicated human prM-specific mAbs facilitated ADE due to their extensive cross-reactivity among four serotypes, and poor neutralising ability [Beltramello et al., 2010]. It is impossible to conclude whether anti-prM is protective or enhance viral infection, more extensive studies on prM are necessary. Nevertheless, the affinities of the antibodies elicited by prM and prM-ED3 were not comparable since the number of doses used for immunisation was different. It was suggested that we should further investigate the differences in protein structure between the prM-ED3 chimeric protein and the native prM and ED3 proteins by x-ray crystallization, and that we should also assess the physical protein-protein interactions between prM and ED3 by a pull-down assay.

The neutralisation potential (10.9%) of the antiserum against the mixture of prM and ED3 proteins was lower than that of anti-prM-ED3 and anti-prM. This could be explained by the conformation differences between prM generated from the prM protein alone and the chermic anti-prM-ED3 protein due to steric hindrance, leading to different immunogenic properties. Additionally, it could be due to the immunogenicity of ED3, which was stronger than that of prM, since ED3 was recognised as the immunoglobulin-like epitope for cell adhesion. There are several factors which influence peptide immunogenicity, including the length and sequence of the protein, the choice of carrier protein and the method of coupling the peptide to the carrier protein [McLean et al., 1991]. In the present study, large proteins instead of small peptides were utilised as immunogens, so carrier proteins and coupling methods could not be the causes for these differences.

Therefore, immunogenicity can probably be attributed to the sequence of protein. Stronger immunogenicity indicated effective peptide presentation, so as to enhance the generation of antiserum against a particular epitope.

It has been well described that ED3 is an antigenic epitope due to its immunoglobulin -like structure for cell adhesion. Interestingly, in the present study, antiserum against the ED3 protein (anti-ED3) demonstrated the lowest antibody titre as well as the lowest neutralisation potential (5.0%) to DEN-2 RSPs among the four antisera. Cells having a fluorescence signal >log 10^0 after anti-ED3 treatment decreased only by 5.0%, which fell between the measurement deviation for detection of the fluorescence signal from two sets of duplicated samples (2.98% and 6.78%). We were unable to define this 5% decrease in fluorescence signal due to the measurement uncertainty since measurement deviation could be influenced by the matrix effect between samples. The lowest neutralisation potential (5.0%) of anti-ED3 against DEN-2 RSP binding among the four antisera could be related to improper folding and the low titre could be due to the single dose of the priming injection which was administered for immunisation . Despite of protein being expressed in the soluble fraction without a re-folding procedure, the native protein folding might not be guaranteed. Steric hindrance could be another cause which buried the critical epitope, provided that native folding of the expressed protein was achieved [Carter, 2003]. Polyclonal antibodies generate a mixture of antibodies with varied specificities, because different regions of the protein can elicit antibodies specifically against a particular domain [Sutcliffe et al., 1980]. Therefore, a failure of recognition of the antiserum to structurally specific proteins might result. Meanwhile, the low antibody titre of anti-ED3 could be

related to the fact that only a single rabbit was immunised, instead of multiple rabbits for the same protein antigen. Some factors which are animal-dependent such as genetic defects or past antigen exposure history may lead to a low immune response or epitope-response bias to the antigen of an individual animal [Hanly et al., 1995].

Interestingly, recent study of de Silva and colleagues demonstrated that anti-E/D3 played a minor role in DV neutralisation [de Silva et al., 2009], but they had a different conclusion from this study. De Silva's team showed evidences the weak neutralising activity of ED3 was not related to either improper folding or masking effect of reactive epitope by the fusion partner. They indicated ED3-binding antibody in human anti-DV sera was less important for neturalising DV. However, this finding must be further investigated since many previous studies have demonstrated and recongised ED3 as a potential epitope for neutralisation. Chin et al [2007] performed a similar study of polyclonal immunisation in mice with the ED3 protein of DEN-1 and -2 serotypes. High neutralisation activities against homologous serotypes were determined by using a plaque reduction neutralisation assay. Complete neutralisation was demonstrated up to an antiserum dilution of 1:16 and in approximately 80% of the antisera with a dilution up to 1:256 [Chin et al., 2007]. Another research group expressed a chimeric tetravalent ED3 protein in *Pichia pastoris* to induce neutralising antibodies in mice. Over 50% inhibition of virus infectivity was demonstrated for all DV serotypes [Etemad et a., 2008]. Although the amino acid sequences (Chin et al.: residues 299-401; Etemad et al.: residues 296-415 for each serotype) of these recombinant proteins were quite similar to the one used in the present study

(residues 296-394); we were not able to compare the neutralisation activity reported in these two studies with that of the present study since different evaluation assays were utilised. The amount of virus applied for the assay was a critical factor in determining the percentage of inhibition of DV infection. Chin et al. [2007] and Etemad et al. [2008] adjusted DV to 500 PFU and 120 PFU, respectively, for their assays, while 10 μg of RSPs instead of DV were used in the present study. For the general neutralisation assay, the concentration of virus was standardised as 100 unit of the reciprocal dilution of virus per unit of volume. This amount of virus should infect 50% of the cells in culture, and is known as $TCID_{50}$ (100 $TCID_{50}$) [Ballew, 2000]. In the present study, no titration of RSPs was prepared since RSPs would not form a plaque or present as a cytopathic effect in cell culture. A large amount of RSPs was necessary for flow cytometry analysis to ensure that a significant fluorescence signal (histogram) was generated by the pre-immunised serum samples and positive control. Thus, the amount of RSPs might be too high to be neutralised to over 50% inhibition by the antiserum.

In spite of the potential of neutralisation of DEN-2 RSPs as demonstrated in our preliminary *in vitro* results using the four antisera, we were not able to make conclusions regarding the neutralisation potential of these antisera since a single animal was immunised instead of multiple animals for each protein fragment. Also, confirmation of neutralisation activities must be further investigated with intact DV. The cross-reactivity of the antisera with all DV serotypes should be studied to explore their potential as vaccine candidates.

4.2.3 Development of a serological diagnostic assay

In the development of a diagnostic tool in the form of a serological assay with the recombinant proteins used as the capture antigen, we discovered a high specificity (>90%) but low sensitivity (<30%) to dengue IgG-positive sera, confirmed by the Panbio Dengue IgG Indirect ELISA kit. Interestingly, prM as the capture antigen was able to detect a relatively higher number of seropositive sera which were confirmed as seropositive by the Panbio Dengue IgG Indirect ELISA assay. Two equivocal plus three known seropositive sera were detected based on the cut-off value calculated from the cut-off calibrator serum, and three sera were detected based on the cut-off value calculated from the local control group. This result might indicate the presence and persistence of anti-prM in the sera of previously DV-exposed individuals. Additionally, it was discovered that the cut-off values calculated based on the absorbance from the Panbio cut-off calibrator serum were similar to the local control group. These findings indicated that the cut-off determined by the Panbio calibrator serum was comparable to that based on the local population. Fine adjustments might be required with an additional negative control group to obtain more accurate cut-off data. Nevertheless, the in-house ELISA assay could be interpreted by means of the Panbio unit.

There was a result discrepancy when ED3 and prM-ED3 were used as the capture antigen which implied that the conformations of the proteins were not similar. However, it is impossible to make conclusions on any interaction between these three proteins, or which protein fragment is more suitable as a capture

antigen for diagnostic tool development, since there were a low number of positive cases in the present study used for assay validation.

For assay validation with acute clinical sera from Brazil, IgG against DV in sera collected during the viremic period was only detected in one sample (one out of 36 sera) by the in-house ELISA assay. However, two-thirds (63.8%) of the dengue acute sera were detected by the Panbio Dengue IgG Indirect ELISA assay. There were thirteen sera not detected by the Panbio ELISA assay which indicated the limitation of the assay for the detection of active dengue infection. The time of sample collection after disease onset was critical for detection. As specified in the instructions of the kit, the ELISA assay kit was not designed to be used as clinical diagnostic tool for dengue, so the results should be interpreted together with related clinical symptoms.

Unexpectedly low sero-sensitivity results were obtained by the in-house ELISA assay, and it was noted that this interpretation of the ELISA results was attributed to the cut-off. Since a high cut-off was determined, a low number of seropositive cases were detected. The high cut-off value might have been due to insufficient purity of the purified protein used as the capture antigen, since interference from impurities would increase the absorbance signal. It was suggested that we should further purify the recombinant protein by the addition of a polishing step, such as gel filtration to achieve higher purity and resolution. Gel filtration is a chromatographic technique which purifies proteins by the size of the molecules. However, it might not suitable to carry out gel filtration in small-scale protein production such as in the present study, since extensive dilution and low

recovery of protein would result. Therefore, a balance between the resolution and recovery of purified protein should be considered since every extra step of protein purification will increase the purity but decrease the yield. In addition, the 6xHis tag and SUMO fusion protein attached to the N-terminus of the recombinant protein might cause a steric effect to prevent IgG from binding. The SUMO fusion protein could be removed by SUMO protease; however, a further polishing step for purifying the recombinant protein from the SUMO protein and SUMO protease by affinity chromatography on a nickel-chelating resin would be necessary. As previously mentioned, every purification step lowers protein yield.

From the results of the ELISA, we discovered that defibrinated plasma from the Hong Kong Red Cross consistently generated a lower absorbance signal from DV IgG-negative serum samples than that of sera collected from the community in Hong Kong (Figure 3.2.13). Additionally, among the eight defibrinated plasma samples which were DV IgG positive, six showed consistently lower absorbance (450nm, reference at 620nm) with the percentage difference from 0.66-2.35%. However, these differences in absorbance would not affect the final interpretation according to Panbio units (Table 3.4.2). This could be attributed to high temperature (RT and 37°C) incubation and serum progressing through repeated freeze-thaw cycles during the defibrination procedures which might degrade immunoglobulins. A similar technique for the conversion of plasma to serum by defibrination was evaluated for several serological tests for syphilis. It was found that >85% of the samples did not show a reduction in antibody titre after defibrination [Castro et al., 2002]. Therefore, more sera are required for accurate validation of our serological assay. In addition, since the samples were

kindly provided by the Hong Kong Red Cross after they finished all screening procedures and laboratory tests following their usual protocols, blood samples might be not have been maintained in proper storage conditions before reaching our laboratory. The quality of sera is a very important factor for correctly detection of antibody.

Regardless of the conclusion as to which recombinant protein could be further developed as a serological assay, our preliminary results indicated that individual recombinant proteins may have different potential as detection antigens for the capture of human anti-dengue IgG for serological assay development, provided that more evaluation steps are performed. Currently, serological assays are commercially available with modifications into a simple format (e.g. Dengue Duo Rapid Strip test [Panbio], Dengue IgG/IgM [Standard Diagnostics]); however, the costs of these serological assays are high (US$ ~10 per test) due the use of whole virus during production. Whole virus utilisation implies many safety issues due to biohazardous materials, which further increase the cost of the assay since special precautions for delivery are necessary. More economical and safer serological tests could be available by the replacement of whole virus with recombinant proteins. In the last decade, the development of serological diagnostic assays for dengue using recombinant proteins has been pursued. Envelope and non-structural proteins (NS1 and NS3) were widely selected as appropriate epitopes for capture antigens [Cuzzubbo et al., 2001; Lemes et al., 2005; AnanadaRao et al., 2005; Dos Santos et al., 2007]. To achieve maximum capture ability for serological detection, multiepitope proteins which are made up of several epitopes from different proteins were synthesised as a polyprotein

connected by linkers [AnanadaRao et al., 2005; Tripathi et al., 2007]. The assay validation with clinical human sera was quite promising, and hopefully this assay will be commercially available in the near future.

4.3 Vector surveillance of *Aedes* mosquitoes collected in Hong Kong by RT-PCR assays

To our knowledge, this pilot study is the first year-round longitudinal study on field-caught mosquitoes for DV screening conducted in Hong Kong. In this pilot study, about one third of the mosquitoes collected belonged to the species *A. albopictus* and higher numbers of this species were found in residential areas. More than half (53.93%) of the mosquitoes were *Culex,* which is not infected by DV. However, *Culex tritaeniorhynchus* and *Culex vishnu*i are vectors of Japanese encephalitis virus which is a member of the *Flavivirus* family. Design of the CDC Wilton trap was not specific for *Aedes* mosquitoes sampling, as described, since multiple mosquito genera were caught, and *Aedes* mosquitoes accounted for only 31.41% of the total field-caught mosquitoes.

The monthly distribution of field-caught *Aedes* mosquitoes in the present study was peak at July 2007 (Figure 3.3.1). This pattern of monthly field-caught *Aedes* mosquitoes was able to correlate with MOI announced by FEHD of the HKSAR, where June 2007 was the peak month. In the present study, shapely decreased in the number of field-caught mosquitoes in August 2007 was related to several thunderstorms during sample collection days and a strong tropical typhoon (Number 1 to Number 8 Gale or Storm Signal) just before the days of mosquito sampling. The number of field-caught mosquitoes including *Aedes* species increased with ambient temperature, since this factor related with distribution, blood feeding activity and adult longevity of mosquito [Kuno, 1997]. However, the positive effect from increasing rainfall was not significant demonstrated in the present study due to raining and thunderstorms during the sampling day restricted

mosquito activity. Besides, the effect of rainfall would not be immediately demonstrated since rainfall indirectly increased mosquito number the by increasing the number of possible breeding sites. Nevertheless, high temperature and high rainfall are well established factors associated with dengue transmission [Kuno, 1997].

Similar mosquito sampling was conducted in December 2004-September 2005 by the Food and Environmental Hygiene Department of the HKSAR government for Japanese Encephalitis virus screening by RT-PCR [Wai et al., 2006, data presented at a conference in Hong Kong]. However, the sampling scale, sites and type of trap for mosquito collection were not specified. All field-caught mosquitoes in the present study were alive prior to storage in -80°C. Live mosquitoes provided the ideal conditions for preserving viral RNA to ensure the accuracy of DV screening. Live mosquito sampling has been emphasised in several studies [Uradaneta et al., 2005; Chung & Pang, 2002; Hung et al., 2001; Chow et al., 1998].

Previous studies revealed that mosquitoes contained a number of contaminants which could inhibit PCR amplification or produce false negative results [Urdaneta et al., 2005; Pankhong et al., 2002; Harris et al., 1998; Chungue et al., 1993]. An extraction method which enabled to eliminate as many contaminants as possible from the mosquito lysate was critical. In the present study, RNA extraction from the mosquito pools was based on a related study for screening of West Nile virus [Lanciotti et al., 2000]. To prepare cell-free lysate by further removal of the cell debris, the mosquito lysate was suspended in RLT

buffer and homogenised with a QIAshredder [Qiagen]. QIAshredders are a convenient tool for homogenization instead of the syringe and needle technique, and is also a sterile and single-use apparatus to help eliminate sample cross-contamination. Complete homogenisation of the mosquito lysate ensured efficient binding of RNA to the membrane of the spin column in the RNA extraction kit. Due to an unknown infection rate and viral load of *Aedes* mosquitoes collected in Hong Kong, sampling preparation was a critical factor to assure the integrity of DV detection. Apart from this commercial RNA extraction kit, a silica-based technique has also been widely used. However, comparison of these two methods was not available. For the method used in present study, BA-1 diluent for suspending the mosquito lysate allowed for immediate inoculation into cell culture for DV isolation if necessary.

Since the presence of a non-specific product consistently appeared in all the mosquito pools amplified by our in-house real-time RT-PCR assay, discrimination between serotypes relied on the melting peak signal rather than the Ct values. The DEN-1 and -3 serotypes were readily discriminated by the specific peak signal detected from the F2 and F3 channels. However, we observed that a weak melting peak signal in the F1 channel from five mosquito pools showed a T_m similar to the specific T_m of serotypes DEN-2 and -4 (Table 3.3.2). This problem led to difficulties in result interpretation. Those suspected cases should have been confirmed by sequence analysis. However, absence of a visible specific gel band following agarose gel electrophoresis indicated that the samples did not yield a detectable quantity of DNA for sequence analysis. It was believed that the fluorescence peak signal was due to the presence of a consistent non-specific

product from mosquito pool, because 1) the fluorescence signal came solely from DEN-2 RNA (Figure 3.3.5A) and 2) intensity of the non-specific product, based on the gel image, was less significant when the mosquito pool spiked was with DEN-2 RNA. These findings implied that the RNA concentration of DV in the mosquito sample was an important factor for DV detection and result interpretation by the in-house RT-PCR assay. The detection limit of DV in the presence of mosquito lysate provided information on the sensitivity of the assay.

Non-specific amplification was not only observed in our in-house assay, but also in the conventional nested PCR assay which acted as a characterised assay for comparison. This conventional nested PCR assay has been applied for mosquito surveillance in endemic countries [Urdaneta et al., 2005; Tuksinvaracharn et al., 2004]. We believed the non-specific amplification was probably due to mosquito RNA since all exacted samples was subjected to DNase digestion prior to the RT-PCR assay. All non-specific amplifications with a similar product size to either serotype were recognised as suspected positive cases and subjected to sequence analysis. Those suspected cases were finally eliminated by the sequencing results.

In summary, DV was not detected in any of the *Ae. albopictus* screened by the two RT-PCR assays. We described the evaluation of RNA recovery after mosquito extraction and the issue of PCR inhibitors. RNA recovery was lower by one log unit after mosquito extraction and treatment with PCR inhibitors in one of the monthly mosquito pools. Similar Ct value between mosquito pools (Figure 3.3.5A) indicated heat-labile inhibitor was absent. Presence of heat-labile inhibitor

rendered inhibitory effect on the early phase of PCR but inhibitory reduced with increasing thermal cycles, so observed Ct value would delay [Kontanis & Reed, 2006]. In spite of non-specific product amplification in the dengue-free mosquito pool, in the presence of DV RNA, the effect of the non-specific product would be masked by the significant fluorescence signal contributed by the product of DV RNA. The signal generated from SGI due to the amplified non-specific product would only lead to earlier Ct detection compared to pure DV RNA samples. Therefore, the in-house real time RT-PCR assay provided a rapid and cost-effective tool which might be used as an alternative method for routine epidemiological surveillance of naturally infected dengue vectors.

There was no mosquito pool in which DV RNA was detected; however, due to the small sample size in this pilot study, we cannot eliminate the possibility of silent dengue transmission among *Ae. albopictus*. The small sample size cannot represent the distribution of mosquitoes in Hong Kong, since there were limitations of mosquito sampling. We were denied permission to sample in some proposed sites which were found to have a high ovitrap index. Furthermore, activities in a few parks such as regular spraying with mosquito pesticides prior to our sampling and restricted locations for placing traps. On some sites, construction work disturbed our sampling. Epidemiological studies on mosquitoes have been carried out in Macao, which is located close to Hong Kong [Almeida et al., 2005]. In contrast to the present study, that study included a large sample size (10,909 mosquitoes) and comprehensive assessments of bioecology (daily biting rate, daily survival rate and parity rate) and the vectorial capacity of *Ae. albopictus*. The distribution of mosquito species in Macao is quite similar to that of Hong Kong,

such that *Ae. albopictus* (77%) is also the only DV vector abundant in Macao [Almeida et al., 2005]. Despite DV not being detected in a Vero cell culture in the Macao study, the outbreak in 2001 had already shown that naturally infected *Ae. albopictus* took part in DV transmission.. Similar outbreaks may occur in Hong Kong if an effective prediction system is not carried out. Therefore, a large-scale study including adult mosquito sampling and an alternative approach of larval sampling using ovitrap with the assistance of the related government department is warranted to obtain a clear picture on the epidemiology of dengue virus vectors in Hong Kong.

4.4 Seroepidemiological study of dengue in the general population of Hong Kong with a commercial ELISA assay

This is the first survey on the seroprevalence of dengue infection conducted in Hong Kong. The overall seroprevalence of dengue infection in the present study was 1.6%, which was much lower than that reported in nearby Asian countries (9.2%-89.0%) [Chen et al., 1997; Singh et al., 2000; Ooi et al., 2001; Teixeira et al., 2002; Wilder-Smith et al.,2004]. The demographic characteristics (Table 3.4.1) of the subjects recruited in the present study were comparable to those of the population of Hong Kong. Therefore, the seroprevalence of dengue in the present study is representative of the population of Hong Kong.

For the indirect IgG ELISA kit utilised in present study, the serological sensitivity (99.2%) and specificity (96.2%) of the kit was highly satisfactory as reported in a previous study [McBride et al., 1998]. It was noted that we obtained comparable ELISA results on serum and defibrinated plasma (a serum analogue) samples based on the acceptable degree of variability (<4%) (Table 3.4.2). A few exceptions were found, including three samples which revealed a larger CV (9.4-11.1%) in Panbio units between serum and defibrinated plasma (Figure 3.4.2). This was likely not related to batch-to-batch variations in the kit since six samples (194-221) were tested together with the same batch of kit reagents. Nevertheless, this difference in Panbio units between serum and defibrinated plasma did not affect the final interpretation. All serum samples recognised as ELISA-positive showed the same interpretation in the corresponding defibrinated plasma samples. Despite the comparable ELISA results on both types of sample, we are not able to identify the serological sensitivity and specificity of the ELISA kit on defibrinated

plasma, since the seropositive sera collected in this study had not been confirmed. A confirmed dengue case should be diagnosed by clinical symptoms together with virus isolation or a four-fold increase in antibody titre in paired serum specimens. Therefore, we were not able to make a conclusion that the ELISA kit was applicable to plasma specimens. The manufacturer of the ELISA kit suggested that it could be applied to plasma samples, but the performance had not been validated by their laboratory [Yorston, 2008].

In the present study, we found that individuals who were not born and did not grow up locally in Hong Kong had a statistically significantly increased risk for dengue seroprevalence. Individuals who came from China or other places were associated with seropositivity. For individuals from other places, except three (30%) who came from the UK and Canada, others came from dengue-endemic areas including Macau, Indonesia and Venezuela. These findings indicated that the original home town was critical for the interpretation of dengue seroprevalence among Hong Kong residents. Introduction of bias might result if the origin of each individual was not well defined. Due to the close relationship between Hong Kong, Macau and China, many residents in Hong Kong moved among the three areas. It was believed that dengue in Macau and China was quite different from that in Hong Kong, although most people were Chinese. Local outbreaks have been recorded in China and Macau and the presence of the *Ae. aegypti* vector has been shown in several provinces in China. However, dengue cases reported in Hong Kong were all imported incidents from nearby endemic countries.

Travelling is a common activity of Hong Kong citizens. According to data from the Travel Industry Council of Hong Kong, more than 70 million outbound trips, which is the equivalent to about 10 journeys per person were reported in 2005 [Ho, 2006]. In the present study, individuals who travelled within the past twelve months (*P*=0.29, OR 2.96 CI 0.36-24.39) and with an increased number of countries visited (*P*=0.15, OR 2.79 CI 0.65-11.98) showed increased risk of seropositivity, but this did not achieve significance. The statistically insignificant association might be due to the small number of positive cases. We understood that the introduction of recall basis and incomplete travel history (one year only) could not directly relate to seropositivity. However, this information gave hints of the travel habits and preferences of individuals in Hong Kong. This is useful information which can be used to correlate travel habits and the seroprevalence of dengue in Hong Kong. Additionally, another purpose of including recent travel history in the questionnaire survey was to determine whether the subject had recently become infected with DV, if their serum sample resulted in a positive ELISA result.

Although a statistically significant association of travel within the past twelve months and number of countries visited with seroprevalence was not achieved, we discovered that individuals who had visited some places in Eastern Asia (China, Macau) or Southeast Asia (Indonesia) during the last year were more likely to have past exposure to dengue fever. Those places which are dengue endemic are the most popular travel venues for Hong Kong citizens. These findings reflected that individuals who have a travel preference to those Asia counties were at a higher risk for dengue infection. Currently, notification of

dengue cases has been found to be steadily increasing in travellers who had returned from a short visit in a dengue endemic area [Cobelens et al., 2002]. It is believed that dengue is expanding worldwide to non-endemic countries due to the incidence of travel-associated infections. In Hong Kong, all confirmed dengue cases were imported from nearby endemic countries; however, an increasing trend of dengue incidence since 2000 enhances the threat of dengue. The mosquito *Ae. albopictu*s, which is abundant in Kong Kong, can be a potential vector for local outbreaks. Therefore, the impact of travel-associated dengue cases should not be underestimated.

In multivariate analysis, only one potential risk factor for individuals who visited Southeast Asia (Indonesia) showed a statistically significant association with seropositivity. The other two potential risk factors were not correlated to each other and considered to be insignificant due to a null value (1.0) included within the 95% CI and a corresponding *P* value greater than 0.05 [Portney & Watkins, 2000].

In spite of a few potential factors which were shown to be statistically significant in association with seroprevalence, we noted a wide range of the 95% CI for these factors (Table 3.4.3). This indicated that there was an insufficient sample size to correlate risk factors for a significant association. Therefore, a larger sample size is essential for the precise estimation of seroprevalence and the identification of potential risk factors.

Since cross-reactivity of antibodies against JE and other *Flavivirus* lead to a false positive result, we included the medical history related to dengue such as 1) past vaccination against yellow fever and JE, 2) past dengue infection and 3) febrile symptoms after recent travel, to eliminate false positive cases. However, dengue is an infection which is usually under-diagnosed, and the information provided by subjects might not completely reflect the real situation. We encountered a discrepancy between a positive ELISA result and past dengue infection history of an individual. Subsequently, the past dengue diagnosis of the individual was traced back for confirmation, and it was discovered that the flu-like clinical symptoms of that subject had not been confirmed as a dengue case by any physician or laboratory test.

In summary, the seroprevalence of dengue among individuals assessed in present study was 1.6%. The sample size represented 0.01% of the total population of Hong Kong. Individuals who were not born and raised in Hong Kong and came from China or Indonesia had a statistically significant association with dengue seroprevalence. Individuals who had travelled multiple countries in Asia were more likely to have a higher risk of previous exposure to dengue. However, an insufficient sample size restricted more appropriate conclusions on the strength of association and estimation of risk factors.

4.5 Conclusion

In this study, two major scopes of research were investigated, including the molecular and epidemiological aspects of dengue infection in Hong Kong. The molecular aspect was sub-divided into two parts: the development diagnostic assays for DV detection and investigations into the neutralisation activities of polyclonal antisera elicited from rabbit immunisation by the three *E. coli*-expressed recombinant dengue proteins. Seroepidemiology for previous DV exposure among the population of Hong Kong and vector surveillance for DV were the epidemiological aspects of dengue infection investigated in Hong Kong.

In summary, a molecular diagnostic assay for DV has been developed. Also, a serological ELISA assay has been set up and preliminarily validated with more than one hundred human serum or plasma samples. The serological ELISA assay must be further modified and verified by additional clinical and control group samples. For the molecular assay, a one-step RT-PCR LightCycler assay (both in kit-based and in-house PCR reagents) has been developed for rapid and simultaneous detection and typing of DV. It has utilised both T_m and colour multiplexing to distinguish all four serotypes of DV. The cost of the assay established in the present study is comparatively cheaper than the TaqMan assay. The cost of the assay has been further reduced by the development of an in-house assay with the use of commercially available PCR reagents to replace the one-step RT-PCR kit (kit-based). The in-house assay was finally reduced to only one-third of the kit-based assay cost with the same PCR profile. Performance of the in-house assay was validated with clinical samples collected from Hong Kong, Mainland

China and Brazil. This assay has a high potential for DV diagnosis including routine screening of clinical samples and vector surveillance.

Three recombinant fusion proteins prM, ED3 and prM-ED3 of DEN-2 were expressed in *E. coli* for the development of a serological assay and investigation of their potential as subunit vaccine candidates. For the serological assay, an indirect ELISA assay format with three individual recombinant proteins of DEN-2 as capture antigen has been set up. This assay was evaluated with sera collected from the community in Hong Kong. Another batch of sera collected from Brazil during the viremic period of the acute phase of dengue fever was also used for assay validation. High sensitivity was not achieved due to the limited number of clinical sera available and insufficient purity of the recombinant proteins for IgG detection due to fusion with a 6xHis-tag and SUMO protein. However, high specificity (all >90%) of the assay was recorded. Preliminary results indicated that the individual recombinant proteins have different potential as capture antigens for serological assay development, but modifications to the purification of recombinant proteins and more extensive evaluation steps must be carried out. It is hopeful that one fragment can be developed as serological assay to replace the expensive and biohazardous serological tests that are now commercially available.

For investigation of their potential as vaccine candidates, purified recombinant proteins were administrated into rabbits for polyclonal antibody immunisation. Antisera were analysed and characterised by immunoblot and ELISA. The neutralisation potential of antisera were investigated. Individual antisera showed varied ability to inhibit DEN-2 RSP binding to Vero E6 cells; and

antiserum against prM-ED3 chimeric protein showed the strongest inhibition of RSP binding among the four tested.

Two parts of the epidemiology studies were also investigated in the present study. For dengue vector surveillance, DV was not detected in the collected mosquitoes. About one-third of the mosquitoes collected belonged to *Ae. albopictus* and were found in relatively higher numbers in the residential areas of urban districts in Hong Kong. However, due to a small sample size in the pilot study, this could not eliminate the possibility of silent dengue transmission among *Ae. albopictus*. A larger scale study is warranted for a clearer epidemiological picture of dengue virus vectors in Hong Kong.

The overall prevalence of DV was 1.61%, which was much lower than that reported by nearby Asian countries. Seroprevalence for the university group was 2.7-fold higher than that of the Red Cross group. Seropositivity was significantly associated with increased risk for subjects who were not born and did not grow up locally in Hong Kong. Individuals with an active travel history and who had visited some areas of Eastern Asia and Southeast Asia (e.g. Indonesia) in the past twelve months were more likely to be dengue seropositive. However, the limited sample size restricted more appropriate conclusions on the estimation of risk factors the strength of association between these risk factors and seroprevalence among the population in Hong Kong.

There were limitations during the study which impeded a more accurate conclusion on some findings. Restriction on locations approved by FEHD for

mosquito sampling affected dengue vector surveillance in Hong Kong. Besides, limited number and prolonged storage of clinical sera of positive dengue cases available in Hong Kong restricted a comprehensive validation of the RT-PCR assays. For study of the neutralizing ability of the antisera, illness of rabbits and lack of availability of a biosafety level-3 laboratory prohibited us from conducting of neutralisation assay against intact DV. It is believed more insight can be addressed if captioned limitations can be overcome

4.6 Future studies

Since there is no vaccine or drug directly targeting DV infection, prevention and control of the morbidity and mortality associated with dengue is an effective strategy to prohibit spread and outbreaks of dengue globally. In the present study, rapid RT-PCR diagnostic assays were developed, which had applications to enable rapid confirmation of dengue case, to mediate prognosis of the disease as well as to deal with the emergence of spreading. The neutralising potential of antisera induced from rabbits by three recombinant protein fragments was demonstrated. An in-house ELISA which utilised the three recombinant proteins as capture antigens was set up. Furthermore, a pilot study of seroepidemiology and vector surveillance for DV demonstrated preliminary figures in Hong Kong. However, the present study should continue since modifications for each part of the study can be made for further investigation.

4.6.1 Molecular diagnostic tools and subunit vaccine candidate development for DV detection

Two molecular PCR assays based on kit-based and self-developed with in-house PCR reagents were developed and validated. However, more clinical samples from different geographic regions for assay validation will be an advantage to generate a more accurate diagnosis.

For serological assay development, it has been suggested that we perform further validation of the self-developed ELISA based on three proteins as capture antigens. We are continuing with clinical sample collection from dengue endemic areas (clinical sera from India are in progress) for assay evaluation purposes. The

cut-off value of absorbance to define sero-negativity and –positivity require more clinical and normal samples to be worked out. Further purification of the recombinant proteins is recommended. For investigation of the neutralising potential of the polyclonal antisera induced from rabbits by three recombinant proteins, it is necessary to carry out the neutralisation assay against intact native dengue virus and other flavivirus to investigate the actual neutralisation potential and the cross-reactivity potential among other DV serotypes. The information arising from advanced study of neutralisation assay may assist in understanding the pathogenesis of dengue infection and thus the aid in the development of vaccines for prevention or treatment.

4.6.2 Epidemiology of dengue infections in Hong Kong

In this study, vector surveillance was a pilot study carried out in Hong Kong. The number of mosquitoes was far from representative of the real situation. A larger scale study is necessary to generate a clearer epidemiological picture of dengue virus vectors in Hong Kong for the prediction of silent transmission of DV. Additionally, a large sample size collected from the general population in Hong Kong is required to generate a more appropriate seroprevalence rate and association between seropositivity for dengue and its related risk factors among the population in Hong Kong. It is believed that the epidemiological data of dengue in Hong Kong enables an in-depth understanding of the transmission dynamics and predictions for the risk of local outbreaks in Hong Kong.

Appendix

Appendix I: Information of clinical serum samples

Information of clinical serum samples from Brazil

City	Sample ID	PCR result[a]	Viral load (copy number/L)[b]	Serotype
Aracaju (n=8)	10	Positive	Not available	2
	24	Positive	Not available	2
	29	Negative	Not available	2
	33	Negative	Not available	3
	45	Negative	Not available	3
	49	Positive	Not available	2
	52	Negative	Not available	3
	53	Negative	Not available	3
Goiânia (n=28)	121	Positive	2.67×10^{10}	3
	149	Positive	8.60×10^{9}	3
	228	Positive	3.74×10^{10}	3
	290	Positive	4.41×10^{10}	3
	344	Positive	1.63×10^{11}	3
	378	Positive	3.21×10^{10}	3
	396	Negative	3.82×10^{7}	3
	423	Positive	3.47×10^{8}	3
	425	Positive	1.89×10^{10}	3
	427	Positive	1.17×10^{10}	3
	429	Negative	4.28×10^{8}	3
	431	Positive	1.35×10^{9}	3
	435	Positive	2.66×10^{9}	3
	436	Positive	1.60×10^{10}	3
	442	Positive	9.74×10^{9}	3
	443	Positive	1.48×10^{10}	3
	445	Positive	1.44×10^{9}	3
	446	Positive	5.53×10^{9}	3
	449	Positive	3.70×10^{10}	3
	842	Negative	6.75×10^{8}	3
	846	Negative	1.17×10^{8}	3
	847	Positive	2.54×10^{9}	3
	855	Positive	7.39×10^{9}	3
	856	Positive	1.30×10^{13}	3
	857	Positive	1.39×10^{10}	3
	858	Positive	1.29×10^{10}	3
	861	Positive	1.08×10^{12}	3
	866	Negative	1.04×10^{8}	3

[a] PCR results according to the in-house RT-PCR LightCycler assay developed in the present study.

[b] Viral load was confirmed by Dengue LC RealArt^{TM} RT-PCR Kit (Artus, Hamburg, Germany) in the Institute of Tropical Medicine, University of São Paulo of Brazil.

Information of clinical serum samples from Guangzhou, China

Sample No.[a] (n=88)	PCR result[b]	Serological assay for dengue[c]	Results of serological assay			
			First serum sample		Second serum sample	
			IgM	IgG	IgM	IgG
3621	Neg	IC	Positive	Positive	/	/
3627	Neg	IC	Negative	Weak positive	Negative	Positive
3628	Neg	IC	Negative	Negative	Negative	Negative
3632	Neg	IC	Weak positive	Negative	/	/
3637	Neg	IC	Negative	Negative	Positive	Positive
3638	Neg	IC	Positive	Weak positive	/	/
3639	Neg	IC	Positive	Weak positive	/	/
3647	Neg	IC	Positive	Positive	/	/
3653	Neg	IC	Negative	Negative	/	/
3656	Neg	IC	Negative	Negative	Negative	Negative
3658	Neg	IC	Positive	Negative	/	/
3664	Neg	IC	Positive	Negative	/	/
3670	Neg	IC	Positive	Positive	/	/
3674	Neg	IC	Weak positive	Positive	/	/
3681	Neg	IC	Positive	Negative	Positive	Positive
3682	Neg	IC	Positive	Positive	/	/
3686	Neg	IC	Negative	Negative	/	/
3689	Neg	IC	Negative	Positive	/	/
3693	Neg	IC	Negative	Positive	/	/
3694	Neg	IC	Positive	/	/	/
3695	Neg	IC	Positive	Negative	/	/
3699	Neg	IC	Positive	Negative	/	/
3701	Neg	IC	Weak positive	/	Positive	Positive
3705	Neg	IC	Positive	/	Positive	/
3708	Pos	IC	Negative	Negative	Positive	Weak positive
3720	Neg	IC	Positive	Negative	/	/
3724	Pos	IC	Negative	Negative	Positive	Positive
3727	Pos	IC	Negative	Negative	Positive	Negative
3732	Neg	IC	Negative	Negative	Positive	Negative
3736	Pos	IC	Positive	Negative	/	/
3741	Pos	ELISA	Positive	Negative	Positive	Negative
3742	Neg	IC	Positive	Weak positive	/	/
3744	Neg	IC	Positive	Negative	/	/
3748	Neg	IC	Positive	Positive	/	/
3774	Neg	IC	Positive	Positive	Positive	Positive

[a] The second serum of the six samples, 3637, 3708, 3732, 3809, 3983 and 4201, which indicated by underline was used for assay validation. In contrast, the first serum of the others was used for assay validation.
[b] PCR results according to the in-house RT-PCR LightCycler assay developed in the present study. Pos, PCR positive; Neg, PCR negative.
[c]Serological assays for dengue was performed in Guangzhou No.8 People's Hospital, China. IC, Immunochromatogrphy assay.

Sample No.[a] (n=88)	PCR results[b]	Serological assay for dengue[c]	Results of serological assay			
			First serum sample		Second serum sample	
			IgM	IgG	IgM	IgG
3790	Neg	IC	Positive	Negative	Positive	Negative
<u>3809</u>	Neg	IC	Negative	Negative	Positive	Negative
3878	Neg	IC	Positive	Positive	/	/
3904	Pos	IC	Negative	Negative	Positive	Negative
3949	Neg	IC	Positive	Negative	/	/
3959	Neg	IC	Weak positive	Negative	/	/
<u>3983</u>	Pos	IC	Negative	Negative	Positive	Negative
3985	Neg	ELISA	Negative	Negative	/	/
3987	Neg	IC	Positive	Negative	/	/
4007	Neg	ELISA	Positive	Negative	/	/
4034	Neg	ELISA	Positive	Negative	/	/
4071	Neg	ELISA	Positive	Positive	/	/
4072	Pos	ELISA	Negative	Negative	Positive	Negative
4106	Neg	ELISA	Positive	Negative	/	/
4124	Neg	ELISA	Positive	Positive	/	/
4135	Neg	IC	Positive	Positive	/	/
4144	Neg	IC	Negative	Negative	Negative	Negative
4146	Neg	ELISA	Negative	Negative	Negative	Negative
4162	Pos	IC	Negative	Negative	Positive	Negative
4164	Neg	IC	Negative	Negative	Weak positive	Negative
4189	Pos	IC	Negative	Negative	Positive	Positive
4190	Neg	IC	Positive	Positive	/	/
<u>4201</u>	Neg	ELISA	Negative	Negative	Positive	Weak
4255	Pos	IC	Weak positive	Negative	Positive	Weak
4273	Neg	IC	Weak positive	Negative	Weak positive	Negative
4281	Neg	IC	Negative	Negative	Negative	Negative
4307	Neg	IC	Negative	Negative	Negative	Negative
4308	Neg	IC	Negative	Negative	Negative	Negative
4336	Neg	IC	Positive	Negative	/	/
4369	Pos	IC	Negative	Negative	Weak positive	Negative
4375	Pos	IC	Negative	Negative	Positive	Negative
4387	Neg	IC	Positive	Negative	/	/
4430	Pos	IC	Weak positive	Negative	/	/
4432	Neg	IC	Positive	Positive	/	/
4461	Neg	IC	Weak positive	Negative	/	/
4463	Pos	IC	Negative	Negative	Positive	Positive
4481	Neg	IC	Positive	Negative	/	/
4506	Pos	IC	Negative	Positive	/	/
4507	Pos	IC	Negative	Negative	Positive	Positive
4571	Pos	ELISA	Positive	Negative	/	/

Table Continued

Sample No.[a] (n=88)	PCR results[b]	Serological assay for dengue[c]	Result of serological assay			
			First serum sample		Second serum sample	
			IgM	IgG	IgM	IgG
4572	Neg	ELISA	Positive	Positive	/	/
4590	Pos	IC	Negative	Negative	Positive	Negative
4620	Neg	IC	Positive	Negative	/	/
4602	Neg	IC	Positive	Negative	/	/
4603	Neg	IC	Positive	Positive	/	/
4605	Neg	IC	Positive	Negative	Positive	Negative
4665	Neg	IC	Positive	Positive	/	/
4668	Neg	IC	Positive	Positive	/	/
4669	Pos	IC	Weak positive	Negative	Positive	Positive
4679	Neg	IC	Weak positive	Negative	Negative	Negative
4681	Neg	IC	Positive	Positive	/	/
4683	Neg	IC	Positive	Negative	/	/
4685	Neg	IC	Weak positive	Negative	/	/

Table Continued

Appendix II: Formula of buffers and reagents

Lysis Buffer

1M KH_2PO_4	0.6 mL
1M K_2HPO_4	9.4 mL
NaCl	4.6g
KCl	1.5g
Glycerol	20 mL
Triton X-100	1 mL
Imidazole	10 mM

Dissolve in 180 mL RO water, mix thoroughly and adjust pH to 7.8
Bring the volume to 200 mL.
Store at 4°C

25% APS (Sigma A3678-100G)

APS	2.5 g

Dissolve in 10 mL
Store at -20°C in 1 mL aliquot

30% acrylamide /0.8% bis-acrylamide solution (37.5:1)

acrylamide	60 g
bis-acrylamide	1.6 g

Dissolve in 100 mL RO water
Bring the volume to 200 mL
Store at 4°C in dark bottle

Stacking gel buffer (4X Tris-HCL/SDS, pH 6.8)

Tris	12.1 g
10% SDS	8 mL

Dissolve in 80 mL RO water
Mix thoroughly and adjust pH to 6.8 with 1N HCl
Bring the volume to 200 mL
Store at 4°C

Separating gel buffer (4X Tris-HCL/SDS, pH 8.8)

Tris	36.34 g
10% SDS	8 mL

Dissolve in 100 mL RO water
Mix thoroughly and adjust pH to 8.8 with 1N HCl
Bring the volume to 200 mL
Store at 4°C

12% SDS-PAGE gel (volume for preparing 4 gels)

Separating gel

30% acrylamide /0.8% bis-acrylamide	5600 µL
Separating gel buffer	3500 µL
25% APS	140 µL
TEMED	14 µL
MilliQ water	4746 µL

Stacking gel

30% acrylamide /0.8% bis-acrylamide	1998 µL
Separating gel buffer	3000 µL
25% APS	120 µL
TEMED	12 µL
MilliQ water	6870 µL

10X Electrophoresis buffer

Tris	30.2 g
Glycine	144 g
SDS	10 g

Dissolve in 1L RO water
Store at 4°C
Dilute into 1X for use

Sample loading buffer (6xSDS)

10%SDS	3 mL
Glycerol	3.6 mL
β-Mercaptoethanol	0.5 mL
4X staking gel buffer (pH 6.8)	1.25 mL
Bromophenol blue	1 mg

Dissolve in 10 mL
Store at -20°C in 1 mL aliquot

Methanol: acetic acid solution (De-stain solution)

Methanol	500 mL
RO water	400 mL
100% acetic acid	100 mL

Mix thoroughly

Store at RT

Coomassie Brilliant Blue stain

Coomassie Brilliant Blue (R250) 0.25 g

Dissolve in 100 mL methanol: acetic acid solution. Mix thoroughly.

Filter the solution through a Whatman No. 1 filter

Store at RT

10X Transfer buffer

Glycine	144 g
Tris	30 g
SDS	3.75 g

Dissolve in 1L RO. Adjust to pH 8.3

Store at 4°C

Add 200 mL Methanol freshly to1X buffer

Bring the volume to 1L.

Appendix III: Information sheet and consent form for seroprevalence study
English version

INFORMATION SHEET

SEROPREVALENCE OF DENGUE IN POPULATION OF HONG KONG

You are invited to participate in a study conducted by Dr Polly Leung and Miss Constance Lo who are Assistant Professor and postgraduate student, respectively, of the Department of Health Technology and Informatics of The Hong Kong Polytechnic University.

Dengue fever is one of the most important mosquito-borne infections associated with dengue virus and prevalent in Southeast Asia. Since vaccination and specific treatment for dengue infection are still unavailable, epidemiological information of dengue is essential for planning of preventive measures. However, such information is lacking in Hong Kong. This study aims to conduct a seroepidemiological survey which determines the dengue antibody prevalence in the population of Hong Kong. This information will reveal the extent of previous exposure to dengue among the population of Hong Kong.

The study will involve collection of a blood sample and completion of a questionnaire, which will take about 30 minutes. The whole procedure will be performed in the University during office hours or in the delegated private laboratory during the business hours of the laboratory. Ten milliliter of blood sample will be drawn by venipuncture by an experienced phlebotomist. Individual blood sample will be tested for antibodies against dengue viruses by commercial reagent set. Mean while, part of the blood samples will be subjected for detection of host genetic marker associated with dengue. It is hoped that this information will help to obtain information of dengue antibody prevalence for planning of preventive measures of dengue infection.

You will experience a mild pain during the procedure of blood sample collection, but it should not result in any undue discomfort. All information related to you will remain confidential, and will be identifiable by codes known only to the researcher. You have every right to withdraw from the study before or during the measurement without penalty of any kind.

If you have any complaints about the conduct of this research study, please do not hesitate to contact Mr Eric Chan, Secretary of the Human Subjects Ethics Sub-Committee of The Hong Kong Polytechnic University in person or in writing (c/o Human Resources Office of the University). If you have any further questions about the study, please do not hesitate to contact Dr. Polly Leung at 3400 8570 or Miss. Constance Lo at 3400 8600.

Thank you for your interest in participating in this study.

Dr Polly Leung & Miss Constance Lo
Chief Supervisor & Investigator

Department of Health Technology and Informatics
The Hong Kong Polytechnic University

CONSENT TO PARTICIPATE IN RESEARCH

SEROPREVALENCE OF DENGUE IN POPULATION OF HONG KONG

I ____ hereby consent to participate in the captioned research conducted by Miss. Lo Lek Hang Constance and Dr. Polly Leung

I understand that information obtained from this research may be used in future research and published. However, my right to privacy will be retained, i.e. my personal details will not be revealed.

The procedure as set out in the attached information sheet has been fully explained. I understand the benefit and risks involved. My participation in the project is voluntary.

I acknowledge that I have the right to question any part of the procedure and can withdraw at any time without penalty of any kind.

Name of participant ____

Signature of participant ____

Name of researcher Lo Lek Hang Constance

Signature of researcher ____

Date ____

Chinese Version

THE HONG KONG
POLYTECHNIC UNIVERSITY
香港理工大學

醫療科技及資訊學系
Department of Health Technology and Informatics

<u>有關資料</u>

香港一般民眾登革熱抗體盛行率研究

誠邀　閣下參加由梁杏媚博士及盧歷衡小姐負責執行的研究計劃。她們分別是香港理工大學醫療科技及資訊學系的助理教授及研究生。

登革熱是其中一種最重要的蚊子媒介傳染病,由登革熱病毒引起及盛行於東南亞地區。監於登革熱目前仍然沒有疫苗及針對性治療,因此血清流行病學所得的數據對計劃登革熱應急預案是非常重要的,可是香港目前缺乏這方面的資料。這項研究的目的是進行一項血清流行病學調查,研究香港一般民眾登革熱抗體盛行率。這些資料有助顯示香港一般民眾曾經受登革熱感染的情況。

這項研究包括一個血液樣本蒐集和一份問卷調查,整個過程需要花費　閣下三十分鐘,並於辦公時間內在理工大學進行或於營業時間內在委託的化驗所進行。　閣下需要提供十毫升血液樣本,抽血過程會由經驗的抽血員負責。每個血液樣本隨後會進行獨立的登革熱病毒抗體測試。希望從這項研究得到的資料有助得知香港一般民眾登革熱抗體盛行率,作為計劃登革熱應急預案的指引。

閣下在血液樣本蒐集過程中會感覺到輕微痛楚,,但不會引起長期不適的感覺。凡有關　閣下的資料均會保密,一切資料的編碼只有研究人員知道。閣下享有充分的權利在研究開始之前或之後決定退出這項研究,　而不會受到任何對　閣下不正常的代遇或責任追究。

如果　閣下有任何對這項研究的不滿,請隨時與香港理工大學<u>人事倫理委員會</u>秘書親自或寫信聯絡(地址:香港理工大學人力資源辦公室 AG426 室轉交)。如果　閣下想獲得更多有關這項研究的資料,請與梁杏媚博士(電話 3400 8570)或盧歷衡小姐(電話 3400 8600)聯系。

謝謝閣下有興趣參與這項研究。

研究員
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Department of Health Technology and Informatics

參與研究同意書

香港一般民眾登革熱抗體盛行率研究

本人 ______________________________ 同意參加由梁杏媚博士及盧歷衡小姐負責執行的研究項目。

我理解此研究所獲得的資料可用於未來的研究和學術交流。然而我有權保護自己的隱私，我的個人資料將不能洩漏。

我對所附資料的有關步驟已經得到充分的解釋。我理解可能會出現的風險。我是自願參與這項研究。

我理解我有權在研究過程中提出問題, 並在任何時候決定退出研究而不會受到任何不正常的待遇或責任追究 。

參加者姓名 ____.

參加者簽名 ____.

研究人員姓名 ____________ Lo Lek Hang Constance ____________

研究人員簽字 ____

日期 ____.

Questionnaire survey for seroprevalence study
English version

SEROPREVALENCE OF DENGUE IN POPULATION OF HONG KONG

Questionnaire

No.________________________

Date:_______________________

* Please circle appropriate item

1. Gender: M / F *
2. Age:______________________________
3. Residential District :___________________
4. Ethnicity: Chinese / Others *, please specify: __________________________
5. Are you Hong Kong Resident? Yes / No *

   _________ year(s) been living in Hong Kong
6. Where did you come from?

   Hong Kong / Mainland China / Others*, pls specify____________________
7. Have you been infected with Dengue or Japanese Encephalitis? Yes / No *
8. Did you have Japanese Encephalitis or Yellow Fever vaccination? Yes / No *
9. Did you travel in the past 12 months? Yes / No *

   Please specify the destination:______________________________________

   Did you have fever within 14 days after travel?Yes / No / Cannot Remember *

Chinese version

香 港 一 般 民 眾 登 革 熱 抗 體 盛 行 率 研 究

問卷

編碼:__________________________

日期:__________________________

*請選擇適用者

1. 性別: 男 / 女*
2. 年齡:__________________________
3. 本港居住地區 (e.g. 沙田):________________________
4. 種族: 華人 / 其他* 請註明: ________________________
5. 請問閣下是否香港居民? 是 / 不是*

   居港年期: ________年
6. 請問閣下來自何地? 香港 / 中國大陸 / 其他* 請註明: ______________
7. 請問閣下有沒有曾經感染登革熱或日本腦炎? 有 / 沒有 *
8. 請問閣下有沒有曾經接受日本腦炎或黃熱病疫苗? 有 / 沒有*
9. 請問閣下在過去十二個月內有沒有外遊? ? 有 / 沒有*

   請註明目的地: ____

   回港後十四日內有沒有出現發燒徵狀? 有 / 沒有 / 忘記了 *

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