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DEVELOPMENT OF PSEUDOTYPED VIRUS ASSAYS FOR THE SEROLOGICAL STUDY OF JAPANESE ENCEPHALITIS FLAVIVIRUS

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Submitted in accordance with the requirements for the degree of
Doctor of Philosophy

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Declaration of Originality

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Abstract

Japanese encephalitis virus (JEV) is one of the primary global causes of viral encephalitis, with approximately 68,000 clinical cases and 20,000 deaths attributed to the virus annually.

Between 30% and 50% of survivors suffer from debilitating neurological sequelae. Despite being a vaccine-preventable disease, no antiviral treatments are licensed and commercially available to counteract JEV infection. In order to quantify the neutralising antibody response raised against antigenic epitopes on flavivirus prME glycoproteins, conventional serological assays such as the plaque reduction neutralisation test (PRNT) can be employed. However, these assays often necessitate the handling of pathogenic wild-type virus in expensive high-biosafety laboratories, restricting the scope of their application, particularly in resource-deprived areas. Chimeric, replication-deficient pseudotype viruses can offer a solution to this problem, as they mimic wild-type virus entry mechanisms, enabling their use in pseudotype virus neutralisation assays (PVNAs). PVNAs bypass high biosafety requirements and permit vaccine evaluation and serosurveillance studies with no risk of inadvertent infection.

This project focuses on the production of functional pseudotype viruses displaying the prM and E surface glycoproteins of the JEV flavivirus, for utilisation in serological neutralisation assays. Subcloning of the prME gene into an appropriate eukaryotic expression vector and insertion mutagenesis to produce prME with 15- and 24-residue upstream signal peptides are shown, before production of JEVpp with either HIV or MLV cores is attempted, *via* the conventional multi-plasmid co-transfection approach or the utilisation of constitutive *gag-pol* expressing cell lines. The impact of additional plasmid-derived furin protease expression and low glucose culture medium, as well as the construction of JEV/VSV chimeric prME glycoproteins and the introduction of Kozak consensus sequences upstream of the prME gene, to enhance the efficiency of JEVpp generation is also explored. Finally, the infectivity of lentiviral pseudotype viruses following lyophilisation, storage and reconstitution is confirmed, thus enabling their affordable global distribution.

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Abbreviations

°C	Degrees centigrade
α	Alpha
Å	Angstrom
β	Beta
β-gal	Beta-galactosidase
Δ	Delta
ΔEG	Delta envelope glycoprotein
ψ	Psi
μg	Microgram
μl	Microlitre
μm	Micrometre
μM	Micromolar
1°	Primary
15SP	15-residue signal peptide
2°	Secondary
24SP	24-residue signal peptide
40S	Eukaryotic small ribosomal subunit
A	Absorbance

AAV-2	Adeno-associated virus type 2
Ab/ab	Antibody
ADE	Antibody-dependent enhancement
ALFV	Alfuy virus
AP	Alkaline phosphatase
AP2	Adapter protein 2
APOBEC	Apolipoprotein B mRNA-editing enzyme catalytic polypeptide
Arg	Arginine
B cell	B lymphocyte
B23	Nucleophosmin/nucleolar phosphoprotein B23
BHK-21	Baby hamster kidney clone 21
BLAST	Basic local alignment search tool
bp	Base pairs
BSL	Biosafety level
C	Capsid gene/protein
C1q	Complement q protein
CAP	Catabolite activator protein
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8

cDNA	Complementary deoxyribonucleic acid
cGP	Chimeric glycoprotein
CHO	Chinese hamster ovary
CLEC5A	C-type lectin domain family 5 member A
CLR	C-type lectin receptor
CMC	Carboxymethylcellulose
CME	Clathrin-mediated endocytosis
CMV	Cytomegalovirus
CNS	Central nervous system
CO ₂	Carbon dioxide
COOH	Carboxylic acid
CPCV	Cacipacore virus
CPE	Cytopathic effect
cPPT	Central polypurine tract <i>cis</i> -active sequence
CPRG	Chlorophenol red-β-d-galactopyranoside
CRD	Carbohydrate recognition domain
CrFK	Crandell-Rees feline kidney
CSF	Cerebrospinal fluid
C-terminus	Carboxyl terminus

CY	Cytoplasmic
DAP12	DNAX-activating protein 12kDa
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DC-SIGNR	Closely related homologue of DC-SIGN
DENV	Dengue virus
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
E	Envelope gene/protein
<i>E.coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid
EG	Envelope glycoprotein
eGFP	Enhanced green fluorescent protein
EIAV	Equine infectious anemia virus
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum

ERA	Evelyn Rokitniki Abseleth
F	Fusion
Fab	Fragment antigen-binding
Fas	First apoptosis signal
FasL	First apoptosis signal ligand
FAVN	Fluorescent antibody virus neutralization
FBS	Foetal bovine serum
Fc	Fragment crystallization region
Fcy	Fc-gamma receptor
FFU/ml	Focus forming units per millilitre
FITC	Fluorescein isothiocyanate
FMDV	Foot and mouth disease virus
Fwd	Forward
g	Grams or g-force
GAG	Glycosaminoglycan
GFP	Green fluorescent protein
GI	JEV genotype I
GII	JEV genotype II
GIII	JEV genotype III

GIV	JEV genotype IV
GV	JEV genotype V
H+L	Heavy and light chain
HA	Haemagglutinin
HCV	Hepatitis C virus
HEK293T	Human embryonic kidney 293 T
HEK293T/17	Human embryonic kidney 293 T clone 17
HFV	Human foamy virus
HI	Haemagglutination inhibition
HIV	Human immunodeficiency virus
HMM	Hidden Markov model
HN	Haemagglutinin-neuraminidase
HPIV-3	Human parainfluenza virus type 3
HSC70	Heat shock cognate protein 70
HSP70	Heat shock protein 70
HSV-1	Herpes simplex virus type 1
HxNx	Haemagglutinin-neuraminidase (influenza subtype)
IC ₅₀	Half maximal inhibitory concentration
IC ₉₀	90% inhibitory concentration

IE	Immediate-early
IF	Immunofluorescence
IFA	Indirect fluorescent antibody
IFN	Interferon
IFN- γ	Interferon gamma
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-10	Interleukin 10
IL-4	Interleukin 4
ITAM	Immunoreceptor tyrosine-based activation motif
JAK-STAT	Janus kinase/signal transducers and activators of transcription
JEV	Japanese encephalitis virus
kb	Kilobase
KCl	Potassium chloride
kDa	Kilodalton
KLD	Kinase-ligase- <i>DpnI</i>
KOUV	Koutango virus
Koz	Kozak (consensus sequence)
KUNV	Kunjil virus

L	Litre
LB	Luria Bertani
LBV	Lagos bat virus
LC-MS/MS	Liquid chromatography tandem-mass spectrometry
log	Logarithm
L-SIGN	Liver/lymph node-specific intercellular adhesion molecule-3-grabbing integrin
LTR	Long tandem repeat
Luc	Luciferase
Lys	Lysine
M	Molar or membrane gene/protein
Mab/mAb	Monoclonal antibody
MAC-ELISA	IgM capture enzyme-linked immunosorbent assay
MALDI-TOF	Matrix-assisted laser desorption/ionization-time of flight
MARV	Marburg virus
mBar	Millibar
MCS	Multiple cloning site
MERS	Middle East respiratory syndrome
mg	Milligram
Mg ²⁺	Magnesium ion

MgCl ₂	Magnesium chloride
MgSO ₄	Magnesium sulphate
ml	Millilitre
MLV	Murine leukaemia virus
mM	Millimolar
MOKV	Mokola virus
MVEV	Murray Valley encephalitis virus
MW	Molecular weight
M β CD	Methyl-beta-cyclodextrin
NaCl	Sodium chloride
NC	Nucleocapsid
NDV	Newcastle disease virus
ng	Nanogram
NLS	Nuclear localization signal
nm	Nanometer
nmol	Nanomolar
NS1	Non-structural gene/protein 1
NS2A	Non-structural gene/protein 2A
NS2B	Non-structural gene/protein 2B

NS3	Non-structural gene/protein 3
NS4A	Non-structural gene/protein 4A
NS4B	Non-structural gene/protein 4B
NS5	Non-structural gene/protein 5
nt	Nucleotide
N-terminus	Amino terminus
NTPase	RNA nucleoside triphosphatase
NY-1	New York-1 hantavirus
ONPG	<i>O</i> -nitrophenyl- β -d-lactopyranoside
p	Plasmid
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDL	Poly-D-lysine
PEI	Polyethylenimine
PFU/ml	Plaque forming units per millilitre
pH	Power/potential of hydrogen
PIP	Pseudo-infectious particle

pmol	Picomolar
PNA	Pseudotype neutralisation assay
pp	Pseudotype particle
prM	Pre-membrane gene/protein
prME	Pre-membrane-envelope gene/protein
PRNT	Plaque reduction neutralisation test
PV	Pseudotype virus
PVDF	Polyvinylidene fluoride
PVNA	Pseudotype virus neutralisation assay
R.luc	Renilla luciferase
RABV	Rabies virus
RdRp	RNA-dependent RNA polymerase
RER	Rough endoplasmic reticulum
Rev	Reverse
RFP	Red fluorescent protein
RH	Relative humidity
RLU/ml	Relative luminescent units per millilitre
RNA	Ribonucleic acid
RNase A	Endoribonuclease A

rNDV	Recombinant Newcastle disease virus
rpm	Revolutions per minute
rRNA	Ribosomal RNA
RSV	Respiratory syncytial virus
RT-PCR	Reverse transcription polymerase chain reaction
RVP	Reporter virus particle
R α H	Rabbit anti-horse
SARS	Severe acute respiratory syndrome
SD	Standard deviation
SDM	Site-directed mutagenesis
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEAP	Secreted alkaline phosphatase
Sf9	<i>Spodoptera frugiperda</i> 9 cells
SFFV	Spleen focus forming virus
SFV	Semliki forest virus
SGR	Subgenomic replicon
SLEV	Saint Louis encephalitis virus
SOC	Super optimal broth with catabolite expression

SRIP	Single-round infectious particle
SV40	Simian vacuolating virus 40
SVP	Subviral particle
T cell	T lymphocyte
T=3	Triangulation number 3
T7	T7 bacteriophage
Ta	Annealing temperature
TAE	Tris-acetate-ethylenediaminetetraacetic acid
Taq	<i>Thermus aquaticus</i>
TBEV	Tick-borne encephalitis virus
TBS-T	Tris buffered saline-Tween 20
TGN	Trans-Golgi network
Th	T helper cell
Tm	Melting temperature
TM	Transmembrane
TMD	Transmembrane domain
TNF α	Tumour necrosis factor alpha
TRIM5 α	Tripartite motif 5 alpha
U	Unit

UC11	Unique segment c 11
USUV	Usutu virus
UV	Ultraviolet
V	Volt
V/O	Vector only
v/v	Volume/volume
VLP	Virus-like particle
VNAAb	Virus neutralizing antibody
VOPBA	Virus overlay protein binding assay
VSV	Vesicular stomatitis virus
VSV-G	Vesicular stomatitis virus glycoprotein
w/v	Weight/volume
WNV	West Nile virus
WPRE	Woodchuck hepatitis virus post-transcriptional regulatory element
X-gal	5-bromo-4-chloro-3-indolyl- β -d-galactopyranoside
YAOV	Yaounde virus
YFV	Yellow fever virus

Chapter 1

Introduction

1.1 Japanese encephalitis virus

Japanese encephalitis virus (JEV) is one of the most prominent causes of viral encephalitis worldwide, with approximately 68,000 symptomatic cases and 20,000 deaths, primarily in children, attributed to the virus each year (Campbell *et al*, 2011; WHO, 2014a). Of those who survive, almost half suffer from irreversible and life-altering neurological damage (Solomon *et al*, 2000). Since its discovery in Japan in the 1870s, JEV has spread considerably and is now endemic across the majority of Asia, putting between 3 and 4 billion people at a tangible risk of infection (Ghosh and Basu, 2009; Yun and Lee, 2014). Despite outbreaks in the Western hemisphere remaining uncommon, rapid globalisation and climate change are respectively facilitating international travel and expanding the habitats of JEV mosquito vectors, thus increasing the likelihood of JEV emerging in geographical regions that were previously unburdened by the virus (Nett *et al*, 2009). The introduction and spread of the phylogenetically similar West Nile virus (WNV) throughout North America since 1999 serves to testify that the threat of the global circulation of JEV has never been more serious, and should not be underestimated (Lanciotti *et al*, 1999; LaBeaud, 2008).

1.1.1 History

Genetic sequence alignment studies predict that JEV originally evolved from an ancestral flavivirus, probably several millennia ago, in the region of the Malay archipelago (Solomon *et al*, 2003; Erlanger *et al*, 2009). However, clinical records of the virus only date back to the early 1870s, when regular summer outbreaks of encephalitis indicative of JEV infection were described in Japan, with substantial incidents occurring periodically at an approximate rate of once every 10 years. Despite other notable epidemics such as the 1904 outbreak dubbed the 'Yoshiwara cold', the first known severe epidemic of the disease took place in 1924, with over

6000 cases and a mortality rate greater than 60% (Miyake, 1964; Solomon *et al*, 2000; Erlanger *et al*, 2009). This outbreak stimulated an increased effort to characterise the pathology and establish the agent responsible for causing the disease. In 1933, pathogen filtered from infected neuronal matter was able to be transmitted to monkeys causing encephalitic symptoms. Soon after this, the virus was successfully isolated from the brain tissue of a fatal Japanese case, and was then officially named the prototype Nakayama strain of JEV in 1935 (Mitamura *et al*, 1936; Miyake, 1964). Subsequently in 1938, JEV was also isolated from *Culex tritaeniorhynchus* mosquitoes, alluding to the role this species plays as a primary vector for virus transmission (Mitamura *et al*, 1938). As it spread to the Korean peninsula and China by 1940, then through many other countries in south east Asia and the Indian subcontinent in the following decades (Figure 1), knowledge of the ecology of JEV and its zoonotic transmission cycles was elucidated (Buescher *et al*, 1959a-b; Scherer *et al*, 1959a-b). By the time the first clinical cases of JEV were recorded in Papua New Guinea and Australia in the mid-1990s, much more information about the virus' biology was established, including its categorisation several years earlier as a member of the *Flavivirus* genus, within the *Flaviviridae* family (Westaway *et al*, 1985).

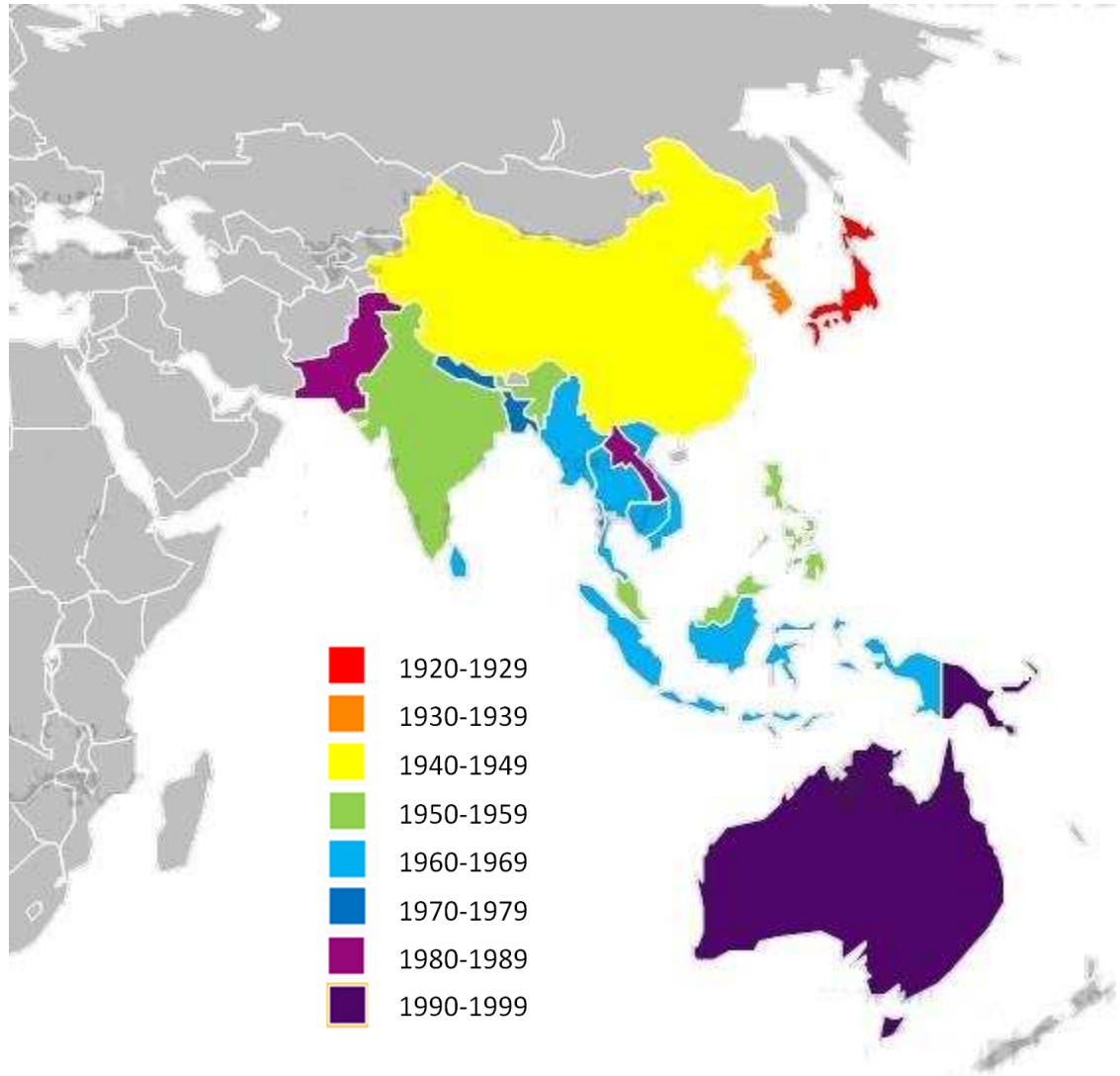


Figure 1. Spread of JEV throughout Asia and Australasia during the 20th century. A choropleth map displaying the years in which the first clinical cases of JEV were reported in Asian and Australasian countries between 1920 and 1999. Clinical case data taken from Erlanger *et al*, 2009. Map drawn using SmartDraw (San Diego, CA, USA) software.

1.1.2 Phylogeny

The *Flaviviridae* family encompasses a wide range of human and animal viral pathogens, and is divided into four genera: *Flavivirus*, *Pestivirus*, *Hepacivirus* and *Pegivirus* (Lindenbach *et al*, 2007). The *Flavivirus* genus is the largest of the four with 74 members, including clinically important viruses such as dengue virus (DENV), yellow fever virus (YFV), tick-borne encephalitis virus (TBEV) and WNV (Kuno *et al*, 1998). Despite the fact that all flaviviruses share common group-reactive epitopes, the genus can be further segregated into 9 antigenic complexes based on serological cross-neutralisation studies, and JEV – another flavivirus – lends its name to one of these (Figure 2). The Japanese encephalitis serocomplex itself comprises nine virus species: Alfuy virus (ALFV), Cacipacore virus (CPCV), JEV, Koutango virus (KOUV), Murray Valley encephalitis virus (MVEV), Saint Louis encephalitis virus (SLEV), Usutu virus (USUV), WNV (including the Kunjin virus clade (KUNV)) and Yaounde virus (YAOV) (Poidinger *et al*, 1996, Mackenzie *et al*, 2002; Thiel *et al*, 2005). Of these viruses, four (JEV, MVEV, SLEV and WNV) are capable of causing severe encephalitic symptoms in humans, and as a collective, the virus members of the JEV serocomplex are geographically distributed across every continent except Antarctica. Despite existing as a single serotype, JEV still exhibits antigenic variation at the more specific classification of immunotype. Initially, the presence of immunological variation amongst JEV strains was demonstrated by Hale and Lee in 1954, who isolated six Malaysian Japanese encephalitis virus strains (as confirmed by histopathological examination of neuronal tissue) before assessing them in a series of cross-neutralisation and complement fixation experiments. In the following years, further investigations were conducted, which immunologically distinguished between the prototype Nakayama strain and the then-recently-isolated G-1 strain of JEV, using the intracerebral protection test, as well as a variety of serological assays. It was concluded that these two strains of the virus may belong in distinct immunotype categories (Kobayashi, 1959; Ogata, 1970). This finding was later corroborated in a study which analysed 26 strains of Japanese encephalitis virus isolated from

Japan and the countries of the Malaya region between 1935 and 1966. Following both conventional haemagglutination inhibition (HI) and antibody-absorption HI testing, as well as the complement-fixation assay, two distinct immunotypes were identified, represented by the Nakayama and Beijing-1 JEV strains (Okuno *et al*, 1968). Since then, a more extensive degree of immunotype diversity has been identified between strains of the virus, with a number of monoclonal antibody-based analytical studies demonstrating at least five distinct antigenic subgroups (Kobayashi *et al*, 1984; Kedarnath *et al*, 1986).

Additionally, JEV exhibits a significant level of genomic variation (Figure 3). The virus can be divided into five different genotypes (GI-V) – four of these genotypes (GI-IV) were originally elucidated by nucleotide sequence analysis of the highly variable prM gene, which was subsequently ratified by similar, more phylogenetic studies involving the E gene and the full-length JEV genome (Chen *et al*, 1990; Chen *et al*, 1992; Williams *et al*, 2000; Solomon *et al*, 2003). Until more recently, the only JEV isolate to fall into GV was the Muar strain in 1952, as determined by E gene phylogeny. As no other strain with particularly high levels of genotypic resemblance to this was isolated for decades afterwards, and also because a confirmatory sequence of a JEV virus of the same strain was absent, virologists working in the discipline were unsure if the distinct diversity of the Muar strain could be attributed to original sequencing errors in a region of its E gene sequence that was actually conserved with other JEV strains from different genotypes (Gould *et al*, 2004). However, in 2009 the JEV strain XZ0934 was isolated from *Culex tritaeniorhynchus* mosquitoes in China – structural gene and complete genome phylogenetic analysis confirmed that this strain also belonged to GV, thus ratifying the authenticity of the genotype, as well as its re-emergence as a circulating genotype of JEV (Uchil and Satchidanandam, 2001; Li *et al*, 2011). Another virus isolate of genotype V JEV was also isolated from a related culicine mosquito species in the Republic of Korea in 2010 (Takhampunya *et al*, 2011).

A correlation is also thought to exist between the presence of particular JEV genotypes in certain geographical regions and climates, and the activity of the virus. Despite some exceptions to this pattern, it is generally observed that JEV strains belonging to GI and GIII circulate primarily in more northern, temperate areas of Asia, whereas GII and GIV strains of JEV are predominantly located in more tropical regions and closer to the equator.

Furthermore, the majority of seasonal, summer epidemics of JEV take place in the temperate regions where the virus circulates, and conversely the tropical countries where JEV is present often experience more endemic strains of JEV. This observation potentially alludes to the fact that particular JEV genotypes could display either epidemic or endemic activity (Chen *et al*, 1990; Chen *et al*, 1992). This geographical pattern of JEV genotypic distribution was also presented in a 2013 study, which also concluded that the products of a division of genotype I into two separate clusters, GI-a and GI-b, were found in geographically distant areas from one another, with GI-a residing primarily in tropical regions and GI-b in countries with more temperate climates (Schuh *et al*, 2013).

It is evident that JEV continues to display a high level of genetic diversity, seeing as since the turn of the 21st century, GI has split into differentiable sub-genotype clusters as well as replacing GIII as the dominant circulating JEV genotype in Asia, and GV has also re-emerged as a circulating genotype (Li *et al*, 2011; Pan *et al*, 2011; Schuh *et al*, 2013; Han *et al*, 2014). This consistent viral evolution poses significant public health risks, especially to the countries in which JEV circulates, as existing vaccines may become less effective and vulnerable populations may possess a lower level of acquired immunity against the newly-emerging drift variants.

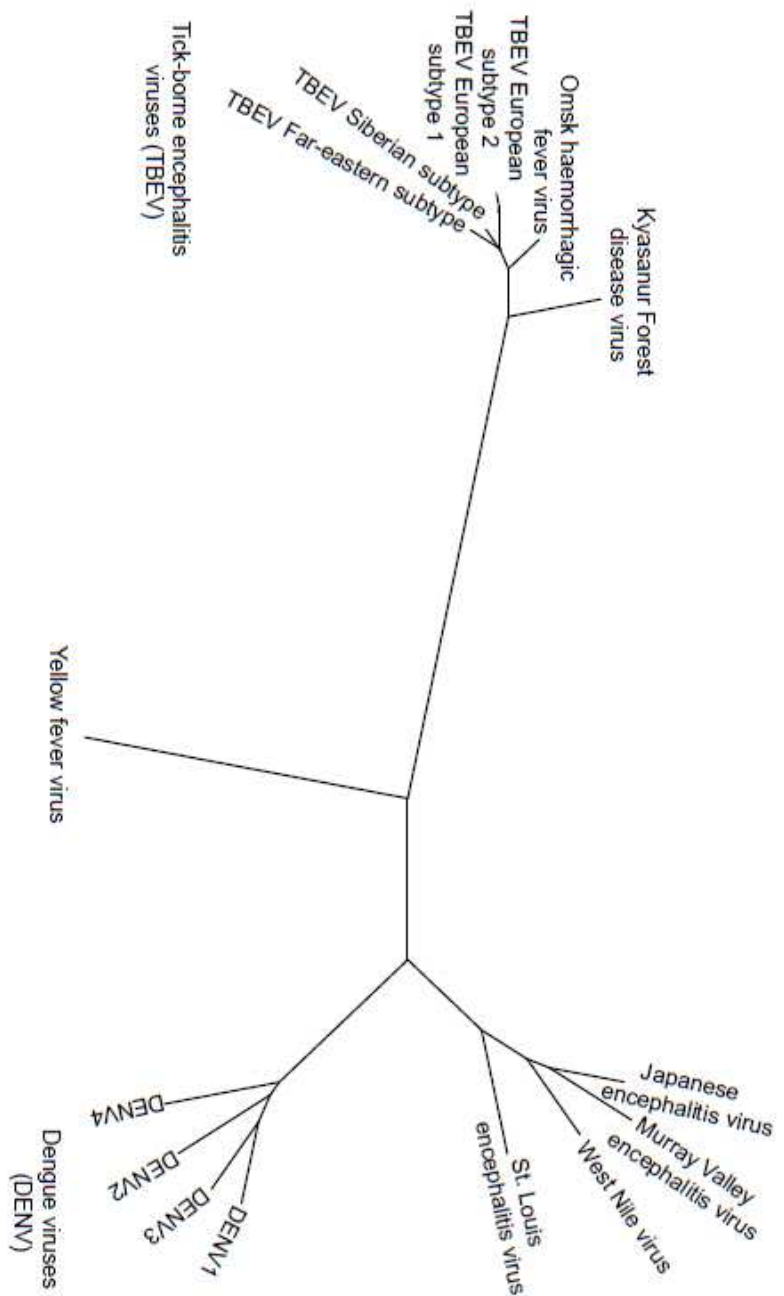


Figure 2. Phylogenetic tree of medically important tick- and mosquito-borne flaviviruses.

Complete polyprotein sequences were aligned, prior to performing phylogenetic reconstruction using PAUP v.4.0b10 and maximum likelihood analysis (figure sourced from Daly and Solomon, 2010).

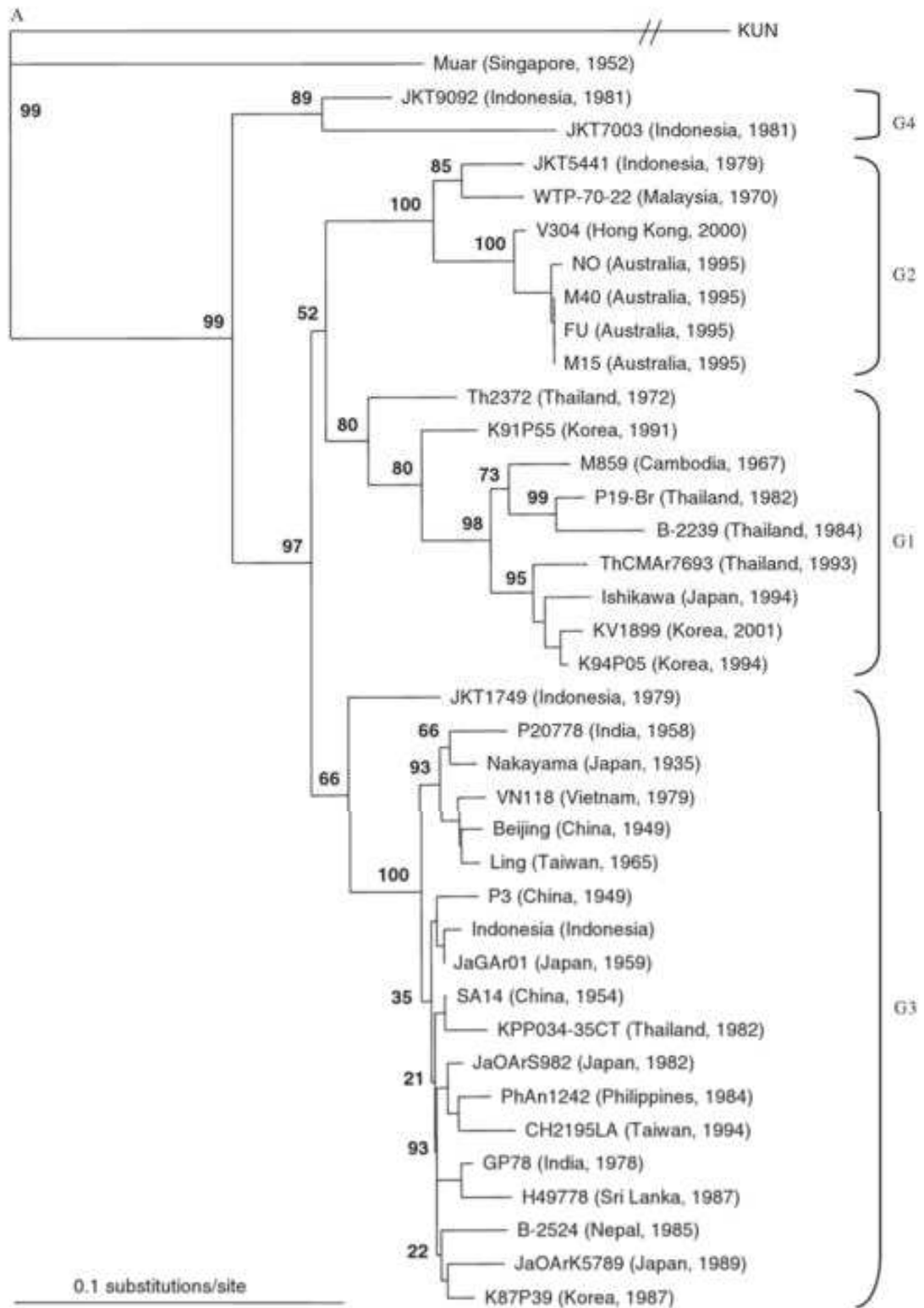


Figure 3. Phylogenetic tree of Japanese encephalitis virus strains. Phylogenetic relationships were predicted using E gene sequence information, and genotypic classifications are indicated (G1-G4). Tree construction was carried out in ClustalX using the neighbour-joining method, and percentage bootstrap calculations are shown at each branch node. The scale at the bottom left hand side of the figure refers to the number of nucleotide substitutions per site. In order to root the tree, E gene sequence information for the KUNV clade was used (figure sourced from Mackenzie *et al*, 2007).

1.1.3 Transmission and epidemiology

JEV is a mosquito-borne arbovirus, sustained within an enzootic cycle between aquatic birds and pigs, and transmitted through the bite of an infected culicine mosquito, primarily the *Culex tritaeniorrhynchus* species (Buescher *et al*, 1959a; Endy and Nisalak, 2002; van den Hurk *et al*, 2009). Pigs and aquatic birds are crucial members of this transmission cycle because they act as asymptomatic amplifying hosts for the virus, which means they develop prolonged and high levels of viraemia sufficient for previously uninfected *Culex* mosquitoes to take up JEV during a blood meal (Buescher *et al*, 1959b; Scherer *et al*, 1959a; Le Flohic *et al*, 2013). Furthermore, water fowl are responsible for efficiently disseminating the virus to new geographical areas, and both pigs and these birds produce a large number of offspring, thus consistently supplying new amplifying hosts to continue the JEV enzootic cycle (Solomon *et al*, 2000). Humans, amongst several other animals, can also be infected with JEV but are considered coincidental 'dead-end' hosts, as viraemia is generally too low and transient to subsequently uphold the virus transmission cycle (Scherer *et al*, 1959b; Le Flohic *et al*, 2013; Figure 4).

Human cases of JEV infection mostly appear in rural or periurban areas of Asia, where communities live in closer proximity to farmland, rice paddy fields and similar environments cohabited by the animals primarily involved in the natural transmission cycle of the virus (Daly and Solomon, 2010). Fluctuations in the numbers of people infected by JEV occur on a seasonal basis in endemic regions – following the monsoon season across large parts of South East Asia, breeding rates of many mosquitoes, including those in the *Culex* genus, increase significantly due to the sudden abundance of standing or stagnant water sites that serve as breeding grounds. Mosquito populations proliferate, leading to accordant rises in amplifying host infection prevalence (Solomon *et al*, 2000). This combination of factors inevitably results in elevated numbers of human infection.

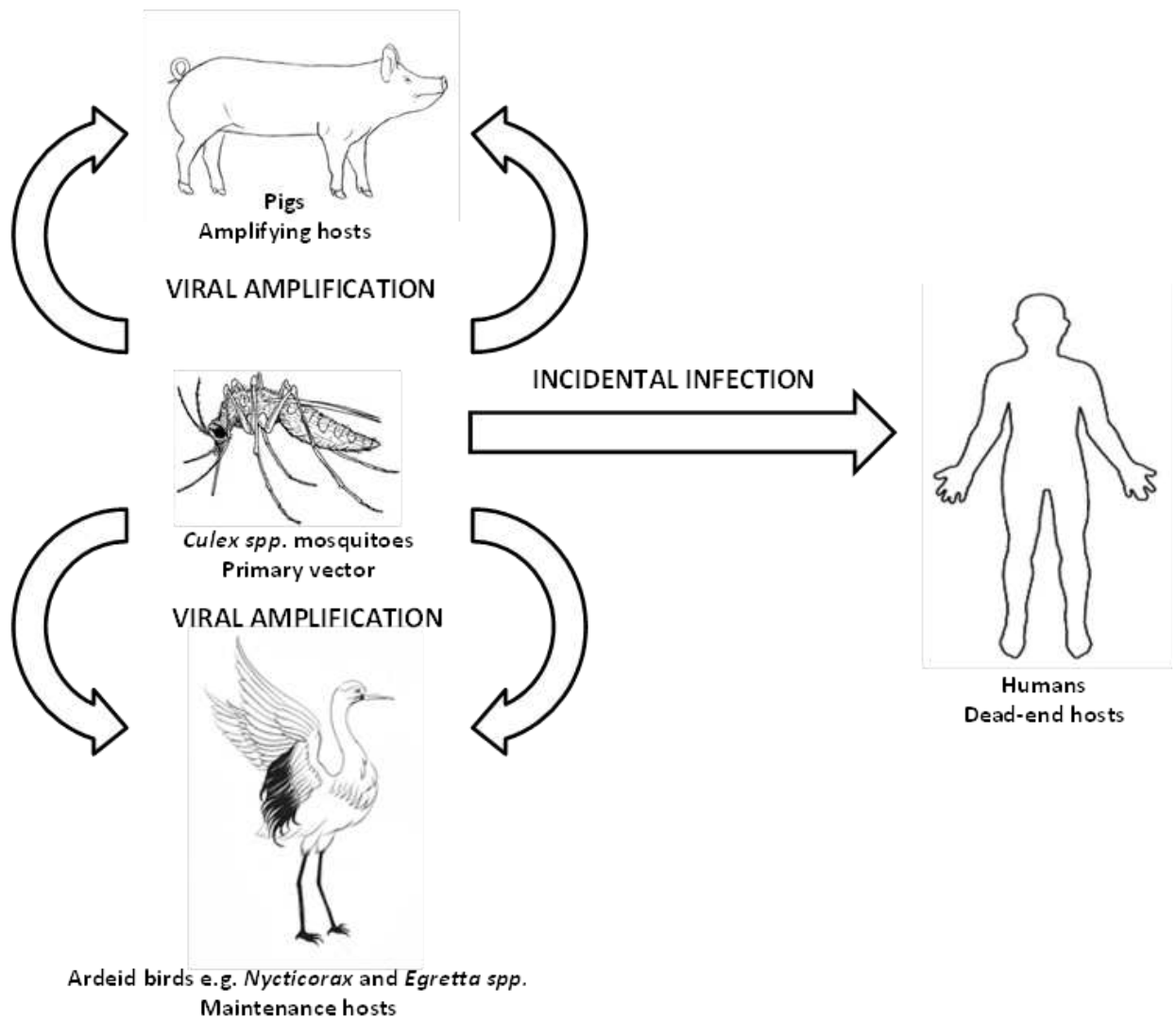


Figure 4. Diagram of the enzootic transmission cycle of JEV. This diagram shows the interaction between the different organisms that primarily constitute the enzootic transmission cycle of Japanese encephalitis virus. The cycle is sustained between culicine mosquitoes, which act as a primary vector and reservoir host, as well as pigs and ardeid bird species, which respectively fulfil the roles of amplifying and maintenance hosts. Humans can only contract JEV through incidental infection and are regarded as 'dead-end' hosts, meaning that the levels of viraemia in infected individuals are not conducive with propagation and continuation of the virus' transmission cycle. Image source URLs: Pig: (<http://www.drawcentral.com/2012/05/how-to-draw-pig.html>); Mosquito: (<http://konkursy.info/mosquito-coloring-page.html>); Heron: (<http://www.dondrup.com/stock-vector/37254-graphicsriver-heron-4529261.html>); Human: (<https://uk.pinterest.com/pin/179651472613171341/>).

1.1.4 Clinical manifestation and disease symptoms

Except for rare, occasional cases of laboratory-acquired infection, contraction of Japanese encephalitis virus is almost invariably caused by the bite of an infected culicine mosquito. At the point of initial infection, JEV breaches dendritic cells (DCs) within the dermis, in close proximity to the bite location. From here, the virus is carried in the antigen-presenting DCs to the peripheral lymph nodes, where the infection spreads to macrophages and other cells of the lymphatic system. A short-lived viraemia of approximately one week subsequently occurs, which is accountable for the generic febrile symptoms characteristic of the initial incubation period of JEV infection (Mackenzie *et al*, 2007). In the vast majority of cases, the virus is cleared by the host immune response at this stage of infection. However, in between 1:200 and 1:2000 cases, JEV manages to cross the blood-brain barrier *via* penetration of the vascular endothelium, thus gaining entry to the central nervous system (CNS) (Ghosh and Basu, 2009). Upon being present in the CNS, the virus can invade the system's neuronal and phagocytic cells, consequentially affecting several tissues in the brain, including central cerebral structures such as the hippocampus, thalamus, substantia nigra and brainstem. Late-stage JEV infection is also witnessed in the temporal lobe and cerebellum, as well as the anterior horn cells of the upper spinal cord. Oedema and an inflammatory infiltrate containing elevated levels of B and T lymphocytes and macrophages are often observed in these infected neuronal regions, and alongside other factors such as neuronal apoptotic activity, these inflammatory responses contribute significantly to the cerebral damage and hallmark neurological presentation of JEV (Mackenzie *et al*, 2007).

In populations where the virus circulates regularly and is considered endemic, symptomatic JEV infection is primarily witnessed in children, since adults have usually developed immunity against the virus throughout their childhood. However, the elderly also display a heightened propensity to develop symptoms attributed to JEV and other flaviviral infections, such as WNV

and MVEV, in comparison to healthy adult cohorts. Furthermore, amongst travellers or those who are newly residing in high-risk regions, the virus can infect individuals regardless of age, due to their naïve immunity.

The clinical disease caused by JEV infection can be neatly ordered into three distinct categories, which can be separated and distinguished between by their levels of severity. As expected, in each category, the symptoms described closely correspond to the clinical pathologies observed during each stage of viral development in an infected patient.

The first of these categories covers non-encephalitic disease: in the vast majority of instances, JEV infection is considered asymptomatic in patients, or they experience a non-specific febrile illness, which is characteristically mild but with the potential to progress into headache, vomiting, diarrhoea and convulsions. This level of illness is almost always self-limiting, and is also known as 'abortive encephalitis'. It is believed that this mild, febrile illness attributed to JEV may well be underdiagnosed, because of the fact that the symptoms appear to resemble several other infections, in both clinical and serological respects, meaning that infection with JEV may not be considered by physicians during diagnosis (Watt and Jongaskul, 2003).

However, in approximately 1 in every 250 cases, acute encephalitic symptoms manifest themselves subsequent to the milder initial presentation, usually after an incubation period of between 7 and 14 days (WHO, 2014a). In these patients, the progression of the disease is characterised by a sudden onset of a more severe fever, and other common symptoms include neck stiffness, convulsions and cranial nerve palsies, which cause a vacant, expressionless face with staring eyes (Figure 5). This clinical presentation links closely to a bad prognosis, with many such cases developing into late-onset encephalitic disease.

Many patients that display the early disease symptoms of encephalitis caused by JEV often progress on to a more severe and life-threatening late-stage encephalitis. Additional

symptoms also emerge in these instances, such as tremor, hypertonia and cogwheel rigidity, as well as a range of motor neuron failures such as erratic facial contortions, twitching and blinking, cerebellar ataxia and seizures. Ultimately, the widespread viral infection throughout the brain causes flaccid paralysis, coma and subsequent respiratory failure (Solomon *et al*, 2000). Between 20% and 30% of those who suffer with this severe encephalitis succumb to the disease, even if the most sophisticated medical facilities are at hand. For the remainder of patients that manage to survive late-onset Japanese encephalitis, the path to recovery is complicated and neurological impairments can take months to subside. Up to 50% of survivors develop and suffer from lifelong neurological sequelae, of which the effects can be twofold: often, physical sequelae are reported, such as varying degrees of paralysis, nerve palsies, blindness, epilepsy, movement disorders and parkinsonism; however, behavioural sequelae can also occur, such as an impaired memory, a loss of the ability to speak, uncharacteristic aggression and emotional lability (Hoke *et al*, 1988).

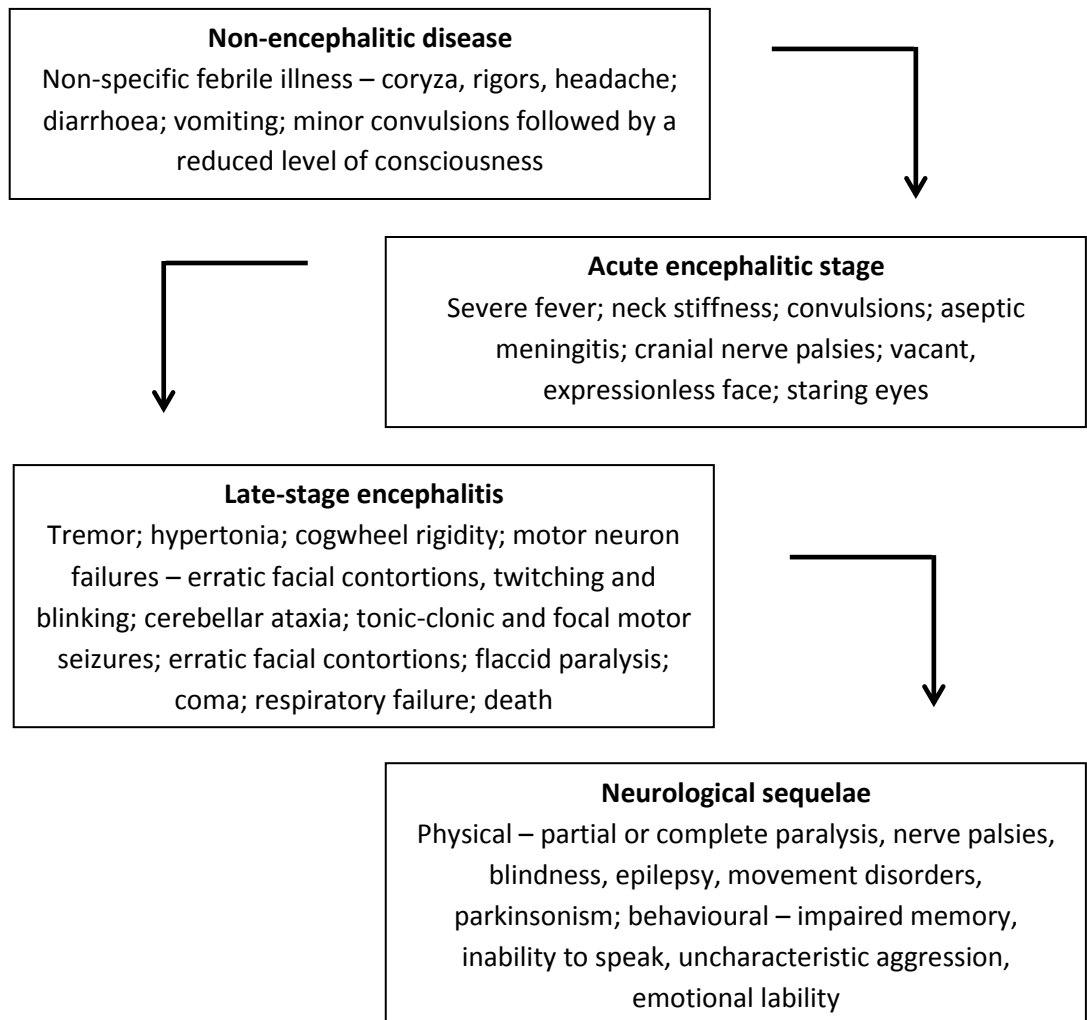


Figure 5. Japanese encephalitis symptoms. A breakdown of the hallmark symptoms of the disease caused by the flavivirus, ranging from the initial non-encephalitic disease, through the acute and late stages of encephalitis to the progression of long-lasting neurological sequelae.

1.1.5 Virion and genome

JEV virions are approximately 50nm in diameter and exist as spherical enveloped particles (Figure 6). The surface of the mature virion contains two proteins – the envelope (E; ~53kDa) and membrane (M; ~8kDa) proteins (Lindenbach and Rice, 2003; Gubler *et al*, 2007). Within the virus envelope, a 30nm diameter nucleocapsid contains the viral genetic material. The ~10.9kb JEV genome exists as a single-stranded, positive-sense RNA molecule, which is processed as one open reading frame and encodes a single polyprotein (Mukhopadhyay *et al*, 2005). The three JEV structural proteins are located at the N-terminus of this genome – capsid (C), premembrane (prM – expressed initially with a precursor element) and envelope (E) proteins – upstream of seven non-structural proteins which are crucial for virus replication (Figure 6).

1.1.5.1 C protein

The C protein is one of the smallest expressed in the JEV genome, with a length of approximately 120 amino acids and a predicted molecular weight (MW) of 12-14 kDa (Chambers *et al*, 1990). It is a basic, highly positively charged protein, with C-terminal and central hydrophobic domains that surround a hydrophilic sequence of amino acids. These regions of strong hydrophobicity or hydrophilicity within the protein are generally conserved among flaviviruses (Abraham *et al*, 2011). It possesses a dimeric structure, with each monomer containing four connected α -helices. The two monomers sit in an anti-parallel orientation, with the $\alpha 2$ and $\alpha 4$ helices of each monomer positioned adjacent to each other, completing the C protein dimer. The function of the capsid protein concerns early JEV assembly, *via* the formation of the nucleocapsid (NC) core and the packaging of the viral genome into the NC (Mukhopadhyay *et al*, 2005). Also, nuclear localisation of the C protein in infected mammalian and insect cells plays a crucial role in JEV replication and pathogenesis (Mori *et al*, 2005).

1.1.5.2 pr and M proteins

The prM protein (~240 amino acids; predicted MW 18.1-19.0 kDa) is a glycoprotein precursor to the structural M protein found in mature JEV virions (Chambers *et al*, 1990). During the early stages of progeny virus biosynthesis and assembly, prM and E form a heterodimeric complex. Within this complex, prM has already acquired its completely folded structure and acts as a chaperone for the folding of the E protein, as well as protecting it from premature, irreversible conformational changes induced by acidic pH as it transits through the intracellular trans-Golgi network (TGN) (Lorenz *et al*, 2002; Heinz and Allison, 2003). Shortly before virus exocytosis and egress from the cell, the prM protein undergoes a proteolytic cleavage step vital for virion maturation. Cleavage is directed to occur immediately after the Arg-X-Arg/Lys-Arg motif in the prM amino acid sequence, which corresponds to the cleavage site of the cellular protease furin, an enzyme which concentrates in the proximity of the TGN. The low-pH environment of the TGN stimulates a conformational change in the prM-E heterodimers of the immature JEV virion, which is generally thought to expose this furin cleavage site, enabling progeny virus maturation to be completed (Mukhopadhyay *et al*, 2005). prM is cleaved into a discarded precursor (pr; ~165 amino acids) fragment and a small, mature membrane (M; ~75 amino acids) protein. In the surface structure of the mature JEV virion, the M protein is partially embedded into the outer lipid membrane of the virus, and possesses a very small ectodomain in comparison with E (Heinz and Allison, 2003; Mukhopadhyay *et al*, 2005). Another important motif in the prM protein is a single N-linked glycosylation site towards the N-terminus of the amino acid sequence, which is highly conserved amongst JEV strains and related flaviviruses, and has a direct influence upon particle release and virus pathogenicity in a mouse model (Kim *et al*, 2008).

1.1.5.3 E protein

The E protein (~500 amino acids; predicted MW ~53 kDa) is the major envelope glycoprotein and the dominant immunogenic antigen on the surface of JEV virions, and is integral for the attachment, membrane fusion and entry of the virus into permissive cells (Chambers *et al*, 1990; Abraham *et al*, 2011). In the mature virion structure, there are 180 individual copies of E which are orientated as head-to-tail, anti-parallel homodimers and lay flat against, rather than protruding from, the lipid bilayer and nucleocapsid, creating a relatively smooth and spikeless virus surface compared to other families of viruses, such as *Orthomyxoviridae* and *Coronaviridae* (Mukhopadhyay *et al*, 2005). Each homodimer is itself arranged in sets of three parallel dimers, thus forming an icosahedral pattern of 30 E protein rafts across the virion surface. It appears inconclusive as to whether flaviviruses conform to T=3 or pseudo T=3 icosahedral symmetry (Caspar and Klug, 1962; Mukhopadhyay *et al*, 2005). Each JEV E monomer consists of three distinct domains: domain III is an immunoglobulin-like domain that creates slight protrusions from the smooth virion surface and is responsible for JEV binding to cellular receptors, as well as for initiating virus fusion and entry into susceptible cells; domain II is involved in maintaining E monomer homodimerisation – this domain is comprised of two ‘finger-like’ structures and also contains a 13-amino acid fusion loop at its distal end, which is hydrophobic and highly conserved amongst JEV strains; these two domain structures are connected by domain I, which possesses a beta-barrel in its centre to facilitate its role as a domain II-III hinge (Wu *et al*, 2003; Pierson and Diamond, 2012). Notably, compared to other species of flaviviruses such as DENV and YFV, the domain II central dimerisation region between constituent monomers within the mature JEV E homodimer possesses a notably short dimer interface. Furthermore, multiple conserved histidine residues in the E protein structure appear to act as important quaternary points of load-bearing contact between monomers in the homodimer complex (Luca *et al*, 2012).

1.1.5.4 Non-structural proteins

The JEV RNA genome expresses seven non-structural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. The ~48kDa NS1 protein in flaviviruses is immunogenic and is capable of stimulating a host protective antibody response. Its function is involved with virus replication, as well as regulation and evasion of the innate immune response directed against the virus (Fan and Mason, 1990; Muylaert *et al*, 1996; Lindenbach and Rice, 1997; Chung *et al*, 2006a; Chung *et al*, 2006b). In addition, NS1', a larger 53kDa NS1-related protein, is also often detected in JEV-infected cells. This protein variant is reported to be brought about *via* ribosomal frameshifting, and plays a role in viral neuroinvasiveness (Chambers *et al*, 1990; Melian *et al*, 2010). NS2A is a viral membrane-associated, hydrophobic protein with a diverse array of functions: it possesses enzymatic activity to cleave the expressed polyprotein at the NS1-NS2A junction, interferes with the host antiviral response by inhibiting the interferon (IFN) signalling pathway, and also contributes to the functioning of the life cycle at several other stages - RNA replication and the viral replicase complex, as well as viral assembly and egress from the host cell (Falgout and Markoff, 1995; Kümmerer and Rice, 2002; Liu *et al*, 2006; Leung *et al*, 2008). NS2B and NS3 remain associated to one another as a heterodimer which anchors itself into the endoplasmic reticulum membrane, where it acts as a crucial factor to the NS2B-NS3 serine protease, which cleaves the expressed JEV polyprotein at several points between non-structural proteins (Lindenbach and Rice, 2003; Abraham *et al*, 2011). The NS3 protein also plays a role in viral replication and assembly due to its enzymatic activity as a RNA helicase, serine protease and NTPase (Bollati *et al*, 2009; Assenberg *et al*, 2009; Pastorino *et al*, 2010). NS4A is another relatively small hydrophobic protein, which possesses a similar role to the NS2A protein, as it is an IFN antagonist. Furthermore, cleavage of the NS4A-4B complex may also mediate host cell cytoplasmic membrane biogenesis, which facilitates efficient RNA replication and intracellular trafficking of viral components (Roosendaal *et al*, 2006; Lin *et al*, 2008). The JEV NS5 protein is the largest of all the proteins expressed from the JEV genome,

with a variety of functions. The protein displays methyltransferase and RNA-dependent RNA polymerase (RdRp) at its N-terminal and C-terminal regions, respectively (Kim *et al*, 2007; Davidson, 2009). It also inhibits the JAK-STAT signalling cascade, *via* inhibiting a phosphorylation step of the pathway, which compromises the antiviral efficiency of IFN (Lin *et al*, 2006).

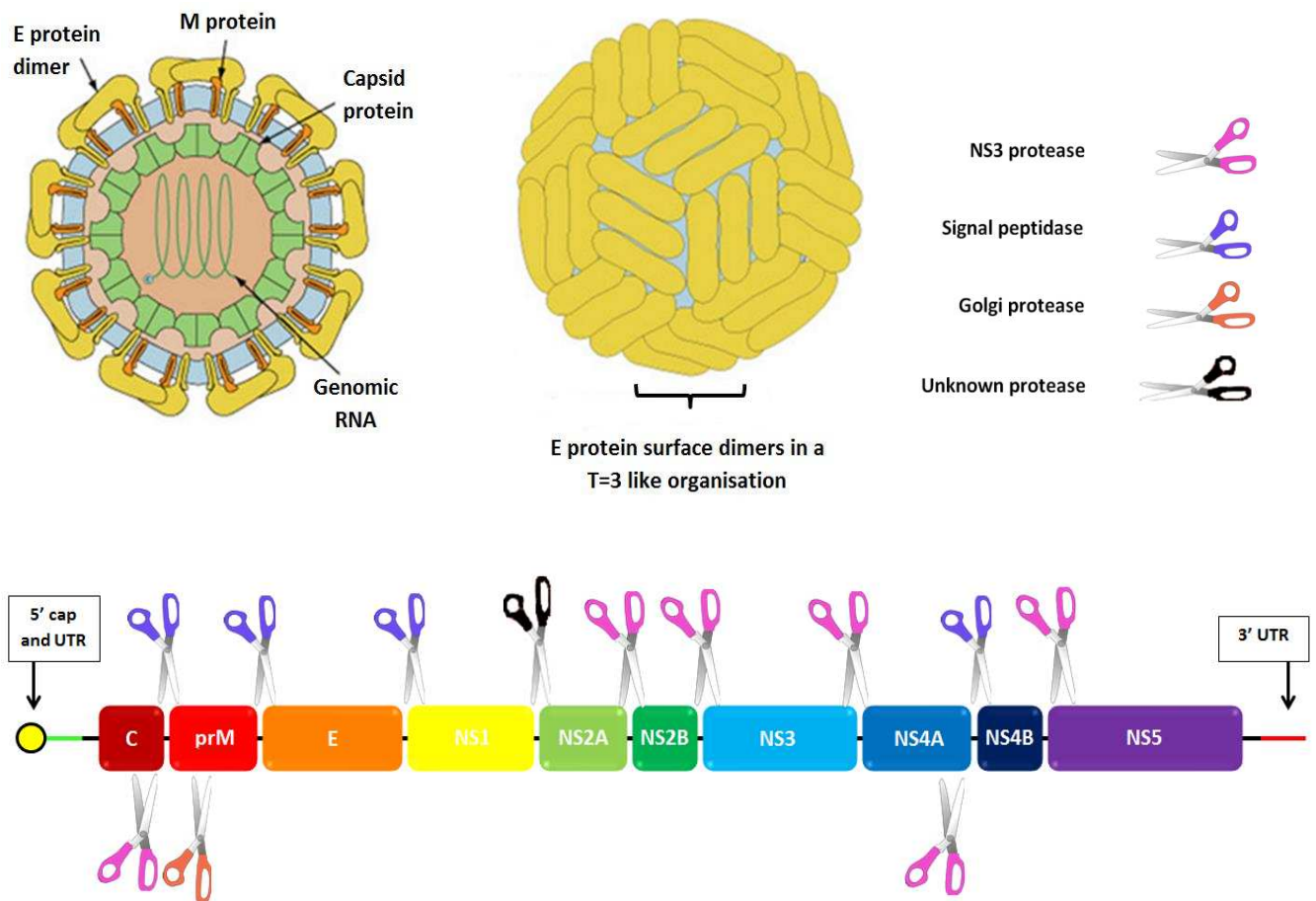


Figure 6. Japanese encephalitis virus virion and genome structure. A schematic of the mature JEV virion is shown, including the orientation and organisation of E protein dimers at the external virus surface, as well as a virion cross-section, with annotations of the membrane and capsid proteins, and the genomic RNA. The JEV genome is also shown, both as a polyprotein with 5' and 3' termini, and with a breakdown of each component gene, alongside the corresponding enzyme that cleaves each junction of the polyprotein during virus assembly (adapted from Viralzone, 2011).

1.1.6 Virus life cycle

1.1.6.1 Attachment, entry and cellular receptors

Entry into living host cells is a vital initiatory step of any viral infection, and the process of entering living cells contributes towards what defines a pathogenic microbe as a virus. Like with virtually all viruses, the JEV infection life cycle begins with entry and penetration of host cells, instigated when domain III of the virus' E protein engages receptors on the host cell surface. Despite significant advancements in recent decades of knowledge concerning the structure and organisation of JEV virions, our comprehension of the molecular interactions that occur when the virus' E protein binds to receptors at the cell membrane is at a much more rudimentary level than that of flavivirus virion structure. However, current understanding of the JEV infectious entry model suggests that at least two distinct cell surface molecules may be actively involved - *attachment factors* that help to stabilise individual virions and concentrate them at the plasma membrane, so that *primary receptors* can be physically bound by viral envelope glycoproteins to initiate entry and infection. Several protein families have been reported in relevant, recent literature as being involved in JEV attachment and entry into host cells (Table 1), and some of the best-characterised of these putative cellular receptors are detailed below:

1.1.6.1.1 Glycosaminoglycans (GAGs) and heparan sulfate

Perhaps the best-established set of molecules demonstrated to interact with the JEV envelope glycoprotein during entry and infection are glycosaminoglycans, otherwise known as GAGs, which are long, unbranched polysaccharides containing sulphate groups, bound and anchored to the plasma membrane by core proteins themselves embedded in the cell surface. These protein-polysaccharide complexes are called proteoglycans (Zhang, 2010). GAGs are the initial point of contact for the JEV E protein with the plasma membrane during virus infection and

host cell penetration, and these large linear structures act as an important attachment factor, by stabilising individual virions and facilitating virus adhesion to the cell surface, in order to bring about binding of the JEV envelope protein to primary cellular receptors (Lee *et al*, 2006). Positively charged amino acid residues on the JEV E protein are able to exploit and interact with the negatively charged sulphate groups of the GAG (Chen *et al*, 1997; Perera-Lecoin *et al*, 2014). Furthermore, GAGs and proteoglycans are ubiquitous molecules found on the extracellular surfaces of all tissues, meaning that JEV can easily utilise them as docking stations for viral adhesion onto a wide variety of cell types (Zhang, 2010). Several other flaviviruses, such as DENV, YFV, WNV and TBEV, also exploit GAG interactions to anchor onto the external face of the cellular plasma membrane, prior to viral entry and host cell fusion (Chen *et al*, 1997; Hilgard and Stockert, 2000; Su *et al*, 2001; Germe *et al*, 2002; Kroschewski *et al*, 2003; Okamoto *et al*, 2012).

1.1.6.1.2 CLEC5A

CLEC5A is a member of the C-type lectin receptor (CLRs) family, which are specialised receptors that possess the ability to recognise carbohydrate profiles on invading pathogens, including viruses aiming to infect host cells, and subsequently play an important role in activating and initiating host cell immune responses against such pathogens. CLRs in general, and more specifically CLEC5A, are commonly expressed at high levels on myeloid cells, such as monocytes, macrophages and dendritic cells – these cell types fall under the tropism of many flaviviruses, and the presence of CLEC5A receptors may be accountable for JEV infection in such myeloid cells. Following carbohydrate motif recognition, CLRs also act as internalisation receptors that target pathogens to acidified intracellular endosomes for enzymatic degradation and disposal from the cell (McGreal *et al*, 2005). Despite the interaction of CLEC5A with JEV virions being demonstrated, as well as with DENV, it has not yet been confirmed whether the receptor interacts with glycans on the envelope glycoprotein of JEV. However, the

literature postulates that CLEC5A functions neither as a primary receptor nor an attachment factor. Rather, due to its lack of a cytoplasmic tail with internalisation motifs, which are observed on other members of the CLR family exploited for flavivirus infection, such as DC-SIGN and L-SIGN, a positively charged amino acid residue on the transmembrane domain of CLEC5A reacts with the DNAX-activating protein 12kDa, otherwise known as DAP12 (Watson *et al*, 2011). DAP12 is an immunoreceptor tyrosine-based activation motif (ITAM)- bearing adapter molecule, which instigates the activation of intracellular signalling pathways (Bakker *et al*, 1999; Colonna, 2003). When binding of CLEC5A by JEV E occurs, the DAP12 molecules present in macrophages and microglia become phosphorylated, thus triggering signal transductions that stimulate the release of proinflammatory cytokines, which cause various pathologies, such as inflammation, vascular leakage and cell death, and serve to significantly increase JEV disease severity (Chen *et al*, 2008; Wu *et al*, 2013). Neutralising antibodies raised against CLEC5A efficiently inhibit these inflammatory pathologies by preventing JEV E binding and subsequent DAP12 activation and signal transduction (Chen *et al*, 2012). Therefore, these findings indicate that CLEC5A plays a vital part in JEV infection, particularly with regards to the pathogenesis of the viral disease and its progression to severe encephalitis.

1.1.6.1.3 High affinity laminin receptor

A recent study, designed by Thongtan *et al* in 2012, aimed to elucidate molecules that were actively associated with the entry and infection of JEV into microglial cells, an integral neuronal cell type distributed throughout the brain and spinal cord tissues. Following a virus overlay protein binding assay (VOPBA) using the pan-specific anti-flaviviral monoclonal antibody HB112 (Henchal *et al*, 1982), to assess which proteins expressed on the microglial cell surface bind the JEV envelope glycoprotein, and liquid chromatography-mass spectrometry analysis (LC-MS/MS), in order to physically separate then gauge the elemental compositions and masses of the candidate JEV-binding proteins, the 37/67 kDa high affinity laminin receptor was

repeatedly and consistently identified in the spectroscopic analyses. Subsequent antibody-mediated inhibition experiments displayed that anti-laminin antibodies significantly neutralised JEV infection and entry into mouse microglial BV-2 cells (Thongtan *et al*, 2012). Therefore, it is postulated that the high affinity laminin receptor is a promising potential cellular receptor for JEV, specifically for microglial entry. However, this study also emphasises the palpable complexity of the interactions between JEV virions and target cellular receptors – the engagement of different receptors, and indeed co-receptors, across the range of cell tropism of the virus is highly likely, and as more knowledge is acquired of JEV cell entry processes, this may be an ongoing obstacle for novel antiviral development.

The 67kDa high affinity laminin receptor is believed to mediate many of the cellular interactions of the major basal membrane component laminin, which is ubiquitous across many mammalian tissue types, and plays a pivotal role in cellular morphology, adhesion and differentiation, amongst other functions (Castronovo *et al*, 1991, Ardini *et al*, 1998).

Interestingly, this receptor has also been demonstrated to definitively act as a cellular receptor for the Sindbis virus in several cell types – importantly, Sindbis virus is a member of the *Alphavirus* genus, a group of viruses that display very similar cell entry and fusion mechanisms as flaviviruses (Wang *et al*, 1992; Morizono *et al*, 2010). This similarity between these two virus genera may be a factor into their mutual exploitation of the high affinity laminin receptor to penetrate cell membranes.

1.1.6.1.4 Heat shock protein 70 (HSP70)

Another protein that is implicated alongside the laminin receptor in Thongtan *et al*'s 2012 study to discover microglial cell surface JEV-binding molecules is the heat shock protein 70 (HSP70). The heat shock family of proteins are upregulated in response to a variety of conditions of cellular stress, such as extreme heat or cold, hypoxia, toxin exposure, starvation and water deprivation. These proteins also act as important intracellular chaperones to ensure

the correct folding and conformation of newly translated proteins takes place, as well as to prevent unwanted protein aggregation following their expression (Whitley *et al*, 1999). In fact, this finding has been independently corroborated in a number of other recent studies, where HSP70 appears to have putative cellular receptor qualities in several other cell types.

Using similar methodologies as observed in other studies aiming to identify proteins with flavivirus-binding characteristics, the HSP70 protein has been flagged as a potential JEV cellular receptor in neuroblastoma cells (Das *et al*, 2009). More specifically, using the mouse-derived cell line Neuro2a, a VOPBA was conducted to isolate membrane-located proteins that were interacting with the JEV E glycoprotein, yielding an approximately 70kDa protein as a candidate, which was then subjected to matrix-assisted laser desorption/ionization-time of flight (commonly abbreviated to MALDI-TOF) mass spectrometry and subsequent MASCOT protein identification software, used to analyse and compare the peptide mass fingerprint of the said protein against established protein databases. The experimental protein showed palpable homology with HSP70, and receptor function and characteristics were confirmed by a range of downstream tests, including infection inhibition and plaque reduction assays using anti-HSP70 polyclonal antibodies, as well as presenting the knockdown of JEV infection in a plaque assay utilising the compound quercetin, a potent HSP70 antagonist (Das *et al*, 2009). Since then, it has also been indicated that the human hepatoma cell line Huh7 is permissible to JEV infection due to HSP70 presence, in association with lipid rafts at the plasma membrane (Zhu *et al*, 2012). Firstly, polyclonal antibody treatment of HSP70 at varying concentrations revealed a specific, dose-dependent decrease of JEV infection into Huh7 cells, and this finding was corroborated by small interfering RNA treatment of the HSP70 receptor, bringing about its depletion throughout the hepatoma cells and in turn, markedly reducing cellular entry of the virus. Binding and co-immunoprecipitation assays were then used to confirm a tangible interaction between the JEV glycoprotein and HSP70, before a series of experiments utilising

the cyclic oligosaccharide methyl- β -cyclodextrin (M β CD) were conducted, which selectively disrupts membrane cholesterol, an important component of membraneous lipid rafts (Ilangumaran and Hoessli, 1998). It was deduced that cholesterol breakdown at the plasma membrane adversely affected JEV entry and infection, and biochemical fractionation of JEV-infected Huh7 cells following M β CD treatment revealed that HSP70 migrates to other areas of the plasma membrane in the absence of intact lipid raft domains, although interestingly this does not affect levels of JEV cell binding. These findings led the authors to elude to HSP70's role as a putative JEV cellular receptor, and conclude that viral entry is facilitated by lipid raft interaction, possibly by clustering and co-localising of JEV virions and HSP70 molecules around membrane cholesterol domains (Zhu *et al*, 2012).

Furthermore, it appears that the very similar heat shock cognate protein 70 (HSC70), which shares a very high percentage homology with HSP70, may act as a cellular receptor for JEV on the *Aedes albopictus* mosquito cell line C6/36, as determined by co-immunoprecipitation and mass spectrometry (White, 1987; Ren *et al*, 2007). Interestingly, this study also speculates on the potential of these heat shock proteins to not only anchor JEV virions to the host cell plasma membrane, but also to fulfil the role of chaperone and facilitate conformational changes of the envelope glycoprotein during virus fusion, in keeping with a primary function of heat shock proteins (Boonsanay and Smith, 2007; Ren *et al*, 2007).

Finally, one of the earliest studies to focus on cell membrane proteins that interact with JEV concluded that a ~74kDa protein specifically present on the plasma membrane of Vero cells binds the JEV envelope glycoprotein, and that JEV monoclonal antibody inhibition significantly prevents cell-virus binding. This study does not speculate or attempt to confirm the identity of this 74kDa protein, but the molecular weight alone begs the question that the protein singled out here could possibly be HSP70 (Kimura *et al*, 1994).

Collectively, these studies provide a convincing body of evidence with which to confidently suggest that heat shock protein 70 could act as a putative cellular receptor for Japanese

encephalitis virus. However, it is worth considering that, if HSP70 were confirmed to be a primary cellular receptor for JEV, developing treatments which would directly antagonise function of the receptor and bring about a knockdown of JEV infection may detrimentally affect the protein's intracellular chaperone functions.

1.1.6.1.5 Vimentin

Perhaps the most recent addition to the list of putative JEV cellular receptors, or proteins which engage the flavivirus at the cell surface, is vimentin – a type III intermediate filament protein expressed in mesenchymal cells. First presented in 2011, the interaction of vimentin with the JEV envelope glycoprotein in neuroblastoma cells was characterised using a virus-protein binding pull-down assay, which yielded a 57kDa protein which was found to interact with virulent and attenuated variants of the NT109 strain of JEV. Following immunoprecipitation, mass spectrometry and BLAST bioinformatics analyses, the detected protein was identified as vimentin. This result was subsequently verified by antibody neutralisation and vimentin expression knockdown experiments, and JEV binding of vimentin was also exploited to perform mutation analyses on the virus envelope glycoprotein, in order to elucidate an amino acid residue which is integral in vimentin binding (Liang *et al*, 2011).

In the same year, the case for vimentin's role as a putative JEV cellular receptor was strengthened, as another article outlining the interactions of the JEV E protein with vimentin at the membrane of PS porcine kidney cells was published (Das *et al*, 2011). Using conventional methodology of a VOPBA, followed by MALDI-TOF mass spectrometry, an approximately 60kDa protein candidate was revealed to be vimentin, which was then further investigated by the utilisation of anti-vimentin monoclonal antibodies, and also with a co-immunoprecipitation assay, which confirmed the co-localisation and tangible interaction of the viral and cellular proteins (Das *et al*, 2011).

1.1.6.1.6 Integrin $\alpha_v\beta_3$

In a study detailing the interaction of West Nile virus with the integrin $\alpha_v\beta_3$ with regards to mediation of Vero cell entry of WNV, it was also discovered that, when comparing the neutralisation potencies of an anti- integrin $\alpha_v\beta_3$ antibody against other flaviviruses, entry of JEV into the Vero cells was also significantly inhibited, albeit to a lesser extent than WNV. This prevention of cellular entry indicates that JEV may exploit the integrin $\alpha_v\beta_3$ protein on plasma membranes to augment its infection efficiency into certain cell types (Chu and Ng, 2004). Integrins are an important family of proteins involved in the attachment and bridging of the cell cytoskeleton to the extracellular matrix, thus acting as the scaffolding for cell-cell interactions (Alberts *et al*, 2002). This set of cell adhesion molecules are in fact extensively implicated as cellular receptors for a range of viruses, including foot-and-mouth disease virus (FMDV) and the hantaviruses NY-1 and Sin Nombre virus (Ren *et al*, 2007). The fact that integrins are repeatedly presented in the literature as putative cellular receptors for evolutionarily distinct viruses implies the feasibility of integrin $\alpha_v\beta_3$ playing a similar role for JEV, alongside other flaviviruses.

1.1.6.1.7 Nucleolin

Nucleolin is a multi-functional phosphoprotein derived from and localised abundantly within the nucleolus of the cell, although it has also been detected in various other organelles of eukaryotic cells, such as the cytoplasm and at the plasma membrane. Expression levels of nucleolin correlate strongly with the rate of functional activity of the nucleolus, and this protein regulates a range of important nucleolar roles, such as: ribosomal RNA (rRNA) synthesis and expression; biogenesis, maturation and folding of ribosomes; manipulation of chromatin structure; cytokinesis; and the synthesis and nucleocytoplasmic trafficking of nascent pre-RNA molecules (Tajrishi *et al*, 2011). In addition, interactions between viral

proteins and nucleolin, as well as the whole nucleolus, have been widely reported in the literature (Masiuk, 2008).

In microglial cells, nucleolin was identified *via* VOPBA and LC-MS/MS spectroscopic analysis as a protein which interacts with JEV at the cell surface, and could be a potential cellular receptor for the flavivirus. This finding was made by Thongtan *et al* and was discovered alongside the aforementioned high affinity laminin receptor. However, unlike the laminin receptor, upon incubation of microglial cells with an anti-nucleolin monoclonal antibody, it was found that JEV infection was not significantly neutralised, indicating nucleolin probably does not possess the role of cellular receptor for the flavivirus into microglial cells (Thongtan *et al*, 2012).

Despite this study concluding that nucleolin does not mediate Japanese encephalitis virus infection, it is certainly feasible to suggest that it could engage the envelope glycoprotein of JEV at the plasma membrane in some way, since it is well-recognised that nucleolin associates with and is exploited by a wide variety of both RNA and DNA viruses (Hiscox, 2002; Salvetti and Greco, 2014). Firstly, the protein acts as a cellular receptor for human parainfluenza virus type 3 (HPIV-3; Bose *et al*, 2004) and human respiratory syncytial virus (RSV; Tayyari *et al*, 2011), and also appears to contribute towards the entry of human immunodeficiency virus (HIV) into both CD4(+) and CD4(-) cells, as determined by the use of nucleolin-specific ligands at the cell membrane, such as midkine, pleiotrophin and lactoferrin, which resulted in competitive inhibition of HIV infection (Callebaut *et al*, 2001; Said *et al*, 2002; Legrand *et al*, 2004; Said *et al*, 2005; Hovanessian, 2006; Masiuk, 2008). Furthermore, nucleolin appears to be integral for the success of several other viruses throughout their life cycles – for instance, the replication and capsid assembly of adeno-associated virus type 2 (AAV-2) is greatly facilitated by nucleolin co-localisation and binding (Qiu and Brown, 1999), and the phosphoprotein is also involved in the life cycle of herpes simplex virus type 1 (HSV-1), including nucleolin recruitment during HSV-1 replication to augment replicative activity, and also by interacting with the C-terminal of

the HSV-1 UC11 protein, to enable one-way directional trafficking of the herpesvirus from the nucleus to the cytoplasm during virus assembly (Callé *et al* 2008; Greco *et al*, 2011).

However, interestingly, nucleolar proteins such as nucleolin have also been reported to possess an active involvement in the life cycle and proliferation of a number of *Flaviviridae* family members, including HCV, DENV, WNV and, most importantly, JEV. Often, these interactions usually occur with the flaviviral capsid protein and revolve around the upregulation of virus replication (Salvetti and Greco, 2014). Indeed, in the case of JEV, select amino acid residues in the C protein appear to have a bearing on nucleolar and nuclear localisation and detection of the virus, which in turn has a beneficial impact on virus propagation, as determined by mutation analyses resulting in impairment and knockdown of JEV production (Mori *et al*, 2005). Also, a similar nucleolar phosphoprotein to nucleolin, named B23, translocates from the nucleoli to the cytoplasm upon JEV infection, subsequently co-localising and binding to the capsid protein, once again with implications for the efficiency of viral replication (Tsuda *et al*, 2006).

To conclude, the breadth of interaction between nucleolin and various viral proteins begs the question, despite a lack of validation in the literature, of whether this nucleolar phosphoprotein may contribute in some way to the cell entry, infection and replication of Japanese encephalitis virus.

Another consideration regarding the search for putative JEV cellular receptors is that, due to the broad host cell range of the flavivirus, a highly conserved receptor may be required to enable the virus to effectively enter many permissible cell types. Therefore, it may be possible that a non-protein-based cellular receptor is involved in cell entry, such as sialic acid residues in influenza cell entry mechanisms, which are ubiquitously found at the plasma membranes of many cell types.

Surface molecule	Molecular properties and function	Cell line	Mechanism of interaction	References
Heparan sulfate	Glycosaminoglycan – linear polysaccharide involved in cellular adhesion, growth regulation and intercellular signalling	BHK-21 – baby hamster kidney fibroblasts	Initial point of cellular contact with virus – attachment factor that stabilises virion at plasma membrane	Lee <i>et al</i> , 2006
CLEC5A	C-type lectin – roles include cell-cell adhesion, pathogen immune responses, apoptosis and myeloid cell signalling pathway activation	Primary human macrophages Primary mouse neurons, glia and microglia	CLEC5A transmembrane domain reacts with DAP12 adapter molecule, activating signalling pathways, releasing cytokines and exacerbating JEV pathogenesis	Chen <i>et al</i> , 2012
37/67 kDa high affinity laminin receptor	Non-integrin cell surface receptor – binds laminin with high affinity	BV-2 – mouse microglia	Putative cellular receptor – plays a role in facilitating the entry of JEV into host cells	Thongtan <i>et al</i> , 2012
HSP70	Heat shock protein – upregulated in response to cellular stress, intracellular chaperones for protein maturation	Neuro2a – mouse neuroblastoma Huh7 – human hepatoma	Putative cellular receptor – important for attachment and entry of JEV into host cells	Kimura <i>et al</i> , 1994; Ren <i>et al</i> , 2007; Das <i>et al</i> , 2009; Zhu <i>et al</i> , 2012
Vimentin	Type III intermediate filament protein	N18 – mouse neuroblastoma HTB-11 – human neuroblastoma PS – porcine kidney	Putative cellular receptor – critical for facilitating cell entry and infection of JEV	Liang <i>et al</i> , 2011; Das <i>et al</i> , 2011
Integrin $\alpha_v\beta_3$	Integrin – involved in bridging of cell cytoskeleton and extracellular matrix, also act as scaffolding for cell-cell interactions	Vero – African green monkey kidney	Putative cellular receptor – shown to interact with JEV E protein at cell membrane, but not as convincingly as WNV	Chu and Ng, 2004
Nucleolin	Nucleolar phosphoprotein – regulates rRNA synthesis and expression, and mediates cellular transport of newly-synthesised pre-RNA molecules	BV-2 – mouse microglia	Engages JEV envelope glycoprotein at the plasma membrane in some way, but no cellular receptor activity	Thongtan <i>et al</i> , 2012

Table 1. Summary of the host cell surface molecules that are reported to interact with Japanese encephalitis virions and facilitate the entry and infection of the flavivirus into host cells. Surface molecules and their functions, as well as target cell lines, mechanisms of interactions and references are listed.

1.1.6.2 Fusion, expression, assembly and egress

Following cellular receptor engagement, JEV undergoes a process known as clathrin-mediated endocytosis (CME) in order to penetrate the host cell plasma membrane. Receptor binding at the cell surface activates the highly conserved adapter protein 2 (AP2), located proximally to the cytoplasmic side of the plasma membrane, to recruit clathrin proteins to the area, which assemble around the activated binding site and create a clathrin-coated pit (Pearse, 1976). Membrane invaginations begin to form at the internalisation site – clathrin acts to stabilise and strengthen the curvature of the plasma membrane, as it gradually expands to create a coated vesicle rather than a pit. A further protein named dynamin congregates at the attached base of the vesicle, and is responsible for its scission and release from the cell membrane. The clathrin molecules subsequently uncoat and are recycled, permitting the vesicle to migrate and incorporate into early endosomes (McMahon and Boucrot, 2011).

Upon exposure to the acidic environment of this prelysosomal endocytic cellular compartment, domain II of the JEV E glycoprotein swings outwards from the domain I-II hinge region and re-orientates itself towards the host endosomal membrane. This induces a rearrangement of the E proteins into a lateral positioning, enabling the fusion peptide at the apex of domain II to insert itself into the host cell. In turn, an irreversible trimerisation of the JEV E glycoprotein takes place. Once this trimerisation has occurred, domain III bends back upon itself, and in so doing draws the viral and endosomal membranes into close proximity with each other, so that hemifusion can take place (Figure 7; Mukhopadhyay *et al*, 2005; Kaufmann and Rossmann, 2011).

After the virus has undergone fusion, the nucleocapsid is released into the cytoplasm of the host cell. A dissociation between the structural capsid protein and its encapsulated viral RNA occurs, in turn releasing the genetic material into the cytosol. From here, replication and translation of the JEV RNA genome is able to take place, expressing the JEV polypeptide as one

complete, intact protein molecule, encoded from a single open reading frame (Lindenbach and Rice, 2003). The polyprotein then undergoes a variety of co- and post-translational enzymatic modifications by a combination of proteases and signal peptidases derived not only from the host cell, but also from the non-structural regions of the expressed JEV genome itself, including the NS2B-NS3 serine protease complex, the NS2 autoprotease and the NS4A protease co-factor. These enzymes collectively cleave the JEV polyprotein at several points, to yield ten individual viral proteins (Murray *et al*, 2008; Daly and Solomon, 2010). Significant proliferation and upregulation of membranous perinuclear organelles, such as the rough endoplasmic reticulum (RER) and the Golgi apparatus, is also observed in early JEV-infected cells, and these subcellular structures may house 'virus factories' to maximise the rate of RNA synthesis, translation and subsequent progeny virion assembly (Hase *et al*, 1990).

In the first instance, immature JEV virions are assembled and bud from the lumen of the ER. The immature JEV virion includes both the E and the full prM proteins, and possesses a somewhat larger structure (600Å external diameter) than its mature counterparts, with 60 irregular, trimeric E protein spikes protruding from the virus surface. This conformation exposes the prM proteins, which sit at the apical tip of each E trimer and are consequentially cleaved by furin-like proteases in the acidic trans-Golgi network (TGN), releasing the precursor section from the remainder of the M protein (Kaufmann and Rossmann, 2011). This cleavage disrupts the E protein spike trimer, enabling the rearrangement of the envelope into the flat, spikeless and smooth icosahedral pattern characteristic of the mature JEV virion (Mukhopadhyay *et al*, 2005).

Subviral particle (SVP) production is routinely observed and is a hallmark trait in JEV infection, as well as with many other flaviviruses. SVPs share the same smooth exterior as mature JEV virions, but have an average diameter of 315Å, and are comprised solely of a lipid membrane and the E and M proteins. Like with the complete virus, JEV SVPs are also assembled at the

endoplasmic reticulum, and following expression and budding, they are subject to the same modifications in the trans-Golgi network. However, they are subsequently non-infectious, since they lack a nucleocapsid. Both mature JEV virions and subviral particles egress from the host cell *via* exocytosis (Mukhopadhyay *et al*, 2005).

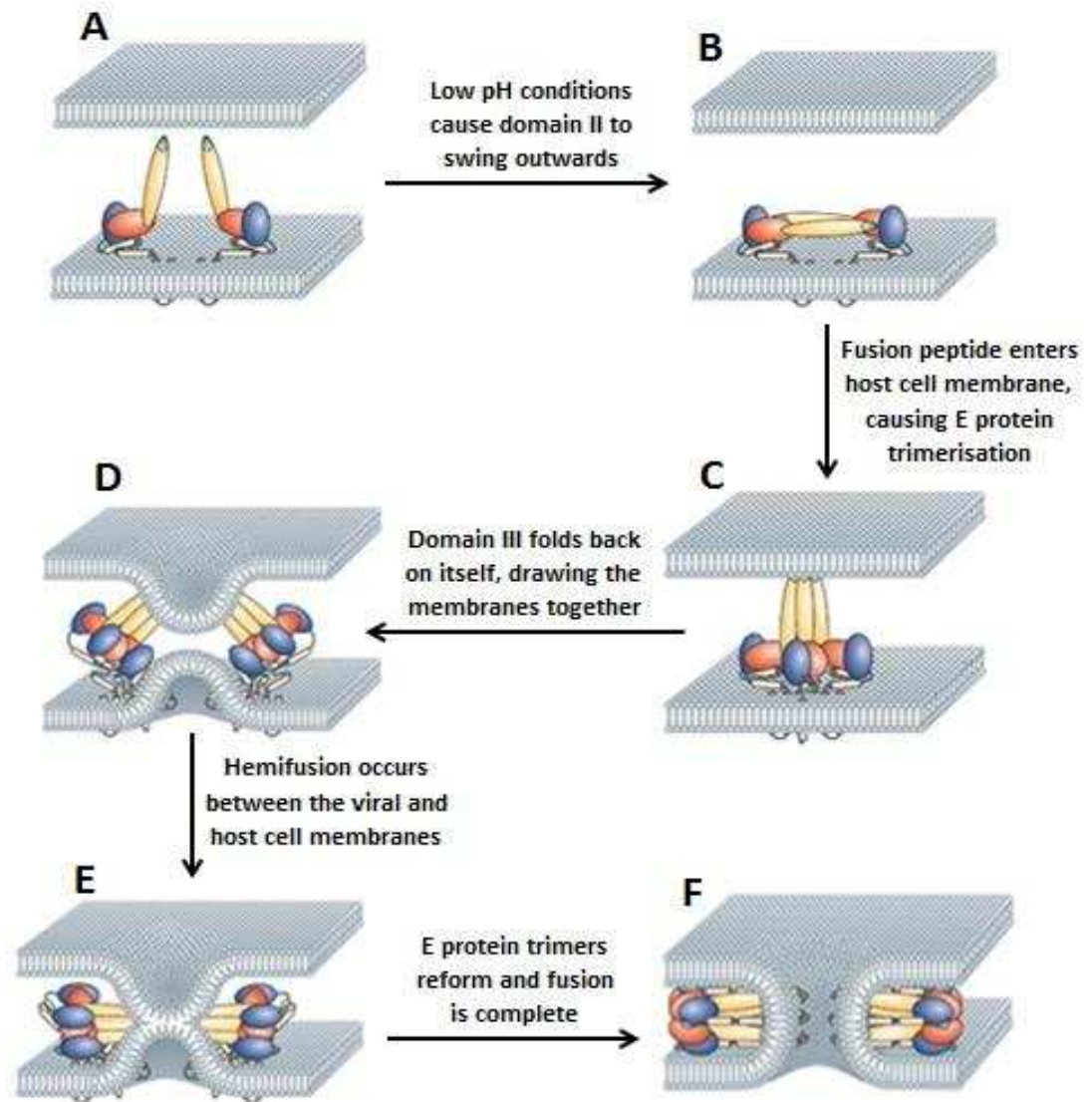


Figure 7. Schematic of the proposed Class II mechanism of fusion between the viral and endosomal membranes necessary for JEV entry into and infection of target cells. A) The dimeric JEV E protein is lying flat on the virus surface, with the fusion peptide (shown in green) buried into the dimer. B) Upon receptor binding and internalisation into the host cell endosome, low pH conditions induce an outward swinging action of domain II (in yellow), most likely at the domain I-II hinge region (domain I in red). C) Lateral rearrangement of the E proteins takes place, enabling fusion peptide insertion into the outer leaflet of the host cell membrane and subsequent E protein trimerization. D) Folding of domain III (in blue) of the E protein back onto itself results in drawing the viral and host cell membranes into close proximity with one another. E) Following the continuing movement of domain III towards domain II, hemifusion occurs between the two membranes. F) An E protein trimer forms, where the transmembrane domain and fusion peptide are near each other (adapted from Mukhopadhyay *et al*, 2005).

1.1.7 Immunity and pathogenesis

1.1.7.1 Humoral immunity

The humoral, antibody-mediated immune response against JEV is well-documented in the literature and known to play a central role in protection against the virus. Upon primary infection with JEV, an IgM antibody response is raised in the patient, usually within 7 days of initial infection, and correlates well with a positive disease prognosis if observed within this timeframe. Maximum serum IgM levels are usually reached about 9 days post-infection onwards (Solomon *et al*, 2000). Presence of IgM at raised levels in both the sera and cerebrospinal fluid (CSF) is often seen in symptomatic, encephalitic patients; however, an IgM response is not usually discernible in the CSF of asymptomatic patients but only in serum samples, further suggesting that viral infiltration of the CNS is concomitant with the development of characteristic JEV symptoms. Isolation of the virus in patient samples has only been achieved when a potent IgM response is absent, as antibody-mediated neutralisation has not curbed the levels of viraemia, and this is associated with patient fatality. Immunoglobulin class switching subsequently takes place in convalescent patients, and by 30 days post-infection, the majority of antibodies present in positive serum or CSF samples are class IgG (Abraham *et al*, 2011).

Indeed, virus neutralising antibodies (VNABs) of either class IgM or IgG, which bind to antigenic epitopes on viral proteins and inhibit their entry into host cells, are primarily elicited against the E glycoprotein of JEV and alone are able to confer strong levels of protective immunity from the virus (Kimura-Kuroda and Yasui, 1988; Zhang *et al*, 1989; Pan *et al*, 2001). Antibody responses have also been observed against a variety of non-structural JEV proteins, but these almost invariably offer negligible to no protection from the virus (Lin *et al*, 2008). VNABs work to inhibit the replication and spread of JEV infection, in turn mediating the damaging cytopathic and encephalitic effects of the virus.

In some instances, flaviviruses are able to exploit the adaptive immune system to their own advantage, with a process known as antibody-dependent enhancement (ADE) of infection. IgM or IgG antibodies bind specifically to viral proteins with their Fab fragments. This engagement is intended to neutralise the virus. However, Fcγ receptors present on immune cells, such as B lymphocytes, macrophages and neutrophils, also bind the antibody-virus complex by the antibody's Fc fragment, which should stimulate the immune cells to initiate phagocytosis or cell-mediated cytotoxicity to destroy the virus, but instead brings the virus particles into close proximity with the surface of the immune cells, leaving them susceptible to infiltration and infection by the flavivirus. Internalisation of the virus can also be brought about by antibody-mediated activation of the classical complement cascade, and consequential binding to the C1q receptor on target cell surfaces. The phenomenon of ADE has been observed for DENV and YFV, as well as for JEV (Gould and Buckley, 1989; Kliks *et al*, 1989).

1.1.7.2 Cell-mediated immunity

Despite being an area of ongoing research and development, our knowledge of cell-mediated immunity against Japanese encephalitis virus is more rudimentary than that of its humoral counterpart. Early studies showed that thymus-deprived mice displayed an impaired anti-JEV antibody response, suggesting a significant role for T cell immune responses in the activation of corresponding B cell immunity against JEV (Mori *et al*, 1970). Furthermore, a certain species of monkey that normally exhibits resistance to JEV became susceptible to the virus when T cell function was suppressed (Nathanson and Cole, 1970). It appears inconclusive whether both CD4+ and CD8+ T-cell responses are both required for protection against JEV – some studies report that both immune populations are necessary, and that depletion of either class results in the loss of functional protective capacity in murine subjects (Murali-Krishna *et al*, 1996); however, other studies conflict with these findings, postulating that CD4+ knockout mice, immunized with an envelope DNA vaccine, experienced an abrogation of immune protection

upon JEV challenge, while this phenomenon was not witnessed in counterpart CD8⁺ knockout mice, concluding that only CD4⁺ T-cells were required to contribute to functional immunity against JEV (Pan *et al*, 2001). Indeed, the activation of CD8⁺ T-cell responses has also been shown to potentially contribute to the pathogenicity of related flaviviruses, such as DENV, as well as MVEV, where a lack of the expression of perforin and Fas/FasL conferred host protection against the virus (Licon Luna *et al*, 2002; An *et al*, 2004).

Since then, both CD4⁺ and CD8⁺ T-cell responses against JEV have been identified in peripheral blood mononuclear cell (PBMC) samples, collected from vaccinated patients who had received a purified JEV vaccine, inactivated with formalin and directed against the virus' structural proteins. Interestingly, vaccine recipients displayed T-cell responses of both subsets, primarily targeting structural proteins, whereas in contrast, patients infected naturally by JEV presented with CD4⁺/CD8⁺ T-cell responses against the capsid and non-structural proteins (Konishi *et al*, 1995).

Indeed, the viral non-structural proteins act as important immunogens in the initiation of T-cell responses, with NS3 eliciting the strongest cell-mediated response. Amino acid residues 193-324 on the JEV NS3 protein act as a dominant epitope for the binding of CD4⁺ and CD8⁺ T-cells, and subsequent IFN- γ production stimulated by NS3-binding suggests that an active, pro-inflammatory Th1 response against this non-structural protein may contribute as a crucial aspect of immune control against JEV (Kumar *et al*, 2004a; Kumar *et al*, 2004b).

A more recent study was the first to conduct a full-breadth analysis of the human T cell response to JEV by systematically mapping JEV epitopes, using a full-length, synthetic peptide library. The study concluded that T-cell responses in healthy, JEV-exposed donors are primarily CD8⁺, and predominantly targeted against the NS3, NS4 and NS5 proteins of the virus, whereas recovered JE patients mostly mount JEV-specific CD4⁺ T-cell responses. Additionally, these responses, in particular the CD8⁺ responses observed in healthy donors, reveal an

extensive level of cross-reactivity with other flaviviruses, such as DENV and ZIKV. Overall, a high-quality, polyfunctional CD4+ T-cell response was associated most strongly with complete recovery from JE infection (Turtle *et al*, 2016).

1.1.8 Vaccines

JEV is a vaccine-preventable disease and a variety of vaccines of different derivations have been developed. The first to be licensed was a mouse brain-derived, killed-inactivated vaccine based on the prototype Nakayama strain of JEV. This was commercially distributed under the name JE-VAX. Although highly efficacious and immunogenic, JE-VAX possessed a number of drawbacks, including high cost, varying levels of side-effects and the necessity to administer multiple primary doses plus vaccine boosters (Yun and Lee, 2014). More recently, inactivated, Vero cell culture-derived vaccines have become more commonplace and are based upon either SA-14-14-2 or Beijing-1 strains of JEV (WHO, 2014b). These vaccines are licensed under several different names, such as ENCEVAC, based on Beijing-1, and IXIARO, which is adjuvanted with an aluminium hydroxide compound and based on SA-14-14-2. These JEV vaccines are advantageous as they are generally considered to be safer than JE-VAX. Also, cell culture-derived vaccines are more amenable for large-scale production than those derived from mouse brain (Yun and Lee, 2014). A recombinant, live-attenuated chimeric vaccine produced by introducing the structural prM and E genes from JEV SA-14-14-2 into the YFV 17D vaccine strain has also recently been licensed under the name ChimeriVax, and is commercially available in some countries, such as Australia and Thailand (Monath *et al*, 2002). Experimental vaccine technologies currently in the pipeline include: the production of virus-like particles displaying particularly immunogenic peptide portions of the E protein of JEV; the use of recombinant vaccinia or canarypox viruses as viral vectors to deliver the genes of JEV antigens to produce *in vivo* protective immunity; and intramuscular immunization with plasmid DNA encoding the JEV prM and E proteins (Daly and Solomon, 2010, Yun and Lee, 2014).

1.1.9 Antivirals

To date, there are no fully licensed antivirals available for treatment of patients infected by JEV. Treatment is purely supportive and involves symptom relief and stabilization of patients with severe encephalitis. However, a number of promising candidates for JEV antiviral therapies have been developed in recent years including minocycline, a tetracycline derivative antibiotic which has also been shown to be protective in cases of flaviviral infection, by alleviating a number of JEV pathologies, such as neuronal apoptosis, microglial activation, caspase activity and release of proinflammatory cytokines (Dutta *et al*, 2010); glucosidase inhibitors situated at the endoplasmic reticulum (ER) that restrict *N*-linked glycosylation and hinder the production of a number of ER-budding viruses; and rosmarinic acid, which is a phenolic compound found in the *Labiatae* family of herbs, which also reduces neuronal apoptosis and cytokine accumulation (Saxena *et al*, 2014). However, all these potential antivirals would have to undergo a rigorous clinical trial system before commercial utilisation.

1.1.10 Diagnosis and serology

As there are several causes of acute encephalitic syndrome, laboratory confirmation of JEV infection is essential for diagnosis. This could be achieved *via* direct virus isolation or reverse transcription polymerase chain reaction (RT-PCR) of viral RNA – however, due to the transient and moderate viral load in symptomatic patients, this can be problematic. Therefore, diagnosis is often retrospectively ascertained by determining antibody levels in patient serum or CSF samples (Solomon *et al*, 2000).

A variety of serological assays that quantify antibody titres raised against viral proteins (in the case of JEV, the majority of antibodies target the E glycoprotein) have been developed for the diagnosis of flaviviruses.

1.1.10.1 Detection of JEV IgM antibodies

Following infection of JEV, the first step of acquired immune response is the production of IgM antibodies against viral antigens, which are usually present at a detectable level at 6 to 14 days post-infection (Maeda and Maeda, 2013). However, this antibody class can persist in convalescent stage patients for up to 4 months post-infection (Roehrig *et al*, 2003; Prince and Matud, 2011). The serological assay primarily used to detect this immunoglobulin class in potentially-infected subjects is the MAC-ELISA (Martin *et al*, 2000; WHO, 2003). For this assay, the viral antigens to calibrate the IgM antibody level in patient serum samples can be acquired from various sources, such as infected tissue culture cells, virus-infected mouse brains, and recombinant virus antigens. The specificity and sensitivity of the MAC-ELISA varies depending on the source of the antigen used, and in some cases when changing the target antigen, the specificity can rise while the sensitivity falls and vice versa. Moreover, the relatively low specificity of this assay makes it difficult to distinguish between similar clinical virus infections. For example, JEV and DENV co-circulate in many regions of India, and WNV has also been shown to be responsible for a proportion of encephalitis cases in India, leading to cross-reactivity issues and low specificity when using the MAC-ELISA (Khan *et al*, 2011). In an attempt to overcome this, two commercially available and commonly-used MAC-ELISA kits, the Panbio® Japanese encephalitis–Dengue IgM Combo ELISA (Alere, Australia) and the Venture Technologies Dengue–JEV modified antigen capture ELISA (Universiti Sains Malaysia, Malaysia), function by simultaneously testing for antibodies to JEV and DENV, with the highest antibody response being indicative of the virus causing the infection (Lewthwaite *et al*, 2010). Another obstacle when utilising this assay is that cerebrospinal fluid (CSF) is the ideal sample to use, in order to diagnose a flavivirus infection with viral encephalopathy as opposed to an incidental peripheral infection, but CSF can be difficult to extract with only limited volumes in children, who are commonly infected with JEV and other encephalitis flaviviruses due to their undeveloped immunity through vaccination or long-term exposure (Anuradha *et al*, 2011).

1.1.10.2 Detection of JEV IgG antibodies

If IgG class antibodies elicited against JEV E protein, or other viral antigens, are present in a patient's serum sample, this is indicative of a long-term infection (Kuno, 2003). In order to detect antibodies of this class, either an IgG-ELISA or an indirect fluorescent antibody (IFA) test is commonly used (Malan *et al*, 2003; Foral *et al*, 2007). A good advantage for the use of these assays is their simplicity, leading to them being commonly used for identifying JEV and other flaviviral infections. However, their low levels of specificity, in a similar vein to the MAC-ELISA, mean that it is difficult to make a specific and definitive diagnosis using these assays alone, and a confirmatory plaque reduction neutralisation test (PRNT) is often required in addition (Maeda and Maeda, 2013).

1.1.10.3 Haemagglutination inhibition (HI)

Haemagglutination inhibition (HI) assays have been consistently used for flavivirus serodiagnosis for many years, as well as for several other viruses from different taxonomic families, including influenza, rabies, and members of the *Alphavirus* and *Bunyavirus* genera (Nagarkatti and Nagarkatti, 1980). The assay works by measuring the total antibody response, including IgM and IgG class antibodies, raised against JEV *via* inhibition of virus-mediated erythrocyte agglutination. It is a cheap assay to perform as it does not require any specialised equipment, instrumentation or expensive antibodies, and a main advantage of this test is that it is not species-specific, meaning that serum samples extracted from a variety of species commonly infected by JEV can be employed in this assay and retrospectively diagnosed. Disadvantages of the HI assay include the results often displaying high levels of cross-reactivity between flaviviral infections, as well as the measurement of binding and not necessarily neutralising antibodies, and quality control problems regarding the species from which the erythrocytes are sourced for the assay (Cha *et al*, 2014).

1.1.10.4 Plaque reduction neutralisation test (PRNT)

The plaque reduction neutralisation test (PRNT) is generally regarded as the 'gold standard' serological assay due to its high level of specificity, enabling it to distinguish not only between different flavivirus infections, but also between immunoglobulin classes (Johnson *et al*, 2009; Maeda and Maeda, 2013). This assay is performed by serially diluting patient samples, such as serum or alternatively cerebrospinal fluid (CSF), and mixing with a constant amount of virus. Following an incubation step of a sufficient duration to allow any antibodies in the patient sample to bind to antigens on the virus surface, the mixture is added to a confluent monolayer of cells permissive to infection by the virus being tested for. After a second incubation step to account for virus attachment and entry into the target cells, the assay medium is aspirated and replaced with a semisolid overlay medium such as agarose or carboxymethylcellulose (CMC), so that once set, progeny viruses released from infected cells are prevented from spreading to and infecting non-adjacent cells in the same well (Calisher *et al*, 1989). This technique results in the formation of plaques or lesions in the cell monolayer caused by virus-induced cytopathic effect (CPE) – these plaques are then used to indirectly quantify virus infection in the assay and are commonly given the unit of plaque forming units per ml (PFU/ml). The antibody concentration which confers a reduction of plaque formation at a given percentage (usually 50% or 90%), compared to a positive virus control sample known to be free of antibodies, enables the calculation of a PRNT₅₀ or PRNT₉₀ neutralising antibody titre (as reviewed in Mather *et al*, 2013). If neutralising antibodies are being detected against a virus that does not cause discernible CPE in target cells, an altered PRNT known as the focus forming assay can be employed, which uses immunostaining with fluorophore- or enzyme-conjugated secondary antibodies. The localised clusters of infected cells can then be counted using fluorescent microscopy and measured as focus forming units per ml (FFU/ml) (Payne *et al*, 2006). Flow cytometry is an alternative, less conventional assay readout approach for viruses that neither cause CPE nor form plaques measurable using the focus forming assay (Kraus *et al*, 2007).

The ability of the PRNT to distinguish between antibodies against different virus species, as well as to determine type-specific antibodies due to its high specificity, is by far the most significant advantage of this assay. However, it also possesses some drawbacks, including the requirement for wild-type infectious virus, necessitating the use of expensive, high-containment tissue culture facilities and extensive personnel training. Also, the assay is laborious and can take up to 7-10 days for definitive plaques to develop, which restricts throughput. Finally, unlike some of the aforementioned serological assays, the PRNT requires a readily culturable cell line which is permissive to infection by the given virus (Maeda and Maeda, 2013).

1.2 Surrogate virus particles for flavivirus serology

Considerable research focus has been invested in developing novel antigenic reagents for serological assays that enable sensitive and specific quantification of neutralizing antibodies raised against flaviviruses, as well as other viral families, whilst addressing persistent dilemmas of high biosafety containment and prohibitive expense associated with traditional methods. These surrogate viruses employed in innovative serological assays are primarily based on the genetic manipulation of the viral genome, especially concerning the genes that encode the structural viral proteins, in order to attenuate pathogenicity but maintain a virus particle with serological value.

1.2.1 Genetically modified and recombinant viruses

Recent advances in technology facilitating the production of recombinant DNA have allowed for the manipulation of flaviviral genomes, to enhance the development and evaluation of novel vaccines, as well as to perform serosurveillance and track the spread of emerging viruses of the *Flaviviridae* family. A chimeric virion, in which the pre-membrane and envelope protein genes of WNV were inserted into the corresponding region of the yellow fever vaccine virus 17D strain genome, was engineered and characterised as a live attenuated vaccine candidate (Arroyo *et al*, 2001). However, this viral chimera was also subsequently utilised as a BSL-2 reagent in PRNT studies, to retrospectively establish the infection profiles of species involved in the 1999 outbreak of WNV in north-eastern USA. A 96% concordance between results obtained by native WNV and the WNV-YFV chimera was observed when evaluating panels of equine and avian sera sampled from the outbreak, with high levels of sensitivity and specificity and comparable virus neutralizing antibody titres recorded (Komar *et al*, 2009). Genetic modifications of the genomes of emerging viruses, such as flaviviruses, could have important ramifications not only for vaccine research and development, but also for use as serological

tools for the quantification of neutralising antibodies, whilst circumventing requirements for high biosafety levels.

1.2.2 Reporter virus particles

One form of low pathogenic, surrogate virus particle which has been constructed and applied widely across the area of flavivirus biology is the reporter virus particle (RVP). For Japanese encephalitis virus, a rapid production method to yield JEV RVPs has been established (Suzuki *et al*, 2014). This methodology initially revolves around the assembly of a sub-genomic replicon (SGR) of viral RNA, possessing all of the necessary viral non-structural genes, but omitting the C, prM and E genes. This SGR can then be transiently co-transfected into producer cells alongside a DNA expression plasmid bearing the corresponding JEV structural genes, ultimately creating by trans-complementation pseudo-infectious virions that encapsidate the SGR. These RVPs are only capable of one round of infection into target cells, since the replicon packaged into the virus particles does not contain the structural viral genes (Suzuki *et al*, 2014). In other studies, RVPs, also known as single-round infectious particles (SRIPs) and pseudo-infectious particles (PIPs), have been produced with reporter genes, such as enhanced green fluorescent protein (eGFP) or renilla luciferase (R. luc) incorporated into the JEV replicon system, usually as a direct substitute to the structural genes, enabling an easier method with which to validate successful SGR packaging, RVP formation and transduction, and for clarity in downstream applications (Li *et al*, 2013). The reporter gene can also be appended to the 3' untranslated region of the replicon, although this is less frequently seen. Once generated, JEV reporter virus particles have been used to facilitate the characterisation of novel genotype V strains of the virus, and as vaccine candidates, due to their immunogenicity (Huang *et al*, 2012; Ishikawa *et al*, 2015). Since replicons are easily manipulated by molecular cloning, it is also possible to incorporate the genes of heterologous antigenic epitopes into the SGR, creating RVPs able to confer dual immune protection in immunized mice upon lethal challenge (Huang *et al*, 2015).

In addition to JEV, a variety of other flaviviruses have been modified and used to create RVPs for subsequent usage in serological assays. Kunjin virus (KUNV) was among the first flaviviruses for which trans-packaging technology was developed for RVP production, and encapsidation was originally achieved following sequential electroporations of the KUNV SGR, followed by a second, recombinant replicon of Semliki Forest virus (SFV) carrying the KUNV structural protein genes. Expression and assembly of the KUNV RVPs was verified by Northern blotting and immunofluorescence, and anti-KUN E monoclonal antibodies were able to potently neutralize the virus-like particles (Khromykh *et al*, 1998). This discovery has been refined and advanced somewhat with the generation of a tetracycline-inducible packaging cell line, which constitutively expresses a Kunjin structural gene cassette, meaning that encapsidation and RVP production can be accomplished solely by transfection of the relevant replicon RNA (Harvey *et al*, 2004). More recently, WNV, the taxonomic 'parent' virus of KUNV, has also successfully been incorporated into the replicon system, with luciferase- and GFP-expressing SGRs created for encapsidation, and a packaging cell line also being developed for ease of RVP production (Pierson *et al*, 2006; Fernández *et al*, 2014). In this case, the WNV RVPs were utilised in downstream assays to assess the neutralizing efficiency of the monoclonal antibody 7H2 (Pierson *et al*, 2006). However, these virus-like particles could be used for other subsequent applications, such as comparative serology. DENV RVPs have been generated in several studies, using the genomes of different subtypes of the virus, as well as introducing a number of reporter genes into the replicon platform (Lai *et al*, 2008; Zou *et al*, 2011; Mattia *et al* 2011). Among these, a panel of GFP-expressing RVPs mimicking all four of the DENV serotypes were developed and employed to quantify neutralising antibody levels in human serum samples. Results correlated strongly with those obtained by the conventional PRNT and were serotype-specific, overcoming a persistent problem with DENV serology (Mattia *et al*, 2011). Other flavivirus RVPs to be successfully produced include TBEV and YFV (Gehrke *et al*, 2003; Jones *et al*, 2005).

Reporter virus particles are a promising solution to the ongoing problems of handling native, wild-type virus, such as high biosafety containment requirements and the associated costs for this infrastructure, as well as being easy to manipulate for mutagenesis and serosurveillance studies. Furthermore, using RVPs, assays are robust and reproducible and issues of low-throughput inherent with PRNT-based studies are resolved.

1.2.3 Virus-like particles produced in insect cells

A series of studies conducted by Yamaji *et al.* in recent years have detailed multiple techniques for the production of JEV virus-like particles using insect cell expression systems (Yamaji and Konishi, 2016). This technology harnesses the characteristic of flavivirus capsid and envelope proteins to self-assemble when expressed into particulate structures which closely resemble authentic virus particles. However, VLPs are replication-deficient and non-infectious, since no incorporation of viral RNA takes place upon particle formation (Noad and Roy, 2003).

Using the established baculovirus-insect cell expression system, JEV VLPs have successfully been generated. First, a recombinant *Bombyx mori* baculovirus is constructed bearing the prM signal sequence and the genes encoding the JEV prM and E proteins. Baculoviral infection of lepidopteran insect cell lines, such as Sf9 or High Five cells, results in high levels of VLP secretion, as determined by Western blotting and ELISA of the JEV E protein, as well as sucrose-density gradient sedimentation analysis to verify the particulate formation of the E antigen molecules. Utilisation of Sf9 cells enables a 10-fold increase in expression of the gene of interest, when compared to corresponding mammalian expression cell lines, such as CHO (Yamaji *et al.*, 2012). A primary advantage of the baculovirus-insect cell system is the ease with which genes can be incorporated into the baculovirus genome for expression, allowing for the manufacture of many antigenic proteins in VLP form in a short space of time.

However, a similar yet distinct alternative to the baculovirus platform is that of stably transformed lepidopteran insect cells. In this method, the structural protein genes of JEV are cloned into a plasmid expression vector, importantly under the control of the *Bombyx mori* polyhedrin promoter, which is responsible for the high expression of antigen observed in both systems. A blasticidin resistance gene is also included, so that transformed High Five cells can be selected for, to achieve a stable lepidopteran insect cell line. When constitutively expressed, higher amounts of expressed E protein were observed, in comparison to the baculovirus infection technique (Yamaji *et al*, 2013; Yamaji and Konishi, 2013). The stably transformed insect cells are more suitable for the production of complex secreted proteins in VLP form, since the machinery necessary for protein synthesis, processing and assembly is not damaged by baculovirus infection.

Insect cell expression systems offer a novel approach for the rapid, large-scale manufacture of VLPs, which can subsequently be used to induce neutralizing antibodies as vaccine candidates, or as antigenic particles in serological assays and clinical diagnostics.

1.3 Pseudotype viruses

1.3.1 Overview and functions

Pseudotype viruses (PVs) are increasingly being used in serological assays for the diagnosis of viral infection or vaccine seroconversion (Table 2). A pseudotype is a chimeric virion that comprises the structural and enzymatic core of one virus and the heterologous envelope glycoprotein(s) of another, which mimics the entry mechanisms of wild-type viruses and can be safely employed in neutralisation assays. Retroviruses are often employed as the core for this technology, with lentiviruses and gammaretroviruses such as HIV and murine leukemia virus (MLV) providing an ideal pseudotype backbone. Rhabdoviruses, such as vesicular stomatitis virus (VSV), and other retroviruses, such as equine infectious anemia virus (EIAV), are also increasingly used as pseudotype cores. Their RNA genomes are manipulated to encode a quantifiable marker gene, which is packaged by retroviral core proteins (Mather *et al*, 2013). Transduction of the target cells by the pseudotype is dependent on the ability of the envelope protein to engage receptors on the cell surface. If entry is successful, the RNA genome is transferred from virus to cell, resulting in reporter gene reverse transcription, genome integration and expression. Levels of marker protein expressed in infected cells can subsequently be measured, which produces a quantitative readout synonymous with the function of the foreign envelope glycoprotein (Temperton *et al*, 2015).

Virus family	Virus genus & members	Core vector system	Reporter protein	Example publication(s)
<i>Orthomyxoviridae</i>	Orthomyxovirus – Influenza A virus (H1, H3, H5 subtypes)	HIV, MLV	Firefly luciferase, GFP	Nefkens <i>et al</i> , 2007; Temperton <i>et al</i> , 2007; Wang <i>et al</i> , 2008
<i>Rhabdoviridae</i>	Lyssavirus - Rabies, Lagos bat virus, Mokola virus, Duvenhage virus, European bat lyssavirus (EBLV) -1/-2	HIV, MLV, EIAV	Firefly luciferase, GFP, β -galactosidase	Mazarakis <i>et al</i> , 2001; Wright <i>et al</i> , 2008, 2009, 2010
<i>Coronaviridae</i>	Coronavirus - SARS	HIV, VSV	GFP, β -galactosidase	Simmons <i>et al</i> , 2004; Fukushi <i>et al</i> , 2005; Temperton <i>et al</i> , 2005
<i>Flaviviridae</i>	Flavivirus – JEV Hepacivirus - hepatitis C	MLV, HIV	GFP, β -galactosidase	Bartosch <i>et al</i> , 2003; Lee <i>et al</i> , 2009
<i>Filoviridae</i>	Filovirus - Ebola, Marburg	HIV	Luciferase	Chan <i>et al</i> , 2000
<i>Bunyaviridae</i>	Hantavirus - Hantaan, Seoul Orthobunyavirus – La Crosse	MLV, VSV	GFP, β -galactosidase	Ogino <i>et al</i> , 2003; Ma <i>et al</i> , 1999
<i>Paramyxoviridae</i>	Henipavirus – Nipah, Morbillivirus – Measles, CDV, PPRV	HIV, VSV	Renilla/firefly luciferase, GFP, secreted alkaline phosphatase (SEAP)	Frecha <i>et al</i> , 2008; Kaku <i>et al</i> , 2009; Tamin <i>et al</i> , 2009; Humbert <i>et al</i> , 2012; Logan <i>et al</i> , 2016a, 2016b
<i>Togaviridae</i>	Alphavirus - Ross River virus, Chikungunya virus	MLV	Luciferase	Salvador <i>et al</i> , 2009; Sharkey <i>et al</i> , 2001
<i>Retroviridae</i>	Lentivirus - HIV	MLV	β -galactosidase	Kim <i>et al</i> , 2001

Table 2. Establishment of the pseudotype platform across several families of emergent RNA viruses (adapted from Mather *et al*, 2013). Along with the conventional retroviral vector system, vesicular stomatitis virus (VSV) core pseudotyping is also commonly utilised. Reporter gene flexibility within the plasmid co-transfection method for pseudotype production enables cost customisation of the pseudoparticles, maximising the scope for laboratories with varying budgets to incorporate the pseudotype system.

1.3.2 Production

PVs are created by simultaneous introduction of the envelope gene, retroviral *gag-pol* genes (responsible for the manufacture and enzymatic processing of the core structural proteins and insertion of the reporter gene into the host chromosome) and the chosen reporter gene into producer cells such as HEK293T cells, using a multi-plasmid co-transfection system (Figure 8). After transcription and translation of the imported genes by the relevant cellular machinery, an RNA dimer of the reporter gene is packaged into the core; these processes are driven by an upstream promoter and a packaging signal, Ψ , respectively. The same packaging signal is omitted from the *gag-pol* construct to prevent replication competence and nullify the potential risk of pathogenic virus proliferation. PV capsids are subsequently induced by further signals in order to transit to the plasma membrane of the producer cell before they bud extracellularly. The virus envelope bearing the heterologous glycoprotein is usually formed from the plasma membrane. This process results in a virus-rich supernatant of culture medium, which can be harvested and titrated on the target cell. The reporter gene is flanked by long tandem repeats (LTRs); these facilitate integration into the target cell genome. Integration is catalysed by the lentiviral polymerase/integrase, which is packaged as part of the pseudotype virion. The titre of the PV is calculated as a function of reporter gene expression (Mather *et al*, 2013; Temperton *et al*, 2015).

Alongside serology, pseudotype viruses have been utilised for a variety of other functions (Temperton *et al*, 2015). These include the identification of virus cellular receptor targets and the elucidation of specific viral entry processes (Wang *et al*, 2004; Simmons *et al*, 2004); the study of innate antiviral processes mediated by post-entry cellular restriction factors, such as TRIM5 α and APOBEC (Bae and Jung, 2014); the screening of novel antiviral compounds to inhibit viral entry and egress mechanisms (Su *et al*, 2008; Basu *et al*, 2014); the delivery of therapeutic genes for clinical treatment purposes (Bischof and Cornetta, 2010); and the

incorporation of pseudotypes as immunogenic antigen in vaccine preparations (Szécsi *et al*, 2006; Breckpot *et al*, 2010).

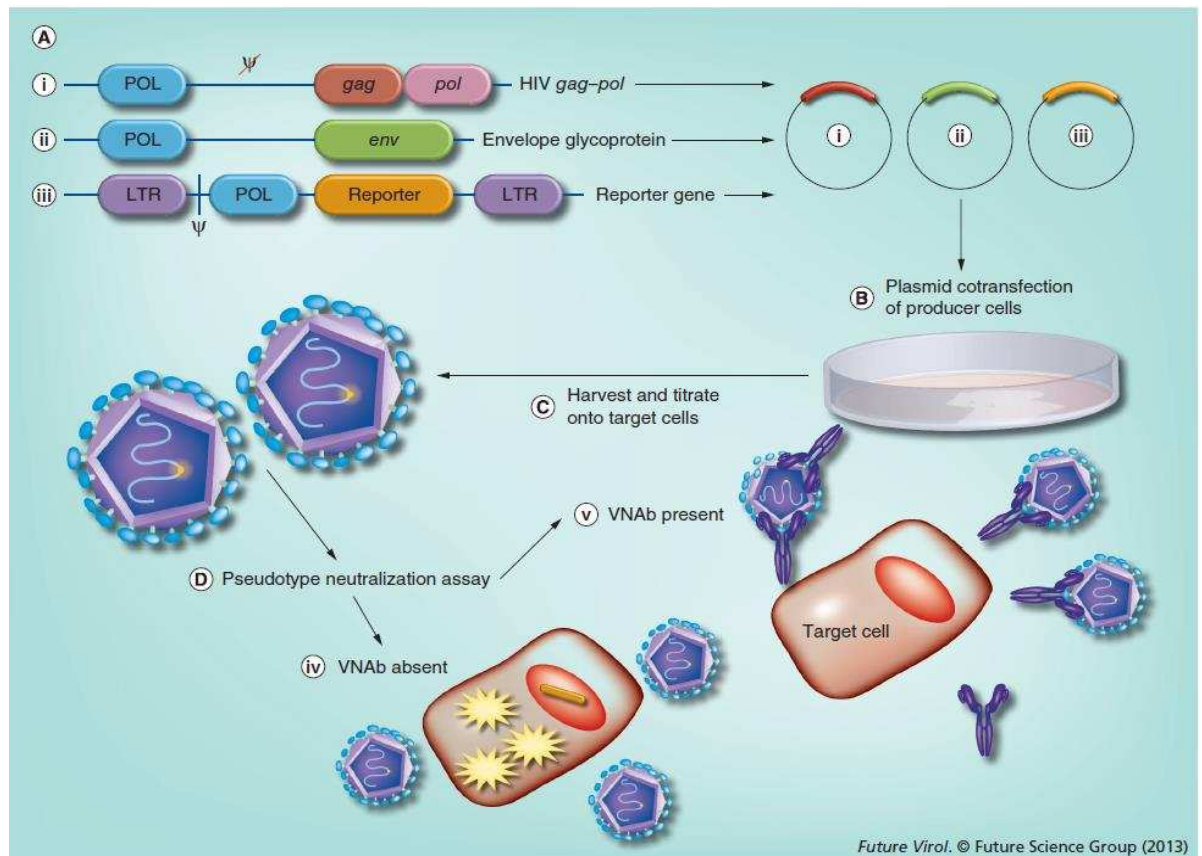


Figure 8. Three-plasmid co-transfection method for pseudotype virus production. (A) Conventional plasmid DNA expression vectors bearing (i) the HIV *gag-pol* gene, (ii) the envelope glycoprotein from the virus of interest and (iii) a reporter gene (e.g., luciferase) are generated. (B) All three plasmids are transfected into ‘producer’ cells (e.g., HEK293 T cells). (C) PV supernatants are harvested and titrated onto permissible target cell lines, to obtain a relative transduction titer. (D) PVs can be subsequently employed as surrogate viruses in pseudotype neutralization assays to quantify VNAb responses. (iv) In the absence of VNABs, the envelope protein of the virus of interest enables entry of the PV into the target cell and the reporter gene is integrated and expressed. (v) Binding of the envelope protein by specific antibodies in the sample blocks entry of the PV into the target cell, thus preventing expression of the reporter gene. The antibody titre can be expressed as the highest dilution of sample that inhibits expression by 50 or 90% (figure sourced from Mather *et al*, 2013).

1.3.3 Advantages

Using pseudotype viruses as serological reagents addresses a number of recurrent disadvantages present in the employment of traditional serological assays. PVs are able to be utilised in neutralisation assays in low bio-containment laboratories, bypassing the need for native, pathogenic virus which dramatically widens the scope of laboratories able to effectively diagnose suspected cases of viral infection (Mather *et al*, 2013).

The flexibility of reporter genes that can be incorporated into the co-transfection stage of pseudoparticle production is another major advantage of the viral pseudotyping approach (as reviewed in Mather *et al*, 2013). The most frequently used reporter genes are GFP, firefly luciferase and renilla luciferase. Luciferase can be regarded as the 'gold standard' reporter gene for the pseudotype platform, with respect to the assay's readout preparation time and quantitative data analysis, although the cost of luciferase reagent kits and the necessity for specialist detection equipment may restrict its widespread application. Use of a GFP reporter does not necessitate additional reagents for reading neutralization titres, but nevertheless requires relatively expensive and time-consuming readout equipment, such as a fluorescent microscope. Alternatively, β -galactosidase substrates can be used to quantify pseudotype and VNAb titers by the introduction of the *lacZ* gene as a reporter (Wright *et al*, 2009). The readout can be obtained in a cost-effective fashion, either by counting cells under a light microscope after incubation with 5-bromo-4-chloro-3-indolyl- β -d-galactopyranoside (X-gal) or by the evaluation of a colorimetric substrate – either *O*-nitrophenyl- β -d-lactopyranoside (ONPG) or chlorophenol red- β -d-galactopyranoside (CPRG) – using an ELISA plate reader or by eye (Figure 9).

In order to maximise the utility of the pseudotype assay system, multiplexing of PVNAs has been demonstrated which permits simultaneous quantification of VNAb responses against several PVs, each harbouring a different reporter gene, in the same assay, sparing valuable

reagents such as serum samples (Wright *et al*, 2010; Molesti *et al*, 2014). For example, Wright *et al*. have explored the potential of multiplexing the pseudotype virus system as a means for simultaneously screening of VNAb responses to more than one virus in diagnostic and serosurveillance studies (Wright *et al*, 2010). Renilla or firefly luciferase reporter genes were incorporated into pseudotyped particles bearing the envelope proteins for Lagos bat virus (LBV) and Mokola virus (MOKV), respectively. LBV and MOKV are phylogroup 2 lyssaviruses that are clinically indistinguishable. A dual PNA was performed in which renilla and firefly luciferase expression was quantified. Results from the duplex assay correlated well with those from PNAs using the individual pseudotypes, and overall seroprevalence of the two lyssaviruses within an *Eidolon helvum* bat reservoir was in accordance with previous studies (Hayman *et al*, 2008; Kuzmin *et al*, 2008). The ability to multiplex the pseudotype platform enables resource-poor laboratories to detect VNABs for several viruses at once, reducing the necessary reagent and sample volumes. The system could be further multiplexed by use of fluorescent markers such as GFP and RFP for additional pseudotype viruses. Ultimately, serological assays could be carried out for whole families of emerging viruses, such as henipaviruses, coronaviruses and hantaviruses that have already been adapted to the pseudotype system.

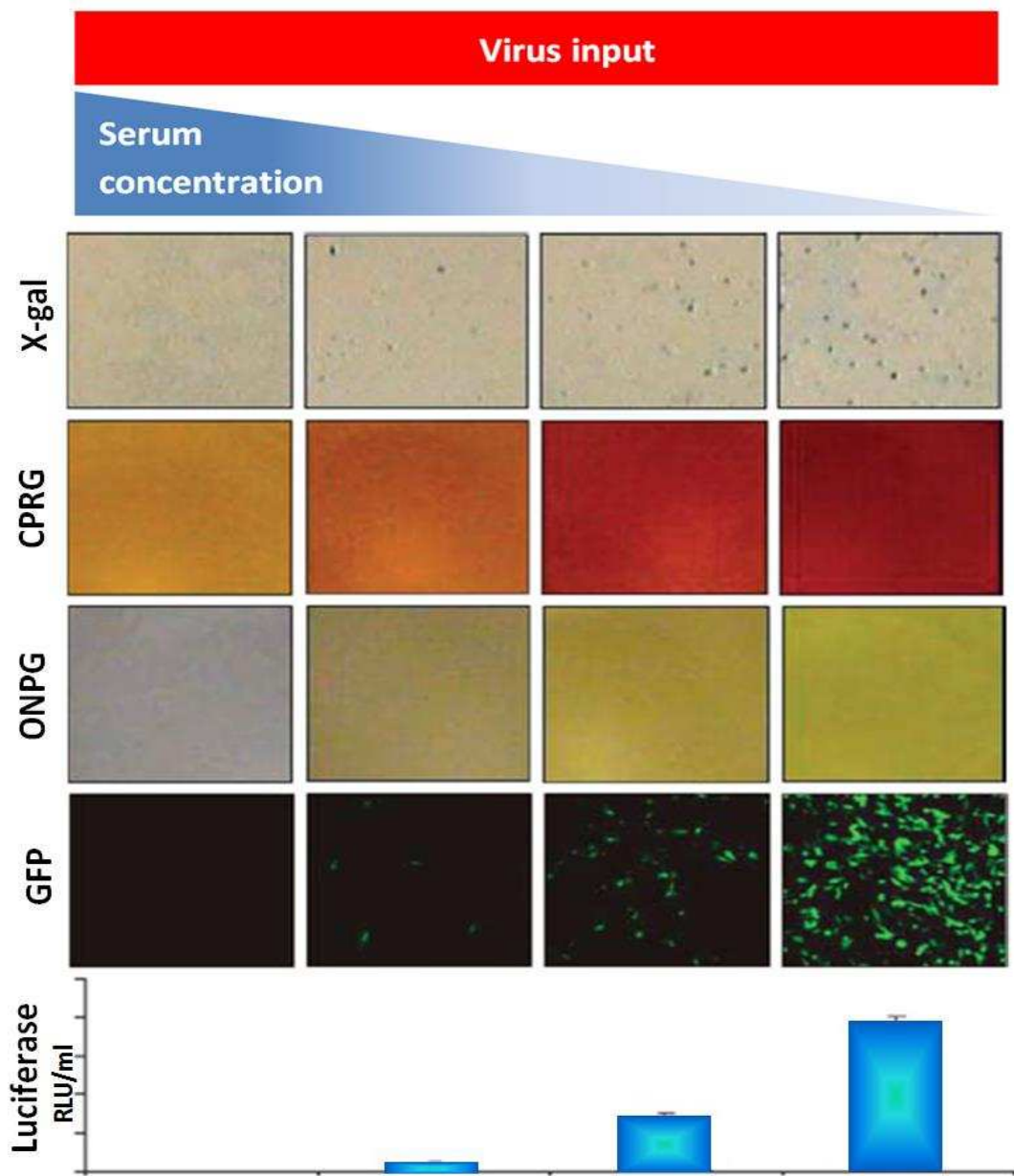


Figure 9. Choice of reporter gene readouts for the measurement of target cell transduction by pseudotype viruses. Images of serial dilutions from neutralisation assays are shown, with heightened levels of pseudotype transduction as serum sample concentration decreases. The expense and accuracy levels of the readout systems increase as you descend through the rows of the figure from X-gal to luciferase (adapted from Temperton and Wright, 2009).

1.4 Aim

The aim of this project is to attempt the production of functional JEV pseudotype viruses, which act as safe, replication-defective surrogates for wild-type, infectious JEV in downstream virus neutralisation assays. Such PVNAs could be used to quantify the VNAb response raised against the JEV antigenic surface glycoproteins, following vaccination or natural infection. This report details a variety of approaches taken to try and induce successful JEVpp generation, as well as addressing several considerations to increase the amenability of the pseudotype virus platform, particularly within resource-deprived areas.

Chapter 2

General materials and methods

2.1 Molecular biology

2.1.1 Plasmids

Commerically synthesized genes were received as cloned inserts into the pUC57 plasmid (Genscript®, cat. n° SD1176). pUC57 (2710bp) is a high-copy pUC19 derivative plasmid, originally isolated from the DH5 α strain of *E.coli*, and is commonly used as a cloning vector (Yanisch-Perron *et al*, 1985). This plasmid possesses a *bla* gene to confer ampicillin resistance and a *lacZ* gene, within which the multiple cloning site (MCS) resides, for blue/white selection during cloning. Also, an origin of replication derived from the pMB1 plasmid is present to enable plasmid amplification in bacterial cells, as well as a CAP protein binding site and a *lac* repressor binding site, which are vital alongside the *lacZ* gene to complete the *lac* operon mechanism for blue/white screening (Figure 10).

During the cloning of the JEV prME envelope glycoprotein genes, pCAGGS was used as a destination vector, and was also used for downstream pseudotype production attempts – this is a high-copy, ampicillin-resistant pUC13-based plasmid, which permits robust, efficient mammalian cell expression of a desired gene insert in a various of eukaryotic cell lines, due to the inclusion of a CAG promoter, consisting of a CMV immediate-early (CMV IE) enhancer, followed by the chicken β -actin promoter. Downstream of the promoter lies the rabbit β -globin gene 3' flanking sequence, which contains a splice acceptor site and a polyadenylation signal. Furthermore, an SV40 origin of replication for prokaryotic expression and a neomycin resistance gene for selection of positively-transfected eukaryotic cells are included in this construct (Figure 11; Niwa *et al*, 1991).

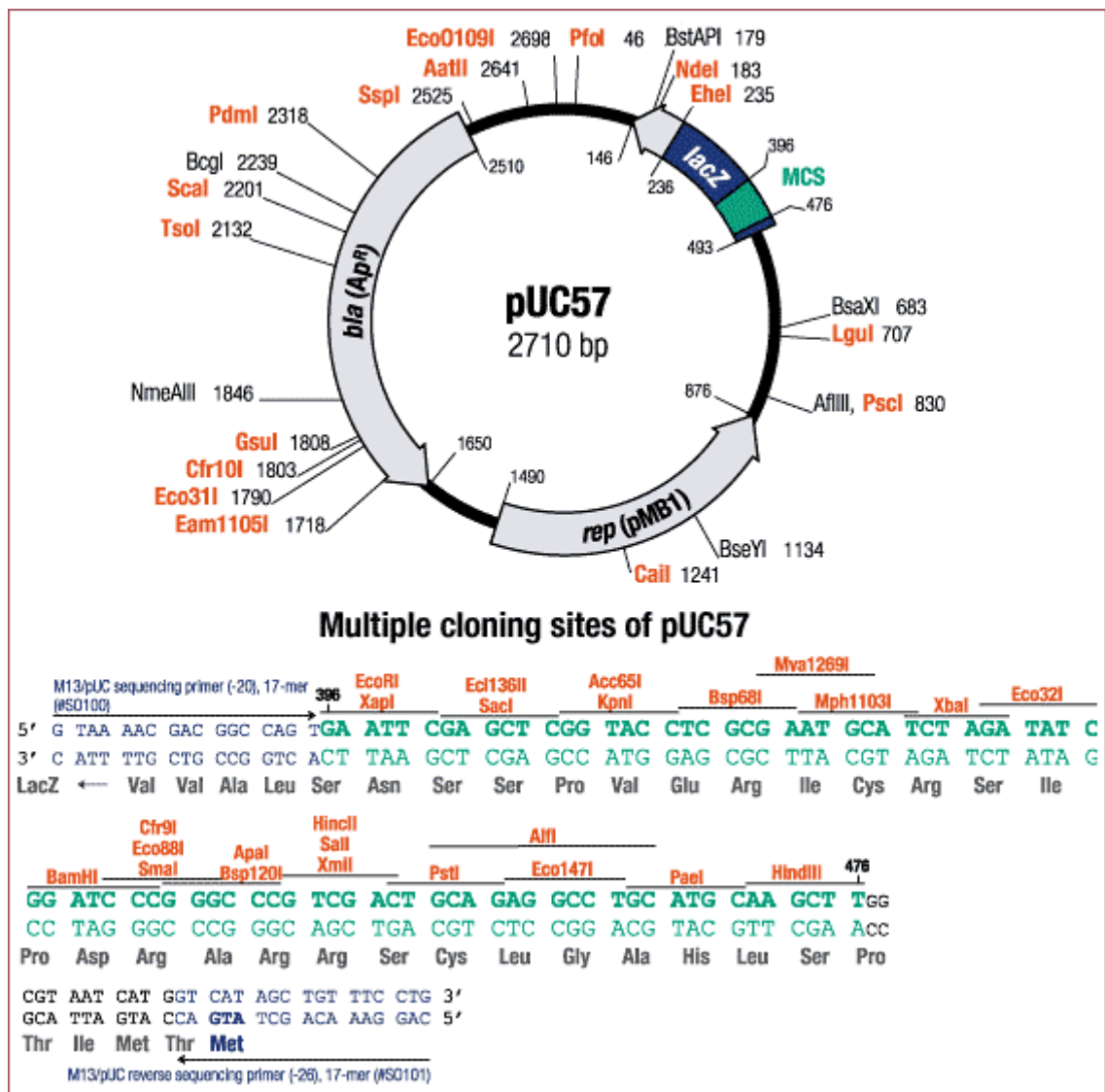


Figure 10. Plasmid map of pUC57. Arrows pointing in an anticlockwise direction indicate that the genetic element is located on the antisense strand of the plasmid DNA. Grey arrows on the plasmid map display the locations of the *bla* ampicillin resistance gene and the pMB1 origin of replication. The blue and grey arrow is representative of the *lacZ* gene location, with the green segment on this arrow denoting the bounds of the multiple cloning site, of which the sequence is displayed in more detail below the plasmid map. This sequence shows each restriction enzyme cleavage site within this region, as well as the annealing sites of the M13 sequence primer pairing (plasmid map sourced from Genscript®).

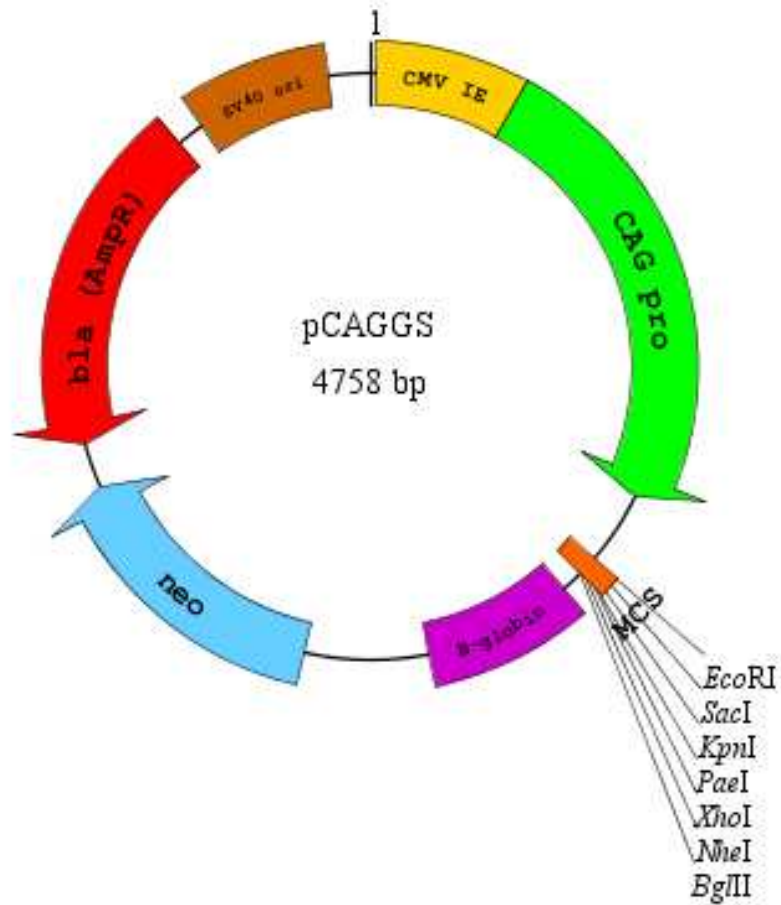


Figure 11. Plasmid map of pCAGGS. The yellow, purple and brown segments present on the plasmid map display the locations of the cytomegalovirus immediate-early enhancer, the rabbit β -globin 3' flanking sequence and the SV40 origin of replication respectively. The green and sky blue arrows pointing in a clockwise direction indicate the sites of the chicken β -actin promoter and the neomycin resistance gene, which are located on the sense strand. The antisense, anticlockwise-pointing red arrow denotes the *bla* ampicillin resistance gene. The small orange segment is the multiple cloning site, with common restriction enzymes featured in a list alongside it. Plasmid map design was carried out using SimVector 4.6 software (PremierBiosoft®, CA, USA).

2.1.2 Liquid and solid bacterial media

To produce stock solutions of antibiotics used in the preparation of bacterial media, either ampicillin sodium salt (Fisher Scientific, cat. n° BP1760) or kanamycin sulphate (Fisher Scientific, cat. n° BP906) were dissolved in UltraPure™ DNase/RNase free distilled water (Gibco®, Invitrogen™, cat. n° 10977-049), to a final stock concentration of 100mg/ml for ampicillin, and 10mg/ml for kanamycin. Ampicillin was diluted to a working concentration of 100µg/ml in liquid bacterial media and 200 µg/ml in solid bacterial media, whereas kanamycin was used in a working solution at the concentration of 50µg/ml in both solid and liquid bacterial media.

When preparing solid and liquid bacterial media, ready-to-dissolve Luria Bertani (LB) agar (Fisher Scientific, cat. n° BP1425 and Sigma-Aldrich, cat. n° L3147) and LB broth (Fisher Scientific, cat. n° BP1426) were respectively used. These powders were dissolved in double distilled water as per the manufacturer's recommendations at the following concentrations: 16g per 400ml for LB agar; 12.5g per 500ml for LB broth. The prepared solutions were then autoclaved, cooled and sealed before use.

Super Optimal broth with catabolite expression, or SOC broth (SOC; Invitrogen™, cat. n° 15544-034), which contains 2% tryptone (w/v), 0.5% yeast extract (w/v), 10mM NaCl, 2.5mM KCl, 10mM MgCl₂, 10mM MgSO₄ and 20mM glucose, was utilised during bacterial transformation as a recovery medium, thus increasing the efficiency of the process.

2.1.3 Transformation of chemically competent *E. coli* cells

In order to propagate plasmid stocks or amplify a cloned plasmid following ligation during molecular cloning, a conventional transformation protocol was followed, utilising Subcloning Efficiency Chemically-Induced Competent *Escherichia coli* DH5α cells (Invitrogen™, cat. n° 18265-017).

Initially, a 12.5µl or 25µl aliquot of DH5α was removed from -80°C cold storage and thawed on ice, before adding 1-2.5µl (within a range of 0.5-10ng) of the desired plasmid DNA. The transformation mixture was then incubated on ice for 30 minutes, before a heat shock step at 42°C for 30 seconds either in a water bath (Jouan, cat. n° 41093014) or an AccuBlock™ Digital Dry Bath (Labnet International, cat. n° D1100-230 V) – the bacterial heat shock temporarily permeabilises the bacterial cell wall and allows entry of intact, circular plasmids. The aliquot was subsequently placed back on ice for 2 minutes to halt the heat shock process. Following this, 250µl of SOC medium was added prior to recovery incubation in a New Brunswick™ Scientific C25KC Incubator Shaker (Eppendorf, Germany) for 1 hour at 37°C and 225rpm. This enables the transformed cells to express the relevant antibiotic resistance protein from its corresponding gene present in the recombinant plasmid, which is essential prior to antibiotic selection on Luria Bertani (LB)-agar (Sigma Aldrich, UK) plates – 50µl of each transformation mixture was plated before overnight incubation (Genlab, cat. n° INC/75) at 37°C.

2.1.4 Plasmid DNA purification from bacterial culture

To amplify and purify plasmid DNA required for transfection into eukaryotic cells, sequencing or analysis following cloning processes, the commercial QIAprep Spin Miniprep Kit (QIAGEN, cat. n° 27104/27106) was followed as per manufacturer's instructions (QIAGEN, 2012), which employs a modified alkaline lysis with sodium dodecyl sulphate (SDS) technique.

Bacterial colonies transformed with a single copy of the desired plasmid were inoculated into 5ml of antibiotic-containing LB broth, before being incubated overnight (12-16 hours) at 37°C and 225rpm in a shaking bacterial incubator. At this stage, 1ml of each bacterial culture was removed and supplemented with 80% (v/v) glycerol to make a 15% glycerol stock and frozen down at -80°C as a stock from which to further inoculate and propagate any required plasmid. The cultures were then pelleted for 3 minutes at 6800 x g using a table-top microcentrifuge (Thermo Fisher Scientific, cat. n° 10524723) before removal of the supernatant. The bacterial

cell pellet was resuspended in 250µl Buffer P1 containing 100µg/ml RNase A until a homogenous bacterial solution was achieved. The RNase A enzyme efficiently breaks down any RNA contamination in the solution, thus helping to create a purer end sample. 350µl of the alkaline Buffer P2 (containing sodium hydroxide and SDS) was then added to lyse the bacterial cell walls and release the cells' contents, including plasmid DNA, before adding 350µl of the neutralisation Buffer N3, which is acidic and contains potassium acetate, to neutralise the alkaline pH of the sample and precipitate out any bacterial macromolecules, such as protein, lipids and chromosomal DNA, as well as SDS from the sample solution. The pH-neutralised and salt-balanced lysate was then cleared of all precipitate by centrifugation for 10 minutes at 17000 x g, pelleting the unwanted contents so that the plasmid DNA-containing supernatant could be loaded onto a QIAprep Spin Column, which possesses a silica membrane that exclusively binds DNA. The loaded spin column was centrifuged for 1 minute at 17000 x g, before flow-through was discarded and two wash buffers were added successively: first, 500µl of Buffer PB, followed by 750µl of Buffer PE. These wash steps were both followed with further centrifugation steps at 17000 x g for 1 minute, and subsequent disposal of flow-through. To ensure the clearance of any ethanol residue from the membrane, a dry centrifugation step of the same duration and speed was performed. In order to elute the plasmid DNA, 50-100µl of nuclease-free water was added to the spin column, incubated for 1 minute to allow for the dissolving of the plasmid DNA into the water, before a final centrifugation step at 17000 x g for 1 minute. The plasmid DNA-rich flow-through was retained in a 1.5ml microcentrifuge tube (Greiner Bio-One, cat. n° 616201), ready for downstream usage.

2.1.5 Calculation of nucleic acid concentration

To calculate the concentration and purity of plasmid DNA, as well as DNA fragments throughout the cloning process, a NanoDrop™ 2000 Spectrophotometer (NanoDrop™

Products, ThermoFisher Scientific) was used, as per manufacturer's instructions. Concentration units were given as ng/ μ l and purity as an absorbance ratio of 260nm/280nm.

2.1.6 Oligonucleotide primers for molecular biology and sequencing purposes

Primers required for molecular biology or sequencing purposes were ordered from Eurofins MWG Operon at a 25nmol synthesis scale and were delivered in a lyophilised, desalted state (unless stated otherwise in the text). Prior to use, the primers were reconstituted in sterile, nuclease-free water to a final stock concentration of 100pmol/ μ l, before further dilution down to a 10pmol/ μ l working concentration.

2.1.7 Sanger chain termination sequencing

To verify the successful incorporation of desired genes into plasmid vectors *via* molecular cloning, recombinant plasmid DNA was sent to GATC Biotech AG for Sanger sequencing, using either the SUPREMERun™ or LIGHTrun™ sequencing systems, depending on the availability of sequencing barcodes and universal primers. For the SUPREMERun™ system, 20 μ l of plasmid at an 80ng/ μ l concentration and 20 μ l of the appropriate sequencing primer were posted in separate tubes to GATC Biotech AG. In some instances, primers were not required to be sent, as GATC Biotech AG could supply an in-house stock of universal primers with which to sequence the plasmid sample (Table 3). For the LIGHTrun™ sequencing system, 5 μ l of 80ng/ μ l plasmid and 5 μ l of the relevant primer were mixed in the same microcentrifuge tube prior to postage.

Primer name	Primer sequence (5' to 3')	No. of nucleotides	T _m (°C)	GC content (%)	Secondary structure/ primer dimers	Primer function
M13 Fwd	GTAAAACGACGGCCAGTG	18	62°C	56%	Low	Amplification of inserts cloned into the pUC57 plasmid
M13 Rev	GGAAACAGCTATGACCATG	19	59°C	48%	Low	Amplification of inserts cloned into the pUC57 plasmid
pCAGGS Fwd	GGTTATTGTGCTGTCTCATC	20	58°C	45%	Low	Amplification of inserts cloned into the pCAGGS plasmid
pCAGGS Rev	GCAGAGGGAAAAAGATCTGC	20	63°C	50%	Low	Amplification of inserts cloned into the pCAGGS plasmid

Table 3. Characteristics of sequencing primers. The M13 and pCAGGS sequencing primer pairings are listed, along with the full sequences and lengths in nucleotides, as well as their percentage GC contents and the level of presence of secondary structures and primer dimers. Additionally, a brief description of the function of each primer is given.

2.1.8 Polymerase chain reactions

In order to amplify genes for routine molecular biology purposes, polymerase chain reaction was used. Since desired prME genes were subcloned from recombinant plasmids following commercial gene synthesis, PCR was used primarily for analytical applications, as opposed to for cloning. Therefore, utilisation of a polymerase enzyme with high levels of amplification fidelity was unnecessary, and so the DreamTaq polymerase was chosen for use (Thermo Fisher Scientific, cat. n° K1081 or K1082), which is designed to consistently offer robust and reliable amplification.

To perform amplification reactions, a 25µl reaction mixture was made up in 0.2µl thin-walled PCR tubes (VWR International Ltd, cat. n° 732-0548) on ice, by mixing 12.5µl DreamTaq Green PCR Master Mix with 1µM of both the forward and reverse primer required in the PCR reaction, as well as approximately 10ng of template plasmid DNA. Nuclease-free, sterile H₂O was then added to achieve a final reaction volume of 25µl. Samples were subsequently

vortexed and transferred to either the Mastercycler Ep Gradient (Eppendorf) or the Mastercycler Ep Gradient S (Eppendorf) thermal cycler, before being run on a PCR program appropriate to achieve substantial amplicon yields.

2.1.9 Restriction endonuclease digestion of DNA

Restriction endonuclease digestion reactions were performed for subcloning purposes in this study, in order to isolate prME gene inserts from original plasmid vectors, as well as to create the necessary compatible nucleotide overhangs at the ends of both expression vector and insert DNA fragments prior to ligation. Furthermore, restriction digest screening to identify successful production of recombinant plasmids was also carried out.

For subcloning purposes, conventional restriction endonucleases (Thermo Fisher Scientific) were generally utilised. In each instance in this study, the two enzymes that were required achieved 100% mutual compatibility in the universal Tango buffer (Thermo Fisher Scientific, cat. n° BY5) at a 2X final concentration. Approximately 800-1000ng of plasmid DNA was mixed with 10U of each enzyme in restriction digest reaction mixtures, before addition of the Tango buffer and sterile, nuclease-free H₂O to a final volume of 20µl. As these restriction endonucleases are suspended in a buffer that contains glycerol, it was important to consider that the overall percentage of glycerol in each reaction mixture was always 10% or less (as recommended by the manufacturers). Reactions were then incubated for 2 hours at 37°C in a waterbath, before use in downstream subcloning purification applications (see sections 2.1.13 and 2.1.14).

When restriction digestion was required for analytical purposes, FastDigest® enzymes (Thermo Fisher Scientific) were used to perform such reactions. Approximately 500ng of plasmid DNA was digested in a total volume of 20µl. When using FastDigest® enzymes, the universal FastDigest® Green Buffer (Thermo Fisher Scientific, cat. n° B72) was able to be used, alongside

10U of each enzyme required in the restriction digest. Final reaction volumes were reached by addition of the appropriate volume of nuclease-free, sterile H₂O. Samples were subsequently transferred to a thermocycler, where they were incubated for 15 minutes at 37°C, followed by an enzyme denaturation step at 80°C for 5 minutes, before being analysed by agarose gel electrophoresis (see section 2.1.12).

2.1.10 DNA ligation

DNA ligation was used in this study to join open, digested expression vectors and JEV prME genes by their complementary restriction enzyme nucleotide overhangs, in the presence of 1U/μl T4 DNA ligase (Thermo Fisher Scientific, cat. n° EL0011) and its corresponding buffer (Thermo Fisher Scientific, cat. n° B69).

Prior to ligation, the concentration and purity of the vector and insert DNA fragments was elucidated using the Nanodrop™ 2000 Spectrophotometer (NanoDrop™ Products, ThermoFisher Scientific). These values guided the calculation of the molar ratio for the ligation reactions, which defines the number of copies of insert and vector DNA incorporated into each reaction sample, using the following formula:

$$\text{Mass of insert (ng)} = \frac{\text{Insert length (bp)}}{\text{Vector length (bp)}} \times \text{Mass of vector (ng)}$$

Ligations were set up at a 1:1 and 1:3 vector to insert molar ratio, in a volume of 10μl or otherwise, the lowest possible volume. A negative control reaction sample, containing only a vector fragment and no insert, was also made up to determine whether any undigested or re-ligated vector was present. The sample reactions were usually incubated overnight (~16 hours), but in some instances over a weekend (~64 hours), at room temperature, allowing a sufficient duration for recombinant plasmids to be formed by ligation.

2.1.11 Colony polymerase chain reaction for screening of recombinant clones

Colony PCR is a rapid, high-throughput method, which in this study enabled fast determination of successful recombinant plasmid DNA in candidate bacterial transformants, *via* amplification of the desired prME gene, following ligation and transformation.

Each selected colony, alongside a positive (colony with vector plus insert) and negative (colony with empty vector) control, was touched lightly with a pipette tip before being streaked onto a designated section of a fresh ampicillin LB-agar gridplate, then into a corresponding tube containing 25µl nuclease-free water. The gridplate was left at 37°C to promote fresh growth of the *E.coli* cells, whilst the inoculum tubes were incubated for 10 minutes to allow bacterial equilibration in the water. The inoculated water samples were then transferred to a thermal cycler and heated to 94°C for 3 minutes to further lyse the the *E.coli* cells and release their contents, including copies of the desired recombinant plasmid, if present. 5µl of each of the heated inocula samples were mixed with 20µl of a Green DreamTaq master mix (Thermo Fisher Scientific, cat. n° K1081 or K1082) cocktail, containing 1µM each of the relevant forward and reverse primer, for each colony being sampled. Subsequently, the samples were run on a PCR program appropriate to achieve strong amplification of the plasmid gene insert.

2.1.12 Gel electrophoresis of DNA

Agarose gel electrophoresis was utilised for analytical purposes to identify the lengths (in nucleotide base pairs) of DNA fragments at several stages throughout the subcloning process, such as after restriction digestion of both insert and vector DNA, as well as post-colony PCR and digest screening. Since the lengths of the desired DNA fragments fall between the range of 500bp and 10kb, a 1% (w/v) agarose (Fisher Scientific, cat. n° BP1356) gel dissolved in 0.5X tris-acetate-ethylenediaminetetraacetic acid (EDTA) buffer (TAE; 50X stock solution, Alpha Laboratories, cat. n° EL0077; or Fisher Scientific, cat. n° BP1332) was used as the

electrophoretic matrix. To achieve fluorescence of DNA bands on the gel under ultraviolet light, either ethidium bromide (Sigma-Aldrich, cat. n° 46067) or Nancy-520 (Sigma-Aldrich, cat. n° 01494) intercalating dyes were added to the molten agarose mixture before pouring, to a final concentration of 0.1µg/ml or 0.5µg/ml, respectively.

It was necessary for loading dye to be added prior to adding the samples onto the gel: for PCR products amplified using the Q5 polymerase, and restriction digest samples cut with conventional restriction endonucleases, 6X DNA loading dye (Thermo Fisher Scientific, cat. n° R0611) was added, diluted to a 1X working concentration; however, when PCR products were amplified using the DreamTaq polymerase or when FastDigest® reactions were performed, the samples were able to be loaded directly onto the gel, since the DreamTaq Green PCR master mix and the FastDigest® Green buffer already contain loading dyes. The GeneRuler 1kb DNA ladder (Thermo Fisher Scientific, cat. n° SM0311) was added to each gel – this molecular weight marker contains an amalgam of DNA fragments of known lengths, which migrate across the electrophoretic gel at different speeds, creating a ‘ruler’ that can be used to approximately measure the lengths of experimental DNA sample fragments.

When a preparative agarose gel needed to be made in order to subsequently excise and extract a DNA fragment, UltraPure™ Agarose (Invitrogen™, cat. n° 6500-500) was used, as a direct substitution for the aforementioned agarose.

All gels were run submerged in a 0.5X TAE electrophoretic running buffer, using a power supply (Consort, cat. n° EV231) and electrophoretic chambers (SCIE-PLAS, cat. n° SVG-SYS Vari-gel MINI; or PEQLAB Biotechnologie GmbH, cat. n° 40-124 or 40-0911; or Sigma-Aldrich, cat. n° EP1101). Analytical gels were run at a voltage of 100-120V, whereas preparative gels were run at 40V to avoid over-heating and re-melting of the gel. Electrophoresis was halted when the loading dye front had migrated across the entire length of the gel.

For observation of gels prior to excision of a DNA band, a trans-illuminator was used (UVItec, cat. n° BXT-26.MX). However, for gels where only images and downstream analysis was required, a G:Box Chemi XT Chemi XT Imaging System (Syngene), along with GeneSnap software (Syngene), was utilised.

2.1.13 Polymerase chain reaction and restriction digest purification of DNA fragments

When PCR and restriction digest reaction samples required purification before subsequent subcloning stages, the QIAquick PCR purification kit (QIAGEN, cat. n° 28104) was used, following manufacturer's instructions. Recommended kit protocols were followed: briefly, Buffer PB was added to the experimental sample at a volume 5 times that of the sample volume, before addition of 10µl of 3M sodium acetate at a pH of 5.0. This enables reaction mixture acidification, acting against buffer PB, as the optimal pH for DNA binding is 7.5. The sample was then placed in a QIAquick column and centrifuged at 17000 x g for 1 minute. At this stage, DNA adsorption to the silica membrane should occur. Flow-through was discarded and 750µl of Buffer PE, which contains ethanol, was added in order to remove any presence of contaminating salts, with another centrifugation step of the same speed and duration. Ethanol residue was then removed from the membrane by a subsequent 2 minute centrifugation, before DNA elution was carried out by adding 30µl of sterile, nuclease-free H₂O at 70°C, incubating at room temperature for 1 minute, then transferring the silica membrane to a fresh 1.5ml microcentrifuge tube and performing a final centrifugation step at 17000 x g for 1 minute.

2.1.14 Agarose gel extraction of DNA fragments

In order to purify fragments of DNA following preparative agarose gel electrophoresis and scalpel excision of the DNA band of interest, agarose gel extraction was carried out, using either the QIAquick Gel Extraction Kit (QIAGEN, cat. n° 28704) or the MinElute Gel Extraction

Kit (QIAGEN, cat. n° 28604). The protocol recommended by the manufacturers was followed: briefly, 3 volumes of buffer QG was added to the sample, corresponding to 1 weight of gel slice. This buffer enables dissolving of the agarose present in the experimental sample when incubated at 50°C for 10 minutes with frequent vortexing. The pH of the sample was then adjusted by adding 10µl of pH 5.0 3M sodium acetate, followed by one sample volume of isopropanol (Fisher Scientific, cat. n° P/7500/17). The sample was subsequently loaded onto either a QIAquick or a MinElute spin column by a 1 minute centrifugation at 17000 x g. Centrifugation-based wash steps with Buffers QG and PE were then performed to ensure complete removal of agarose and salt traces, followed by a dry centrifugation of the column at 17000 x g for 2 minutes to remove ethanol residue. To elute, 30µl of sterile, nuclease-free H₂O at 70°C was added to the spin column being used, before a final incubation for 1 minute at room temperature and a 1-minute centrifugation at the same speed, into a clean 1.5ml microcentrifuge tube. Use of sterile water instead of an elution buffer ensures there is no possibility of EDTA presence in DNA samples, which can hinder downstream applications.

2.1.15 Site-directed mutagenesis of DNA using Q5 polymerase

When it was necessary to alter the nucleotide sequence of a prME envelope glycoprotein gene, site-directed mutagenesis was carried out. In all instances, the Q5 site-directed mutagenesis kit (New England Biolabs, MA, USA; Cat. No. #E0554) was utilised, due to the high fidelity of the polymerase and its ability and versatility to incorporate large insertion mutations into existing plasmid constructs. The mutagenesis system works by whole-plasmid PCR amplification elongating from mutagenic primers – designed using the NEBaseChanger™ program – before direct treatment with a ‘KLD’ enzyme mix which contains a kinase and ligase to 5’ phosphorylate and circularise the PCR product, as well as the *DpnI* restriction enzyme which digests and enables removal of methylated template DNA. For large insertions (>6nt), both primers are required to possess half of the mutagenic sequence each, with the 3’ end of

the insertion incorporated into the forward primer, and the 5' of the insertion incorporated into the reverse primer.

Reaction sample mixtures to perform Q5 site-directed mutagenesis are compiled by mixing 12.5µl of the 2X Q5 Hot-Start High Fidelity PCR Master Mix with the forward and reverse mutagenic primers to a final concentration of 0.5µM each, and also a mass of template plasmid DNA equivalent to 1-25ng. Addition of sterile, nuclease-free H₂O takes place to achieve a final reaction volume of 25µl. Samples are then subjected to the following 30-cycle PCR program: an initial 30 second denaturation step at 98°C, followed by 30 cycles of a 10 second denaturation step at 98°C, a 30 second annealing stage at a temperature dictated by the properties of the mutagenic primers, and then an elongation step of 72°C for 30 seconds/kb of plasmid length. A final extension stage of 2 minutes at 72°C is added to the end of the PCR program.

1µl of each amplified sample is carried forward to the KLD stage of the mutagenesis methodology. Reaction mixtures are compiled by assembling the following reagents: 1µl of PCR product, 5µl of the 2X KLD Reaction Buffer, 1µl of the 10X KLD Enzyme Mix and 3µl of sterile, nuclease-free H₂O to reach a total reaction volume of 10µl. Samples are mixed well and incubated at room temperature for at least 5 minutes, before downstream transformation, colony PCR and sequence verification can take place, to assess correct mutagenic alteration of the template sequence.

In all instances, mutagenesis was carried out on pUC57 recombinant plasmids harbouring a prME gene, due to the relatively short length of the pUC57 plasmid. As the Q5 mutagenesis system employs amplification of the whole plasmid to incorporate mutations, amplifying a smaller plasmid sequence reduces the possibility of random mutations occurring in the PCR product.

2.2 Cell culture

All cell culture maintenance and procedures were carried out in a MSC-Advantage™ Class II Biological Safety Cabinet (Thermo Fisher Scientific, cat. n° 51028226). Cultures were incubated in a Heracell™ 150i humidified CO₂ Incubator (Thermo Fisher Scientific) at 37°C and 5% CO₂.

2.2.1 Maintenance and characteristics of cell lines

Human embryonic kidney 293T clone 17 cells (HEK293T/17; Pear *et al*, 1993) were used for transfections to attempt production of JEV pseudotype viruses, as well as to determine expression and intracellular localisation of the prME proteins. Also, HEK293T/17 cells were used as a potential target cell line in JEV transduction experiments, along with baby hamster kidney 21 cells (BHK-21; Stoker and Macpherson, 1964), Crandell-Rees feline kidney cells (CrFK; Crandell *et al*, 1973) and Vero cells (Yasumura and Kawakita, 1993), as they are generally considered to be permissive to JEV infection and are commonly used in studies involving cellular entry of JEV.

TECeB6 and TECeB15 are packaging cell lines modified from TE671 cells (ATCC HTB-139) to express MLV *gag-pol* and produce replication-defective MLV gammaretrovirus cores, which can subsequently be pseudotyped with a heterologous envelope glycoprotein. In addition, TELCeB6 cells express the vector MFG-*nlslacZ* which incorporates the β -galactosidase reporter into the MLV cores. Despite this, different reporter genes can be incorporated into these cores, providing the correct packaging signals are active. These packaging cells were used for transfections to attempt production of JEV-pseudotyped MLV, using a slightly different technique to conventional multi-plasmid co-transfection.

In all instances, cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) with high glucose and GlutaMAX™ (Gibco®, Invitrogen™, cat. n° 31966-021), or an equivalent DMEM medium (Sigma-Aldrich, cat. n° D6429, or PAN-Biotech, cat. n° P04-04510),

supplemented with 15% (v/v) European Union approved origin heat-inactivated Fetal Bovine Serum (FBS; Gibco®, Invitrogen™, cat. n° 10500-064, or PAN-Biotech, cat. n° P30-8500) and 1% (v/v) 10000 U penicillin/10mg/ml streptomycin (Sigma-Aldrich, cat. n° P4333).

All cell cultures were grown in T75 culture flasks (Thermo Fisher Scientific, cat. n° 156499), except for HEK293T/17 cells, which were maintained in 10cm sterile Nunclon® surface cell culture dishes (Thermo Fisher Scientific, cat. n° 150350).

When subculturing was necessary, old medium was aspirated from the cell monolayer, before rinsing with 2ml of 0.05% (w/v) Trypsin-0.53mM EDTA solution (Sigma-Aldrich, cat. n° T3924, or PAN-Biotech, cat. n° P10-040100), then incubation with 2ml of fresh trypsin-EDTA solution at 37°C and 5% CO₂, until complete detachment of the cells from the culture vessel took place. This trypsinisation reaction was quenched by the addition of 6ml 15% FBS-DMEM medium, in which the cells were thoroughly resuspended, and a fraction of this cell suspension was seeded into a fresh 15% FBS-DMEM containing culture vessel, at an appropriate dilution to achieve a designated subculturing ratio (these ranged from 1:4 to 1:12 for different cell lines).

2.2.2 Freezing and thawing of cell lines

Upon reaching 80% confluency, cell lines were frozen down to create stock aliquots, from which subsequent cultures of the cell line can be propagated. Cell monolayers were detached from the culture vessel by the aforementioned trypsinisation process, then centrifuged at 1000 x g for 5 min to pellet the contents of the cell suspension. The old medium supernatant was discarded before addition of 5ml of fresh freezing DMEM medium, which includes 10% (v/v) dimethyl sulfoxide (DMSO; VWR International Ltd, BDH Prolabo GPR RECTAPUR®, cat. n° 282164K). Three cryovials of cell solutions to be frozen are then produced and placed in a Mr. Frosty™ Freezing Container (Thermo Fisher Scientific, cat. n° 5100-0001), inside a -80°C freezer. This freezing container acts as an isopropanol bath for the cryovials of cells, enabling

their temperature to reduce down to -80°C at a rate of 1°C/minute, and thus ensuring their continued viability. Frozen cell aliquots were then stored long-term inside a cryobox (Thermo Fisher Scientific, cat. n° 1417563).

In order to thaw frozen aliquots of cell lines to begin a new culture, cryovials were added to a 37°C waterbath to thaw the contents as quickly as possible. The frozen cells were then transferred to a 15 ml tubes (Greiner Bio-One, cat. n° 188271) containing 7 ml of the appropriate culture medium, before centrifugation at 1000 x g for 5 minutes and removal of the supernatant to ensure there are no traces of cytotoxic DMSO carried forward to the established culture. Cell pellets were then resuspended in 5-10ml of fresh 15% FBS-DMEM medium and transferred to the appropriate culture vessel, before placing in the humidified incubator at 37°C and 5% CO₂ to permit cell adhesion and monolayer formation before subculturing and culture maintenance begins.

2.3 Pseudotype production

2.3.1 Plasmids used for pseudotype production

The following core and reporter gene plasmid constructs were used in pseudotype virus production attempts, alongside recombinant pCAGGS-prME plasmids subcloned in earlier methodology in this study (see Section 2.1.1):

p8.91, which was originally named pCMV-ΔR8.91 (Zufferey *et al*, 1997), is the primary HIV core plasmid used in this study. It was kindly provided by Dr Nigel Temperton (Universities of Greenwich and Kent, Medway, UK). This plasmid is a second generation lentiviral packaging plasmid construct which expresses the HIV *gag-pol* genes, driven by a human CMV promoter. The HIV-1 accessory genes *vif*, *vpr*, *vpu* and *nef* have been deleted from this construct. The other HIV *gag-pol* plasmid construct used to attempt pseudotype production is psPAX2 (Addgene).

Plasmids encoding the MLV *gag-pol* genes used in one technique to attempt MLV-JEV pseudotype generation were pCMVi and pHCMV-MLVgagpol. These plasmids were a kind gift from Dr Alexander Tarr, *via* Dr Barnabas King, University of Nottingham.

pCSFLW, which was also kindly provided by Dr Nigel Temperton, is a self-inactivating lentiviral vector, which possesses a Ψ packaging signal, as well as an internal promoter derived from the U3 segment of the long terminal sequence of the spleen focus forming virus (SFFV); a Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) which enhances the expression levels of the reporter gene; and the the central polypurine tract cis-active sequence (cPPT). The pCSFLW plasmid was modified from the similar pCSGW plasmid (originally known as pHR'SIN-cPPT-SE; Demaison *et al*, 1992). However, the pCSGW plasmid contains an eGFP reporter gene system, instead of a corresponding firefly luciferase one. In this study, pCSFLW and pCSGW were used as reporter gene plasmids for incorporation into the pseudotyping system, to attempt to produce HIV-JEV pseudotypes with firefly luciferase and GFP readouts, respectively. Gammaretroviral reporter genes used in this study for attempted generation of MLV-JEV pseudotype viruses were pMLVluc for luciferase (provided by Dr Nigel Temperton), and pCNCG (provided by Dr Nigel Temperton) and pHCMV-MLVgfp (also kindly received from Dr Alexander Tarr, University of Nottingham) for GFP.

2.3.2 Protocol for pseudotype production

Multi-plasmid co-transfections were used for all pseudotype production attempts in this study. 24 hours prior to transfection, approximately 8×10^5 HEK293T/17 producer cells (except for the MLV packaging cell line experiments, in which case either TELCeB6 or TECeB15 cells were used as producers) were seeded into sterile 6-well tissue culture plates (Nunclon®, Thermo Fisher Scientific).

On the day of transfection, a DNA mix containing a selected *gag-pol*, reporter gene and prME construct was set up at either a 1:1.5:1 (with masses of 500µg, 750µg and 500µg) or 1:1.5:3 (core:reporter:envelope) ratio, and topped up with sterile, nuclease-free H₂O to a volume of 15µl. In a separate tube, 18µl of either the FuGENE® 6 (Promega, cat. n° E2692) or the polyethylenimine/PEI (Sigma Aldrich, cat. n° 408727) transfection reagent was directly diluted in 200µl OptiMEM® I reduced serum medium (Gibco®, Invitrogen™, cat. n° 31985-047), ensuring no contact was made with the inside walls of the microcentrifuge tube, as this can hinder the efficiency of transfection. After 5 minutes of incubation at room temperature, the DNA mix was added to the transfection reagent mix, and incubated for 15 more minutes, to allow for formation of complexes between the DNA and transfection reagent. The mixture was then added drop-wise to a well of the 6-well tissue culture plate, containing an 80% confluent monolayer of the required producer cell line. The transfection plate was then placed back into the incubator at 37°C and 5% CO₂.

At 24 hours post-transfection, the media was replaced for 1.5ml of fresh 15% FBS-DMEM. Pseudotype supernatants were harvested at 48 hours after transfection by removal of the cell culture medium using a 5ml syringe, and by passing through a 0.45µm mixed cellulose ester membrane filter (Merck Millipore, cat. n° SLHA033SB), before storing at -80°C until required for use in titration assays.

2.3.3 Titration assay for pseudotype viruses

For all titration assays, a starting volume of 100µl of each viral pseudotype candidate supernatant was 1:2 serially diluted across a white 96-well tissue culture plate (Nunc Microwell, Thermo Scientific, UK) before addition of 1×10^4 desired target cells and incubation in a humidified tissue culture incubator. In all instances where a luciferase reporter gene was incorporated into pseudotype particles, titration assay plates were incubated for 48 hours, prior to measuring relative luminescent units per ml (RLU/ml), using the Bright-Glo™ assay

system (Promega, cat. n° E2650) and GloMax Multi detection system luminometer (Promega, cat. n° E7031 and E7041) to quantify luciferase reporter expression. Pseudotype transduction titres were calculated by converting RLU readout values at a range of assay dilutions into RLU/ml, before determining the arithmetic mean and standard deviation (SD). When GFP reporters were packaged into pseudotype viruses, the titration assay was incubated for 72 hours prior to optical determination of transduction by visualising cells under a fluorescent microscope.

2.3.4 Pseudotype virus neutralisation assay

When assessing the ability of serum samples to neutralise and inhibit the infectivity and transduction of functional pseudotype viruses into permissible target cell lines, the pseudotype virus neutralisation assay (PVNA) assay.

To perform the PVNA, 2-fold serial dilutions of the experimental serum samples are performed across the 96-well assay plate, starting with 5µl of neat serum in a 100µl mixing volume. In the last serial dilution, 50µl of the mixing volume is discarded. The assay plate was then centrifuged for 1 minute at 500 x g, using an ELMi CM-6MT Centrifuge and rotor 6M04, before addition to each experimental well of 50µl the relevant pseudotype virus supernatant, adjusted to contain exactly 1×10^6 RLU. A viral input control (containing no serum) and a cell only control (containing no virus or serum) were also included on the assay plate, which was centrifuged again for 1 minute at 500 x g, before placing in the humidified incubator for 1 hour. This allotted time frame enables binding of any neutralizing antibodies in the antiserum sample to antigenic epitopes exposed on the pseudotype virus surface or envelope glycoprotein. Finally, the last addition to the assay plate is 1×10^4 cells of the target cell line being employed in the experiment, in a volume of 50µl, before a final centrifugation step of the same speed and duration, before incubation at 37°C and 5% CO₂.

48 hours later, the knockdown of firefly luciferase expression was quantified by addition of 50µl of Bright-Glo™ solution and, after 5 minutes' incubation at room temperature, reading the plate using the GloMax luminometer.

Analysis of resultant assay data was performed using Microsoft® Excel 2011 and GraphPad Prism® version 6 (GraphPad Software). To measure serum sample neutralization potency, RLU values, as originally determined from the titration assay, were normalised and presented as a percentage neutralisation value. Normalisation was achieved by defining 0% and 100% neutralisation as the arithmetic mean of the viral input and cell only controls, respectively. Percentage neutralisation was calculated by working out the percentage reduction of luminescence, and in turn of subsequent pseudotype entry inhibition into target cells, between these two neutralisation bounds. Half maximal and 90% inhibitory concentrations could then be deduced, expressed as assay serum dilution factors, by using a non-linear regression analysis system (log [inhibitor] vs normalised response – variable slope).

2.4 Validation of protein expression

2.4.1 Immunocytochemistry/immunofluorescence

In order to identify the intracellular localisation of any expressed prME protein, indirect immunofluorescent staining was employed. In advance, poly-D-lysine (PDL) coated coverslips (Vitrocam, UK) were placed into wells of a 6-well plate and UV sterilised in a tissue culture cabinet for 30 minutes. 8×10^5 HEK293T/17 cells were then seeded into the 6-well tissue culture plates and placed in a humidified incubator for 24 hours to reach ~70-80% confluence, including on the coverslip. Cells were transfected with 1µg of either pCAGGS-15SPprME or a pl.18 plasmid encoding the haemmagglutinin (HA) envelope glycoprotein of A/equine/Sussex/89 H3N8 influenza, using PEI, and incubated for a further 48 hours – a sufficient duration for expression of the glycoproteins to occur. (The HA of this equine

influenza isolate was used as a positive control, as it is able to create successful HIV pseudotypes, and the primary polyclonal sera used for immunostaining was extracted after vaccination with both JEV and equine influenza vaccines. This means that the same primary and secondary antibodies could be used for experimental samples and positive controls). Following the transfection and incubation, culture medium was aspirated and the cell monolayers were washed in a 3 x 5 minute schedule with 1ml PBS (Sigma Aldrich, UK). To fix and permeabilise the transfected cells, 1ml of 100% methanol was added to each well and the plate incubated on ice for 10 minutes. A 1:200 dilution (in PBS) of neat serum from an equine vaccinated against JEV (and H3 influenza) was used as the primary antibody sample, and a FITC-conjugated, rabbit anti-horse IgG (H+L) secondary antibody (Sigma Aldrich, UK) was utilised at a 1:1000 dilution (in PBS). NucBlue Live Fixed Cell Nuclear Stain (Life Technologies, UK) was also added to the secondary antibody stock at a 1 drop/3ml concentration. Sample wells were thoroughly washed with PBS (in the same schedule as previously) after the fixing, permeabilisation and both antibody incubation stages of the immunofluorescent staining protocol. Coverslips were mobilised from sample wells and mounted onto microscope slides using Mowiol 4-88 anti-fade coverslip mounting medium (Sigma Aldrich, UK). Once mounted and set, the coverslips were scrutinised using a fluorescent microscope, selecting blue (DAPI) and green (FITC) filters for nuclear stain and expressed prME antigen visualisation, respectively.

2.4.2 SDS-PAGE and Western blotting

Examination of prME protein expression was achieved in this study using SDS-PAGE and Western blotting. Similar to the immunofluorescence experiments, 8×10^5 HEK293T/17 cells were seeded into 6-well plates and incubated for 24 hours to reach ~70-80% confluence, including on the coverslip. Cells were transfected with 0.5 μ g or 1.5 μ g of either pCAGGS-15SPprME or pCAGGS-24SPprME, using PEI, and incubated for a further 48 hours. Following

removal of DMEM and addition of 1ml lysis buffer (20mM Tris Base, 137mM NaCl, 0.2% Triton X-100 plus protease inhibitors), cell monolayers were detached *via* scraping, transferred to a 1.5ml Eppendorf tube and incubated on ice for 20 minutes. Cell lysates were then centrifuged at 14,000 rpm and 4°C for 10 minutes and the peptide-rich supernatant was retained. Protein samples, including a sample of an IXIARO® inactivated vaccine (Valneva) as a positive control, were mixed with 4x Laemmli sample loading buffer (BioRad, CA, USA) then heated for 5 minutes at 95°C to denature all peptides, before loading onto a 12% polyacrylamide resolving gel. The gel was completely submerged in 1x SDS-PAGE running buffer (50mM Tris Base, 0.38M Glycine, 0.1% SDS (w/v)) and ran at 200V for 60 minutes, in order to separate cellular peptides by their molecular weight in kDa. Protein transfer onto a PVDF membrane (Immobilon P, Micropore, UK) was achieved by submersion in a transfer buffer (25mM Tris Base, 192mM Glycine, 20% methanol) and application of a 30V electrical voltage for 2 hours. Both the protein electrophoretic and transfer stages were carried out using the Mini-PROTEAN Electrophoresis System (BioRad, CA, USA). Next, membranes were blocked in a 3% Marvel dried skimmed milk solution prepared in TBS-T (25mM Tris Base, 137mM NaCl, pH 7.5 plus 0.05% Tween20) for 1 hour at room temperature to prevent antibody binding of non-specific epitopes. Mouse JEV ab81193 (Abcam, UK) monoclonal antibody was used as a primary antibody to blot for domain III of the JEV E glycoprotein, produced a band of ~38kDa on downstream blot images. Mouse anti- β -actin monoclonal antibody (Licor, NE, USA) was also used for primary antibody binding to β -actin, serving as an internal control to determine blotting success. As both primary antibodies were murine-raised, one secondary antibody was utilised in this study: IRDye 800CW-conjugated, polyclonal goat anti-mouse IgG (H+L) (Licor, NE, USA). All antibodies were 1:1000 diluted in TBS-T + 1% Marvel solution before membranes were added and incubated on a rocking platform for 1 hour at room temperature. Membrane wash steps at a 3 x 5 minute schedule in TBS-T were undertaken between each of the blocking

and antibody binding stages of the protocol. Blotted membranes were visualised using the Odyssey CLx System for Infrared Fluorescent Western blots (Licor, NE, USA).

Chapter 3

Manipulating transfection and assay parameters to attempt production of functional retroviral pseudotypes bearing JEV envelope glycoproteins

3.1 Introduction

As previously mentioned (see Section 1.3.3), the pseudotype virus platform offers a variety of benefits for utilisation in virus biology and serology experimentation, such as the opportunity to bypass high biosafety containment requirements, the flexibility of reporter gene packaging dependent upon laboratory financial constraints, multiplex pseudotype assays to reduce the cost-per assay burden, and the ability to swiftly incorporate different virus envelope protein genes into the co-transfection pool for the production of pseudotype particles for serosurveillance, mutagenesis, antigenic drift and other studies (Mather *et al*, 2013).

Consequently, highly pathogenic members of several families of emergent RNA viruses have been pseudotyped, including *Orthomyxoviridae*, *Rhabdoviridae*, *Coronaviridae*, *Filoviridae* and *Bunyaviridae* (for a full breakdown, see Table 2). Likewise, pseudotyping technology has also been applied to various members of the *Flaviviridae* family, to facilitate clinical research without the necessity to handle native, pathogenic virus.

Hepatitis C virus (HCV), a member of the *Hepacivirus* genus of *Flaviviridae*, is an infection of high clinical importance, as it infects several hundred million people worldwide, and is a common causative agent of chronic liver disease and hepato-cellular carcinoma. Historically, the study of HCV is hampered by the lack of an efficient cell culture system, capable of supporting virus replication *in vitro* (Bartosch *et al*, 2003a). To address this issue, HCV pseudotypes have been developed where the E1-E2 glycoproteins are displayed on both lentiviral and murine retroviral cores, with packaged GFP and luciferase reporter genes for

reliable and rapid determination of infectivity (Bartosch *et al*, 2003a; Hsu *et al*, 2003). Subsequently, these pseudotype particles have been employed in an array of downstream applications, such as determining neutralization epitopes on the HCV envelope glycoproteins, understanding the extent of genetic quasispecies variation in patients suffering from chronic liver disease, and elucidating the pH-dependent entry mechanisms of the virus, mediated by E1-E2 complexes (Bartosch *et al*, 2003a; Hsu *et al*, 2003; Bartosch *et al*, 2003b; Tarr *et al*, 2007; Dreux and Cosset, 2009; Bartosch and Cosset, 2009). More recently, the infectivity phenotype of many HCV pseudotypes isolated from chronically-ill patients was improved by comprehensive optimisation of many parameters of the viral pseudotype entry assay. In some instances, isolates which previously proved refractory to pseudotyping were incorporated into the chimeric pseudotype particles much more efficiently following assay optimisation (Urbanowicz *et al*, 2016).

Within the *Flavivirus* genus, DENV pseudotypes for all four serotypes have also been created, consisting of the structural and enzymatic core of HIV harbouring a luciferase reporter gene, and displaying the heterologous prME glycoproteins of DENV. Interestingly, in order to achieve successful interaction between the HIV core and DENV envelope components, which is crucial in the generation of functional pseudotype particles, a chimeric glycoprotein was constructed, where the transmembrane domain of the DENV E protein was replaced with the cytoplasmic and transmembrane domains of the VSV-G protein. High infectivity titres into target cells were recorded, which could be effectively inhibited by lysosomotropic agents and mutations to crucial amino acids located in the fusion loop of the DENV E protein, indicating a pH-dependent, fusogenic mechanism of entry (Hu *et al*, 2007).

Using different production approaches, pseudotype particles displaying the prM-E glycoproteins of Japanese encephalitis virus have also been generated. For instance, the TELCeB6 packaging cell line, which constitutively expresses the MLV *gag-pol* genes to encode

the viral nucleocapsid, can be employed as producer cells to create MLV-JEV pseudotypes, when transfected *via* the calcium phosphate method with JEV prME and β -galactosidase expression vectors. Resultant JEV pseudotypes could efficiently transduce permissible target cells, and were potently neutralized by positive JEV antisera, with results correlating closely with those obtained by PRNT (Lee *et al*, 2009; Lee *et al*, 2014).

The VSV pseudotype platform, which is a common alternative to retroviral pseudotyping, has also successfully yielded functional chimeric virus particles possessing heterologous JEV envelope glycoproteins. VSV-JEV pseudotypes were generated initially by construction and production of a recombinant VSV lacking the G protein and possessing in its place a luciferase reporter gene (VSV-G/Luc-*G), followed by infection of VSV-G/Luc-*G into HEK293T cells or Huh7 cells transiently expressing the foreign JEV prME proteins. The pseudotype viruses produced were subsequently used to elucidate the inhibitory effects of cholesterol and the enhancing effects of ceramide on the infectivity, entry and proliferation of JEV (Tani *et al*, 2010; Tani *et al*, 2012). JEV pseudotypes with VSV cores were also used to develop more understanding of the involvement of cyclophilin B in viral replication (Kambara *et al*, 2011).

Additionally, utilisation of a recombinant TRIP lentiviral vector bearing the codon-optimised structural prME proteins was able to create viable JEV virus-like particles with a lentiviral core, which were immunogenic and able to seroconvert immunized piglets and BALB/c mice (de Wispelaere *et al*, 2015). However, from current relevant literature, it appears that no lentiviral or gammaretroviral pseudotype viruses displaying JEV envelope glycoproteins have successfully been produced using the conventional 3-plasmid co-transfection system.

Therefore, the aim of this chapter is to explore and manipulate various cloning, transfection and assay parameters, in an attempt to generate JEV retroviral pseudotypes of a functionally high infectivity titre, so that they can be utilised in downstream serological applications.

3.2 Materials and Methods

3.2.1 Molecular biology

3.2.1.1 Genes

The JEV isolate used to attempt pseudotyping in this chapter was the Beijing-1 strain (Genbank accession number L48961). The adjacent prM and E genes from Beijing-1 were custom synthesised by Genscript, NJ, USA and cloned within the pUC57 plasmid. The synthesised prME gene was designed to include the restriction sites for *EcoRI* and *BglII* at the 5' terminus, and the sites for *XhoI* and *NotI* restriction enzymes at the 3' terminus. Additionally, the sequence encoding the last 15 residues from the C-terminus signal peptide of the JEV C gene (immediately upstream of the prM gene in the JEV genome) was also included in the synthesised gene construct.

3.2.1.2 Restriction digests

Restriction digests were performed during the subcloning process to isolate the prME gene from its original pUC57 vector, as well as to enable ligation of the pCAGGS expression vector and the prME insert, and for digest screening to identify successful pCAGGS-prME recombinant plasmids. Either conventional or FastDigest® *EcoRI* and *XhoI* restriction enzymes with corresponding buffers, were used. More information on restriction digest reaction conditions can be found in Section 2.1.9.

3.2.1.3 Gel electrophoresis

Agarose gel electrophoresis was utilised in this chapter throughout the subcloning process, such as following restriction enzyme digests of prME and pCAGGS DNA, as well as after colony PCR and digest screening processes. In all cases, 1% agarose gels were used for DNA separation, prepared in 0.5x (TAE) buffer containing ethidium bromide (Sigma Aldrich, UK) at a

1:100,000 working dilution. 0.5x TAE was also used as an electrophoretic running buffer. More information regarding the loading dyes and DNA ladders used, as well as the electrophoretic voltages and visualisation techniques, can be found in Section 2.1.12.

3.2.1.4 PCR purifications and gel extractions

PCR purification was used to clean up and purifying the endonuclease-digested pCAGGS expression vector, before it could be used for ligation. The QIAquick PCR purification kit was used for this purpose, according to manufacturers' instructions, and more information is given in Section 2.1.13.

Following the digestion of the original pUC57-prME plasmid to release the prME gene, the sample was run on an Ultrapure agarose gel to separate out the vector (~2.7kb) and insert (~2.1kb) DNA fragments. The prME gene fragment could then be excised from the gel with a scalpel, aided by a benchtop UV transilluminator, before weighing the gel slice. To isolate the insert DNA and remove remnants of the TAE-agarose gel, the sample was processed through the MinElute Gel Extraction Kit (Qiagen, Netherlands) following manufacturer's instructions (see Section 2.1.14).

3.2.1.5 Ligations

pCAGGS and prME DNA fragments were joined together by their complimentary restriction enzyme nucleotide overhangs by ligation. This process was catalysed by 1U/ μ l T4 DNA ligase (Thermo Scientific, UK) and its corresponding buffer.

Prior to ligations, the concentration (in ng/ μ l) and purity (as a A[260/280] ratio) of digested and appropriately purified pCAGGS vector and prME insert DNA samples was quantified using the Nanodrop 2000 (Thermo Scientific, UK). This information was used to perform molar ratio calculations, before ligations with a 1:1 and 1:3 vector:insert molar ratio, as well as a vector only negative control sample, were set up and incubated overnight at room temperature,

allowing a sufficient duration for recombinant pCAGGS-prME plasmids to be potentially ligated (for more details, see section 2.1.10).

3.2.1.6 Transformations

Subsequent to ligation, 2.5µl of the ligation samples (1:1, 1:3 and vector only) were each transformed into a separate 25µl aliquot of Subcloning Efficiency DH5α Competent *E.coli* cells (Life Technologies, UK). A detailed bacterial transformation protocol can be found in Section 2.1.3. Antibiotic selection could then take place on Luria Bertani (LB)-agar (Sigma Aldrich, UK) plates containing 100µg/ml ampicillin - 50µl of each transformation mixture was plated before overnight incubation at 37°C.

The resulting numbers of transformant colonies on each plate were assessed to determine the success of the subcloning procedure. The negative control plate (transformation of ligation mixture lacking the insert DNA fragment) acted as a background, as every colony on this plate will have most likely been formed by transformation of a completely uncut or partially-digested and self-ligated pCAGGS plasmid vector.

A proportion of the colonies present on the vector:insert plates were taken forward to be screened by colony PCR and in some instances, digest screening. If the number of vector only colonies was high compared to those on the insert plates, then the digested pCAGGS vector underwent alkaline phosphatase treatment (Thermo Scientific Fast AP, UK) following manufacturer's instructions, which 5' dephosphorylates the cut vector sequence, minimising the potential for vector self-circularisation during digestion.

3.2.1.7 Colony PCR

Colony PCR was used as a screening process to assess whether transformant colonies from the 1:1 and 1:3 vector:insert molar ratio ligations harboured a recombinant pCAGGS-prME plasmid. This general protocol is outlined in Section 2.1.11. The forward and reverse primers

used to recognise the prME gene termini were as follows: (JEVprMEFwd primer: GAGAATTCAGATCTCATGTGGCTCGC; JEVprMERev primer: ATTGGTAGCTAAGAACACGACGACACCTCC). PCR samples were then run on a 30x thermal cycle with a 30-second denaturation step at 94°C, a 30-second annealing step at 50°C and a 2-minute extension step at 72°C, before running on a 1% agarose gel to determine positive or negative plasmid clones. The colony PCR positive control amplified the original pUC57-prME plasmid, whereas the negative control was an identical PCR master mix but containing no template DNA.

3.2.1.8 Overnight cultures

Positive pCAGGS-prME clones, as determined by colony PCR, were further propagated by growth in overnight starter cultures. These 5ml cultures of LB growth medium (Sigma Aldrich, UK) contained 50µg/ml ampicillin – to select for recombinant plasmid-containing *E.coli* – and were inoculated by one of the positive colonies each, grown from the colony PCR LB-agar gridplates. The cultures were incubated overnight (12-16 hours) at 37°C and 225rpm in a shaking bacterial incubator.

3.2.1.9 Plasmid purification

All plasmid stocks grown up in DH5α *E.coli* overnight cultures were purified using the QIAprep Spin Plasmid Miniprep Kit (Qiagen, Netherlands), as per manufacturer's instructions (a more detailed breakdown of this protocol can be found in Section 2.1.4). In all instances, 4ml of bacterial culture was taken forward into the plasmid purification process, with the remaining 1ml being supplemented with 15% glycerol and frozen down at -80°C as a stock from which to further inoculate and propagate any required plasmid.

3.2.1.10 Site-directed mutagenesis

In order to increase the length of the JEV C-terminal capsid signal sequence from 15 to 24 residues, Q5 site-directed mutagenesis was employed. The general methodology of this mutagenesis platform, as well as reaction mixtures, volumes and PCR thermal cycling programs, can be found in Section 2.1.15.

The mutagenic primer design, as carried out by the NEBaseChanger program, and the strategy of mutagenesis employed to insert the extra 27 nucleotides of the JEV capsid signal sequence, are shown in Figure 12. The mutagenic sequence is split across the two primers, so that the 3' 13 nucleotides of the sequence to be inserted is included on the 5' terminus of the forward oligonucleotide primer, and the beginning 14 mutagenic nucleotides at the 5' terminus of the sequence for insertion are found at the 5' end of the complementary reverse primer, which will amplify in an antisense orientation. This creates a 9 amino acid residue insertion, directly before the start of the JEV prM sequence, and immediately after the 5' overhang, restriction enzyme sites and starting methionine codon located at the 5' terminus of the insert gene in the pUC57-prME plasmid construct.

Following mutagenic PCR and KLD enzymatic treatment, then positive colony screening, purification and sequence verification of the extended, full signal peptide JEVprME, the gene was subcloned into pCAGGS before downstream usage.

Current construct sequence (around 5' prME terminus):

GGTACCTCGCGAATGCATCTAGATAATGAGAATTCAGATCTCATGTGGCTCGCGAGCTTGGC

pUC57 vector sequence 5' overhang, EcoRI site, BglII site and starting Met

Start of JEV prM New sequence to be introduced

Sequence being introduced (first 9 residues of JEV signal peptide):

GGCAAGAGAAGATCAGCAGGCTCAATC

Desired construct sequence:

GGTACCTCGCGAATGCATCTAGATAATGAGAATTCAGATCTCATGGCAAGAGAAGATCAGCAGGCTCAATCTGGCTCGCGAGCTTGGC

Forward primer:

GGTACCTCGCGAATGCATCTAGATAATGAGAATTCAGATCTCATGGCAAGAGAAGATCAGCAGGCTCAATCTGGCTCGCGAGCTTGGC

5' AGCAGGCTCAATCTGGCTCGCGAGCTTGGC 3'

Reverse primer:

GGTACCTCGCGAATGCATCTAGATAATGAGAATTCAGATCTCATGGCAAGAGAAGATCAGCAGGCTCAATCTGGCTCGCGAGCTTGGC

5' GATCTTCTTGCCCATGAGATCTGAATTCTCATTATCTAGATGC 3'

Figure 12. Outline of Q5 mutagenic primer design to produce full JEV C-terminal capsid signal sequence upstream of prME gene. The Q5 site-directed mutagenesis kit works by amplifying the whole pUC57 plasmid (the section directly upstream of the 5' prME terminus highlighted in light blue) using specific mutagenic primers (mutagenic primer sequence highlighted in orange). In this strategy, the desired additional stretch of nucleotides (in purple) would be introduced directly in front of the start of the JEV prM sequence (including the shorter 15-residue signal peptide; in yellow) and the restriction sites and starting methionine residue (in green). The desired construct sequence and primer binding sites (bordered in black) are also shown.

3.2.2 Production of pseudotype viruses and pseudotype-based assays

3.2.2.1 Multi-plasmid co-transfection to produce pseudotype viruses

Three- and four-plasmid co-transfections were used repeatedly in this chapter for all attempts to produce pseudotype viruses displaying the prME glycoproteins of the JEV virus, such as for candidate HIV-JEV and MLV-JEV pseudotype supernatants with each combination of core and reporter plasmid construct, and also for the furin introduction and low glucose environment experiments.

Candidate transfections were carried out as described in more detail in Section 2.3.2, except for in the following instances: a pCAGGS-furin plasmid was included in the DNA mix during transfection for these experiments (as described in more detail in Section 3.3.4); the regular DMEM medium formulation was replaced with a low glucose formulation in the study investigating the effect of glucose on JEV PV transduction (as described in more detail in Section 3.3.5); the HEK293T/17 cell line was replaced with the TELCeB6 and TECeB15 cell lines as producer cells, and the inclusion of pCMVi or pHCMV-MLVgagpol in the DNA mix for transfection was not required, in the MLV packaging cell line approach to MLV-JEVpp production (as described in more detail in Section 3.3.6).

3.2.2.2 Titration assays

Pseudotype titration assays were used in this chapter to establish whether successful production of function of HIV- or MLV-JEV pseudotypes had taken place. As described in more detail in Section 2.3.3, 100µl of each viral pseudotype candidate supernatant was serially diluted before addition of 1×10^4 target cells: for these experiments, either HEK293T/17, BHK-21, CrFK or Vero. Assay incubation durations, readout systems and data analysis methods are also given in Section 2.3.3.

3.2.3 Validation of protein expression

3.2.3.1 Immunofluorescence

To enable further investigation of the intracellular localisation of expressed prME protein when pCAGGS-prME plasmids are transfected into PV producer cells, indirect immunofluorescent staining was utilised. HEK293T/17 cells grown on poly-D-lysine coated coverslips in 6-well tissue culture plates were transfected with pCAGGS-15SPprME, before being incubated for 48 hours then fixed with 100% methanol. A seropositive polyclonal equine serum was used as a primary antibody sample, followed by staining with the FITC-conjugated, rabbit anti-horse secondary antibody to enable fluorescent visualisation of any expressed prME, so that its location could be gauged. The protocol followed to perform this technique can be found in detail in Section 2.4.1.

3.2.3.2 SDS-PAGE and Western blotting

Further examination of the expression of the prME protein was achieved by use of SDS-PAGE and Western blotting. 70-80% confluent HEK293T/17 monolayers with pCAGGS-15SPprME or pCAGGS-24SPprME, before 48 hours' incubation. The cells were then lysed and lysates were harvested, prior to running on a 12% polyacrylamide gel. Cellular proteins separated by their molecular weights in kDa were then transferred to a PVDF membrane, before blocking, binding with monoclonal primary antibodies and IRDye 800CW-conjugated secondary antibodies before visualisation of resultant blot images. A more detailed version of the protocol followed for this experiment can be found in Section 2.4.2. For this Western blot, a sample of an IXIARO® inactivated vaccine (Valneva) was also included as a positive control.

3.3 Results

3.3.1 Subcloning of 15SPprME gene into pCAGGS expression vector

To enable eukaryotic expression of the JEV 15SPprME gene, the coding region was subcloned from pUC57 into the pCAGGS expression vector. This expression plasmid was chosen for subcloning primarily due to the presence of its chicken β -actin promoter – preliminary attempts to create plasmid stocks with existing prME constructs in pl.18 and phCMV expression vectors were repeatedly unsuccessful (data not shown). Simultaneously, the insert (pUC57-prME) and vector (pCAGGS) plasmids were digested by *EcoRI* and *XhoI* restriction enzymes, prior to alkaline phosphatase treatment and PCR purification of pCAGGS. As DNA fragments of two different sizes were produced in the pUC57-prME restriction digest, these had to be electrophoretically separated in order to gel extract and purify the prME gene (Figure 13A). After NanoDrop quantification of prME and pCAGGS to determine DNA concentration and purity, ligations were set up at a 1:1 and 1:3 (vector:insert) molar ratio, as well as a vector only (V/O) control. Following transformation and plating of ligation samples, one, ten and zero colonies were observed on the 1:1, 1:3 and V/O plates, respectively (Figure 13B-D). Nine of the colonies on the 1:3 plate were screened using colony PCR, revealing colonies #2, #4, #6 and #9 as positive pCAGGS-prME clones (Figure 13E). These colonies were subsequently cultured and plasmid DNA purified, before undergoing digest screening, which reinforced successful subcloning of the prME gene (Figure 13F). Finally, pCAGGS-prME clone #2 was sequence verified (GATC Biotech), confirming 100% identity with the official Genbank sequence for JEV Beijing-1 prME.

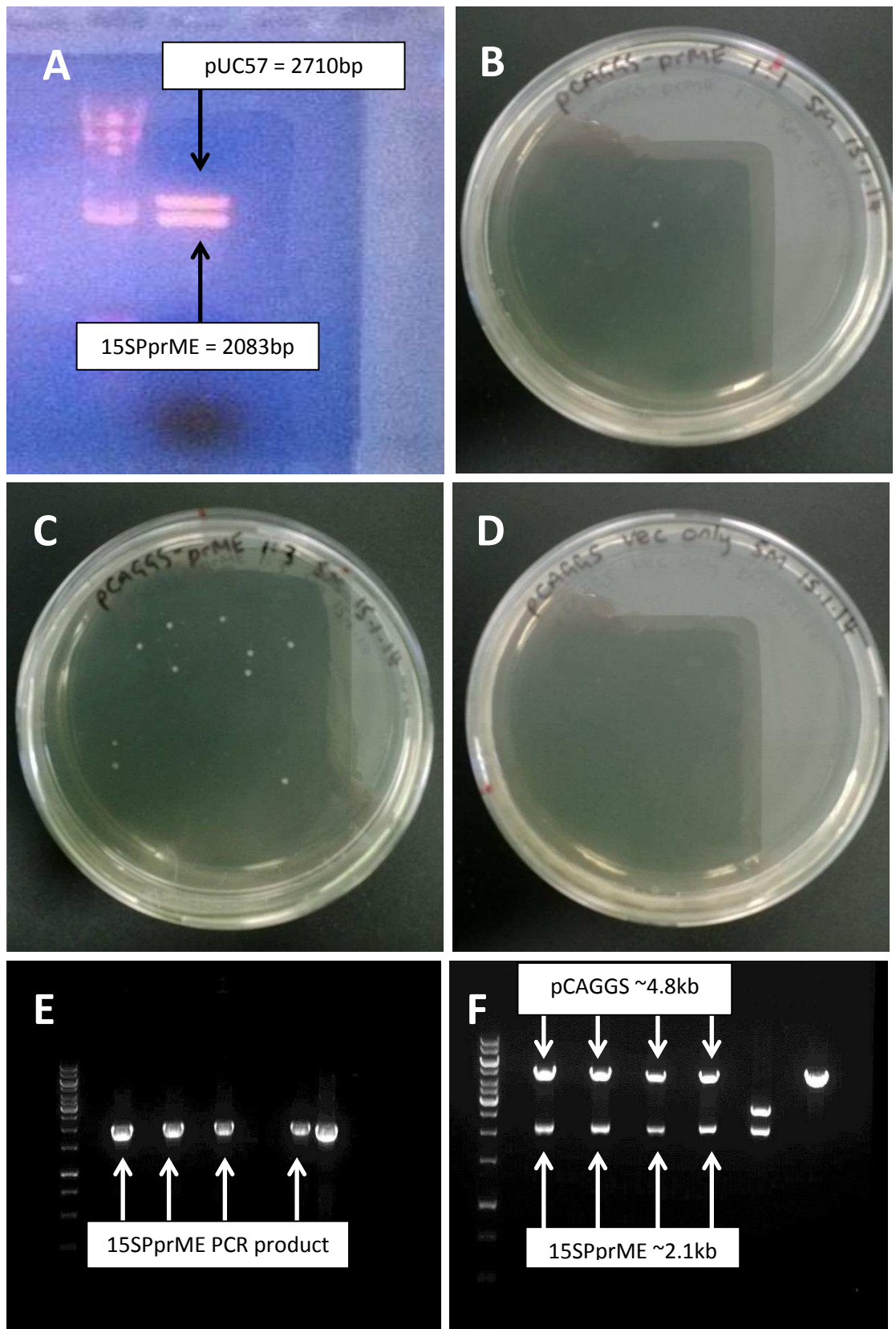


Figure 13. Subcloning of 15SPprME into pCAGGS. A) Ultrapure agarose gel displaying ~ 2.1 kb 15SPprME to be extracted, following digestion from pUC57. B-D) Transformation plates of pCAGGS-prME ligations at 1:1 (B) and 1:3 (C) molar ratios, and vector only (D) control. E) Agarose gel of colony PCR screen, confirming pCAGGS-prME clones #2, #4, #6 and #9 as positive. ~ 2.1 kb band at far right of gel is original pUC57-prME positive control. F) Restriction digest screen (*EcoRI/XhoI*) of pCAGGS-prME plasmid DNA purified from previously determined positive clones. Far right lane on gel is empty pCAGGS negative control; penultimate right lane is pUC57-prME.

3.3.2 Site-directed insertion mutagenesis to create 24SPprME

In order to ensure correct signal peptidase cleavage at the JEV C-prM junction, in order to direct the transit and modification of expressed prME protein through the ER-Golgi complex, the truncated 15-residue signal peptide upstream of the prM gene – originally included in the synthesised JEV prME – was extended to produce the full, 24-residue signal peptide, using the Q5 SDM kit (New England Biolabs, MA, USA). prME mutagenesis was carried out within the smaller pUC57 vector, as opposed to pCAGGS, in order to reduce the chance of introducing non-specific mutations or polymerase ‘slippage’ in the insert or vector during whole plasmid amplification. The mutagenesis thermal cycling program included 25 cycles: denaturation at 98°C for 10 seconds, annealing at 70°C ($T_{\text{annealing}} = \text{highest primer } T_{\text{melting}} + 3^{\circ}\text{C}$) for 30 seconds, and elongation at 72°C for 2 minutes (10-20 seconds per kb of plasmid). After rapid ligation of the amplicon and template removal *via* KLD treatment, mutagenic plasmid DNA was transformed into DH5α *E.coli* cells, propagated by overnight culture and purified. Sanger sequencing (GATC Biotech) of nucleic acid derived from two separate transformant colonies confirmed that the 27 N-terminal nucleotides of the signal peptide were correctly inserted into the prME construct (Figure 14). 24SPprME was then subcloned from pUC57 into pCAGGS (employing aforementioned methodology) before downstream usage.

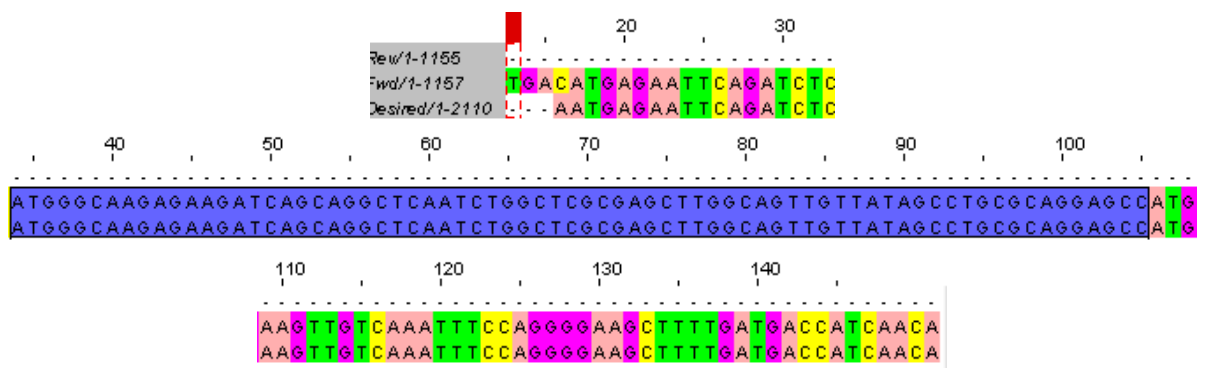


Figure 14. Multiple sequence alignment (Jalview, UK) of the 24SPprME construct (top row) and the JEV Beijing-1 prME Genbank database sequence (bottom row). Nucleotides 34-105 (shaded in blue) encode the 24-residue C-terminal signal peptide of the JEV C gene.

3.3.3 Production of JEV pseudotype viruses with HIV and MLV cores

Following successful construction and subcloning of 15SPprME and 24SPprME, the production of retroviral pseudotypes was attempted, using the multi-plasmid co-transfection system. A variety of candidate JEV pseudotype supernatants were harvested: for lentiviral pseudotypes, the HIV *gag-pol* was expressed from p8.91 or psPAX2 plasmids, whereas for gammaretroviral pseudotypes, the pCMVi plasmid encoded the MLV *gag-pol* genes. Furthermore, pCSFLW or pMLVluc luciferase plasmids, and 0.5µg or 1.5µg of either pCAGGS-15SPprME or pCAGGS-24SPprME were transfected into HEK293T/17 producer cells during JEVpp production. The candidate HIV-JEVpp and MLV-JEVpp supernatant samples were titrated onto four target cell lines – HEK293T/17, BHK-21, CrFK and Vero – and incubated for 48 hours, before quantifying luciferase expression (in RLU/ml) as an indirect measurement of virus infection (Figures 15-16). All titration assays included two negative controls: Δ envelope glycoprotein (ΔEG), which is a PV bearing no viral envelope glycoprotein, and a non-transduced cell only control. In every instance, relative transduction titres of JEVpp experimental samples were not significantly higher than those of ΔEG, indicating that no significant pseudotype particles bearing the envelope glycoproteins of JEV were produced. Additionally, the same candidate panel of JEV pseudotypes harbouring a GFP gene, which is a less sensitive system than luciferase, were also produced, but no GFP reporter gene expression was observed either, when these JEVpp production candidates were titrated.

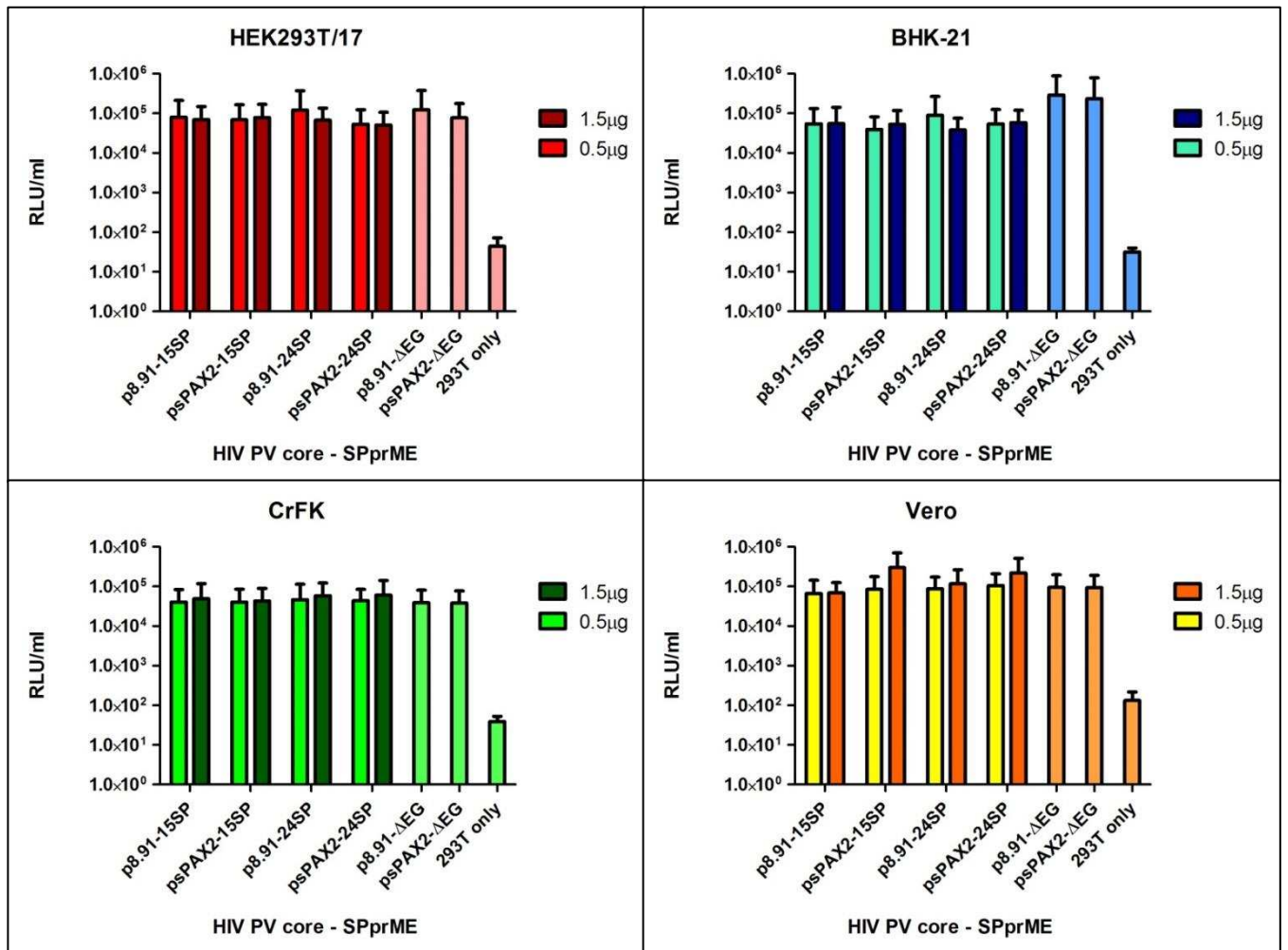


Figure 15. Infectivity of JEV-pseudotyped lentiviral vector candidates onto HEK293T/17, BHK-21, CrFK and Vero target cell lines. Pseudotype transduction titres are expressed as mean \pm SD (data retrieved in triplicate) of relative luminescent units per ml (RLU/ml). Δ envelope glycoprotein and target cell only negative controls are also shown. '15SP' and '24SP' denotes the length in amino acid residues of the signal peptide upstream of the prME gene. '0.5 μ g' and '1.5 μ g' refers to the mass of pCAGGS-15SPprME or pCAGGS-24SPprME transfected during JEVpp production attempts.

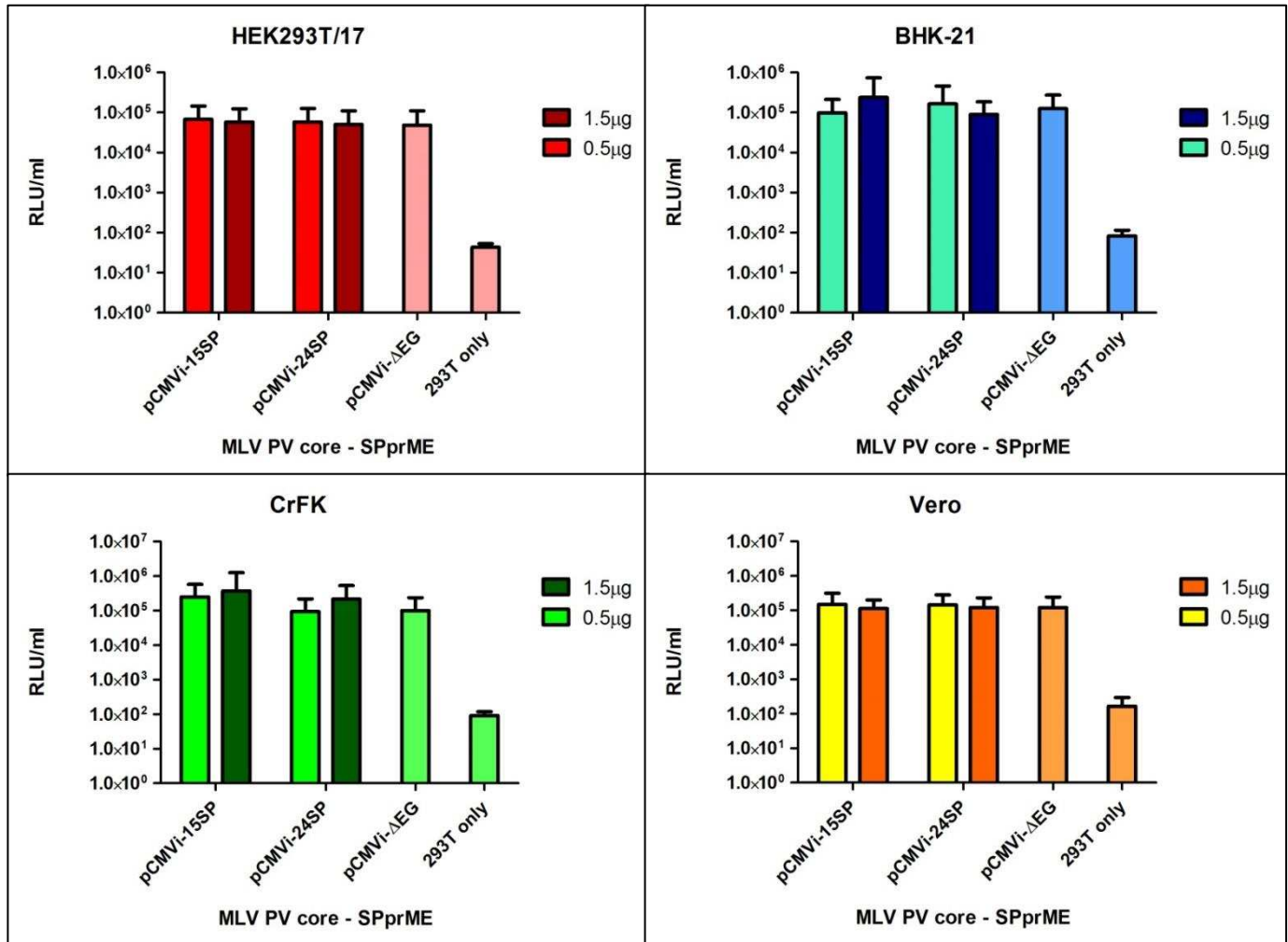


Figure 16. Infectivity of JEV-pseudotyped gammaretroviral vector candidate supernatants onto HEK293T/17, BHK-21, CrFK and Vero target cell lines. Pseudotype transduction titres are expressed as mean \pm SD (data retrieved in triplicate) of relative luminescent units per ml (RLU/ml). Δ envelope glycoprotein and target cell only negative controls are also shown. '15SP' and '24SP' denotes the length in amino acid residues of the signal peptide upstream of the prME gene. '0.5 μ g' and '1.5 μ g' refers to the mass of pCAGGS-15SPprME or pCAGGS-24SPprME transfected during JEVpp production attempts.

3.3.4 Introduction of furin to increase infectivity of JEV pseudotype viruses

As furin induces maturation of nascent JEV virions in the Golgi apparatus *via* cleavage of the M protein precursor, JEVpp production was attempted in the presence of additional furin protease expressed from a pCAGGS-furin plasmid, with masses of 0.25µg, 0.5µg, 0.75µg and 1µg added alongside p8.91/pCMVi (0.5µg), pCSFLW/pMLVluc (0.75µg) and pCAGGS-24SPprME (0.5µg) during transfection. Pseudotype virus supernatant was harvested before titration onto HEK293T/17, BHK-21, CrFK and Vero cell lines to gauge JEVpp infectivity, measured in RLU/ml. Production of JEV pseudoparticles appeared to be unsuccessful in all cases, regardless of the mass of furin protease transfected or whether an HIV or MLV core was employed (Figure 17). Relative transduction titres of JEVpp preparations with additional furin protease were at equivalent levels to PV harvests lacking the furin plasmid and ΔEG negative controls for each of the target cell lines, implying no transduction mediated by JEV prME envelope glycoproteins took place.

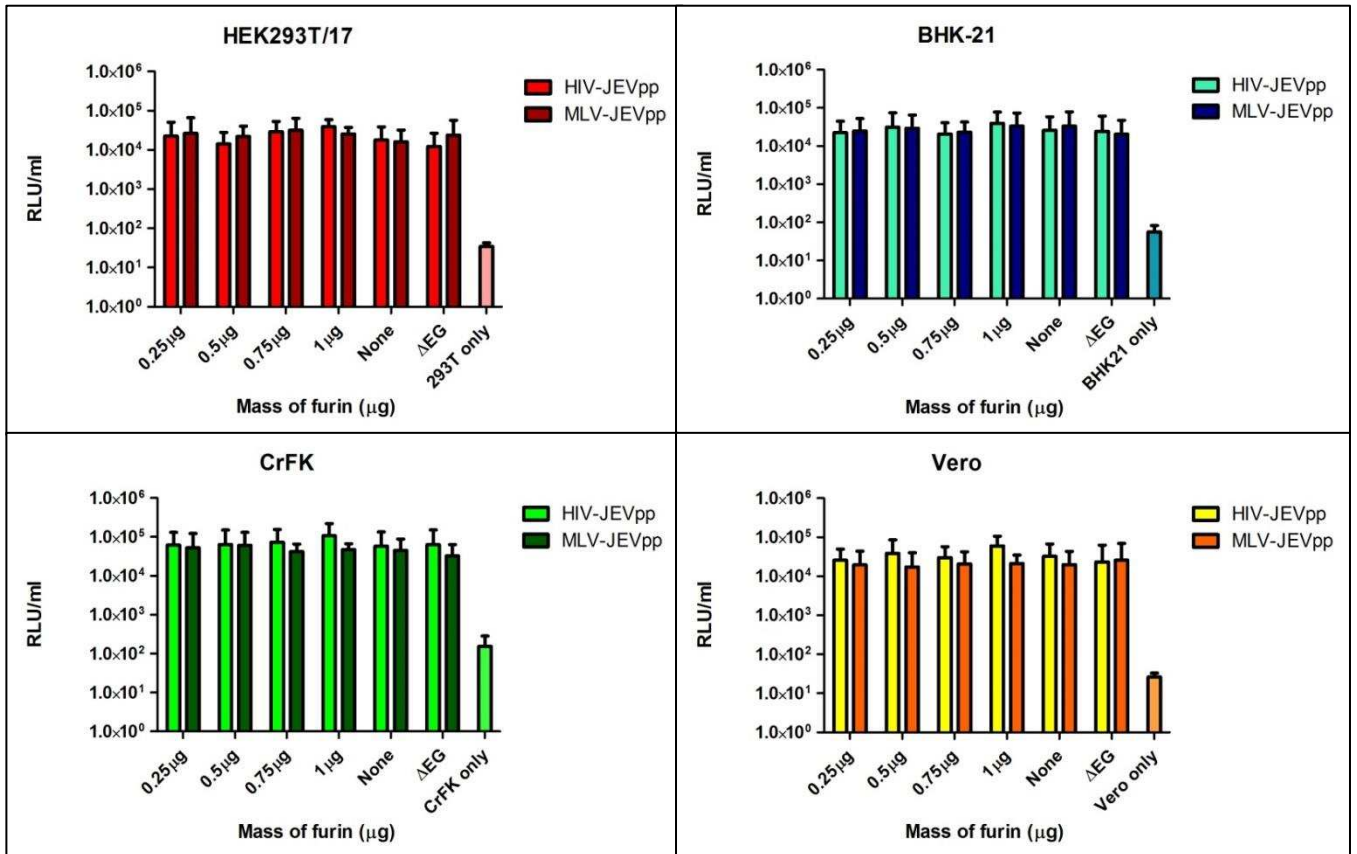


Figure 17. The influence of plasmid-derived furin protease expression on production of JEV-pseudotyped retroviruses. Candidate JEVpp preparations were titrated onto HEK293T/17, BHK-21, CrFK and Vero target cell lines. Pseudotype transduction titres are expressed as mean \pm SD (data retrieved in triplicate) of relative luminescent units per ml (RLU/ml). Δ envelope glycoprotein and target cell only negative controls are also shown. The x-axis values of '0.25 μ g', '0.5 μ g', '0.75 μ g' and '1 μ g' refer to the mass of pCAGGS-furin transfected during JEVpp production attempts.

3.3.5 Low glucose environment for generating JEV pseudotype viruses

Previous literature has reported that glucose can disrupt the mechanism of action of the C-type lectin DC-SIGNR, which acts as an attachment factor on permissive target cells for a number of flaviviruses. Virion engagement by the carbohydrate recognition domain (CRD) of DC-SIGNR stabilises prME glycoprotein binding to a given cellular receptor, thus facilitating flavivirus fusion and entry. The binding and competitive inhibition of monosaccharides, such as glucose and mannose, to the CRD domain of DC-SIGNR, perturbs virus attachment and negatively impacts upon the success of flavivirus infection (Obara *et al*, 2013). To investigate whether this phenomenon effects HIV- and MLV-JEV pseudotype transduction of target cells, transfections and titration assays to produce and quantify the infectivity of JEV pseudotype viruses were carried out in a low glucose (1g/L) DMEM culture medium formulation.

Transduction titres (measured in RLU/ml) were compared between JEVpp preparations (using pCAGGS-24SPprME) in low and high glucose DMEM (4.5g/L – typical glucose concentration in DMEM culture medium), with no significant improvement of infectivity observed by lowering the glucose concentration in the culture medium (Figure 18). All pseudotype virus titres were of comparable levels to Δ EG negative controls, indicating negligible production of functional JEV pseudoparticles.

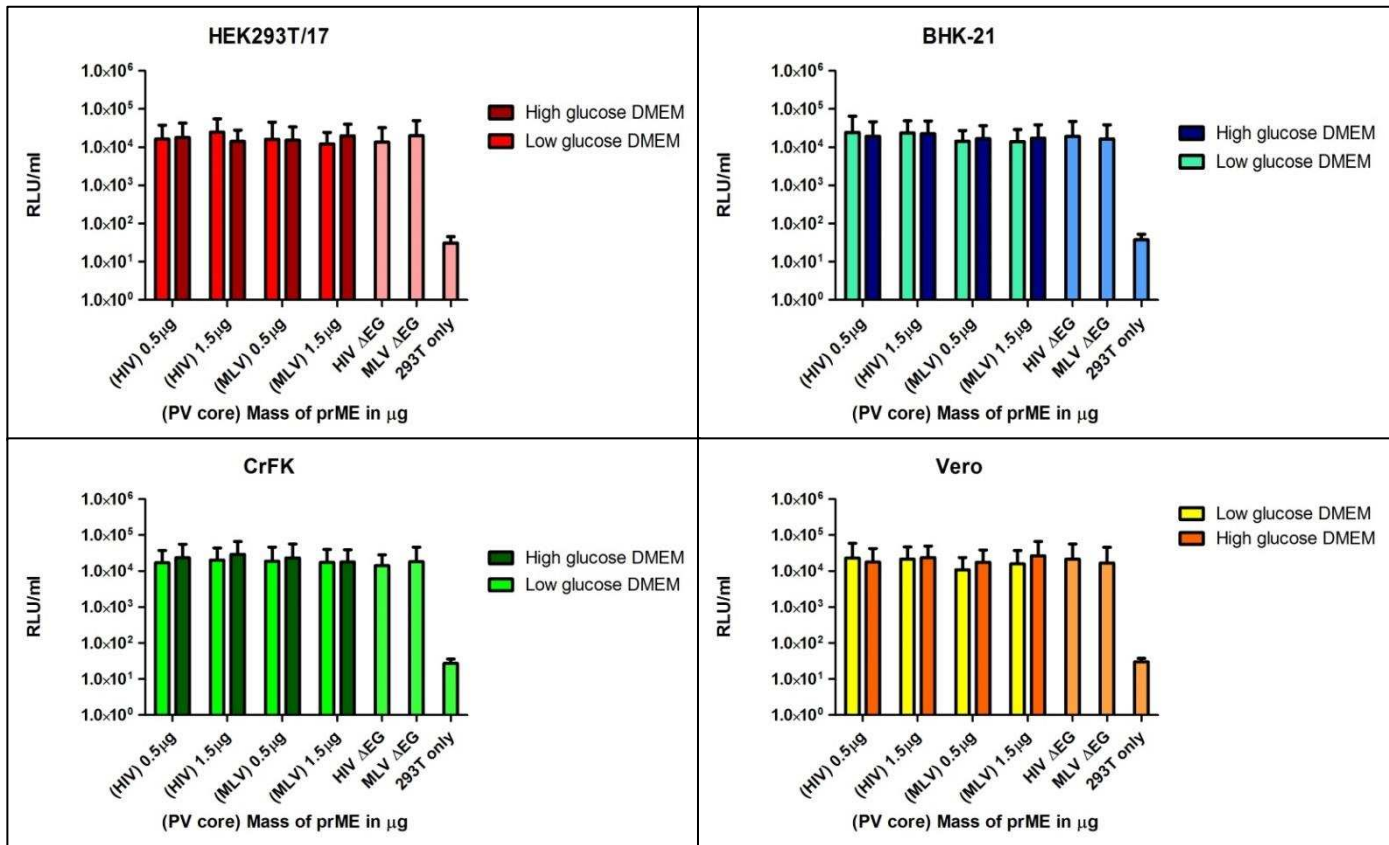


Figure 18. Comparison of JEV pseudotype virus preparations in low and high glucose DMEM culture medium. Candidate JEVpp samples (with HIV and MLV cores) were titrated onto HEK293T/17, BHK-21, CrFK and Vero target cell lines. Pseudotype transduction titres are expressed as mean \pm SD (data retrieved in triplicate) of relative luminescent units per ml (RLU/ml). Δ envelope glycoprotein and target cell only negative controls are also shown. 'Low glucose' and 'high glucose' are defined as 1g/L and 4.5g/L glucose in DMEM culture medium, respectively. '0.5 μ g' and '1.5 μ g' refer to the mass of pCAGGS-24SPprME introduced into mammalian producer cells to create JEVpp particles.

3.3.6 Utilisation of MLV packaging cell lines to attempt JEV pseudotype production

Successful production of JEV-pseudotyped MLV virus particles has been reported previously, using the MLV *gag-pol* packaging cell line TELCeB6 as opposed to transfecting the necessary components for functional pseudoparticle generation (Lee *et al*, 2009; Lee *et al*, 2014). Both TELCeB6 and TECeB15 cell lines constitutively express replication-defective MLV pseudotyping cores, and thus can be exploited to produce pseudotype viruses by transient transfection of a heterologous envelope glycoprotein and a chosen reporter gene. In a bid to mimic the reported JEV pseudotype formation, both of these packaging cell lines were utilised as producer cells in lieu of HEK293T/17, and were each simultaneously transfected with pCNCG (GFP-encoding gammaretroviral vector plasmid) and either 1µg or 3µg of pCAGGS-15SPprME using Fugene 6 transfection reagent (Promega, UK). After harvesting of the candidate pseudotype supernatant from the packaging cell line monolayers and filtration to remove cellular debris, the JEVpp preparations were titrated onto HEK293T/17, BHK-21 and CrFK target cell lines. Retroviral pseudotypes bearing the VSV-G glycoprotein and harbouring a GFP reporter gene were used as a positive control in the titration assays, which were incubated for 72 hours, before assessing levels of GFP expression under a fluorescent microscope. As can clearly be seen from Figure 19, no observable green fluorescence is present in any of the experimental sample wells, despite witnessing highly efficient transduction and GFP expression in the VSV-G positive control wells. This is a convincing indication that no successful JEVpp particles have been generated and in turn, no target cell transduction, reporter gene integration and expression have occurred.

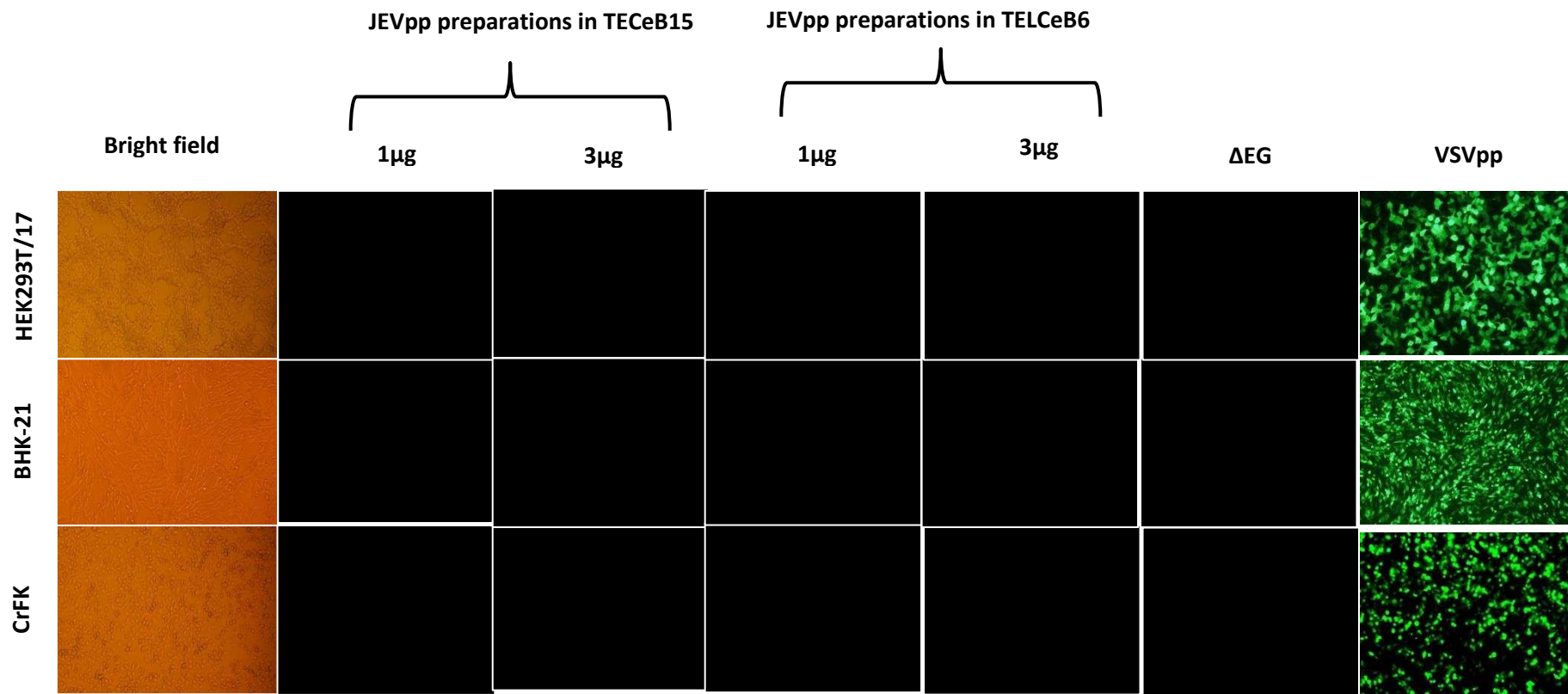


Figure 19. Utilisation of MLV packaging cell lines TECEB15 and TELCEB6 to attempt JEV pseudotype production. Representative green fluorescent images are displayed following titration assays with JEVpp samples generated in TECEB15 and TELCEB6. Bright field images of each cell line, as well as VSVpp positive control and ΔEG negative control images are also shown. '1µg' and '3µg' refer to the mass of pCAGGS-15SPprME transfected during JEVpp production attempts.

3.3.7 Immunofluorescence to determine prME intracellular localisation

As the alteration of a number of methodological parameters was not successful in the generation of functional pseudotype viruses bearing the JEV prME glycoproteins, indirect immunofluorescent staining was undertaken to ascertain the subcellular distribution of any expressed prME antigen. HEK293T/17 cells were transfected with 1 μ g of either pCAGGS-15SPprME or pl.18-Sussex89H3 and incubated for 48 hours. Following fixation/permeabilisation of transfected cells and antibody staining, sample coverslips were mounted onto glass slides and visualised under a fluorescent microscope. A selection of negative controls was employed: to gauge whether any non-specific primary antibody binding was taking place, untransfected HEK293T/17 cells were stained with equine polyclonal 1 $^{\circ}$ Ab, rabbit-anti-horse (R α H)-FITC 2 $^{\circ}$ Ab and NucBlue nuclear stain (Figure 20E); in order to rule out non-specific secondary antibody binding, 293T cells transfected with either the prME (Figure 20B) or HA (Figure 20D) plasmids were stained only with the R α H-FITC 2 $^{\circ}$ Ab (PBS in place of 1 $^{\circ}$ Ab) and NucBlue nuclear stain. From here, samples with the full combination of antibodies could be reliably compared to negative controls to ascertain immunostaining mediated by binding to virus glycoprotein. As expected for the HA positive control (Figure 20C), the strongest immunostaining can be observed as green 'halos' at the edges of individual cells, which is indicative of plasma membrane-localised HA expression, and suitably explains why successful pseudotyping with this heterologous envelope glycoprotein occurs. However, the immunofluorescence image for JEV prME expression (Figure 20A) appears to contain perinuclear green 'patches', which could be due to glycoprotein localisation at the endoplasmic reticulum. This result suggests that moderate levels of prME expression may be taking place, but that this glycoprotein is retained intracellularly, and is not being incorporated into retroviral pseudoparticles – possibly due to contrasting native budding sites of flaviviruses and particular retroviruses.

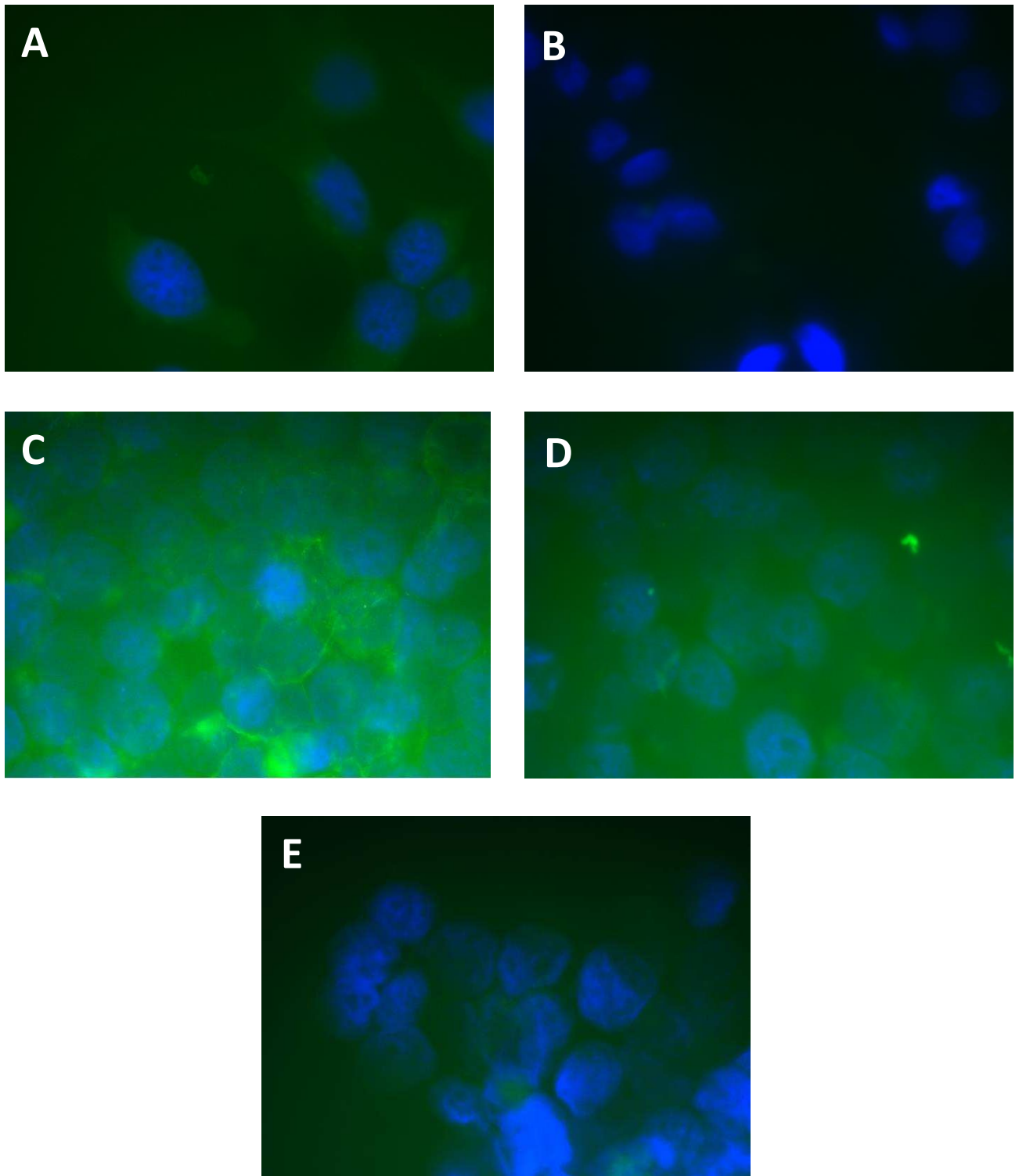


Figure 20. Indirect immunofluorescent staining images to determine JEV prME intracellular localisation. A) HEK293T/17 cells transfected with pCAGGS-15SPprME and stained with equine polyclonal 1° Ab, RαH-FITC 2° Ab and NucBlue nuclear stain. B) HEK293T/17 cells transfected with pCAGGS-15SPprME and stained with PBS, RαH-FITC 2° Ab and NucBlue nuclear stain. C) HEK293T/17 cells transfected with pI.18-H3/A/equine/Sussex/89 and stained with equine polyclonal 1° Ab, RαH-FITC 2° Ab and NucBlue nuclear stain. D) HEK293T/17 cells transfected with pI.18-H3/A/equine/Sussex/89 and stained with PBS, RαH-FITC 2° Ab and NucBlue nuclear stain. E) Untransfected HEK293T/17 cells stained with equine polyclonal 1° Ab, RαH-FITC 2° Ab and NucBlue nuclear stain.

3.3.8 Western blotting to validate prME expression

Due to the persistent inability to produce retroviral pseudotype viruses displaying the prME envelope glycoproteins, SDS-PAGE and Western blotting was carried out to validate whether any prME expression is taking place when transfecting the pCAGGS-15SPprME and pCAGGS-24SPprME constructs into HEK293T/17 cells. Either 0.5µg or 1.5µg of the 15SP or 24SP prME plasmids were transfected and incubated for 48 hours, prior to cell lysis, protein separation by SDS-PAGE, transfer onto a PVDF membrane, and blotting for desired antigens. As an internal control, β-actin was also immunoblotted for, to determine whether the protocol was functioning correctly. Blot images taken using the Odyssey CLx machine revealed that β-actin was present in all samples, as expected, but band size and clarity varied, indicating a relatively functional but inconsistent procedure. For prME expression, the JEV monoclonal antibody 81193 was utilised in Western blotting, which binds to domain III of the E protein and yields a ~38kDa band. According to the blot image, no mAb81193 binding or prME expression were detected in any of the sample wells (Figure 21). Furthermore, JEV E protein was also not detected by the mAb81193 in the IXIARO positive control lane, suggesting a shortcoming in the protocol used to produce the Western blot, such as poor protein transfer to the PVDF membrane. It is possible that this result may be legitimate – however, this protocol was not completely refined and could require further optimisation before an actual lack of prME expression could be confidently concluded. Potential further protocol alterations include: standardisation of the mass of expressed prME added prior to SDS-PAGE peptide separation; the percentage of the polyacrylamide resolving gel used for SDS-PAGE; the method of protein transfer from gel to PVDF membrane; and the affinity of the primary antibody utilised for prME blotting.

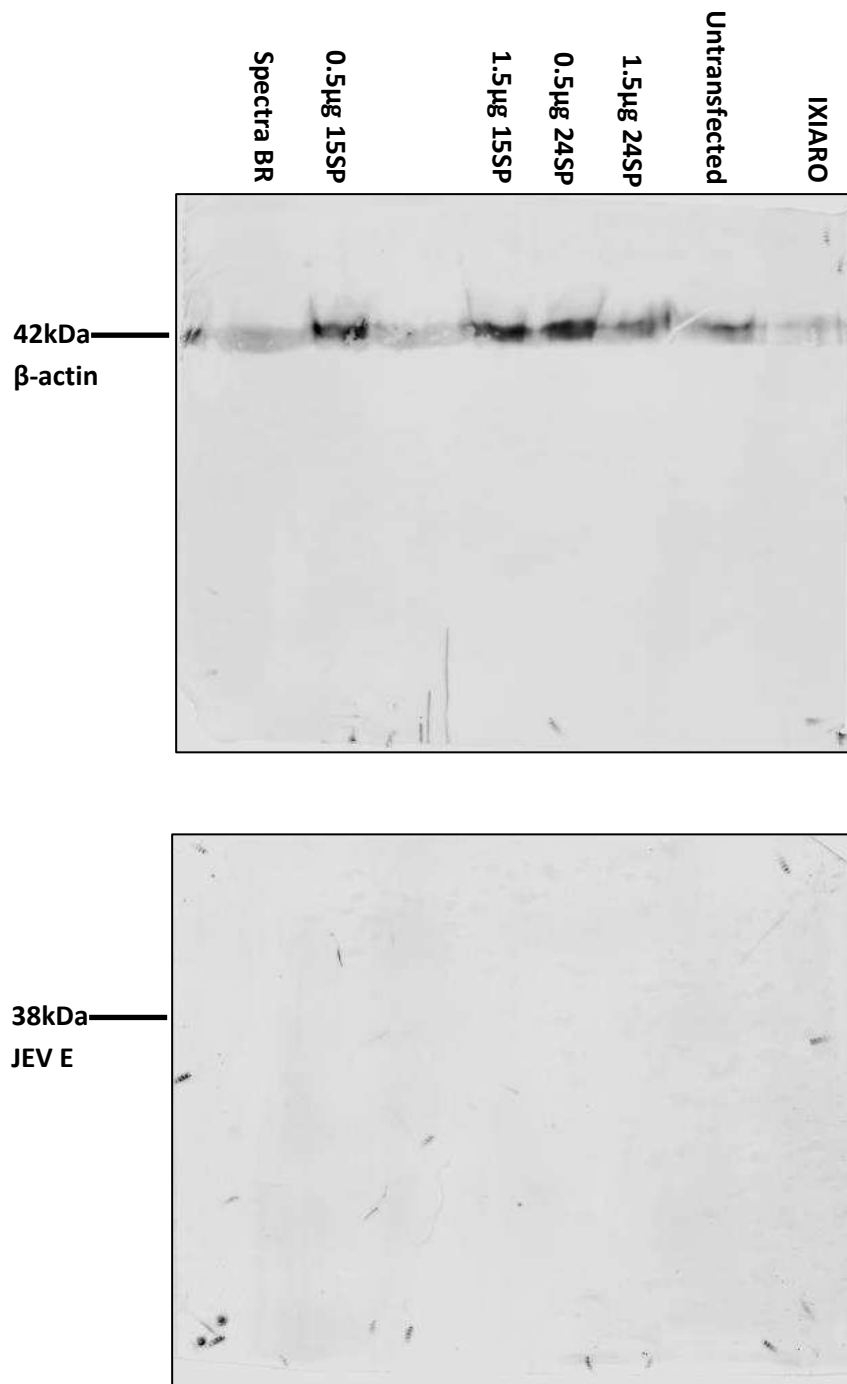


Figure 21. Western blot images of β -actin (~42kDa) and JEV prME (~38kDa) to validate protein expression in HEK293T/17 cells. A 12% polyacrylamide resolving gel was used to separate cell lysate peptides, prior to protein transfer and blotting for desired antigens. Spectra BR ladders with molecular weights (kDa) are also displayed at their approximate position on the PVDF membrane, since they were not visualised by the Odyssey CLx (Licor, NE, USA) machine. '0.5µg' and '1.5µg' refer to the mass of pCAGGS-15SPprME ('15SP') or pCAGGS-24SPprME ('24SP') transfected into HEK293T/17 cells before SDS-PAGE and Western blotting.

3.4 Discussion

As can clearly be seen from the set of results displayed in this report, retroviral pseudoparticles displaying the prM and E envelope proteins of JEV have not been successfully generated, despite the exploration of several variable parameters that could have had an impact on pseudotype production. This is not attributed to the prME gene and its manipulations, which were carried out at the start of the project – the subcloning process of the custom-synthesised gene yielded positive results throughout, with gel electrophoresis images indicating successful digestion and liberation of the prME insert, and positive pCAGGS-prME clones being identified from colony PCR and restriction digest screens. Furthermore, recombinant pCAGGS plasmids containing the 15SPprME or the extended 24SPprME genes were sequence verified, ensuring that unwanted mutations could not alter the structure of expressed prME and subsequently hinder pseudotype virus assembly.

In downstream transfections to generate JEV pseudotype viruses (PVs), both HIV and MLV cores were utilised, as well as employing two distinct approaches to produce the PVs: the conventional multi-plasmid co-transfection method, and the use of MLV packaging cell lines that stably express the *gag-pol* core proteins for coating with heterologous viral envelopes. This enabled the comparison of two distinct retrovirus cores to interact with JEV prME proteins and assemble functional pseudoparticles. However, neither core was able to initiate PV production. Additionally, despite reports of TELCeB6 cells effectively acting as an MLV pseudotype backbone (Lee *et al*, 2009; Lee *et al*, 2014), results from this project conflict with those findings. Overall, it appears that the choice of core retrovirus employed was not a dependent factor on the success of pseudotyping JEV prME in this instance. However, the inclusion of a strong positive control in these experiments would increase the strength of these data.

The carboxyl (COOH) terminus signal peptide of the C protein is located at the C-prM junction of the JEV genome, and directs the translocation of the prM protein through the secretory pathway in eukaryotic cells, once cleaved by signal peptidases (Stocks and Lobigs, 1998). In the relevant literature, signal peptide lengths of both 15 and 24 amino acid residues have been reported (Davis *et al*, 2001; Lin *et al*, 1998). Initially, the synthesised pUC57-prME construct for this study contained a 15-residue (or 45-nucleotide) signal peptide upstream of the prM gene. However, in order to maintain the correct signal peptidase cleavage and ensure the COOH C protein signal peptide spans the ER membrane properly, a prME gene construct with the full 24 residue (or 72 nucleotide) signal peptide was produced. Despite this, transfections with either the 15SPprME or 24SPprME genes did not produce functional JEV pseudotype viruses, indicating that the length of signal peptide and subsequent efficiency of prM and E protein trafficking through the Golgi apparatus was not a causal factor for the lack of successful JEVpp production.

A variety of target cell lines were also exploited to monitor transduction of any functional JEVpp and subsequent reporter gene expression: HEK293T/17, BHK-21, CrFK and Vero. Of these, BHK-21 and Vero are extensively used in virology to study entry and fusion mechanisms of JEV, and thus are generally acknowledged as being permissive to JEV infection (Ding *et al*, 2011, Makino and Jenkin, 1975; Su *et al*, 2002; Nawa *et al*, 2003). CrFK cells have also been utilised as target cells for JEV entry (Lee *et al*, 2009; Cochran *et al*, 1991). As these permissible cell lines were not infected by putative JEVpp preparations at significantly higher levels than HEK293T/17 (which is not usually employed in JEV entry studies), or indeed at all, it can be confidently concluded that the inability to develop pseudotype-based assays for JEV is not attributed to the lack of a specific cellular receptor on target cell lines.

Furin-like proteases are known to cleave the precursor segment from the JEV M protein during virus egress, inducing a conformational change at the virus envelope to create mature,

infectious virions (Davis *et al*, 2006; Mukhopadhyay *et al*, 2005). Reports of endogenous furin protease expression in HEK293T/17 cells vary between publications (Xu *et al*, 2002; Tay *et al*, 2012; Tse *et al*, 2014), so an additional range of masses of furin plasmid were transfected into producer cells during JEVpp generation, in an attempt to stimulate this proteolytic reaction and create JEV pseudotype viruses with a structurally mature envelope. Regardless of plasmid mass, the heightened eukaryotic expression did not appear to play a critical role in JEVpp production, maturation and infectivity. However, to further investigate this phenomenon, plasmid-derived furin protease expression could be quantified by Western blot, and a cell type confirmed to endogenously express higher levels of furin protease, such as Huh7 (Tay *et al*, 2012), could also be employed as JEVpp producer cells.

The potential inhibitory impact of using high glucose DMEM culture medium to produce JEV pseudoparticles was also explored. Glucose and similar monosaccharides (from approximately $\geq 1\text{mM}$ concentration) are proven to competitively inhibit the carbohydrate binding domain of DC-SIGN, which can act as a stabilising attachment factor on host cell plasma membranes, facilitating entry and infection for a number of flaviviruses (Obara *et al*, 2013). Despite hypothesising that this phenomenon may play a role in preventing JEVpp target cell transduction, the infectivity titres of JEV pseudotypes generated and titrated in low or high glucose DMEM were not significantly different, suggesting that glucose concentration is not a variable that can detrimentally influence JEVpp infection.

A critical factor for success in general pseudotyping, and more specifically for JEV pseudotype viruses, is to validate that the necessary plasmid-borne envelope glycoproteins are being efficiently expressed within producer cells such as HEK293T/17. Due to the customisation of plasmids designed for optimal eukaryotic expression, it would be logical to hypothesise that recombinant protein production is taking place, driven by the efficient promoter region upstream of the cloned gene. Furthermore, indirect immunofluorescent staining was used to

identify the intracellular localisation of expressed prME antigen, with results suggesting that there is identifiable expression of JEV envelope glycoproteins following transfection with the pCAGGS-xSPprME constructs, but that it is retained in perinuclear regions of the cell. This finding is consistent with the possibility of endoplasmic reticulum retention of prME, and failure of the HIV and MLV cores to recruit the JEV envelope during their egress. Despite this, the immunofluorescence images are extremely faint, the Western immunoblotting images clearly did not detect any expressed prME protein and it is evident from these results that no substantial JEV prME expression has taken place. A further consideration for future work related to this project would be to obtain a definitive verdict of the levels and locations of intracellular prME expression within producer cells, as this would offer valuable insight for subsequent JEVpp production attempts.

Chapter 4

Genetic modification of the Japanese encephalitis virus pre-membrane and envelope proteins to augment eukaryotic expression and stimulate pseudotyping with heterologous nucleocapsids

4.1 Introduction

Despite its many advantages, the pseudotype virus platform also poses some significant challenges. For instance, the majority of viruses that have to date been adapted into pseudotypes possess a single envelope glycoprotein, though the expression of two distinct envelope proteins may be a prerequisite for the successful generation of a PV, such as with the routinely-pseudotyped HCV. This can lead to a more complicated process for achieving functional PV production (Bartosch *et al*, 2003a; Mather *et al*, 2013). Furthermore, discrepancies can occur between the density of envelope glycoproteins displayed on the surface of a pseudoparticle and of a native, wild-type infectious virus (Kolokoltsov and Davey, 2004). This can be problematic, since a reduction of glycoprotein density can result in the loss of quarternary epitopes created by the tight packing of envelope protein complexes (Kaufmann *et al*, 2010). Conversely, over-expression and excessive display of glycoproteins on the pseudotype surface can in fact mask important antibody epitopes that are accessible and immunologically recognised on the native virion surface (Nelson *et al*, 2008). Therefore, to avoid substantial serological implications, close scrutiny of assay data when conducting comparative serology is vital.

However, a major challenge and perhaps the most relevant with regards to this study, is that critical processes in the assembly and maturation of the envelope proteins in the wild-type virus may be lost in the generation of a PV. In particular, retroviral pseudotyping lends itself more readily to the incorporation of heterologous envelope proteins from external-budding

viruses. This is because particle formation of retroviruses occurs at the plasma membrane, whereas for internal-budding viruses such as flaviviruses, it occurs at the endoplasmic reticulum. Although some internal-budding viruses can be successfully pseudotyped, such as SARS and HCV (Bartosch *et al*, 2003a; Hsu *et al*, 2003; Simmons *et al*, 2004; Temperton *et al*, 2005; Fukushi *et al*, 2006), in many instances, this mismatch in the subcellular locations of virus maturation can inhibit the creation of functional PVs.

One approach for counteracting this obstacle to successful pseudotyping is *via* the construction of a chimeric glycoprotein (cGP), which can encourage interaction between the nucleocapsid core and virus envelope protein during the production, assembly and egress of pseudoparticles. This technology has been successfully employed in the generation of PVs for each of the four DENV serotypes, where the transmembrane (TM) domain of the DENV E was replaced with the TM and cytoplasmic (CY) domains of the VSV-G glycoprotein (Hu *et al*, 2007). Importantly, the ectodomain of the native glycoprotein gene (i.e. DENV E) is not amended, so that when expressed, resultant PVs do not possess any structural alterations from an external perspective. The VSV envelope protein is an ideal template for cGP construction as it efficiently and reliably creates high-titre retroviral pseudotypes, which are frequently employed as positive controls in other viral pseudotyping studies, due to their wide host range and cell tropism (Cronin *et al*, 2005; Temperton *et al*, 2015). Also, the VSV-G TM and CY domains contain a membrane-targeting signal (Rose and Whitt, 2001), which enhanced the intracellular trafficking and plasma membrane expression of the DENV prME, thus enabling the generation of functional DENV PVs. This finding was confirmed by Western blot of producer cell lysates and viral supernatant (Hu *et al*, 2007). Moreover, it has also been reported that an effective method for overcoming problems concerning the infectivity titre of RABV lentiviral pseudotypes is through the creation of a chimeric glycoprotein. In this case, the construct consisted of the external and TM domains of RABV fused to the CY domain of VSV-G, and

resulted in increased incorporation of envelope glycoproteins onto HIV-1 lentiviral cores, as well as increased *in vitro* infectivity into HEK293T cells (Carpentier *et al*, 2011).

Another modification that can be made to the genes of virus envelope proteins in a bid to promote successful incorporation into pseudotype particles is the upstream inclusion of a Kozak consensus sequence. Originally in 1978, Kozak described a scanning model for the initiation of translation in eukaryotic cells. Along with elucidating the mechanism of how the 40S ribosomal subunit binds to the 5' end of an mRNA and scans along it for an AUG (methionine) codon before initiating translation, it was also proposed that if a particular sequence of nucleotides is located directly upstream of the AUG codon, the efficiency of translation initiation can be enhanced, which in turn has the effect of significantly boosting protein expression levels (Kozak, 1978; Kozak, 1986; Kozak, 2002; Nagakawa *et al*, 2008). Efficient expression of the heterologous envelope glycoprotein is a crucial criterion in the generation of high-titre, functional pseudotype viruses – it has been presented in some pseudotyping studies concerning the internal-budding SARS and MERS coronaviruses that augmented 'overexpression' of the envelope protein gene can result in glycoprotein 'leakage' to the plasma membrane and subsequent incorporation onto retroviral nucleocapsids as they bud from the cell (Simmons *et al*, 2004; Perera *et al* 2013; Temperton *et al*, 2015). This phenomenon could translate to other families of internal budding viruses, such as flaviviruses. The heightened protein expression conferred by the incorporation of a Kozak sequence into the envelope gene plasmid may increase the glycoprotein abundance at the plasma membrane, and in turn the likelihood of successful pseudotype virus formation.

In this chapter, the construction of chimeric virus glycoproteins featuring the JEV E ectodomain, as well as the introduction of distinct Kozak consensus sequences upstream of the prME gene, will be investigated in order to promote the production of functional retroviral pseudotypes displaying the JEV envelope glycoproteins.

4.2 Materials and Methods

4.2.1 Mutagenic primer design and synthesis

The design of primers to be employed for both the construction of the JEV/VSV chimeric glycoproteins and the introduction of Kozak consensus sequences was conducted using the NEBaseChanger online program (<http://nebasechanger.neb.com/>; New England Biolabs, MA, USA). This tool is recommended by New England Biolabs for generation of primer sequences and calculation of an optimal thermal cycling program, when using the Q5 Site-Directed Mutagenesis Kit. All Kozak and chimeric glycoprotein primers were synthesised to order by Eurofins MWG Operon, Germany.

4.2.2 Polymerase chain reaction

Polymerase chain reaction is used as an integral part of the protocol for all site-directed mutagenesis carried out in this chapter. The Q5 Hot-Start High Fidelity DNA Polymerase is utilised in this kit, as part of a 2X concentrated Master Mix, which also contains a polymerase buffer, dNTPs and Mg²⁺ ions. Once mixed with the relevant plasmid template DNA and mutagenic primers, it is subjected to a thermal cycling program, causing exponential amplification of the template and incorporation of the required mutations into the subsequent amplicon material. The pUC57 plasmid was chosen as an initial starting template for all mutagenesis presented in this chapter, since it is a shorter construct than the pCAGGS expression vector used in subcloning, minimising the potential for polymerase replication slippage and nucleotide discrepancies to occur in the PCR product. Information pertaining to the generic materials used for performing PCR can be found in Chapter 2 of this thesis. Further details of the mutagenic PCR reaction mixture and thermocycling program are shown in Tables 4 and 5.

Q5 site-directed mutagenesis – PCR reaction mixture – total volume 25µl	
Component	Volume (µl)
2X Q5 PCR Master Mix	12.5µl
Fwd primer (10µM)	1.25µl
Rev primer (10µM)	1.25µl
Template plasmid DNA (diluted to 1-25ng/µl)	1µl
Nuclease-free H ₂ O	9µl

Table 4. Q5 site-directed mutagenesis PCR reaction mixture. Volumes are stated in µl for each component in the reaction mixture, to a total volume of 25µl. The final concentration of the oligonucleotide primers in the mixture was 0.5µM.

Q5 site-directed mutagenesis – standard thermal cycling program		
Cycle stage		Temperature (°C) and duration (mins/secs)
Initial denaturation		98°C for 30 seconds
Denaturation	x25 cycles	98°C for 10 seconds
Annealing		50-72°C for 30 seconds
Extension		72°C for 2 minutes
Final extension		72°C for 2 minutes
4°C until removal from thermocycler		

Table 5. Standard thermal cycling program for Q5 site-directed mutagenesis kit. Each cycle stage is given, with corresponding temperature (in °C) and duration (in mins/secs). The optimal annealing temperature varies in each mutagenic amplification taking place and is calculated by the NEBaseChanger online tool, depending on the melting temperature (T_m) of the forward and/or reverse primers. Recommendations range from 50-72°C.

4.2.3 Kinase-ligase-DpnI (KLD) enzyme treatment

Once exponential amplification is complete, the next stage of the Q5 Site-Directed Mutagenesis kit, used for all mutagenesis attempted in this chapter, is the KLD enzymatic reaction. The enzyme mix contains a blend of kinase, ligase and DpnI enzymes. This treatment harnesses the catalysing abilities of the three enzymes, to bring about phosphorylation and intramolecular ligation of the whole-plasmid PCR product, as well as DpnI digestion of methylated template DNA. Mutagenic amplicons produced during the production of JEV/VSV chimeric glycoproteins and the insertion of Kozak sequences into the JEV prME gene construct underwent KLD treatment, with incubation for 5 minutes at room temperature, before proceeding with transformation. The KLD reaction mixture is as follows (detailed in Table 6):

Q5 site-directed mutagenesis – KLD treatment reaction mixture – total volume 10µl	
Component	Volume (µl)
PCR product	1µl
2X KLD buffer	5µl
10X KLD enzyme mix	1µl
Nuclease-free H ₂ O	3µl

Table 6. Q5 site-directed mutagenesis KLD reaction mixture. Volumes are stated in µl for each component in the reaction mixture, to a total volume of 10µl.

4.2.4 Transformation into DH5 α *E.coli* cells

Following KLD treatment, the whole-plasmid mutagenic amplicons should be successfully phosphorylated and circularised, as well as in a template-free solution, thanks to the presence of DpnI digesting unwanted, methylated DNA. Therefore, the intact mutant plasmids can be transformed into DH5 α competent *E.coli* cells for propagation. In all instances, transformation was carried out as is described in Chapter 2 of this thesis, with the only difference being the plasmid DNA introduced into the competent *E.coli* cells was different from sample to sample. Potential transformants, harbouring Kozak- or chimeric glycoprotein-mutated plasmids, were streaked onto ampicillin-LB agar plates to promote growth of resistant colonies.

4.2.5 Colony PCR

If bacterial colonies grow and produce distinct, isolated colonies on their respective ampicillin-LB agar plates, this indicates that transformation of the particular mutated pUC57-prME plasmid into DH5 α cells has been successful, due to the ampicillin resistance gene, located on the pUC57 vector backbone. In order to confirm specific uptake of the desired mutagenic plasmid, colony PCR was used, which amplifies a particular gene or stretch of nucleotides, enabling the screening of a plasmid with a desired insert to take place, without the prior need for further culturing and purification steps. All colony PCR in this chapter, for both Kozak and chimeric glycoprotein experiments, was performed using the M13 Fwd and Rev universal sequencing primers, which bind to the pUC57 plasmid in the arms of the vector, flanking the insert gene for amplification. Furthermore, the 'JEV50SCREEN' thermal cycling program used to carry out the colony PCR amplification is detailed in Table 7. A more detailed protocol, which includes information on the DreamTaq Green polymerase used, as well as positive and negative control samples, can be found in Chapter 2 of this thesis.

Colony PCR – ‘JEV50SCREEN’ thermal cycling program	
Cycle stage	Temperature (°C) and duration (mins/secs)
Initial denaturation	94°C for 2 minutes
Denaturation	x30 cycles
Annealing	
Extension	
Final extension	72°C for 5 minutes
4°C until removal from thermocycler	

Table 7. ‘JEV50SCREEN’ thermal cycling program for colony PCR screening. Each cycle stage is given, with corresponding temperature (in °C) and duration (in mins/secs). Once finished, the samples were stored in a refrigerated state in the thermocycler until removed completely.

4.2.6 Restriction enzyme digest screening

To corroborate findings determined by colony PCR, especially in instances when successful transformation of particular Kozak or chimeric glycoprotein mutant pUC57-prME plasmids cannot be definitively confirmed, a further screening process of restriction enzyme digestion was utilised. This reaction uses either conventional or FastDigest *EcoRI* and *XhoI* restriction enzymes, which cleave the recombinant pUC57-prME plasmids in the multiple cloning site (MCS) of the plasmid backbone, thus accurately removing and separating out an insert, if it is present. The length of the resulting vector and insert fragments can then be calculated *via* gel electrophoresis (see section 4.2.7) to assess whether the desired insert gene is possessed. Details of the endonuclease reactions, including incubation durations and temperatures, can be found in section 2.1.9. Representative conventional and FastDigest restriction digestion reactions are respectively shown in Tables 8 and 9.

Restriction enzyme digest screening – conventional REs – reaction mixtures – total volume 20µl			
Component	Sample reaction volumes (µl) or masses (ng)		
	①	②	③
pUC57-prME plasmid DNA	–	~500ng	~500ng
pUC57 Kozak/cGP-prME mutant plasmid DNA	~500ng	–	–
<i>EcoRI</i> enzyme	1µl	1µl	–
<i>XhoI</i> enzyme	1µl	1µl	–
10X stock Tango buffer	4µl	4µl	4µl
Nuclease-free H ₂ O	Up to 20µl	Up to 20µl	Up to 20µl

Table 8. Conventional restriction enzyme digest screening reaction mixture. Each component of the reaction mixture is detailed, along with its volume (in µl) or mass (in ng) for each of the experimental samples: reaction ① is designed to cleave a Kozak or cGP mutagenesis plasmid, whereas ② is the positive control containing the sequence-verified pUC57-prME plasmid, and ③ is an uncut negative control containing no conventional restriction endonucleases.

Restriction enzyme digest screening – FastDigest REs – reaction mixtures – total volume 20µl			
Component	Sample reaction volumes (µl) or masses (ng)		
	①	②	③
pUC57-prME plasmid DNA	–	~500ng	~500ng
pUC57 Kozak/cGP-prME mutant plasmid DNA	~500ng	–	–
FD <i>EcoRI</i> enzyme	1µl	1µl	–
FD <i>XhoI</i> enzyme	1µl	1µl	–
10X FD Green buffer	2µl	2µl	2µl
Nuclease-free H ₂ O	Up to 20µl	Up to 20µl	Up to 20µl

Table 9. FastDigest restriction enzyme digest screening reaction mixture. Each component of the reaction mixture is detailed, along with its volume (in µl) or mass (in ng) for each of the experimental samples: reaction ① is designed to cleave a Kozak or cGP mutagenesis plasmid, whereas ② is the positive control containing the sequence-verified pUC57-prME plasmid, and ③ is an uncut negative control containing no FastDigest restriction endonucleases.

4.2.7 Gel electrophoresis

When the results of colony PCR or restriction enzyme digest screening reactions needed to be visualised, gel electrophoresis was employed. 10µl and 20µl, for colony PCR and restriction digest screening respectively, of each sample was loaded onto a 1% (w/v) agarose gel, with 0.5X TAE used as the gel solvent and electrophoretic buffer. A 120V voltage was subsequently applied, until the gel dye front had migrated to the foot of the gel. Further information concerning the visualisation of the gel, as well as the details and volumes of the ladder used on all agarose gels as size standard for the measurement of experimental DNA bands, is located in Chapter 2.

4.2.8 DNA sequencing

As a final screening process to validate whether successful introduction of Kozak consensus sequences or construction of JEV/VSV chimeric glycoproteins had taken place, pUC57-prME plasmids that had been subjected to Q5 site-directed mutagenesis were sent to GATC Biotech, in order to undergo Sanger sequencing and accurately determine the insert nucleotide sequence. Either pCAGGS or universal M13 forward and reverse sequencing primers were utilised for sequencing purposes, depending on whether the plasmid vector was pUC57 or pCAGGS, respectively. More details, including sample preparation for the SUPREMERun™ and LIGHTrun™ sequencing systems, is present in Section 2.1.7.

4.2.9 Transmembrane domain prediction

A crucial aspect of logically designing a series of chimeric glycoprotein constructs is to accurately predict the location of transmembrane and cytoplasmic domains in both the native and template virus envelope glycoproteins being used – in this case, JEV E and VSV-G, respectively.

In order to elucidate the most likely positions of the two transmembrane domains towards the JEV E C-terminus, the amino acid sequence of the envelope glycoprotein was processed through a variety of online transmembrane topology prediction programmes (Table 10):

- HMMTOP: This automatic server for transmembrane helices and protein topology utilizes a hidden Markov model (HMM) to study amino acid distributions within sample sequences, against a large database of known membrane-spanning, cytoplasmic and extracytoplasmic protein segments, to predict transmembrane topology (Tusnády and Simon, 1998; Tusnády and Simon, 2001).
- TMHMM: This transmembrane topology predictor also uses a hidden Markov model and builds upon very similar HMM architecture to HMMTOP. TMHMM is able to perform specialised modelling of various membrane protein regions, and is particularly well suited to TM domain prediction because it incorporates several parameters, such as amino acid hydrophobicity, charge bias and helix length into one sophisticated estimation model (Sonnhammer *et al*, 1998; Krogh *et al*, 2001).
- Phobius: The strength of this transmembrane topology predictor is that it is also a signal peptide predictor, and has the ability to discriminate between TM domains and SPs. This is particularly advantageous, as their hydrophobic characteristics can lead to incorrect cross-prediction between these two motifs (Käll *et al*, 2004).
- TMPred: The TMPred program is able to make a prediction of the location and orientation of a protein's membrane-spanning regions, using an algorithm which examines an array of established membranous protein segments extracted from the UniProt KB/Swiss-Prot database. A variety of biological parameters are taken into account in the algorithm, such as putative transmembrane sequences, amino acid hydrophobicity, flanking region sequences and protein taxonomy (Hofmann and Stoffel, 1993).

- Sosui: This prediction system offers a fast, accurate determination of protein topology, with the unique ability to distinguish membrane-bound and soluble proteins from amino acid sequences, as well as identifying transmembrane domains and elucidating secondary and tertiary protein structures (Hirokawa *et al*, 1998; Mitaku and Hirokawa, 1999; Mitaku *et al*, 2002).

Following the processing of the JEV E protein sequence through these online transmembrane topology prediction programs, a consensus was drawn based upon the results yielded from each program. From here, the cGP constructs could be compiled in amino acid form and subsequently reverse transcribed to yield nucleotide sequences.

Determination of JEV E transmembrane domains using TMD topology programs		
TMD topology program	Website URL	References
HMMTOP	http://www.enzim.hu/hmmtop/html/document.html	Tusnády and Simon, 1998; Tusnády and Simon, 2001
TMHMM	http://www.cbs.dtu.dk/services/TMHMM/	Sonnhammer <i>et al</i> , 1998; Krogh <i>et al</i> , 2001
Phobius	http://phobius.sbc.su.se/	Käll <i>et al</i> , 2004
TMPred	http://embnet.vital-it.ch/software/TMPRED_form.html	Hofmann and Stoffel, 1993
Sosui	http://harrier.nagahama-i-bio.ac.jp/sosui/	Hirokawa <i>et al</i> , 1998; Mitaku and Hirokawa, 1999; Mitaku <i>et al</i> , 2002

Table 10. Transmembrane topology prediction programs for the determination of JEV E TM domains. A full list of the transmembrane topology programs utilised to predict the TM domains of the JEV envelope glycoprotein are displayed, including the website URLs of the programs, and their references.

Utilisation of the transmembrane domain and the cytoplasmic tail of the VSV-G protein in relevant literature is relatively established and their nucleotide sequences have been presented in several publications, with slight sequence variation observed amongst the

defined sequences (Cleverley and Lenard, 1998; Lagging *et al*, 1998; Buonocore *et al*, 2002; Köhl *et al*, 2004; Lei *et al*, 2010; Gravel *et al*, 2011). For this study, the amino acid residues that constitute the VSV-G TM domain and C-tail were defined by alignment and comparison of previous iterations of the protein regions, before an informed estimation was made, based upon the usual length, hydrophobicity and charge capping of the two glycoprotein motifs. Once a consensus was reached for the TMD and C-tail for VSV-G, these defined sequences could then be input into the amino acid sequences of the designed chimeric glycoprotein constructs.

4.3 Results

4.3.1 Design of JEV chimeric glycoprotein constructs using a VSV-G template

In an attempt to induce an interaction between the JEV envelope glycoproteins and retroviral *gag-pol* cores in pseudotype production, the construction of several chimeric glycoproteins was attempted. These maintain the E ectodomain of JEV on the external, extraviral surface, but the transmembrane domains (TMDs) of JEV are altered by introduction of, or swapping with, the TMD and cytoplasmic tail (C-tail) of vesicular stomatitis virus G protein (VSV-G). A VSV-G template was chosen to be employed in the production of JEV chimeric glycoproteins, since this envelope glycoprotein pseudotypes retroviral cores with high efficiency and has been shown to enhance the capability of other virus envelopes producing functional HIV pseudotypes (Carpentier *et al*, 2012). The following JEV prME/VSV-G chimeric glycoproteins (cGP) constructs were considered for design and utilisation in JEV pseudotyping experiments (see Figure 22 for schematics of all constructs):

Chimeric glycoprotein #1: Replacing second JEV E TMD with the C-tail of VSV-G

The simplest chimeric glycoprotein approach involves the substitution of the second JEV E TMD with the C-tail of VSV-G. The VSV-G C-tail is essential for its interaction with retroviral *gag*

and subsequent glycoprotein coating during pseudotype production, so has been included in this cGP to stimulate similar connections for JEV envelope glycoproteins. However, a main concern with this cGP construct is that pseudotype viruses have to successfully engage with target cells and induce entry *via membrane fusion* – for VSV-G, it has been confirmed that its TMD is vital for fusion with permissible target cell membranes (Cleverley and Lenard, 1998), whereas the unique hairpin motif between the two flavivirus TMDs is crucial for fusion and cellular entry for this family of viruses (Fritz *et al*, 2011). The absence of the complete TMD segment from either virus may prevent the success of this cGP construct.

Chimeric glycoprotein #2: Replacing both of the JEV E TMDs with the TMD and C-tail of VSV-G

JEV/VSV cGP #2 involves the replacement of both JEV TMDs with the VSV TMD and C-tail.

Although this construct possesses the intact VSV transmembrane and cytoplasmic sections so should be fusion competent, the unique feature of flavivirus envelope proteins is that they contain a double TMD with a hairpin. It is possible that interactions between the ectodomain and transmembrane regions of JEV could be hindered by the removal of the distinctive JEV TMD segment. Furthermore, heterodimeric interactions between JEV prM and E proteins have been previously reported (Lin and Wu, 2005; Peng and Wu, 2014), and the absence of the E TMDs from this chimeric glycoprotein may prevent their intra-membrane contact with the prM transmembrane residues, possibly resulting in reduced prM-E heterodimerisation and inhibition of JEV structural conformational change and maturation.

Chimeric glycoprotein #3: Appending the VSV-G TMD and C-tail onto the C-terminus of full length, wild-type JEV E

The third cGP involves appending the VSV-G TMD and C-tail onto the C-terminus of the full-length JEV E protein. This construct will result in the expression of three TMDs: the JEV E double TMD hairpin followed by the VSV-G TMD. The presence of the intact JEV

transmembrane segment should allow for interactions between E and prM, and subsequent virion envelope maturation; interaction with HIV or MLV *gag* proteins, and in turn pseudotype virus formation should occur, owing to the VSV-G TMD/C-tail. If successful PV production indeed takes place, the pseudoparticles should be fusion-competent with target cell membranes, mediated by either virus' transmembrane domains. However, a potential disadvantage of this construct is that the addition of an extra, heterologous transmembrane-spanning domain may physically block and hinder intra-membrane prM-E interactions.

Chimeric glycoprotein #4: Appending a duplicate first JEV E TMD and the VSV-G C-tail onto the C-terminus of wild-type, full length JEV E

Chimeric glycoprotein construct #4 is similar to #3, but involves the duplication of the first JEV E TMD, in place of the VSV-G TMD, whilst retaining the VSV-G C-tail. This approach may create lower levels of interaction inhibition between the prM and E TMDs, as the extra domain embedded in the membrane is native to JEV. Nonetheless, the presence of this duplicated TMD may cause steric hindrance and subsequent envelope protein conformational distortion, as well as potentially inducing incorrect interactions with other prM and E TMDs.

Chimeric glycoprotein #5: Inserting an anti-parallel tandem repeat of the VSV-G C-tail between the two JEV E TMDs

JEV/VSV cGP #5 involves the insertion of an anti-parallel tandem repeat of the VSV-G cytoplasmic tail between the two JEV E TMDs. As the VSV-G C-tail is all that is required to interact with retroviral core proteins during pseudotype assembly, it is possible that the TMD is obsolete for chimeric glycoprotein construction, as fusion of pseudoparticles into target cells can be mediated by the full JEV E TMD segment. If this is the case, then construct #5 would not alter the transmembrane topology of the JEV envelope proteins. As the VSV-G C-tail is naturally monomeric, inclusion of a single C-tail would probably result in hairpin loop

formation and attachment of its C-terminus to the second JEV TMD, restricting its interaction with retroviral *gag*. Inclusion of two anti-parallel C-tails should enable them to sit adjacent to one another and more exposed to the cytosol, facilitating interaction with the pseudotyping cores.

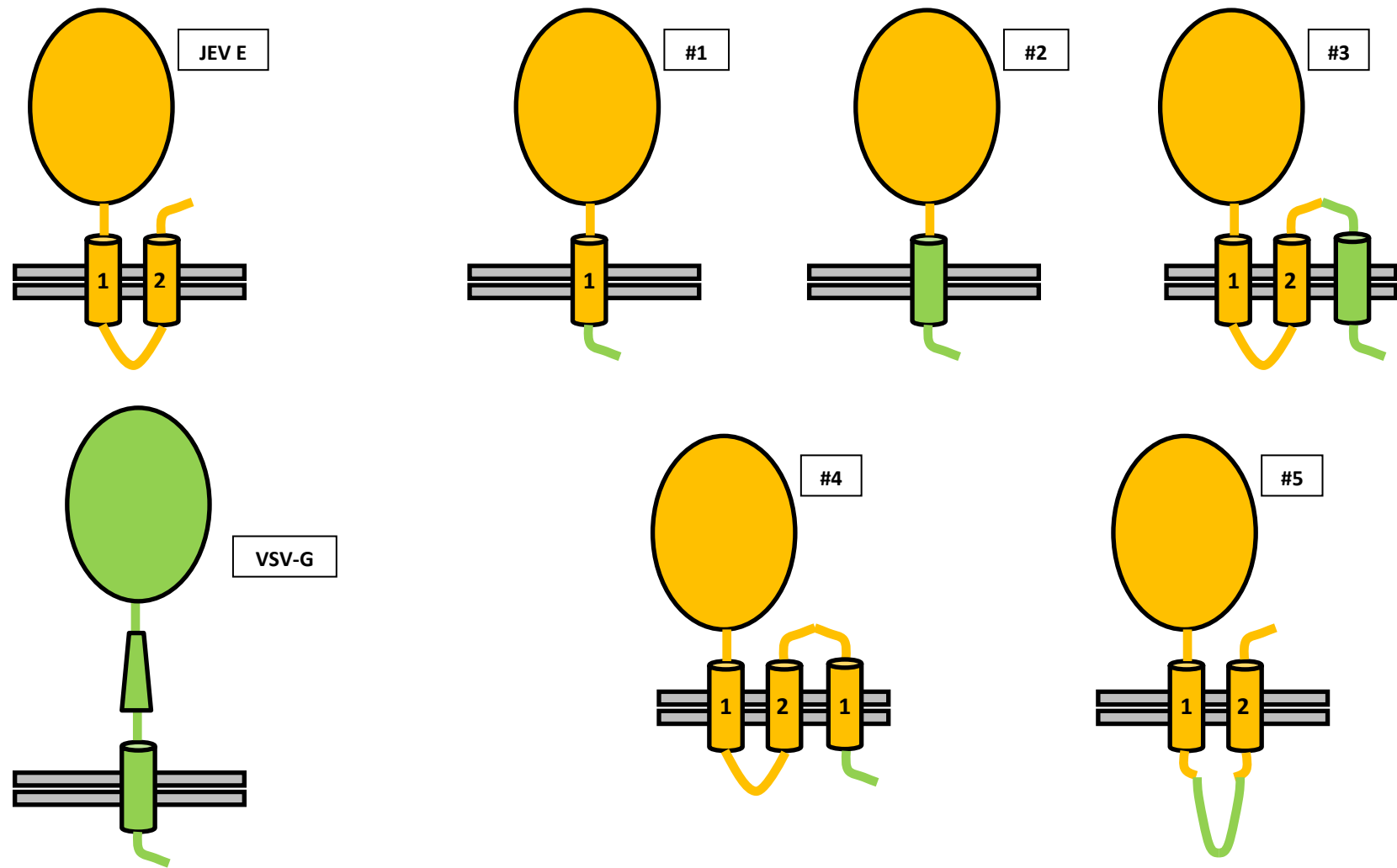


Figure 22. A selection of schematics to represent the five JEV/VSV chimeric glycoprotein constructs. #1) Replacing second JEV E TMD with the C-tail of VSV-G. #2) Replacing both of the JEV E TMDs with the TMD and C-tail of VSV-G. #3) Appending the VSV-G TMD and C-tail onto the C-terminus of wild-type, full length JEV E. #4) Appending a duplicate first JEV E TMD and the VSV-G C-tail onto the C-terminus of wild-type, full length JEV E. #5) Inserting an anti-parallel tandem repeat of the VSV-G C-tail between the two JEV E TMDs. Cartoon depictions of the native VSV-G and JEV E proteins are also displayed.

4.3.2 Prediction and definition of JEV E TM domains and VSV-G TM domain and C-tail

Once the designs for the potential VSV-JEV chimeric glycoproteins were finalised, it was then necessary to accurately define the amino acid regions of the JEV and VSV envelope glycoproteins that would constitute the transmembrane domains and, in the case of VSV, the cytoplasmic tail. Confident prediction of these segments would then enable amino acid sequence compilation of the chimeric constructs, and subsequent primer design for the site-directed mutagenesis required to produce the cGPs.

As previously mentioned, the transmembrane domain and cytoplasmic tail of the VSV-G protein have been detailed in several previous publications (Cleverley and Lenard, 1998; Lagging *et al*, 1998; Buonocore *et al*, 2002; Köhl *et al*, 2004; Lei *et al*, 2010; Gravel *et al*, 2011) and the exact definitions of their sequences vary to a certain degree, particularly concerning the length and N-terminal cut-off point of the transmembrane domain. Therefore, to identify the optimum sequences for each domain, the candidate amino acid sequences were aligned and comparative analysis was performed, which involved examining sequence length, hydrophobicity and the presence of charged amino acids, which tend to act as 'capping' residues at either extremity of a TM domain (Figure 23). The analysis revealed that the C-tail sequence is consistently defined in every aligned sequence as the final 29 residues of the C-terminus of the VSV-G protein, and so this exact sequence was used as the VSV-G cytoplasmic tail in this study, as shown in Figure 23. Following assessment of the candidate transmembrane domains extracted from previously published articles, sequence #6 was originally deemed to be the most suitable defined TMD sequence, as at 21 amino acids long, it conforms to the typical length of a membrane-spanning segment of a protein, and this stretch of residues also begins with a positively-charged lysine, which would be likely to act as a capping residue (Cleverley and Lenard, 1998). However, in this instance, the decision was made to include an extra 6 amino acids upstream of the lysine, to act as a 'flexible linker' between the VSV-G TMD

and the JEV E sequence, as well as to allow for any potential inaccuracies in the visual prediction of the TMD sequence itself. Therefore, the final defined VSV-G TMD sequence selected for this study is actually identical to the candidate sequence #5 listed in Figure 23 (Buonocore *et al*, 2002).

In order to distinguish the locations of the two transmembrane domains at the C-terminus of the JEV E protein, a variety of online resources were utilised, which are designed to assess the transmembrane topology of proteins and identify intramembranous areas of submitted amino acid sequences (full details given in section 4.2.9). The prME proteins of a representative strain from genotypes I to IV of JEV were inputted into each of the TMD topology programs, to check for any structural or topology-based variability of a genotype-specific nature. Once the results were given, the predicted transmembrane domains from each request were compared across the different JEV strains and TMD topology programs used. A high level of consistency was observed between all the predictions, with the locations of the two transmembrane domains unanimously defined as between residues 631-655 and 661-682, respectively (Figure 24). This finding appears accurate when assessed in the context of the structure of JEV and flavivirus E proteins in mature virions, as the two TM domains reside in close proximity to one another at the C-terminus of the E sequence (Mukhopadhyay *et al*, 2005). In addition to the two corroborated transmembrane domain predictions, the TMPred topology program also recognised a third stretch of amino acids (residues 599-620) as possessing the potential to be membrane-spanning (Figure 24). However, upon further inspection, this result was disregarded, as the sequence corresponds to the location of the second stem helix of JEV E, which lies flat against the JEV envelope and makes contact with the phospholipid bilayer in the final virion structure. These contacts with the lipid membrane of the virus, which are important in its function of preventing electrostatic repulsions throughout the E protein in its mature conformation, require a higher presence of hydrophobic amino acids in this sequence

(Mukhopadhyay *et al*, 2005). The subsequent hydrophobicity and length of this stem helix is most likely the cause of its inclusion in one of the predictions made by the TMPred program.

Candidate VSV transmembrane domain and cytoplasmic tail sequences - references	
Candidate sequence number (#)	Corresponding reference
#1	Lei <i>et al</i> , 2010
#2	Köhl <i>et al</i> , 2004
#3 (C-tail only)	Köhl <i>et al</i> , 2004
#4	Lagging <i>et al</i> , 1998
#5	Buonocore <i>et al</i> , 2002
#6	Cleverley and Lenard, 1998
#7	Gravel <i>et al</i> , 2011

Candidate VSV TMD and C-tail sequences

```
#1 GDTGLSKNPIELVEGWFSWKSSIASFFFFIIGLIIGLFLVLRVGIHLCIKLKHTKKRQIYTDIEMNRLGK [70]
#2 IELVEGWFSWKSSIASFFFFIIGLIIGLFLVLRVGIHLCIKLKHTKKRQIYTDIEMNRLGK [61]
#3 RVGIHLCIKLKHTKKRQIYTDIEMNRLGK [29]
#4 GLIIGLFLVLRVGIHLCIKLKHTKKRQIYTDIEMNRLGK [39]
#5 GWFSSWKSSIASFFFFIIGLIIGLFLVLRVGIHLCIKLKHTKKRQIYTDIEMNRLGK [56]
#6 KSSIASFFFFIIGLIIGLFLVLRVGIHLCIKLKHTKKRQIYTDIEMNRLGK [50]
#7 FFFIIGLIIGLFLVLRVGIHLCIKLKHTKKRQIYTDIEMNRLGK [44]
```

Defined VSV TMD and C-tail sequences

TMD: GWFSSWKSSIASFFFFIIGLIIGLFLVLR [27]

C-tail: RVGIHLCIKLKHTKKRQIYTDIEMNRLGK [29]

TMD & C-tail:

GWFSSWKSSIASFFFFIIGLIIGLFLVLRVGIHLCIKLKHTKKRQIYTDIEMNRLGK [56]

Figure 23. Comparative analysis for the definition of the VSV-G transmembrane domain (TMD) and cytoplasmic tail (C-tail). Candidate amino acid sequences for the VSV-G TMD and C-tail were aligned before definition of the C-tail and prediction of the TMD were confirmed, based upon sequence length, hydrophobicity and the presence of charged ‘capping’ residues. The finalised and defined amino acid sequences, as well as the corresponding references for each candidate sourced from previously published material, are also shown. The VSV TM domain is coloured in purple, and the C-tail in yellow.

<i>TMD topology prediction program</i>	<i>JEV E strain</i>			
	Beijing-1	Ishikawa	FU	SA14
HMMTOP	638-655 662-680	638-655 662-680	638-655 662-680	638-655 662-680
TMHMM	633-655 662-681	633-655 662-681	633-655 662-681	633-655 662-681
PHOBIUS	634-655 662-680	634-655 662-680	634-655 662-680	634-655 662-680
TMPred	599-620 638-655 662-680	638-655 662-680	599-620 638-655 662-680	599-620 638-655 662-680
SOSUI	631-653 661-682	631-653 661-682	631-653 661-682	631-653 661-682
Consensus (<i>amino acid residue number</i>)	631-655 661-682	631-655 661-682	631-655 661-682	631-655 661-682

Defined JEV TMD sequences

TMD1: **GAFRTLFGGMSWITQGLMGALLLWMGVNAR** [30]

TMD2: **DRSIALAFLATGGVLVFLATNVHA** [24]

Figure 24. Determination of the transmembrane domains (TMDs) located at the C-terminus of the JEV E protein. Five TMD topology programs were used – HMMTOP, TMHMM, Phobius, TMPred and Sosui – to predict the membrane-spanning sites of E for four virus strains from distinct JEV genotypes, before a general consensus was drawn. The amino acid sequences for both JEV E TMDs are also defined. JEV E TMDs 1 and 2 are coloured in green and blue, respectively.

Wild-type JEV Beijing-1 prME sequence

MWLASLAVVIACAGAMKLSNFQGLLMTINNTDIADVIVPTSKGENRCWVRAIDVGYMCEDTITYECPKLTMGNDPEDVDCWCDNQE
VYVQYGRCTRTRHRSKRSRRSVSVQTHGESSLVNKEAWLDSTKATRYLMKTENWI IRNPGYAFLAAI LGWMLGSNNGQRVVFTI LLLL
VAPAYSFNCLGMGNRDFIEGASGATWVDLVLEGDSCLT IMANDKPTLDVRMINIEASQLAEVRSYCYHASVTDI STVARCPTTGEAHN
EKRADSSVYCKQGFTDRGWGNGCGLFGKGSIDTCAKFSCTSKAIGRTIQSENIKEYEVGIFVHGTTTSENHGNYSAQVGASQAAKFTVT
PNAPSITLKLGDYGEVTL DCEPRSGLNTEAFYVMTVGSKSFLVHREWFHDLALPWT PPSSTAWRNRELLMEFEEAHATKQSVVALGSQ
EGGLHQALAGAI VVEYSSSVKLTSGHLKCR LKMDK LALKGT TYGMCTEKF SFAKNPADTGHGT VVI ELSYSGSDGPKIPIVSVASLN
DMTPVGR LVTV NPFVATSSANSKVLVEMEPFPGDSYIVVGRGDQ INHHWYKAGSTLGKAFSTTLKGAQR LAALGDTAWDFG SIGGVF
NSIGKAVHQVFG **GAFRTLFGGMSWITQGLMGALLLWGMINARDRSIALAFLATGGVLVFLATNVHA**

Construct #1 – Replacing second JEV E TMD with the C-tail of VSV-G

MWLASLAVVIACAGAMKLSNFQGLLMTINNTDIADVIVPTSKGENRCWVRAIDVGYMCEDTITYECPKLTMGNDPEDVDCWCDNQE
VYVQYGRCTRTRHRSKRSRRSVSVQTHGESSLVNKEAWLDSTKATRYLMKTENWI IRNPGYAFLAAI LGWMLGSNNGQRVVFTI LLLL
VAPAYSFNCLGMGNRDFIEGASGATWVDLVLEGDSCLT IMANDKPTLDVRMINIEASQLAEVRSYCYHASVTDI STVARCPTTGEAHN
EKRADSSVYCKQGFTDRGWGNGCGLFGKGSIDTCAKFSCTSKAIGRTIQSENIKEYEVGIFVHGTTTSENHGNYSAQVGASQAAKFTVT
PNAPSITLKLGDYGEVTL DCEPRSGLNTEAFYVMTVGSKSFLVHREWFHDLALPWT PPSSTAWRNRELLMEFEEAHATKQSVVALGSQ
EGGLHQALAGAI VVEYSSSVKLTSGHLKCR LKMDK LALKGT TYGMCTEKF SFAKNPADTGHGT VVI ELSYSGSDGPKIPIVSVASLN
DMTPVGR LVTV NPFVATSSANSKVLVEMEPFPGDSYIVVGRGDQ INHHWYKAGSTLGKAFSTTLKGAQR LAALGDTAWDFG SIGGVF
NSIGKAVHQVFG **GAFRTLFGGMSWITQGLMGALLLWGMINARVGIHLICIKLKHKKRQIYTDIEMNRLGK**

Construct #2 – Replacing both of the JEV E TMDs with the TMD and C-tail of VSV-G

MWLASLAVVIACAGAMKLSNFQGLLMTINNTDIADVIVPTSKGENRCWVRAIDVGYMCEDTITYECPKLTMGNDPEDVDCWCDNQE
VYVQYGRCTRTRHRSKRSRRSVSVQTHGESSLVNKEAWLDSTKATRYLMKTENWI IRNPGYAFLAAI LGWMLGSNNGQRVVFTI LLLL
VAPAYSFNCLGMGNRDFIEGASGATWVDLVLEGDSCLT IMANDKPTLDVRMINIEASQLAEVRSYCYHASVTDI STVARCPTTGEAHN
EKRADSSVYCKQGFTDRGWGNGCGLFGKGSIDTCAKFSCTSKAIGRTIQSENIKEYEVGIFVHGTTTSENHGNYSAQVGASQAAKFTVT
PNAPSITLKLGDYGEVTL DCEPRSGLNTEAFYVMTVGSKSFLVHREWFHDLALPWT PPSSTAWRNRELLMEFEEAHATKQSVVALGSQ
EGGLHQALAGAI VVEYSSSVKLTSGHLKCR LKMDK LALKGT TYGMCTEKF SFAKNPADTGHGT VVI ELSYSGSDGPKIPIVSVASLN
DMTPVGR LVTV NPFVATSSANSKVLVEMEPFPGDSYIVVGRGDQ INHHWYKAGSTLGKAFSTTLKGAQR LAALGDTAWDFG SIGGVF
NSIGKAVHQVFG **GWFSWKSIIASFFFIIGLIIGLFLVLRVGIHLICIKLKHKKRQIYTDIEMNRLGK**

Construct #3 – Appending the VSV-G TMD and C-tail onto the C-terminus of wild type, full length JEV E

MWLASLAVVIACAGAMKLSNFQGLLMTINNTDIADVIVPTSKGENRCWVRAIDVGYMCEDTITYECPKLTMGNDPEDVDCWCDNQE
VYVQYGRCTRTRHRSKRSRRSVSVQTHGESSLVNKEAWLDSTKATRYLMKTENWI IRNPGYAFLAAI LGWMLGSNNGQRVVFTI LLLL
VAPAYSFNCLGMGNRDFIEGASGATWVDLVLEGDSCLT IMANDKPTLDVRMINIEASQLAEVRSYCYHASVTDI STVARCPTTGEAHN
EKRADSSVYCKQGFTDRGWGNGCGLFGKGSIDTCAKFSCTSKAIGRTIQSENIKEYEVGIFVHGTTTSENHGNYSAQVGASQAAKFTVT
PNAPSITLKLGDYGEVTL DCEPRSGLNTEAFYVMTVGSKSFLVHREWFHDLALPWT PPSSTAWRNRELLMEFEEAHATKQSVVALGSQ
EGGLHQALAGAI VVEYSSSVKLTSGHLKCR LKMDK LALKGT TYGMCTEKF SFAKNPADTGHGT VVI ELSYSGSDGPKIPIVSVASLN
DMTPVGR LVTV NPFVATSSANSKVLVEMEPFPGDSYIVVGRGDQ INHHWYKAGSTLGKAFSTTLKGAQR LAALGDTAWDFG SIGGVF
NSIGKAVHQVFG **GAFRTLFGGMSWITQGLMGALLLWGMINARDRSIALAFLATGGVLVFLATNVHA GWFSWKSIIASFFFIIGLIIGL
LFLVLRVGIHLICIKLKHKKRQIYTDIEMNRLGK**

Construct #4 – Appending a duplicate 1st JEV E TMD and the VSV-G C-tail onto the C-terminus of wild type, full length JEV E

MWLASLAVVIACAGAMKLSNFQGLLMTINNTDIADVIVPTSKGENRCWVRAIDVGYMCEDTITYECPKLTMGNDPEDVDCWCDNQE
VYVQYGRCTRTRHRSKRSRRSVSVQTHGESSLVNKEAWLDSTKATRYLMKTENWI IRNPGYAFLAAI LGWMLGSNNGQRVVFTI LLLL
VAPAYSFNCLGMGNRDFIEGASGATWVDLVLEGDSCLT IMANDKPTLDVRMINIEASQLAEVRSYCYHASVTDI STVARCPTTGEAHN
EKRADSSVYCKQGFTDRGWGNGCGLFGKGSIDTCAKFSCTSKAIGRTIQSENIKEYEVGIFVHGTTTSENHGNYSAQVGASQAAKFTVT
PNAPSITLKLGDYGEVTL DCEPRSGLNTEAFYVMTVGSKSFLVHREWFHDLALPWT PPSSTAWRNRELLMEFEEAHATKQSVVALGSQ
EGGLHQALAGAI VVEYSSSVKLTSGHLKCR LKMDK LALKGT TYGMCTEKF SFAKNPADTGHGT VVI ELSYSGSDGPKIPIVSVASLN
DMTPVGR LVTV NPFVATSSANSKVLVEMEPFPGDSYIVVGRGDQ INHHWYKAGSTLGKAFSTTLKGAQR LAALGDTAWDFG SIGGVF
NSIGKAVHQVFG **GAFRTLFGGMSWITQGLMGALLLWGMINARDRSIALAFLATGGVLVFLATNVHA GAFRTLFGGMSWITQGLMGALL
LWGMINARVGIHLICIKLKHKKRQIYTDIEMNRLGK**

Construct #5 – Inserting an anti-parallel tandem repeat of the VSV-G C-tail between the two JEV E TMDs (without flexible linker between C-tail repeats)

MWLASLAVVIACAGAMKLSNFQGLLMTINNTDIADVIVPTSKGENRCWVRAIDVGYMCEDTITYECPKLTMGNDPEDVDCWCDNQE
VYVQYGRCTRTRHRSKRSRRSVSVQTHGESSLVNKEAWLDSTKATRYLMKTENWI IRNPGYAFLAAI LGWMLGSNNGQRVVFTI LLLL
VAPAYSFNCLGMGNRDFIEGASGATWVDLVLEGDSCLT IMANDKPTLDVRMINIEASQLAEVRSYCYHASVTDI STVARCPTTGEAHN
EKRADSSVYCKQGFTDRGWGNGCGLFGKGSIDTCAKFSCTSKAIGRTIQSENIKEYEVGIFVHGTTTSENHGNYSAQVGASQAAKFTVT
PNAPSITLKLGDYGEVTL DCEPRSGLNTEAFYVMTVGSKSFLVHREWFHDLALPWT PPSSTAWRNRELLMEFEEAHATKQSVVALGSQ
EGGLHQALAGAI VVEYSSSVKLTSGHLKCR LKMDK LALKGT TYGMCTEKF SFAKNPADTGHGT VVI ELSYSGSDGPKIPIVSVASLN
DMTPVGR LVTV NPFVATSSANSKVLVEMEPFPGDSYIVVGRGDQ INHHWYKAGSTLGKAFSTTLKGAQR LAALGDTAWDFG SIGGVF
NSIGKAVHQVFG **GAFRTLFGGMSWITQGLMGALLLWGMINARVGIHLICIKLKHKKRQIYTDIEMNRLGK KGLRNMIEDTYIQRKKT
HKLIKICLHIGVDRSIALAFLATGGVLVFLATNVHA**

Figure 25. Amino acid sequences of wild type JEV Beijing-1 prME and the five JEV/VSV chimeric glycoprotein constructs. The two JEV E transmembrane domains, as well as the VSV transmembrane domain and cytoplasmic tail, are highlighted in colour (in accordance with Figures 4.2 and 4.3). In construct #5, the two lysine residues shaded in grey indicate the middle of the VSV-G C-tail anti-parallel tandem repeat.

4.3.3 Mutagenic primer design for the construction of VSV/JEV chimeric glycoproteins

Following the determination of the amino acid sequences for the JEV E TMDs, as well as the VSV-G TMD and C-tail, compilation of the complete amino acid sequences for the VSV/JEV chimeric glycoprotein constructs could take place (Figure 25). Subsequently, these full sequences were then used to enable the design of oligonucleotide primers necessary to carry out Q5 site-directed mutagenesis for cGP construction.

Primer design was achieved through use of the NEBaseChanger program – in order to successfully produce effective primer sequences, the program requires the fulfilment of several criteria. Firstly, the amino acid sequences of the template (native JEV Beijing-1 24SPprME) and desired (relevant cGP construct) proteins had to be reverse transcribed into nucleotide sequences, prior to entering into the NEBaseChanger system. The other parameters required were the nature of the mutagenesis i.e. insertion or substitution, and identification of the nucleotides which flank the mutagenic region. Following the completion of this information, NEBaseChanger is then able to generate specific forward and reverse primer sequences to enable the desired mutagenesis, and also offers important additional properties of each primer, such as the length in nucleotides, the GC% and the melting temperature (T_m). Furthermore, the program calculates a recommended paired annealing temperature (T_a) for the primers being used together in a mutagenesis reaction, which equals 3°C higher than the lowest T_m of the two primers. Primer sequences and characteristics are given in greater detail in Tables 11 and 12, respectively.

In chimeric glycoprotein construct #4, a duplicated first JEV E TM domain is present directly upstream of the appended VSV-G cytoplasmic tail. Unfortunately, this means that it cannot be feasibly generated using the Q5 site-directed mutagenesis kit, due to the fact that non-specific primer-binding and subsequent inaccurate amplification would occur. Therefore, it was decided that the best way to proceed for the momentum of this study was to cease pursuing

the production of this construct and prioritise the other chimeric glycoprotein constructs. It may be possible to achieve cGP construct #4 *via* an alternative cloning method, such as Gibson Assembly (New England Biolabs, MA, USA; Cat. No. #E5510S) – however, due to time constraints attached to the project, this experiment could regrettably not be carried out.

JEV-VSV chimeric glycoprotein construction – primer sequences			
Primer name	Chimeric GP construct	Starting plasmid/ cGP construct	Primer sequence (green mutagenic tail; bold red primer binding region)
JEV_VSV_cGP#1_FW	#1	pUC57-24SpPrME	AAGACGATTTATACAGACATAGAGATGAACCGACTTGGAAAG TAGCGGCCGCTCGAGCAT
JEV_VSV_cGP#1_RV	#1	pUC57-24SpPrME	TTCTTGGTGTGCTTAAATTAATGCAAAAGATGGATACCAA CTGTGTGCGTTGATGCCCATC
JEV_VSV_cGP#2_FW	#2	cGP#1 (pUC57-24SpPrME-cGP#1)	CTTTATCATTAGGGTTAATCATTGGACTATTCTTGGTTCT CGAGTTGGTATCCATCTTTTG
JEV_VSV_cGP#2_RV	#2	cGP#1 (pUC57-24SpPrME-cGP#1)	AAAAAAGAGCAATAGAGCTTTTCCAAC TACTGAACCAACCACCAACA CTTTGGTGAAC
JEV_VSV_cGP#3.1_FW	#3.1	pUC57-24SpPrME	CTTTATCATTAGGGTTAATCATTGGACTATTCTTGGTTCT CTAGCGGCCGCTCGAGCAT
JEV_VSV_cGP#3.1_RV	#3.1	pUC57-24SpPrME	AAAAAAGAGGCAATAGAGCTTTTCCAAC TACTGAACCAACAGCATG CA CATTGGT AGCTAAAGAAC
JEV_VSV_cGP#3.2_FW	#3.2	cGP#3.1 (pUC57-24SpPrME-cGP#3.1)	AAGACGATTTATACAGACATAGAGATGAACCGACTTGGAAAG TAGCGGCCGCTCGAGCAT
JEV_VSV_cGP#3.2_RV	#3.2	cGP#3.1 (pUC57-24SpPrME-cGP#3.1)	TTCTTGGTGTGCTTAAATTAATGCAAAAGATGA TACCAACTG GA GAACA CAAGAA TAGT CCAATGATTAA CCCTATG
JEV_VSV_cGP#4_FW	#4		
JEV_VSV_cGP#4_RV	#4		
JEV_VSV_cGP#5.1_FW	#5.1	pUC57-24SpPrME	AAGACAGATTTATACAGACATAGAGATGAACCGACTTGGAAAG GA CCGATCAATTGCTTTGGC
JEV_VSV_cGP#5.1_RV	#5.1	pUC57-24SpPrME	TTCTTGGTGTGCTTAAATTAATTAATGCAAAAGATGA TA CCAACTG GTG GTGCGTTGATGCC CAAT
JEV_VSV_cGP#5.2_FW	#5.2	cGP#5.1 (pUC57-24SpPrME-cGP#5.1)	AAAAGACCCACAAGTTAAAAAATTTGGCTTCA TATCGGGT TCGAGACCGATCA ATTGCTTTG
JEV_VSV_cGP#5.2_RV	#5.2	cGP#5.1 (pUC57-24SpPrME-cGP#5.1)	TTTCTCTGAATATATGTGTCTATCTCCATGTTTCGAAGTCCCTT CTTTCCA AGTGGGTT CAATC

Table 11. Primer sequences employed in Q5 site-directed mutagenesis for the construction of JEV/VSV chimeric glycoproteins. The given primer names and their corresponding constructs are listed, along with the template plasmid and the full primer sequence, generated by the NEBaseChanger program. The colouration on each primer distinguishes the mutagenic tail (in green) and the primer binding region (in red).

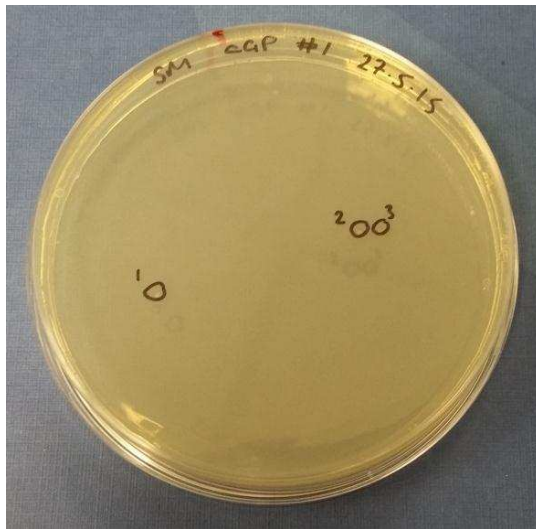
JEV-VSV chimeric glycoprotein construction – primer characteristics					
Primer name	Primer length (nt)	GC content (%)	Primer binding site GC content (%)	T _m /melting temperature (°C)	Paired T _a /annealing temperature (°C)
JEV_VSV_cGP#1_FW	61	20	67	71°C	69°C
JEV_VSV_cGP#1_RV	63	17	58	66°C	
JEV_VSV_cGP#2_FW	60	15	45	58°C	60°C
JEV_VSV_cGP#2_RV	59	14	44	57°C	
JEV_VSV_cGP#3.1_FW	58	21	67	71°C	69°C
JEV_VSV_cGP#3.1_RV	66	17	44	66°C	
JEV_VSV_cGP#3.2_FW	61	20	67	71°C	70°C
JEV_VSV_cGP#3.2_RV	78	17	38	67°C	
JEV_VSV_cGP#4_FW					
JEV_VSV_cGP#4_RV					
JEV_VSV_cGP#5.1_FW	63	16	50	62°C	65°C
JEV_VSV_cGP#5.1_RV	62	16	56	65°C	
JEV_VSV_cGP#5.2_FW	61	13	44	56°C	59°C
JEV_VSV_cGP#5.2_RV	63	14	47	58°C	

Table 12. Characteristics of oligonucleotide primers used in Q5 site-directed mutagenesis to produce JEV/VSV chimeric glycoproteins. Each row presents a variety of properties corresponding to each mutagenic primer, including primer length, overall and primer binding site GC percentage, as well as the melting temperature and subsequent paired annealing temperature.

4.3.4 Q5 site-directed mutagenesis for the production of VSV/JEV chimeric glycoproteins

Once the primers were successfully designed, their synthesis was ordered using Eurofins MWG Operon (details in section 4.2.1). After their arrival and reconstitution to the appropriate working concentration (see section 2.1.6), they were incorporated into mutagenesis PCR reaction mixtures, in an attempt to produce the candidate VSV/JEV chimeric glycoproteins.

Initially, Q5 site-directed mutagenesis was carried out to produce cGP constructs #1, #3.1 and #5.1, as these mutagenic reactions required the standard pUC57-24SPprME plasmid as a template, unlike construct #2, #3.2 and #5.2, which require existing, successfully-mutated cGP construct plasmids as templates. The mutagenic PCR reactions were set up as described in Section 4.2.2. In these instances, the mutagenesis for both constructs #1 and #3.1 could be performed simultaneously, as they both required an annealing temperature of 69°C, whereas for construct #5.1, an annealing temperature of 65°C was necessary (Table 12). From here, 1µl of each amplified mutant PCR product was taken forward into the KLD reaction and subsequent transformation, before plating onto ampicillin-containing LB-agar plates. Resulting colony numbers and images of the streaked LB-amp plates can be observed in Figure 26. The three colonies residing on the VSV/JEV cGP #1 plate and the six present on the VSV/JEV cGP #5.1 plate were then subjected to colony PCR screening, to validate the presence of the mutated, chimeric VSV/JEV prME glycoprotein.



Transformation of candidate VSV/JEV cGPs	
Transformation plate	Number of colonies
VSV/JEV cGP #1	3
VSV/JEV cGP #3.1	0
VSV/JEV cGP #5.1	6



Figure 26. Transformant colony numbers of mutagenic pUC57-prME VSV/JEV chimeric glycoprotein candidate constructs. Following transformation of the cGP plasmid constructs and subsequent streaking onto LB-amp agar plates, the numbers of colonies present on each plate, after an overnight 37°C incubation, were recorded. Images of each of the antibiotic plates are also shown, to validate the identification of the distinct colonies.

4.3.5 Screening of potential mutant VSV/JEV chimeric glycoprotein plasmid clones

The potential positive VSV/JEV cGP plasmid clones identified on the bacterial transformation plates subsequently underwent a colony PCR screening process, to calculate the size in nucleotide base pairs of the insert in the transformed, mutagenic plasmid, and thus ascertain if the mutagenesis to construct each chimeric glycoprotein has been successful. Each plated colony from constructs #1 and #5.1 was lysed, before mixing with a DreamTaq polymerase master mix and undergoing colony PCR, using the 'JEV50SCREEN' thermal cycling program (Table 7). Once complete, sample PCR products were run on a 1% (w/v) agarose gel at 120V, prior to UV visualisation. As can be seen from Figure 2.7, each of the three construct #1 colonies (samples #1-3) successfully amplified a ~2.1kb DNA insert, and out of the six construct #5.1 colonies (samples #4-9), all of the samples except #6 also appear to be positive, with strong amplification of the desired fragment. In addition to the experimental samples, a positive control (pUC57-24SPprME plasmid) and a negative control (empty pUC57 vector) were included on the agarose gel – interestingly, amplicon DNA was not apparent in the positive control lane. However, due to the convincing positive results observed in the vast majority of the experimental cGP lanes, a representative sample was taken forward for plasmid purification and Sanger sequencing, to check for successful mutagenesis and correct formation of the cGP construct.

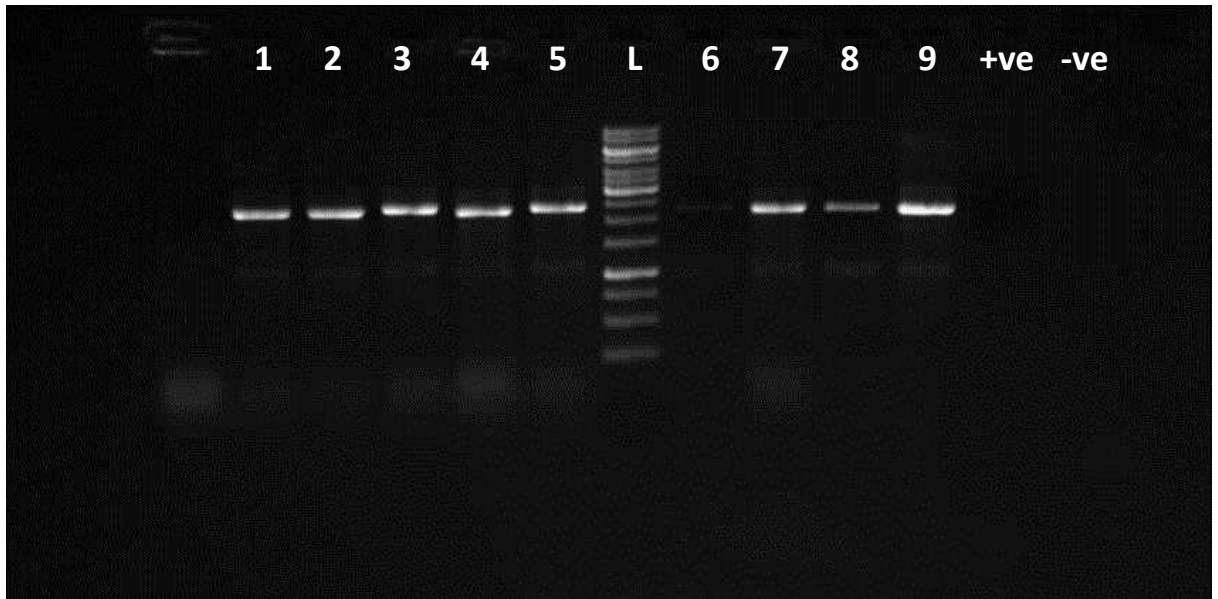


Figure 27. Gel electrophoresis image following colony PCR screening of candidate VSV/JEV chimeric glycoprotein constructs. Experimental samples #1-3 (for cGP construct #1) and #4-9 (for cGP construct #5.1) were run in their corresponding, numbered lanes, alongside the GeneRuler 1kb DNA ladder (in lane L) and positive and negative controls (in lanes +ve and -ve, respectively). Strong amplified DNA bands of a length of ~2.1kb, which equates to the approximate lengths of the cGP plasmid prME inserts, can be seen in lanes 1-5 and 7-9, with a faint PCR product of the same size also witnessed in lane 6.

4.3.6 Plasmid purification and Sanger sequencing of positive VSV/JEV chimeric glycoprotein clones

On the basis of strongest amplicon production during colony PCR and the brightest bands visualised on the following gel image, samples #2 and #9 were selected for purification and sequencing. These two sample colonies were picked from the re-streaked and incubated bacterial plate prepared during the colony PCR process, before overnight growth in an LB broth starter culture, pelleting by centrifugation and subsequent plasmid purification (as detailed in Section 2.1.4). Concentrations and purities of the pUC57-prME cGP plasmids were measured using the Nanodrop 2000 spectrophotometer (ThermoFisher Scientific, UK), with the resulting values presented in Table 13.

cGP plasmid construct	Concentration (ng/μl)	Purity (A260/280)
pUC57-prME cGP #1	201.9	1.81
pUC57-prME cGP #5.1	117.2	1.84

Table 13. Measurement of pUC57-prME chimeric glycoprotein plasmid DNA concentration and purity. Prior to Sanger sequencing, the concentration and purity of each cGP plasmid construct was measured. Concentrations and purities are given in ng/μl and absorbance ratio of 260/280nm, respectively, alongside the corresponding plasmid construct name.

Each plasmid construct was then mixed with the M13 reverse primer and sent for sequencing, using the LIGHTrun Sanger sequencing system (GATC Biotech, Germany). Unfortunately, following the receipt of the sequencing results and analysis of each chromatogram, it was evident that the prME C-terminus of both the candidate cGP #1 and #5.1 plasmids was identical to that of the native pUC57-prME insert C-terminus, confirming that in fact the mutagenesis to create the VSV/JEV chimeric glycoproteins was unsuccessful. Therefore, as a consequence, further mutagenesis to complete the construction of cGP candidates #3 and #5, as well as to produce construct #2, was also unable to be carried out.

4.3.7 Selection of Kozak consensus sequences and subsequent mutagenic primer design

In a bid to stimulate ribosomal activity and initiate translation of JEV viral RNA transcripts, thus considerably boosting downstream expression levels of prME proteins, the mutagenic insertion of three distinct, putative Kozak sequences was attempted. Out of the many short nucleotide stretches that have previously been defined as effective Kozak sequences, the three sequences decided upon to incorporate directly before the initial methionine (ATG) codon of the 15SP- or 24SP-prME genes were GTCAAA (Etheridge *et al*, 2014), CACAAA (Jackson *et al*, 2011) and GCCACC (Babaie *et al*, 2011). This decision was made not only because of previous publications that have detailed the functional ability of these three sequences to initiate translation, but also because there is empirical evidence from previous studies carried out within the same laboratory group, indicating that each of these sequences have successfully contributed to high expression levels in the same batch of HEK293T/17 producer cells used in this study.

In a similar fashion as with the chimeric glycoprotein primers, the design of mutagenic primers intended for the insertion of Kozak sequences was carried out using the NEBaseChanger program. Details of the nature and location of the mutation, as well as the template and desired sequences, were submitted to the program, resulting in the generation of six different forward primers, depending on the Kozak sequence being inserted, and also upon the length of the signal peptide upstream of the prME gene in the relevant construct. In each instance, the same reverse primer was generated as the 3' terminus of the pUC57-prME insert was unchanged and required no alteration in this series of mutations. The NEBaseChanger system also provided additional primer information and corresponding annealing temperatures for each downstream mutagenesis reaction (Table 14). All designed primers were subsequently ordered from Eurofins MWG Operon and reconstituted to the correct stock and working dilutions, prior to application in Q5 site-directed mutagenesis.

Kozak insertion mutagenesis primer characteristics						
Primer number	Primer name	Primer sequence	Length (nt)	GC content (%)	Tm (°C)	Ta (°C)
#1	JEV_15SPprME_GTCAAA_Fwd	ATTCAGATCTGTCAAAATGTGGCTCGCGAGC	31	48	57	60
#2	JEV_24SPprME_GTCAAA_Fwd	ATTCAGATCTGTCAAAATGGGCAAGAGAAGATCAG	35	40	55	58
#3	JEV_15SPprME_CACAAA_Fwd	ATTCAGATCTCACAAAATGTGGCTCGCGAGC	31	48	57	60
#4	JEV_24SPprME_CACAAA_Fwd	ATTCAGATCTCACAAAATGGGCAAGAGAAGATCAG	35	40	55	58
#5	JEV_15SPprME_GCCACC_Fwd	ATTCAGATCTGCCACCATGTGGCTCGC	27	56	57	60
#6	JEV_24SPprME_GCCACC_Fwd	ATTCAGATCTGCCACCATGGGCAAGAGAAG	30	50	57	60
#7	JEV_SPprME_Kozak_Rev	TTCATTAATGCTCGAGC	18	44	56	As Fwd

Table 14. Sequences and properties of oligonucleotide primers used for the mutagenic insertion of Kozak sequences into pUC57-prME plasmids. Alongside each given primer name, the full primer sequence and relevant properties are displayed, such as the length in nucleotides, the percentage GC content, as well as the melting and resultant annealing temperatures. The red section on each coloured primer corresponds to the mutagenic region, whereas the green sections are where the primer binds.

4.3.8 Mutagenesis, transformation and screening of potential Kozak-mutated plasmids

To attempt the production of each of the six desired Kozak sequence insertions, Q5 site-directed mutagenesis was carried out. The relevant template plasmids – either pUC57-15SPprME or pUC57-24SPprME – were mixed with the Q5 polymerase master mix and the primers required to insert each candidate Kozak sequence, as detailed in Table 4.1. Based upon the recommendations given by the NEBaseChanger program, reactions involving forward Kozak primers #1, #3, #5 and #6 required an annealing temperature of 60°C, whereas for mutagenesis with forward primers #2 and #4, the recommended annealing temperature was 58°C. All other temperatures and durations of the Q5 PCR thermal cycling program were identical for all Kozak mutagenesis, and are displayed in Table 5.

Subsequent to the mutagenic PCR reactions, 1µl of each PCR product was then carried forward into kinase-ligase-*DpnI* (KLD) treatment. Samples were incubated for a sufficient duration for the enzymes to take effect, before 2.5µl of each KLD-treated sample was transformed into 25µl aliquots of competent DH5α *E.coli* cells, streaked onto ampicillin-containing LB-agar plates and incubated overnight at 37°C. The resultant colony numbers observed on these bacterial plates are shown in Table 15.

Transformation plate	No. of colonies
pUC57-15SPprME-GTCAA AKoz	1
pUC57-24SPprME-GTCAA AKoz	0
pUC57-15SPprME-CACAA AKoz	0
pUC57-24SPprME-CACAA AKoz	0
pUC57-15SPprME-GCCACCKoz	16
pUC57-24SPprME-GCCACCKoz	1

Table 15. Transformant colony numbers of potential Kozak mutated pUC57-prME plasmid clones.

Subsequent to transformation, streaking onto LB-ampicillin agar plates and overnight incubation, individual *E.coli* colonies potentially harbouring the desired Kozak mutant plasmids were counted and presented in a tabulated format.

In order to ascertain whether the colonies grown on the transformation plates possess the desired mutagenic plasmid with an inserted Kozak sequence, the 18 candidate colonies underwent colony PCR screening, using the M13 forward and reverse primers and amplifying with the 'JEV50SCREEN' thermal cycling program (Table 7), before undergoing gel electrophoresis and UV visualisation. However, none of the prME inserts of the candidate Kozak plasmids were successfully amplified, as well as the positive control included on the gel (pUC57-24SPprME plasmid). Despite repeating this colony PCR experiment with a fresh preparation of the DreamTaq polymerase master mix, as well as altering the annealing temperature to stimulate improved primer binding to the PCR template, amplification of the desired ~2.1kb insert bands was not achieved. Therefore, restriction digest analysis was performed as an alternative screening process on a candidate colony from the potential pUC57-15SPprME-GTCAAANKoz, pUC57-15SPprME-GCCACCKoz and pUC57-24SPprME-GCCACCKoz plasmids.

Restriction digest analysis was performed using *EcoRI* and *XhoI* restriction enzymes. In this instance, gel band lengths of ~2.7kb (pUC57) and ~2.1kb (Koz-prME) were desired. Furthermore, both conventional and FastDigest restriction enzymes were utilised in this reaction, for comparative purposes. Reaction mixtures for both sets of enzymes were prepared as detailed in Tables 8 and 9, before being placed in a thermocycler for incubation. Sample reactions #1-5, containing conventional enzymes, and samples #6-10, which included FastDigest enzymes, were incubated as described in Section 2.1.9. All samples were then run on an agarose gel (see Figure 28 for sample details), to assess the resulting DNA fragment lengths and quality of digestion. Both the conventional and FastDigest enzymes successfully digested the pUC57-24SPprME and Kozak mutant pUC57-prME plasmids, though a higher quality of digestion and clarity was observed for the FastDigest restriction enzymes. These data also confirm the presence of a prME insert in all of the mutagenic Kozak pUC57-prME plasmids, as bands of the approximate lengths of ~2.7kb and ~2.1kb were present in each of the experimental samples, as well as for the positive control. These DNA fragment lengths correspond to the known lengths of the pUC57 empty vector and the prME insert, respectively (Figure 28).

From here, each of the three candidate colonies – pUC57-15SPprME-GTCAAkKoz, pUC57-15SPprME-GCCACCKoz and pUC57-24SPprME-GCCACCKoz – were cultured, before plasmid purification and LIGHTrun Sanger sequencing took place, using the universal M13 forward primer. Unfortunately, once the sequencing results were returned and the chromatogram files were analysed, the mutagenesis was also revealed to be unsuccessful for the insertion of the Kozak consensus sequences upstream of the prME gene, as the aligned experimental sequences were identical at the 5' terminus to the original 15SP- and 24SP-prME genes, meaning that downstream studies to assess prME glycoprotein levels during JEV pseudotype production, in the presence and absence of distinct Kozak sequences, were unable to be performed.

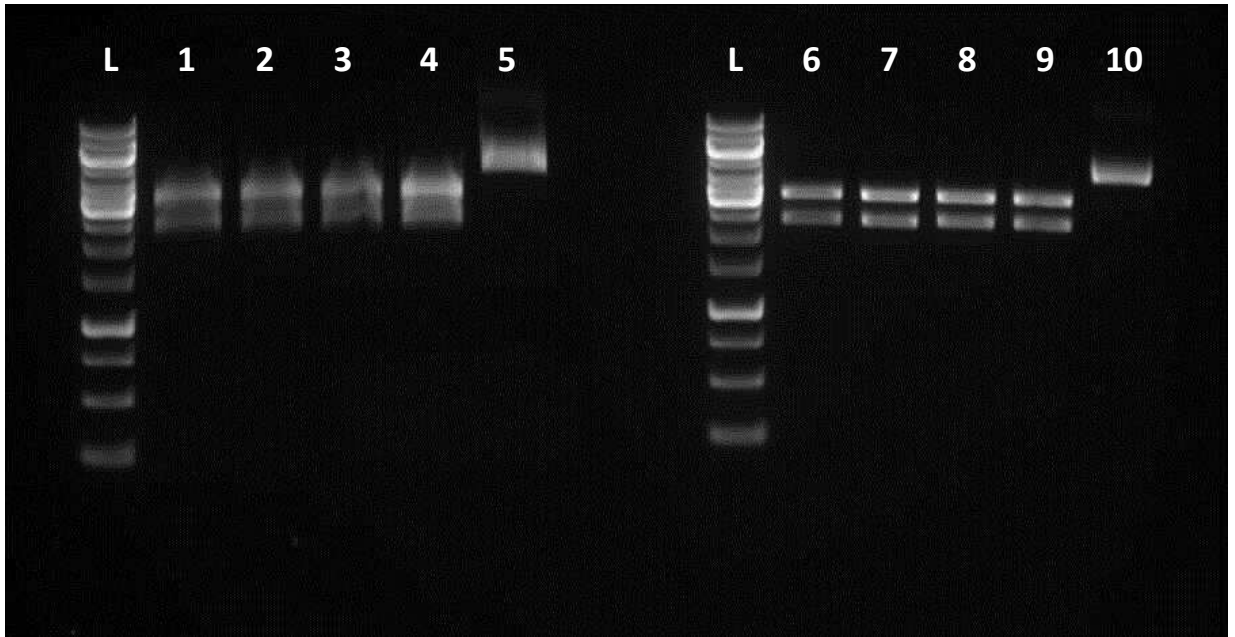


Figure 28. Gel electrophoresis image following restriction enzyme digest screening of candidate Kozak insert mutagenic plasmids. Experimental samples #1 and #6 (for pUC57-15SPprME-GTCAA AKoz), #2 and 7 (for pUC57-15SPprME-GCCACCKoz) and #3 and #8 (for pUC57-24SPprME-GCCACCKoz) were run in their corresponding, numbered lanes, alongside the GeneRuler 1kb DNA ladder (in lanes marked L) and positive (pUC57-24SPprME) and negative (uncut pUC57-24SPprME) controls (in lanes #4 and #9, and #5 and #10, respectively). All sample reactions containing restriction endonucleases successfully digested the plasmids, to achieve DNA fragment bands of ~2.7kb (pUC57) and ~2.1kb (prME), indicating the presence of potentially-mutated Kozak prME inserts.

4.4 Discussion

The theme of this chapter was to explore various methods which could potentially increase the likelihood of producing functional-titre pseudotype viruses bearing the JEV envelope glycoproteins. There were two primary approaches to the experimental work presented in this chapter. Firstly, the attempted construction of VSV/JEV chimeric glycoproteins was explored, in a bid to induce an interaction between the JEV envelope proteins and the lentiviral *gag-pol* core proteins. This technique has been successfully employed previously to stimulate the assembly of other heterologous viral envelopes onto lentiviral cores, resulting in effective production and increased titre of pseudotype particles (Hu *et al*, 2007; Carpentier *et al*, 2011). Secondly, the presence of particular, short nucleotide stretches known as Kozak consensus sequences have been widely reported (Kozak, 1978; Kozak, 1986; Kozak, 2002; Nagakawa *et al*, 2008). Kozak sequences, when located directly upstream of the starting methionine codon of a gene, serve to enhance the initiation of RNA translation, often resulting in a boost in protein expression levels. This mechanism could be harnessed to increase the intracellular expression of JEV viral envelopes for this study, and indeed, the three Kozak sequences selected for insertion into the pUC57-prME plasmid constructs – GTCAAA, CACAAA and GCCACC – have all been presented as effective translation initiators in recently published literature, for a variety of downstream applications (Babaie *et al*, 2011; Jackson *et al*, 2011; Etheridge *et al*, 2014).

Unfortunately, the full effects of the VSV/JEV chimeric glycoprotein and the Kozak insertion mutant plasmid constructs could not be explored when applied to the production of JEV pseudotype viruses, as the Q5 site-directed mutagenesis utilised to perform the required nucleotide changes and form these genetic alterations was unsuccessful on both counts. The cause of the ineffective mutagenesis is currently unknown – however, following scrutiny of the protocols and methodologies used, it is possible that a failed KLD treatment could be accountable. An issue occurring at this step could mean that methylated template DNA was

not properly digested, and as a consequence, was carried forward to transformation and subsequent screening stages of the experiments. This eventuality may explain the sequencing results confirming that no mutagenesis took place, despite positive results in the previous colony PCR and restriction digest screening steps of the studies. Furthermore, false positive results may have occurred in screening stages involving colony PCR, due to non-specific primer binding, although methylated template DNA contamination seems more feasible.

The Q5 mutagenesis kit was used for the experiments detailed in this chapter because it had promptly and successfully been able to elongate the JEV C-terminal capsid signal peptide by 9 residues, or 27 nucleotides, as shown in Chapter 3 of this thesis. Also, preliminary experiments using other similar site-directed mutagenesis kits had not been fruitful. However, a disadvantage to using the Q5 SDM kit is that there is a limited scope to optimise or troubleshoot the PCR and KLD stages of the kit's protocols. This is due to the requirement of the NEBaseChanger program, which accurately designs oligonucleotide primers necessary to create the necessary insertion or substitution – it would be counter-intuitive to manipulate these sequences as the exact mutagenic sequence would not be incorporated into the PCR amplicon. Furthermore, other properties concerning the primer of the PCR thermal cycling program are recommended, such as the paired annealing temperature of the mutagenesis amplification. These recommendations are founded on detailed analysis of the primer sequence given by the same program, and are likely to produce an optimal result in downstream site-directed mutagenesis attempts.

To conclude, if either of the approaches explored in this chapter resulted in the successful production of high-titre JEV pseudotype titres, it may enable their reliable utilisation in serological or virus biology-based study, whilst bypassing the considerable drawbacks of having to handle native, pathogenic JEV, and the high infrastructure and training expenses associated to working in high biosafety environments.

Chapter 5

Lyophilisation of lentiviral pseudotype viruses for the development and distribution of a neutralisation assay-based diagnostic kit

5.1 Preface

The ability to effectively distribute and store assay reagents in a stable state is a vital aspect of the development of any assay, when the ambition is present to disseminate a diagnostic kit for clinical purposes, especially on an international or global scale. If the kit is designed to be utilised at the point of care, in a resource-deprived area and/or a region with a hot, arid or tropical climate, it is especially important that the reagents, in this instance pseudotype viruses, maintain a high level of storage stability and retention of virus titre. In this chapter, lyophilisation is explored as a means of preserving pseudotype viruses in medium- to long-term storage, to enable their viability for cost-effective transit, end-point ambient incubation, reconstitution and use in downstream assays, with minimal detriment to their quality and efficiency. Unfortunately, since functional pseudotype viruses bearing JEV envelope glycoproteins were not successfully produced, a variety of high-titre RNA virus pseudotypes from different families were used as substitutes.

This work constitutes a peer-reviewed article in the *Journal of Virological Methods* (Mather *et al*, 2014).

5.2 Introduction

In recent years, the rate of emergence and re-emergence of viral diseases has significantly increased, creating a concordant rise in the level of importance and impact on the global, clinical public health scale. Influenza (family Orthomyxoviridae, genus Influenzavirus A, species Influenza A virus) continues to be amongst the viruses with the highest propensity to cause morbidity and mortality in human populations – in 1997 and 2013 respectively, the H5N1 and H7N9 influenza subtypes caused notably severe outbreaks, and the highly transmissible H1N1 virus caused a pandemic in 2009, fortunately mitigated by its low pathogenicity (Yuen *et al.*, 1998; WHO, 2010; Gao *et al.*, 2013). The threat of influenza persists as novel subtypes, such as H6N1 and H10N8, cross species barriers to infect humans for the first time, and the recent emergence and discovery of H17N10 and H18N11 in Guatemalan bat reservoirs, which display a high level of diversity from other, more established influenza A subtypes (Tong *et al.*, 2013; Wei *et al.*, 2013; To *et al.*, 2014). Similarly, rabies (family Rhabdoviridae, genus Lyssavirus, species Rabies virus) is a virus that possesses global ubiquity, with cases reported frequently on every continent except Antarctica. The lyssavirus is responsible for an annual mortality rate in excess of 60,000 people, of which the victims are primarily children and infants in resource-deprived regions in Africa and Asia (WHO, 2013). Upon the onset of symptoms, rabies has an almost 100% case fatality rate, which is the highest of any known viral infection. Only very few patients have survived following the development of symptomatic pathology, with most of these cases resulting in a number of neurological sequelae (Jackson, 2013). Isolated outbreaks of Marburg virus (family Filoviridae, genus Marburgvirus, species Marburg marburgvirus) in the Democratic Republic of the Congo in 1999-2000, and then in Angola in 2004-2005, generated respective mortality rates of 83% and 90%, and more recent outbreaks in Uganda also act as a reminder that spillover events into human populations from unexpected, zoonotic viral sources can rapidly bring about serious public health concerns (Brauburger *et al.*, 2012). Indeed, towards the end of 2014, the related Zaire ebolavirus, which is another prominent

member of the *Filoviridae* family, was the pathogen responsible for causing an outbreak which spread primarily through the West African countries of Guinea, Liberia and Sierra Leone, finally resulting in 28,652 cases throughout the next two years, with 40% of these proving fatal (Kaner and Schaack, 2016). Therefore, it is vitally important to consider options for monitoring the spread and curtailing the outbreak severity of such pathogenic viruses.

Serological assays that are able to quantitatively measure the levels of antibody responses raised against immunogens, such as antigenic viral glycoproteins, allow for the experimental evaluation of novel vaccine and antiviral treatments, as well as permitting serosurveillance to track and monitor the epidemiological spread of viruses, which actively contributes towards international public health initiatives. Serology compliments other branches of viral diagnostics, such as direct virus isolation or reverse transcription polymerase chain reaction (RT-PCR) diagnosis, through being able to retrospectively identify cases of acute viral infection once the transient viraemic stage has passed in the patient (Papenburg *et al*, 2011).

Alongside their benefits, most conventional serological assays possess different disadvantages that have the propensity to negatively affect their reliability and efficiency.

However, conventional serological assays possess drawbacks which detrimentally affect their efficiency. Importantly, most require the use of infectious wild-type virus, necessitating expensive, specialized biosafety level 3 or 4 (BSL-3 or -4) laboratories which are not readily available, especially in resource-limited areas. Hemagglutination inhibition (HI) assays, used routinely for influenza, suffer from variability caused by different erythrocytes and inhibitory factors, as well as low sensitivity. ELISA-based assays do not require the use of wild-type virus, but are also hindered by low sensitivity and cross-reactivity between samples. Furthermore, both HI and ELISA cannot differentiate between virus neutralising and non-neutralising antibody responses (as reviewed in Mather *et al.*, 2013). Virus neutralisation assays, such as plaque reduction neutralisation test (PRNT) and fluorescent antibody virus neutralisation

(FAVN) assay, can measure virus neutralising antibody (VNAb) responses with high sensitivity and specificity levels but also require high biosafety for assay preparation, and in some cases are time-consuming and suffer from low-throughput (Cliquet *et al.*, 1998; as reviewed in Mather *et al.*, 2013).

A potential solution to these issues is the utilisation of retroviral pseudotype viruses (PVs). PVs are composed of the structural and enzymatic core of one virus combined with heterologous envelope glycoproteins (Temperton *et al.*, 2015). Manipulations to the genomic RNA of the lentiviral core create a replication-defective PV that encapsulates a quantifiable reporter gene. Transduction of a permissible target cell line is dependent upon the ability of the envelope glycoprotein to engage its cellular receptor in a process that mimics wild-type virus entry mechanisms. If this is successful, the reporter gene can be integrated into the host cell genome and subsequently expressed. Resultant levels of reporter protein in transduced cells can be measured, giving a readout equivalent to viral titre. Pseudotype virus neutralisation assays (PVNAs) attain comparable, if not higher, sensitivity and specificity results than many traditional serological assays (Desvaux *et al.*, 2012).

In order to maximise the utility of the pseudotype assay system, multiplexing of PVNAs has been demonstrated which permits simultaneous quantification of VNAb responses against several PVs (each harbouring a different reporter gene i.e. renilla and firefly luciferase, or GFP and RFP) in the same assay, sparing valuable reagents such as serum samples (Wright *et al.*, 2010). The flexibility of reporter genes that can be incorporated into PVs further customises the assay. Luciferase and GFP reporters enable highly quantitative readouts but require expensive reagents and/or equipment. However, infection by PVs that encapsulate lacZ (expressing β -galactosidase) or secreted alkaline phosphatase (SEAP) reporter genes can be quantified by adding colorimetric substrates such as ONPG, CPRG or p-nitrophenyl phosphate

and measuring color change with an ELISA plate reader or by eye (Wright *et al.*, 2009; Kaku *et al.*, 2012).

Multiplexing, as well as selecting 'low-cost' reporter genes, considerably reduces the cost-per-assay burden of the pseudotype platform. However, the high expenses associated in optimal transportation and storage can be an inhibitory obstacle in the international distribution of PVNAs. Despite pseudotype studies being conducted on field serum from resource-poor tropical countries, and reports of viruses that circulate in tropical regions being successfully pseudotyped (Wright *et al.*, 2009; Kishishita *et al.*, 2013), there appear to have been no published studies involving the carrying out of pseudotype neutralisation assays in tropical countries, especially in rudimentary laboratories without air-conditioning or access to reliable freezer units.

The aim of this study was to ascertain the viability of lyophilising pseudotype viruses with a view to developing a PVNA-based kit. Pseudotype stability was monitored after subjection to environmental conditions likely experienced in the production, transit and usage of such a kit, especially to tropical countries. PV titres were also assessed subsequent to lyophilisation and immediate reconstitution, as well as incubating freeze-dried pellets at a variety of temperatures and humidities before reconstitution.

5.3 Materials and Methods

5.3.1 Viruses and cells

The virus isolates pseudotyped in this study were influenza A/H5N1/Vietnam/1194/2004 strain (Genbank accession number ABP51976), rabies virus (RABV) strain Evelyn Rokitniki Abseleth (ERA; UniProtKB/Swiss-Prot code ABN11294) and the Lake Victoria strain of Marburg virus (MARV; Genbank accession number DQ447649). Previously, the influenza HA gene and RABV G gene of these isolates were both sub-cloned into the pl.18 expression vector (Cox *et al.*,

2002). The Marburg GP gene within the pCAGGS expression vector was a kind gift from Graham Simmons (Blood Systems Research Institute, San Francisco, CA, USA).

Human embryonic kidney 293T clone 17 (HEK293T/17; ATCC CRL-11268) (Pear *et al.*, 1993) cells were used for all transfections and as a target cell line for titration and neutralisation assays involving H5 pseudotype virus. Baby hamster kidney 21 cells (BHK-21; ATCC CRL-10) (Stoker and MacPherson, 1964) were used as a target cell line for RABV and MARV pseudotype virus assays. Both cell lines were cultured at 5% CO₂ in Dulbecco's Modified Eagle Medium (DMEM) + GlutaMAX (Life Technologies, UK) supplemented with 15% foetal bovine serum (FBS) and 1% penicillin/streptomycin (Sigma Aldrich, UK).

5.3.2 Serum samples

For use in H5 PVNAs, a sample from a panel of ten sera extracted from chickens vaccinated with an inactivated, monovalent, adjuvanted H5N2 vaccine (A/chicken/Mexico/232/94/CPA strain) was selected. Previous studies have confirmed its seropositivity by HI (a titre of 1:1024 with a homologous H5N2 test antigen) and PVNA, against an H5 A/Vietnam/1194/2004 luciferase PV (Terregino *et al.*, 2010; Molesti *et al.*, 2013). To neutralise RABV pseudotypes, serum was used from a human subject vaccinated on days 0, 7 and 21 with the inactivated Rabipur vaccine (Novartis Vaccines, Germany).

5.3.3 Production of pseudotype viruses

The generation of all lentiviral pseudotype viruses was performed as detailed previously (Temperton *et al.*, 2007; Wright *et al.*, 2008). 24 hours prior to transfection, approximately 4x10⁶ HEK293T/17 cells were seeded into sterile 10 cm tissue culture dishes (Nunc™ Thermo Scientific, UK). The HIV *gag-pol* plasmid, pCMV-Δ8.91 (Zufferey *et al.*, 1997) and the firefly luciferase reporter construct pCSFLW – (Capecchi *et al.*, 2008) based on pHR'SIN-cPPT-SGW outlined in (Demaison *et al.*, 2002) – were transfected simultaneously with either the influenza

HA, rabies G or Marburg GP expression vectors at a ratio of 1:1.5:1 (core:reporter:envelope) using the Fugene6 lipid-based reagent (Promega, UK). At 24 hours post-transfection, the cells were incubated with fresh media. For H5 transfections, exogenous recombinant neuraminidase from *Clostridium perfringens* (Sigma Aldrich, UK) was also added at this stage. Pseudotype supernatants were harvested at 48 hours after transfection and passed through a 0.45µm pore filter (Millex®, Millipore, Billerica, MA, USA), before being prepared for lyophilisation. Remaining supernatant was aliquoted and stored at -80°C.

5.3.4 Lyophilisation of pseudotype viruses

Individual samples of pseudotype virus were mixed with a sucrose-PBS cryoprotectant solution at a 1:1 v/v ratio to a 1M-0.1M range of molarities. Importantly, all lyophilisation was carried out in low surface-tension polypropylene microcentrifuge tubes (Caesa Lab, Canada), to prevent binding of the virus glycoproteins to the inside surface of the tubes, and subsequent loss of pseudotype titre, during freeze-drying. Once prepared, virus samples were pre-frozen at -80°C. Immediately prior to lyophilisation, a second, pierced lid, made of standard polypropylene, was applied to each sample tube to allow for moisture release. All lyophilisation was carried out overnight in a FreeZone 2.5 litre freeze-drying chamber (Labconco, Kansas City, MO, USA) at a temperature of -50°C and a pressure of <0.133mBar. If the lyophilised pellets were stored for a sustained length of time after freeze-drying, the standard polypropylene pierced lid was removed from the sample tube, and the original low surface-tension polypropylene lid was replaced. Likewise, in the instances where the pellets were stored at a constant humidity as well as temperature, the sample tubes were kept in a sealed, humidified incubator unit, controlled by a humidistat. DMEM + GlutaMAX (with the same supplementation as for the cell culture) were attempted for all reconstitution of lyophilised pellets, except for Figure 35 where distilled, nuclease-free H₂O was also used.

5.3.5 Pseudotype titration and neutralisation assays

Titration and neutralisation assays were performed in 96-well plates and based upon previously described protocols (Temperton *et al.*, 2007; Wright *et al.*, 2009; Scott *et al.*, 2012), but adapted for the use of reconstituted, lyophilised pseudotype. For titration assays, 1:2 serial dilutions of reconstituted pseudotype were incubated with 1×10^4 HEK293T/17 or BHK-21 cells for 48 hours before measuring relative luminescence units per ml (RLU/ml). For the neutralisation assay, serum samples were serially diluted (ranging from 1:40 to 1:81920) and incubated with 1×10^6 RLU of reconstituted pseudotype (as calculated from the titration assay) for 1hr at 37°C to permit antibody attachment to surface virus glycoproteins. 1×10^4 HEK293T/17 or BHK-21 cells were then added to each well and incubated for 48 hours, prior to taking a chemiluminescent readout. In all instances, Bright-Glo luciferase assay reagent (Promega, UK) and a Glomax 96 luminometer (Promega, UK) were used to quantify luciferase reporter expression.

5.3.6 Statistical analysis

Pseudotype transduction titres were calculated by converting RLU readout values at a range of assay dilutions into RLU/ml, before determining the arithmetic mean and standard deviation. PVNA raw data was normalised as % neutralisation between mean values for a virus only control (equivalent to 0% neutralisation or 100% infection) and a cell only control (equivalent to 100% neutralisation or 0% infection), then IC_{50} and IC_{90} values were calculated using non-linear regression analysis (log [inhibitor] vs normalised response – variable slope). All data manipulation was performed on GraphPad Prism 5 (GraphPad software, San Diego, CA, USA).

5.4 Results

5.4.1 Production of lentiviral pseudotypes

High titre lentiviral pseudotype particles were generated bearing the envelope glycoproteins from influenza A/H5N1/Vietnam/1194/2004, rabies ERA and Marburg Lake Victoria strains. Transduction efficiency of the pseudotypes into HEK293T/17 cells (for influenza H5) and BHK-21 cells (for RABV and MARV) was evaluated, and luciferase expression was observed at 2.04×10^{10} , 8.21×10^9 and 7.46×10^9 RLU/ml, respectively (Figure 29). All titration assays included two negative controls: Δ envelope glycoprotein (Δ EG), which is a PV bearing no viral envelope glycoprotein, and a non-transduced cell only control.

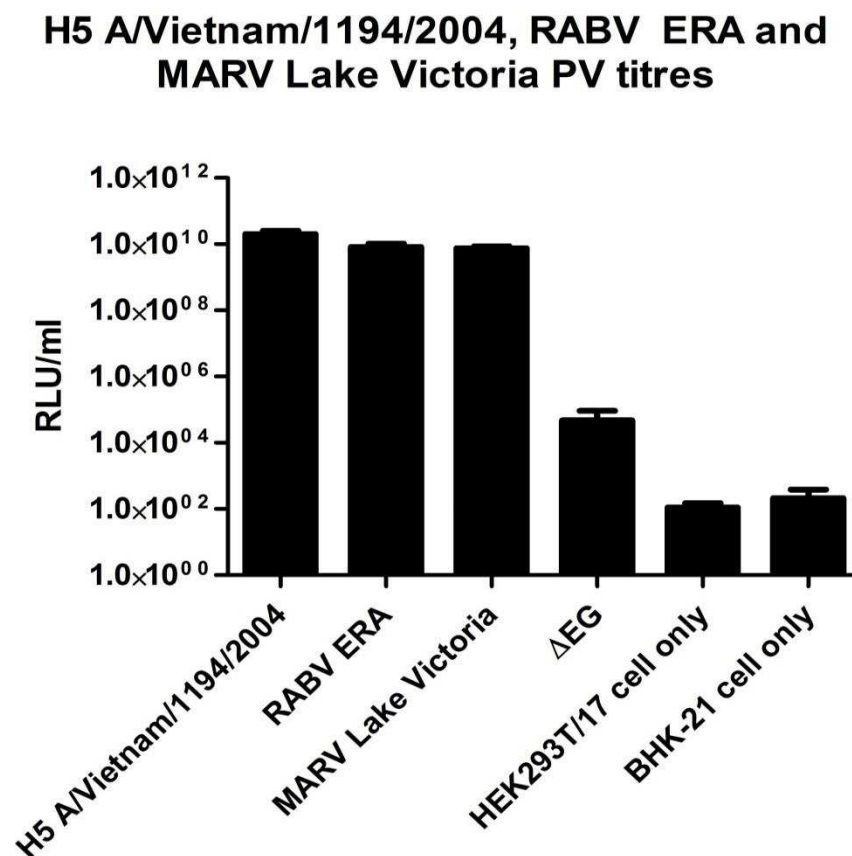


Figure 29: Infectivity of pseudotyped lentiviral vectors displaying influenza H5 A/Vietnam/1194/2004 HA, RABV ERA G and MARV Lake Victoria GP glycoproteins. Pseudotype transduction titers are expressed as mean \pm SD of relative luminescent units per ml (RLU/ml). Δ envelope glycoprotein, HEK293T/17 cell only and BHK-21 cell only negative controls are also shown.

5.4.2 Lyophilisation of lentiviral pseudotypes

Next, pseudotype supernatants were mixed with a stepwise dilution series of sucrose-PBS solutions (1M, 0.5M, 0.25M, 0.1M) which acts as a cryoprotectant during lyophilisation.

Supernatant was also lyophilised in pure PBS solution containing no sucrose, which is referred to as 0M sucrose-PBS. After overnight freeze-drying, lyophilised pellets were immediately reconstituted and transduction efficiency measured in a titration assay. Less than $1\log_{10}$ of decrease in viral titre, measured in RLU/ml, was observed with H5, RABV and MARV pseudotypes at all cryoprotectant concentrations, when compared to their non-lyophilised counterparts (Figure 30A-C). Levels of titre retention are therefore sufficient for these lyophilised PVs to be taken forward into PVNA assays. As PV titre was retained following reconstitution of recently lyophilised pellets, regardless of sucrose-PBS concentration, freeze-drying for subsequent experiments was carried out at 1M, 0.5M and 0M cryoprotectant molarities.

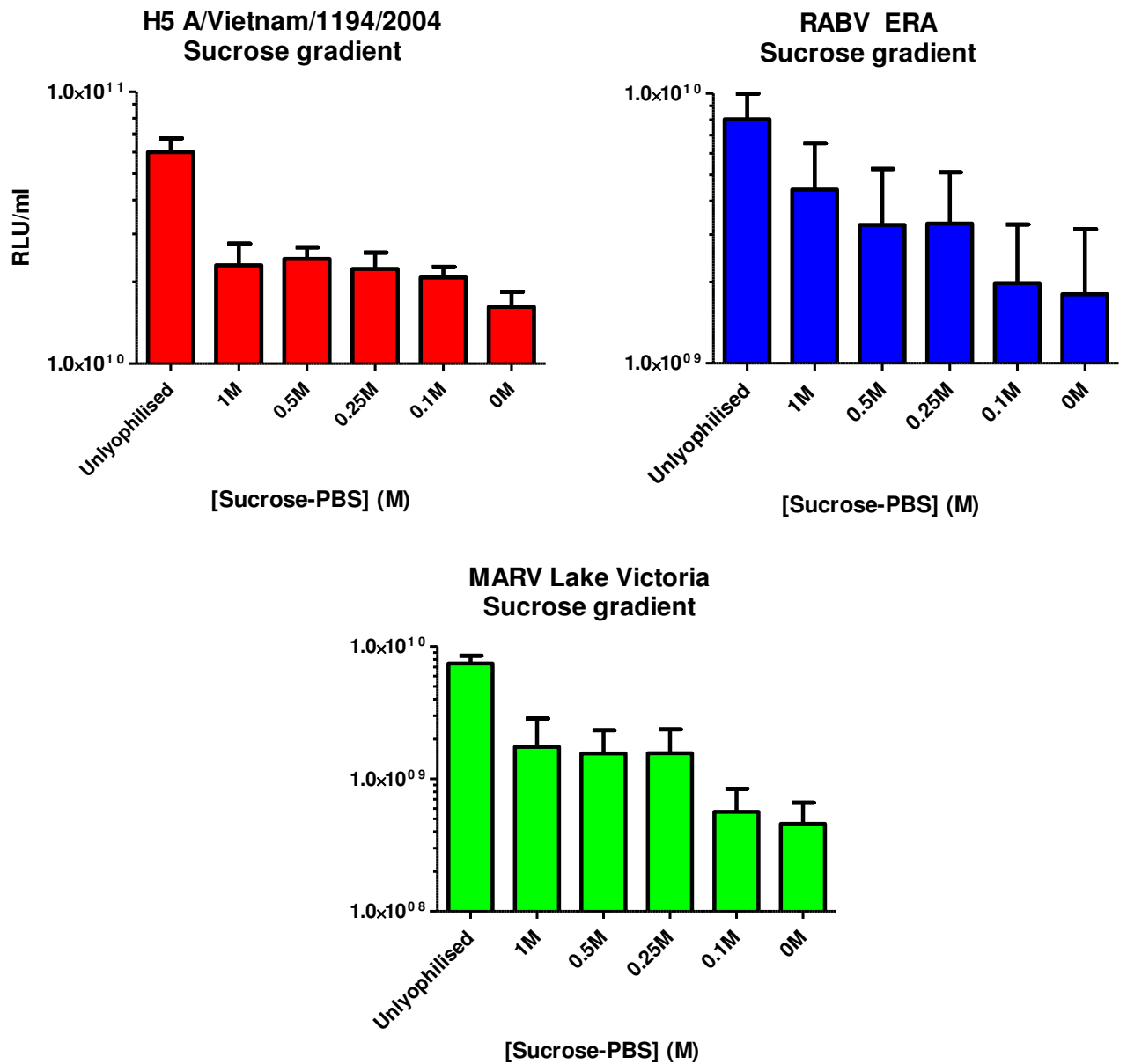


Figure 30: Transduction retention of pseudotype viruses with (A) influenza H5 A/Vietnam/1194/2004 HA, (B) RABV ERA G and (C) MARV Lake Victoria GP envelope glycoproteins following lyophilisation at a gradient of sucrose-PBS cryoprotectant molarities. Relative PV transduction titers are shown as mean \pm SD of relative luminescent units per ml (RLU/ml).

5.4.3 Durability of lyophilised pseudotypes

To ascertain the stability of PV aliquots stored in the freeze-dried state, individual lyophilised pellets in 1M, 0.5M and 0M cryoprotectant were incubated for varying durations at the following temperatures: -80°C, -20°C, +4°C, +20°C, +37°C/70% relative humidity (RH) and +37°C/95% RH. After 1, 2 and 4 weeks, freeze-dried pellets of PV were reconstituted and titrated as previously described (subsections 2.4 and 2.5) to calculate viral titre in RLU/ml. Generally, PV titre retention was high for all lyophilised H5 (Figure 31A-C), RABV (Figure 32A-C) and MARV (Figure 33A-C) samples that were stored at the lowest temperatures, but as the storage temperature increased, PV samples freeze-dried in the absence of cryoprotectant degraded significantly, with transduction efficiency decreasing to that of Δ EG. Interestingly, relative humidity (RH) seems to play a role in viability of lyophilised PV pellets, with 1M- and 0.5M-cryoprotected samples stored for 4 weeks generally retaining functional virus titre up to 37°C and 70% RH, but heavily degrading in a 95% humidified atmosphere at the same temperature.

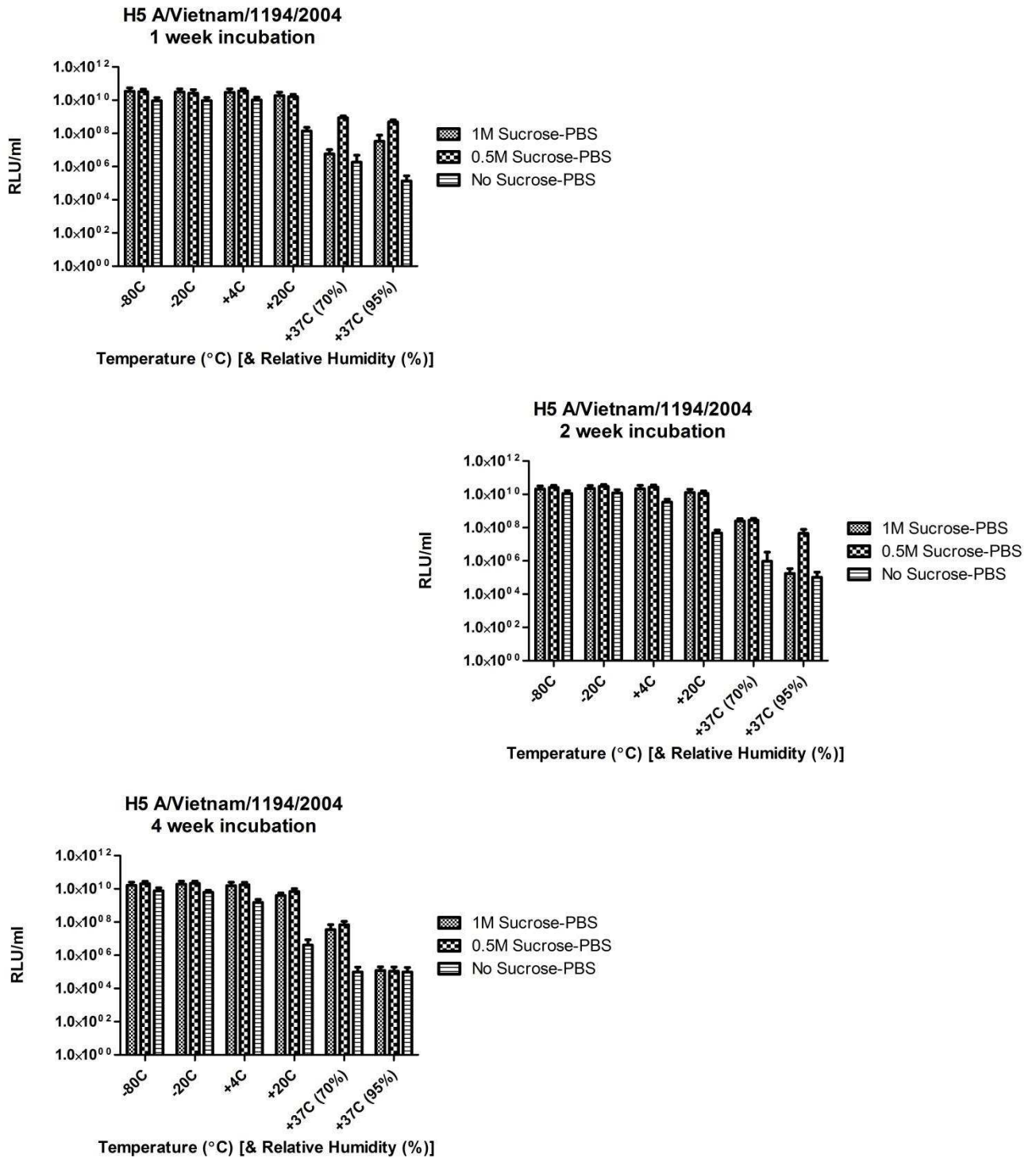


Figure 31: Effect of lyophilisation and pellet incubation on infectivity of H5 pseudotyped lentiviral vectors. Freeze-dried PVs displaying H5 A/Vietnam/1194/2004 HA were stored in either 1M, 0.5M or 0M sucrose-PBS cryoprotectant at a variety of temperatures and humidities for (A) 1 week, (B) 2 weeks and (C) 4 weeks before reconstitution and employment in a titration assay. Pseudotype transduction titres are displayed as mean \pm SD of relative luminescent units per ml (RLU/ml).

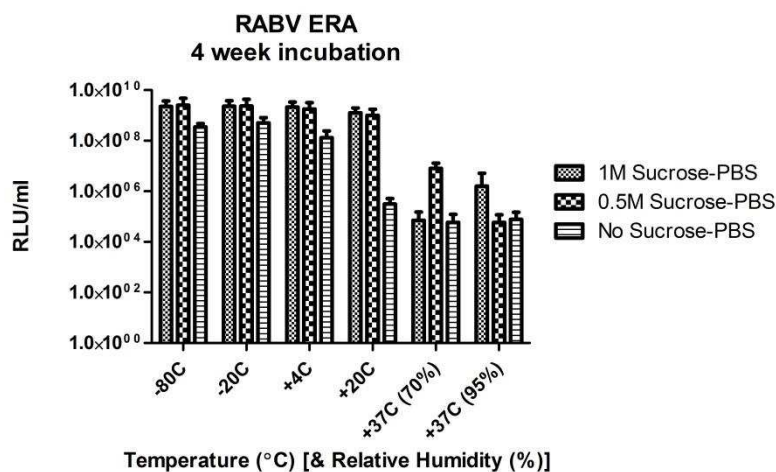
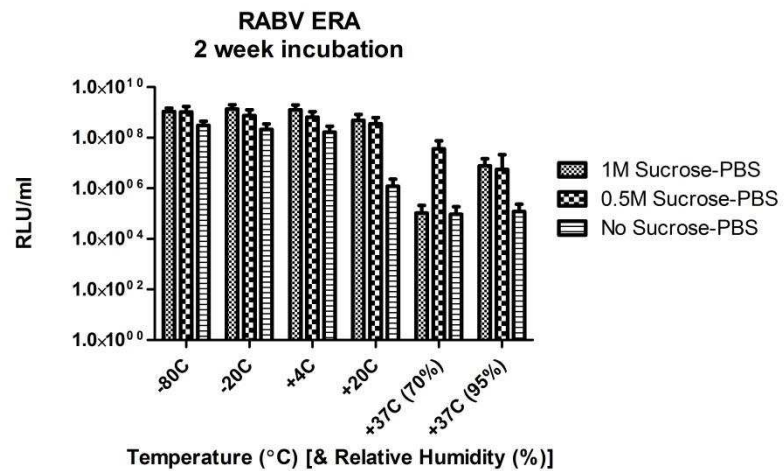
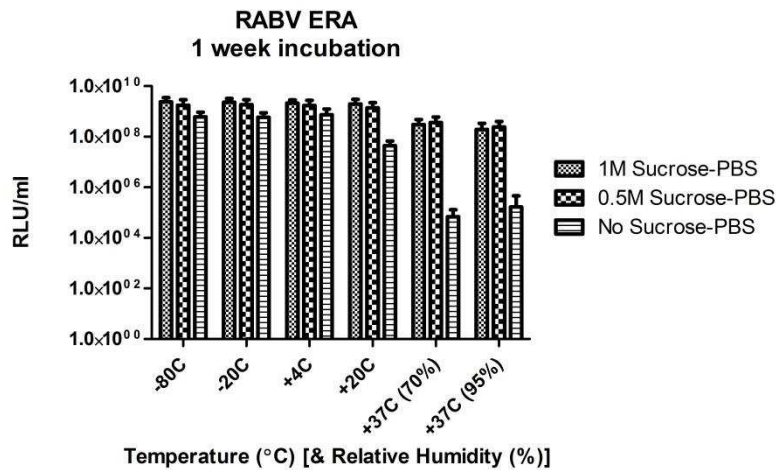


Figure 32: Effect of lyophilisation and pellet incubation on infectivity of RABV pseudotyped lentiviral vectors. Freeze-dried PVs displaying RABV ERA G glycoproteins were stored in either 1M, 0.5M or 0M sucrose-PBS cryoprotectant at a variety of temperatures and humidities for (A) 1 week, (B) 2 weeks and (C) 4 weeks before reconstitution and employment in a titration assay. Pseudotype transduction titres are expressed as mean \pm SD of relative luminescent units per ml (RLU/ml).

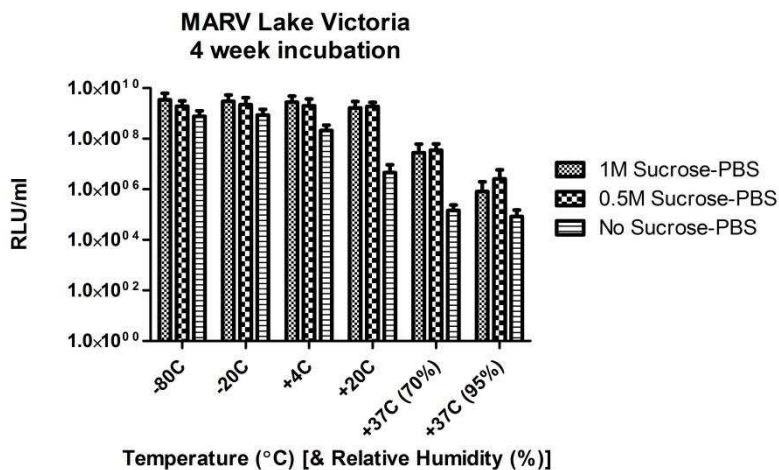
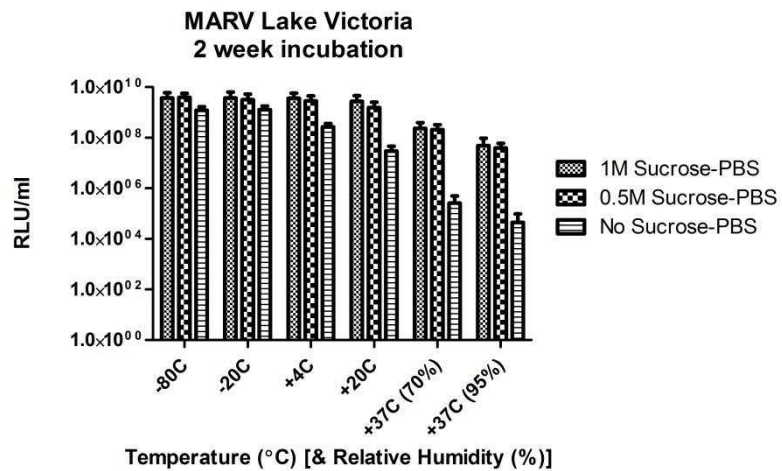
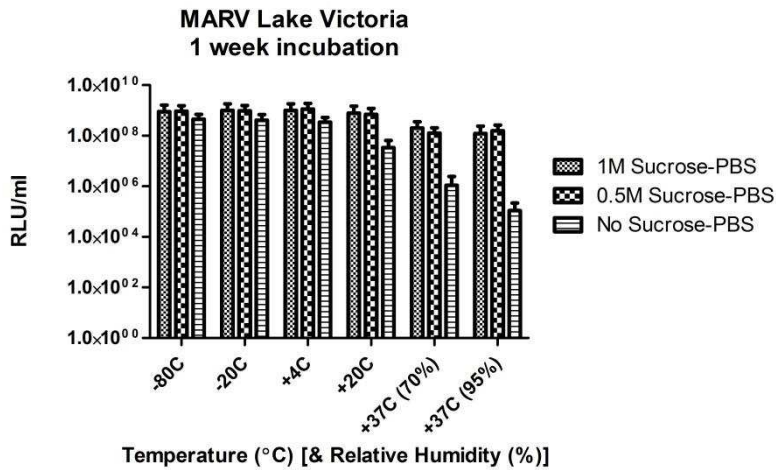


Figure 33: Effect of lyophilisation and pellet incubation on infectivity of MARV pseudotyped lentiviral vectors. Freeze-dried PVs displaying MARV Lake Victoria GP glycoproteins were stored in either 1M, 0.5M or 0M sucrose-PBS cryoprotectant at a variety of temperatures and humidities for (A) 1 week, (B) 2 weeks and (C) 4 weeks before reconstitution and employment in a titration assay. Pseudotype transduction titres are shown as mean \pm SD of relative luminescent units per ml (RLU/ml).

5.4.4 Reconstitution of lyophilised pseudotypes

It is possible that reconstituting in supplemented DMEM results in an accumulation of soluble culture medium components in the pseudotype sample which may affect downstream employment in serological assays. To address this issue, we reconstituted H5, RABV and MARV pseudotypes (immediately after lyophilisation, in the presence of 0.5M sucrose-PBS) with distilled, nuclease-free H₂O and DMEM (with supplementation described in subsection 2.1), before comparing their transduction ability into corresponding target cell lines with a titration assay (Figure 34). Levels of pseudotype titre retention were very similar with either reconstitution solution, indicating that possible culture medium nutrient accumulation when using DMEM to reconstitute lyophilised pseudotypes does not have an adverse effect on pseudotype infectivity. However, water could viably be used as an alternative solution for resuspension of freeze-dried pseudotypes, but it is uncertain whether this would detrimentally affect the health of the target cell lines in titration and neutralisation assays, due to insufficient volumes of fresh DMEM.

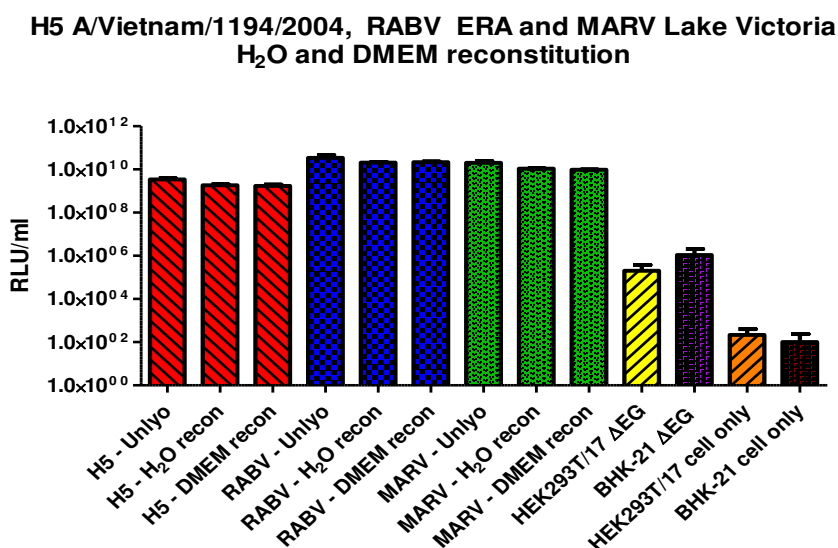


Figure 34. Influence of reconstitution solution on H5, RABV and MARV pseudotyped lentiviral vectors. Freeze-dried PVs with influenza H5 A/Vietnam/1194/2004 HA, RABV ERA G or MARV Lake Victoria GP envelope glycoproteins were reconstituted in either distilled, nuclease-free H₂O or supplemented DMEM culture medium before utilisation in a titration assay. Pseudotype transduction titres are shown as mean \pm SD of relative luminescent units per ml (RLU/ml). Unlyophilised pseudotype positive controls, and Δ envelope glycoprotein, HEK293T/17 cell only and BHK-21 cell only negative controls are also shown.

5.4.5 Neutralisation of pseudotypes post-lyophilisation

The ability for lyophilised PVs to transduce target cells indicates that the influenza A, RABV and MARV envelope glycoproteins do not structurally deteriorate during the freeze-drying process, especially in the receptor-binding domains. However, in order to assess the structural integrity in the antigenic epitopes of the glycoproteins, neutralisation assays were also carried out using serum samples confirmed as antibody-positive against H5 and RABV strains. VNAb IC₅₀ and IC₉₀ titres (the reciprocal of the highest serum dilution still able to confer 50% and 90% virus neutralisation) were compared between lyophilised and immediately reconstituted H5 and RABV pseudotypes, and their ‘fresh’, unlyophilised counterparts, with no discernible reduction in the capability of antibody-mediated neutralisation observed (Table 16).

Pseudotype virus (PV)	IC ₅₀	IC ₉₀
H5 A/Vietnam/1194/2004 - lyophilised	2560-5120 (4679)	1280-2560 (1390)
H5 A/Vietnam/1194/2004 - unlyophilised	2560-5120 (3919)	1280-2560 (1345)
RABV ERA – lyophilised	40960-81920 (44072)	5120-10240 (6381)
RABV ERA – unlyophilised	20480-40960 (27324)	5120-10240 (6222)

Table 16: Comparison of neutralising antibody titres against untreated and lyophilised pseudotyped lentiviruses. Half maximal inhibitory concentration (IC₅₀) and 90% inhibitory concentration (IC₉₀) values of confirmed antibody-positive antisera against H5 influenza and RABV pseudotypes before and after lyophilisation. VNAb titres were calculated using GraphPad Prism 5 software and are displayed as serum assay dilutions. Exact IC₅₀ and IC₉₀ values are also shown in parentheses.

5.5 Discussion

It has been well-documented that retroviral pseudotypes are valid, reliable alternatives to wild-type virus for serological applications (Temperton *et al.*, 2015). Advantageous qualities of the PVNA platform include the ability to conduct the assay in BSL-1 laboratories, as well as the availability to perform multiplex assays and incorporate 'low-cost' reporter genes into pseudoparticles, which all reduce the cost implications and increase the potential ubiquity of pseudotyping (Wright *et al.*, 2009, 2010; Kaku *et al.*, 2012). However, the current necessity to store aliquots of PV supernatant at -80°C and to maintain the cold-chain during PV transportation present serious monetary obstacles for laboratories to acquire such reagents, especially if on a limited budget.

Here, the viability of lyophilisation has been demonstrated as an alternative, cost-effective state for the storage and distribution of pseudotype viruses. In the presence of cryoprotectant, H5 influenza, rabies and Marburg PV supernatant retain very high levels of infectivity following freeze-drying and reconstitution. Subsequent freeze-dried pseudotype pellets can generally withstand incubation for 4 weeks at a range of temperatures up to 37°C, and incubation in a 'tropical climate' (37°C and 95% relative humidity) for 2 weeks, whilst maintaining a viral titre sufficient for employment in downstream neutralisation assays. This confirms the stability and glycoprotein integrity of lyophilised PVs throughout environmental conditions likely to be experienced within the production, dissemination and storage of a PVNA-based kit.

Furthermore, both H5 influenza and rabies reconstituted pseudotypes were neutralised by VNAb-positive serum samples to the same potency as their 'fresh', unlyophilised counterparts, indicating that antigenic epitopes on each virus glycoprotein do not structurally deteriorate during lyophilisation, thus ratifying the suitability of freeze-dried PVs from a serological viewpoint.

With regard to the wider implications for PVNA-based serology kits, the findings reported in this study are also encouraging. The survival and usability of somatic cells after freeze-drying and reconstitution has already been established, with positive implications for many areas of biomedicine (Loi *et al.*, 2008). Indeed, the opportunity to produce samples of pseudotype virus and candidate cell line as stable, dried pellets would considerably facilitate global distribution of a multi-component PVNA kit, at a fraction of the current expenditure for overseas shipping and storage on dry ice. Additionally, the high cost of purchasing frozen cell line ampoules from certified repositories can be avoided by incorporating lyophilised cells into such a kit. Overall, the utilisation of PVNA-based kits would significantly ameliorate logistic dilemmas surrounding vaccine evaluation and serological surveillance, especially for laboratories situated in resource-poor countries where many emerging viral infections are prevalent.

Virus lyophilisation as a stable means of storage is certainly not a novel phenomenon, with the process being acknowledged for decades (Tyrrell and Ridgwell, 1965). Studies involving wild-type virus freeze-drying generally concur with this one in several aspects by, for instance, demonstrating the ability to store lyophilised foot and mouth disease virus at 4°C for 1 year (Fellowes, 1965) and freeze-dried poliovirus preparations at 37°C for 5 days (Berge *et al.*, 1971). Infectivity tests were also undertaken on pseudorabies virus lyophilised in a number of suspension media, with glutamate formulations mixed with sucrose or dextran proving the most cryoprotective (Scott and Woodside, 1976). Furthermore, the viability of freeze-dried viral vector formulations has been investigated for gene therapy applications. Retroviral vectors have recovered with more than 90% infectivity post-lyophilisation in the presence of sucrose cryoprotectant (Shin *et al.*, 2010), with adenoviral vectors only showing negligible drops in titre following freeze-drying and storage at ambient temperatures (Croyle *et al.*, 2001). Likewise, lyophilised influenza virosomes retained both structure and function after 12 weeks' storage at 4°C (Wilschut *et al.*, 2007). In comparison, pseudotyped retroviral vectors rapidly decreased in titre following three to five freeze-thaw cycles (Higashikawa and Chang,

2001), which further reinforces how preferable lyophilisation is for employment in VNAb-based serological kits.

Relative humidity (RH) – the percentage saturation of water vapour in air – plays a pivotal role in storage stability of dried pseudotype pellets, with high RH levels proving detrimental to PV recovery and transduction potential. Certainly for wild-type influenza virus, it has been confirmed that both virus transmission and infectivity are significantly decreased in highly humid atmospheres, which could be an attributive factor for its seasonal fluctuation (Lowen *et al.*, 2007; Noti *et al.*, 2013). It has previously been postulated that viruses with a high lipid content are more sensitive to high RH (Assar and Block, 2001).

Investigation of further parameters would be necessary before a robust, reliable PVNA-based kit could be trialled and clinically utilised. Firstly, existing data would need to be extrapolated by testing freeze-dried pellet storage stability over longer durations i.e. six months, one year and three years, as well as comparing other candidate suspending media to sucrose-PBS to ensure maximum efficiency of cryoprotection. Employing freeze-dried pseudotypes in PVNAs against larger panels of sera, before drawing comparisons against not only unlyophilised pseudotypes, but also established serological assays using live virus, would be vital to assess accordance in VNAb titres between assays. To increase PVNA kit flexibility and customisation, it would also be important to assess the sensitivity of other commonly used pseudotype virus cores to lyophilisation and subsequent stability studies. Another consideration is to accurately simulate conditions during an international transit journey, thus ascertaining the ability for lyophilised PVs to cope with harsh temperature and atmospheric fluctuations between, for example, an aeroplane cargo deck and tropical climate conditions.

To conclude, in this study it is shown H5 influenza, rabies and Marburg pseudotype viruses can be stably stored in a lyophilised state for 4 weeks at temperatures up to 37°C, in the presence of at least 0.5M sucrose-PBS as a cryoprotectant, and retain much of their infectivity once

reconstituted and employed in virus neutralisation assays. This confirms the viability of producing a freeze-dried PVNA-based kit, which would considerably facilitate the execution of vaccine evaluation and sero-surveillance studies, especially in countries without access to BSL-3/-4 containment laboratories or constant cold-chain storage facilities, and ultimately permit the development of improved serological control measures for many emerging viral infections.

Chapter 6

Final discussion and conclusions

In this thesis, a wide variety of approaches were attempted to produce retroviral pseudotype viruses bearing the heterologous JEV prME glycoproteins, which could be utilised in several downstream applications, such as evaluation of novel vaccines and antiviral treatments, or serosurveillance studies to track the virus' geographical spread, ultimately in order to enhance the serological study and cell entry processes of the flavivirus.

Chapter 1 of this thesis provides an introduction and literature review, which aims to help identify the importance of the data presented in subsequent chapters, in the context of the wider bank of literature in the discipline. This chapter outlines the clinical impact of JEV and of flaviviruses as a genus of viruses, before dissecting various stages of the JEV life cycle that are highly relevant to the body of work of this thesis. Furthermore, the role of pseudotype viruses as surrogates for their native, pathogenic counterparts is introduced, along with their advantages when compared with other surrogate virus particles used for flaviviral serology in low biosafety environments. Subsequently, Chapter 2 describes all of the general materials and methods routinely utilised throughout the studies which constitute the practical work of the following results chapters.

As detailed in Chapter 3, the exploration of various experimental parameters was undertaken, related to the transfection and titration stages of JEV pseudotype virus production attempts. This work involved gauging JEV PV titres when manipulating target cell lines, transfected plasmid masses, use of low glucose culture media, plasmid-derived protease expression and employment of different transfection producer cell systems, before attempts to validate glycoprotein expression. Despite not being able to successfully generate functional JEV pseudotype preparations, the immunofluorescence experiment carried out in this chapter,

which indicates the intracellular retention of expressed prME protein at the endoplasmic reticulum membrane, was important in prompting the directions taken regarding the experiments conducted in the next chapter.

The premise of Chapter 4 of this thesis was to increase the likelihood of JEV pseudotype production *via* genetic alteration of the prME plasmid constructs. This objective was attempted by construction of chimeric prME glycoproteins with mutagenic insertions or substitutions of the VSV-G transmembrane domain and/or cytoplasmic tail, in a bid to induce interactions between the JEV envelope proteins and the retroviral *gag-pol* core proteins; and also by mutagenesis to insert Kozak consensus sequences upstream of the prME gene, to augment the initiation of RNA translation and consequently boost intracellular expression levels of the JEV envelope glycoprotein. Although the mutagenesis in this chapter was not successful, meaning that the cGP and Kozak mutant constructs could not be employed in JEVpp production trials, aspects of the experimental design within this chapter represent an attempt to produce novel knowledge not currently present in the virological discipline. For example, following searches of current, relevant literature, it appears that panels of VSV-JEV chimeric glycoproteins with designs similar to those included in the results of this chapter have not been previously published.

The data presented in Chapter 5 highlights the importance of maximising the utility and amenability of the pseudotype virus platform, particularly for laboratories in resource-deprived areas. Confirmation that lentiviral pseudotype viruses bearing envelope glycoproteins from distinct RNA virus families can be lyophilised, stored at a variety of temperatures, humidities and durations, and then reconstituted to retain functional to high virus titres which can be effectively neutralised by positive serum samples, furthers our knowledge of the durability of pseudotype viruses and has important implications for the global distribution and storage of a PVNA-based kit package.

Throughout the laboratory work carried out for this project, several contingency strategies had to be followed, due to the inability to produce JEV PVs in previous experiments, meaning that the incorporation of JEV pseudotype viruses in downstream applications was unable to occur. A likely reason for the inability to produce retroviral pseudotypes bearing JEV glycoproteins is due to the mismatch in subcellular localisations of any expressed prME protein, which resides in the endoplasmic reticulum membrane, and HIV *gag-pol* cores, which directly exit the cell from the external plasma membrane, during viral assembly and budding. However, if generation of functional JEV pseudoparticles had been successful, they could be employed in a distinct range of downstream applications. For instance, comparative serology studies could be performed, where the results of a JEV PVNA were analysed alongside those of a more established serological assay, such as PRNT. If a high correlation between the two datasets was observed, this would indicate that the pseudotype-based neutralisation assay may be suitable for use in clinical serology studies, circumventing the requirement to handle pathogenic virus. Furthermore, pseudotyping of other flaviviruses, such as WNV and Zika viruses especially, but also other members such as TBEV, SLEV and MVEV, could be carried out, which could pave the way for the development of similar studies of these pathogens and potentially to develop a multiplexing system for flavivirus pseudotypes. Such a system enables the simultaneous screening of antibody samples against two viruses in the same sample well of an assay plate, saving both money and valuable reagents, such as experimental serum. Other subsequent applications for JEV pseudotype viruses include their utilisation to evaluate screening programs for novel antiviral treatments, and to aid the elucidation of potential cellular receptors for attachment and entry of the flavivirus into host target cells. Further considerations for the production of JEV PVs are discussed below.

The utilisation of HIV and MLV structural and enzymatic proteins as backbones for JEV pseudoparticle production has been persistently fruitless throughout this project. Due to this, the exploitation of distinct pseudotyping cores for the generation of JEVpp should be explored.

Firstly, the employment of VSV as a pseudotyping core, primarily produced *via* the VSV ΔG^* system in which the native VSV glycoprotein is replaced with a reporter gene, has also been successfully shown in combination with a variety of heterologous envelope glycoproteins, such as Nipah virus, Hantaan and Seoul hantaviruses, and the ER-budding SARS coronavirus (Kaku *et al*, 2009; Tamin *et al*, 2009; Ogino *et al*, 2003; Fukushi *et al*, 2006). Due to its success at pseudotyping other internal-budding RNA viruses, the VSV backbone system was viewed as a promising option for JEVpp generation. However, functional VSV-JEV pseudotype viruses have already been reported by one group (Tani *et al*, 2010), and routine application of the manipulated VSV genome necessitates containment level 2 practices (Whitt, 2010), requiring adherence to relatively stringent biosafety guidelines and official approval from institutional Health and Safety boards. Such laboratories are not readily available for the implementation of this project work.

Also, human foamy virus (HFV) has been considered as a core for pseudotype flaviviruses, due to its regular use as a gene therapy vector (Mergia and Heinkelein, 2003). HFV is another member of *Retroviridae*, so by definition would be capable of reverse transcribing a reporter gene RNA dimer, meaning that the conventional multi-plasmid co-transfection system for pseudotype generation would not have to be drastically altered. Furthermore, this spumavirus possesses a unique morphology within the wider retrovirus family, as it buds from the endoplasmic reticulum rather than the plasma membrane. As HFV and JEV bud from the same membranous organelle, this could circumvent the issue of mismatched virus budding locations potentially experienced during HIV-JEVpp and MLV-JEVpp production (Mergia and Heinkelein, 2003; Trobridge, 2009).

As an alternative to VSV, the use of recombinant Newcastle disease virus (rNDV) as a viral vector expressing foreign JEV prME is being investigated, with co-operation from Dr Subbiah Elankumaran, University of Maryland (Huang *et al*, 2003; Zhao and Peeters, 2003; Wen *et al*,

2013). This process would involve the removal of the haemagglutinin-neuraminidase (HN) and fusion (F) proteins from the full-length NDV genome and replacement with a luciferase (or other) reporter gene, to create an rNDV- Δ HN/F-Luc genome. This manipulated genome would then be cloned into a suitable DNA expression plasmid and transfected into pseudotype producer cells, alongside either the JEV 15SP- or 24SPprME plasmid construct. Additionally, this system functions under the transcriptional control of T7 RNA polymerase – this additional mandatory element can be introduced into HEK293T/17 producer cells *via* infection with a fowlpox-T7 helper virus, or alternatively by utilisation of BSR-T7 cells, which constitutively express the T7 RNA polymerase. An overview of the recovery systems available for recombinant NDV production from cDNA, which form the basis for the methodology to produce rNDV-JEV pseudotypes, can be found in Huang *et al*, 2003.

Lastly, in collaboration with Professor Yvonne Perrie (Aston University, UK), another consideration to induce functional generation of JEV pseudotype viruses is *via* the exploitation of liposomes. Providing they were in the cleaved, mature conformation, recombinant prME glycoproteins of JEV could be incorporated into the external surface of liposomal vesicles. Upon introduction onto a producer cell monolayer either transiently transfected by, or constitutively expressing, a desired retroviral *gag-pol* and reporter gene, these liposomes could theoretically fuse and merge with the cells' plasma membrane phospholipid bilayers, resulting in mature JEV envelope proteins coating the retroviral cores following budding and egress. If this approach was successful, it would circumvent the necessity for transfection of a prME plasmid, and subsequent expression, assembly and maturation, as a novel methodology for pseudotype virus generation.

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